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THE FUNCTION OF PS INTEGRINS
IN DROSOPHILA EMBRYO AND WING MORPHOGENESIS

by
Marc Christophe Brabant

A Dissertation Submitted to the Faculty of the
DEPARTMENT OF BIOCHEMISTRY
In Partial Fulfillment of the Requirements
For the Degree of
DOCTOR OF PHILOSOPHY
In the Graduate College
THE UNIVERSITY OF ARIZONA

1995
As members of the Final Examination Committee, we certify that we have read the dissertation prepared by Marc Christophe Brabant entitled THE FUNCTION OF PS INTEGRINS IN DROSOPHILA EMBRYO AND WING MORPHOGENESIS and recommend that it be accepted as fulfilling the dissertation requirement for the Degree of Doctor of Philosophy.

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Final approval and acceptance of this dissertation is contingent upon the candidate's submission of the final copy of the dissertation to the Graduate College.

I hereby certify that I have read this dissertation prepared under my direction and recommend that it be accepted as fulfilling the dissertation requirement.

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The position-specific (PS) integrins of *Drosophila* are homologous in structure and function to vertebrate integrins, a family of transmembrane receptors that facilitate mechanical linkage and signal transduction between extracellular molecules and the cytoskeleton. Most integrins, including PS integrins, are receptors for components of the extracellular matrix. Integrins are αβ heterodimers, and structural domains within both subunits typically influence ligand specificity. PS1 and PS2 integrins are composed of unique α subunits, αPS1 or αPS2, and share a common β subunit (βPS). *myospheroid (mys)* encodes βPS, and the characterization of *mys* phenotypes indicates that PS integrins function during tissue morphogenesis; e.g., PS integrins are required for adhesion of embryonic muscles to their body wall attachments, and for adhesion between the two surfaces of the pupal wing blade. During metamorphosis, the flat bilayered adult wing blade is formed from a single layered larval wing disc, and removal of PS integrins from this process causes wing "blisters". Since wing morphogenesis is a lengthy and dynamic process, the temporal requirement for integrin function was unclear. Using heat-shock inducible *mys* transgenes, the wing blister phenotype of animals with compromised integrin function was rescued. By varying the times of transgene induction, I find that
integrin function is required from very early in metamorphosis until at least the last 24-48 hours of wing development. Since the inflated (if) gene encodes $\alpha_{PS2}$, characterization of if phenotypes promised to reveal the developmental requirements specific for PS2. New if lethal alleles were generated and found to retain little or no wild-type $\alpha_{PS2}$ function. As seen for mys mutants, if mutant embryos show extreme defects in somatic muscle attachments and in midgut morphogenesis. Unlike mys, however, there is no dorsal herniation in if mutant embryos. With respect to wing morphogenesis, clonal analysis experiments demonstrate that if+ function is required only in cells of the ventral wing surface. To examine the in vivo functions of distinct integrin structures, transgenes were created to express either PS2 subtype, PS2m8 or PS2c. I find that PS2m8 or PS2c rescues the if- muscle phenotype. In contrast, ectopic PS2m8 (but not PS2c) activity dominantly disrupts wing morphogenesis.
CHAPTER 1
INTRODUCTION TO FIELD OF INTEGRIN RESEARCH

The integrin family of cell surface receptors performs essential functions in multicellular animals. A full understanding of how integrins function promises to provide fundamental insights into a wide variety of complex biological phenomena. Of particular interest to developmental biologists, integrins function during tissue morphogenesis in animals. This dissertation presents my research which analyzes the requirements for integrins during specific morphogenetic events in Drosophila melanogaster.

Integrins: a Family of Homologous Receptors

In 1986, the term "integrin" was coined to describe an integral membrane complex involved in the transmembrane association between the extracellular matrix and the cytoskeleton (Tumkun et al., 1986). Because this integrin complex is structurally related to other receptors for extracellular ligands, the definition of an integrin has been expanded to encompass any member within this family of homologous receptors (reviewed by Hynes, 1987). All integrins are $\alpha\beta$ heterodimers created by the noncovalent pairing of two large glycoprotein subunits, one $\alpha$ (120-180kD) and one $\beta$ (90-110kD) as diagrammed in Figure 1.1. Originally,
FIGURE 1.1  Diagram of an integrin. Integrins are integral membrane heterodimers composed of noncovalently associated α and β subunits. The globular "head" domain binds extracellular ligands (typically components of the extracellular matrix), and the cytoplasmic "tails" interact with cytoskeletal proteins.
it was thought that each \( \alpha \) subunit was capable of forming heterodimers with only one of several different \( \beta \) subunits expressed within a cell. This observation led to the grouping of vertebrate \( \alpha \) subunits according to which \( \beta \) subunit they are capable of pairing with as shown in Table 1.1. Although these groupings are still largely valid, it has become clear that a few \( \alpha \) subunits form heterodimers with more than one type of \( \beta \) subunit (e.g. see \( \alpha_\gamma \) in Table 1.1).

The current nomenclature designates each integrin subunit with a unique subscript (e.g. \( \alpha_3 \) and \( \beta_1 \)) and the particular receptor formed from the pairing of subunits as simply the "\( \alpha_x\beta_y \)" integrin (e.g. \( \alpha_3\beta_1 \)). The integrin family of homologous receptors is operationally defined by the high degree of amino acid sequence identity shared between all \( \alpha \) subunits and between all \( \beta \) subunits. In addition to the set of vertebrate integrins presented in Table 1.1, integrins have also been found in invertebrates including Caenorhabditis elegans and Drosophila melanogaster (Table 1.2).

**Biological Role of Integrins**

The apparently ubiquitous distribution of integrins throughout the multicellular animal kingdom suggests that integrins perform important functions in most, if not all, metazoans (e.g. see Marcantonio and Hynes, 1988). Indeed, integrins have been shown to influence a variety of
<table>
<thead>
<tr>
<th>Integrin</th>
<th>Ligands Recognized</th>
<th>RGD Binding Site?</th>
</tr>
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<tbody>
<tr>
<td><strong>β1 Integrins</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>α1β1</td>
<td>CG, LM</td>
<td>NO</td>
</tr>
<tr>
<td>α2β1</td>
<td>CG, LM</td>
<td>NO</td>
</tr>
<tr>
<td>α3β1</td>
<td>FN, LM, CG, EG</td>
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</tr>
<tr>
<td>α4β1</td>
<td>FN, VCAM-1</td>
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</tr>
<tr>
<td>α5β1</td>
<td>FN</td>
<td>YES</td>
</tr>
<tr>
<td>α6β1</td>
<td>LM</td>
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</tr>
<tr>
<td>α7β1</td>
<td>LM</td>
<td>NO</td>
</tr>
<tr>
<td>α8β1</td>
<td>?</td>
<td>?</td>
</tr>
<tr>
<td>αvβ1</td>
<td>VN, FN</td>
<td>NO</td>
</tr>
<tr>
<td><strong>β2 Integrins</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>αLβ2</td>
<td>ICAM-1, ICAM-2</td>
<td>NO</td>
</tr>
<tr>
<td>αMβ2</td>
<td>C3b, FB, FX, ICAM-1</td>
<td>NO</td>
</tr>
<tr>
<td>αXβ2</td>
<td>FB, C3b</td>
<td>NO</td>
</tr>
<tr>
<td><strong>β3-β8 Integrins</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>αIIbβ3</td>
<td>FB, FN, W, VN, T</td>
<td>YES</td>
</tr>
<tr>
<td>αvβ3</td>
<td>VN, FB, W, T, FN, O, CG</td>
<td>YES</td>
</tr>
<tr>
<td>α6β4</td>
<td>LM?</td>
<td>?</td>
</tr>
<tr>
<td>αvβ5</td>
<td>VN</td>
<td>YES</td>
</tr>
<tr>
<td>αvβ6</td>
<td>FN</td>
<td>YES</td>
</tr>
<tr>
<td>α4β7</td>
<td>FN, VCAM-1</td>
<td>NO</td>
</tr>
<tr>
<td>αIELβ7</td>
<td>?</td>
<td>?</td>
</tr>
<tr>
<td>αvβ8</td>
<td>?</td>
<td>?</td>
</tr>
</tbody>
</table>

See review by Hynes (1992) for specific references. Abbreviations for ligands: CG, collagen(s); LM, laminin; FN, fibronectin; EG, epiligrin; VN, vitronectin; C3b, a component of complement; FB, fibrinogen; FX, factor X; W, von Willebrand factor; T, thrombospondin; O, osteopontin; VCAM-1, ICAM-1 and ICAM-2 are Cell Adhesion Molecules.
### TABLE 1.2 Invertebrate integrins and their adhesive properties

<table>
<thead>
<tr>
<th></th>
<th>C. elegans:</th>
<th>D. melanogaster:</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Integrin</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(αF54G8.5β?)^1</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Ligands Recognized</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>?</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>RGD Binding Site?</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>?</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Integrin</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>βPS integrins</td>
<td></td>
<td></td>
</tr>
<tr>
<td>αPS1βPS</td>
<td>LM^2</td>
<td>NO^2</td>
</tr>
<tr>
<td>αPS2βPS</td>
<td>TG^3, (VN, FN)^4</td>
<td>YES^3,4</td>
</tr>
<tr>
<td>(αPS3?βPS)^6</td>
<td>?</td>
<td>?</td>
</tr>
<tr>
<td><strong>other Drosophila integrins</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(α2βν)^7</td>
<td>?</td>
<td>?</td>
</tr>
</tbody>
</table>

^1The F54G8.5 genomic sequence was submitted to the EMBL sequence data base (accession #Z19155) by the "C. elegans sequencing project" (see Sulston et al., 1992); sequence analysis which identified the open reading frame encoding an integrin α subunit homologue performed by Werhli et al., (1993).

^2Gotwals et al., (1994); Tom Bunch personal communication.

^3Fogerty et al., (1994).

^4Bunch and Brower (1992)

^5A novel Drosophila α subunit (αPS3?) has been recently isolated from proteins immunoprecipitated with anti-βPS antibodies indicating that a third PS integrin (αPS3?βPS) exists (K. Stark, personal communication).

^7Yee and Hynes (1993)

Abbreviations for ligands: LM, Drosophila laminin (but not vertebrate laminin); TG, Drosophila tiggrin; VN, vitronectin (vertebrate); FN, fibronectin (vertebrate).
biological phenomena in higher organisms including cell signaling, cell migration and cell differentiation events. As cell surface receptors, integrins mediate these cellular events through interactions with extracellular ligands. With few exceptions, integrin ligands are specific components of the extracellular matrix (see e.g. collagen, laminin and fibronectin in Tables 1.1 & 1.2).

The extracellular matrix is a complex assemblage of various secreted macromolecules composed of polysaccharide and protein (e.g. see review by McDonald, 1988). Many components of the extracellular matrix are known to play critical roles during cell differentiation and tissue morphogenesis, although the details of these interactions are poorly understood (reviewed by Adams and Watt, 1993). Since the integrin superfamily represents the major class of receptors for extracellular matrix components, integrins are thought to function as conduits for communication between cells and their extracellular matrix environment (Hynes, 1992).

Integrin intracellular domains are thought to interact with the actin cytoskeleton (see cytoplasmic "tails" in Figure 1.1). This hypothesis is supported by numerous light and electron microscopic studies which show that integrins are often localized to areas of the plasma membrane which have dense accumulations of cytoskeletal components (reviewed by Burridge et al., 1988). In the cases where these
Cytoskeletal connections have been characterized, integrins appear to interact with actin microfilaments through cytoplasmic linker proteins such as talin, vinculin and $\alpha$-actinin (e.g. see Horwitz et al., 1986). Therefore, integrins provide physical linkage between two large molecular superstructures: the extracellular matrix and the intracellular cytoskeleton.

Considering this capacity to physically connect intra- and extra-cellular superstructures, it is not surprising that integrin mediated biological events are often manifested as cell adhesion events (reviewed by Hynes, 1992). Clearly, the regulation of cell adhesion events is a fundamental requirement for the morphogenesis of specialized tissues. One important mechanism for regulating integrin mediated cell adhesion appears to be the differential expression of structurally distinct integrins possessing unique adhesion properties.

In addition to their roles in modulating the adhesive properties of cells, it has become clear that integrins also function as true receptors with signal transduction functions (reviewed by Humphries et al., 1993). Specifically, integrin mediated adhesion events can influence the proliferative state of cells, and moreover, interactions between integrins and their ligands have been shown to elicit specific changes in gene expression. Theoretically, integrin mediated signaling could be an indirect result of cell shape changes.
associated with altered adhesion properties, or signals could be directly transduced through ligand induced changes in integrin structure. There are considerable data to implicate the latter mechanism as several integrins have been shown to exist in alternative conformations which correlate with active and inactive ligand binding states. Presumably, information from the extracellular environment is transmitted to cells through ligand mediated stabilization of the active conformation, and this active structural state affects the binding of receptor associated molecules within the cell. Intriguingly, integrins appear to transmit signals bidirectionally as signals generated from within the cell can also influence the conformational state (and ligand binding activity) of integrins.

Integrins are therefore considered to be cell adhesion molecules possessing the ability to transmit signals back and forth between the extracellular and intracellular environments. Interestingly, many gene products which were originally described as being exclusively adhesion or signaling molecules are now recognized as having both functions; in fact, arguments have been made that many, if not most, adhesion molecules also function in signaling pathways (e.g. see review by Bunch and Brower, 1993). Integrins are distinguished from other adhesion molecules by virtue of their role as the primary receptors for the diverse group of molecules which make up extracellular matrices.
Because of these observations, it seems probable that integrins function during many of the fascinating biological interactions which occur between animal cells and their extracellular matrices.

Structure of Integrins

The primary defining characteristic of all integrins, that their protein subunits share striking sequence identity, indicates that these are homologous proteins. In fact, comparisons of integrin sequences from worm, fly and human genomes indicate that a common integrin structure arose prior to the divergence of the phylogenetic lines leading to these organisms (>500 million years ago) (Wehrli et al., 1993), sufficiently early in evolution to be consistent with the hypothesis that integrins could have been necessary to maintain multicellularity in the first metazoans (Hynes, 1992). Although integrin sequence similarities are generally observed over entire subunit lengths, all subunits also contain specific regions of exceptionally high sequence identity. These highly conserved structural domains are thought to reveal a basic structural framework through which most (if not all) integrins perform homologous functions.

To fully understand the relationship between integrin structure and function, it will be necessary to determine the structure of integrins at a level far beyond our current knowledge. Unfortunately, the elucidation of integrin
tertiary structure at very high resolution (e.g. at the atomic level) promises to be a most formidable task. In addition to the difficulty of performing biophysical and biochemical studies with high molecular weight glycoprotein heterodimers, structural analysis of integrins is further complicated because each subunit resides in three distinct subcellular environments: the cytosol, the plasma membrane and the extracellular space.

For purposes of simplicity, it is useful conceptually to subdivide $\alpha$ and $\beta$ subunits into three gross structural regions: the huge extracellular domains which associate with ligands (>100kD for $\alpha$ subunits, and >75kD for $\beta$ subunits), the ~26 amino acid transmembrane spanning sequences and the small (generally <60aa) intracellular domains which associate with cytoskeletal proteins (Figure 1.1). Although these structural subdivisions are artificial, there are data to suggest that treating the largest of these domains as an independent functional unit can be a fruitful experimental approach. For example, truncated forms of $\alpha$ and $\beta$ subunits, which lack their transmembrane and cytosolic domains, have been shown to form heterodimers and retain authentic ligand binding specificity (Dana et al., 1991; Bodary et al., 1991). In the following sections the extracellular, transmembrane and intracellular domains are discussed independently with particular attention to the presumptive functions of homologous sequence motifs found within each domain.
The Extracellular Domain: The large external domain of integrins, where ligand binding occurs, appears to be subdivided into distinct "stalk and head" structures. Specifically, electron micrographs of integrins show a large globular head attached to two parallel stalks 12-14 nM in length, and these stalks are thought to position the head this distance from the plasma membrane as diagrammed in Figure 1.1 (Nermut et al., 1988). The two stalks appear to be extended regions of the $\alpha$ and $\beta$ subunits; while the highly folded globular head appears to be formed from the N-terminal domains (40-50kD) of both subunits.

The unique ligand binding properties of a particular integrin are thought to be a function of sequences from both the $\alpha$ and $\beta$ subunits. This hypothesis is supported by the observation that exchanging either subunit for an alternative one results in a receptor with altered ligand binding properties. For example, $\alpha_5\beta_1$ and $\alpha_6\beta_1$ integrins differ drastically in their ligand specificity as $\alpha_5\beta_1$ and $\alpha_6\beta_1$ bind fibronectin and laminin respectively; while $\alpha_\gamma\beta_5$ and $\alpha_\gamma\beta_6$ integrins also recognize different ligands (Table 1.1). These observations, when taken together with chemical crosslinking indicating that amino acid residues from both subunits interact with ligands (D'Souza et al., 1988; Marcantonio et al., 1988), support models which propose that
an interface of \( \alpha \) and \( \beta \) sequences constitutes a significant part of the ligand binding surface (Figure 1.1).

When looking at the sequence identities between integrin subunits, it is clear that the extracellular regions of all \( \alpha \) subunits have seven repeats of a homologous domain containing approximately 25 amino acids. Importantly, three to five of these repeats always contain one copy of an aspartic acid rich motif DXDXDXGXXD (where \( X \) is an uncharged amino acid) which shares significant sequence identity with known divalent cation binding motifs found in other proteins such as calmodulin (Kirchhofer et al., 1991). These sequences are found in the N-terminal portion of each \( \alpha \) subunit suggesting a role in ligand binding (Figure 1.2). Since the \textit{in vitro} affinity and specificity of ligand binding by integrins is highly dependent on the concentrations of divalent cations in the media (reviewed by Hynes, 1992), these repeats potentially provide one mechanism through which integrin ligand binding affinity might be modulated \textit{in vivo}.

Additionally, extracellular domains have numerous cysteines occupying conserved positions along each subunit;
 FIGURE 1.2 Diagram of the primary structure of PS integrin subunits showing features conserved with other integrins. Conserved cysteine residues (56 for \( \beta \)'s and 16 for \( \alpha \)'s) are indicated with dashed lines. In \( \beta_{PS} \), stippled boxes \((W,X,Y,Z)\) indicate the position of tandem repeats found in all \( \beta \) subunits. In \( \alpha_{PS1} \) and \( \alpha_{PS2} \), black boxes \((I-VII)\) indicate the positions of homologous domains found in all \( \alpha \) subunits. Hatched boxes indicate transmembrane domains.
at least 16 cysteines are conserved in each \( \alpha \) subunit, and \( \beta \) subunits typically display 56 conserved cysteines (Figure 1.2). Importantly, these residues appear to create a regular pattern of intrasubunit disulfide bridges, and these bridges are thought to stabilize tertiary structures common to many (if not all) integrin extracellular domains (Calvete et al., 1989; 1991). For example, all \( \beta \) subunits contain a tandem array of four related cysteine rich domains (each \(~40\) amino acid domain contains 8 cysteines with nearly identical spacing) which are homologous to sequences within the short arm of laminin, a region of laminin which forms an elongated rod-like structure (Nermut et al., 1988). Such observations, combined with the fact that these cysteine rich repeats are located near the transmembrane domain (Figure 1.2), have led to the hypothesis that these sequences constitute much of the \( \beta \) subunit's stalk region (Figure 1.1). The other disulfide bridges found in \( \alpha \) and \( \beta \) subunits are generally located more distally (the N-terminal portion of each subunit), and these bridges undoubtedly add stability to the globular head structure.

The Transmembrane Domain: Integrin transmembrane domains consist of approximately 26 nonpolar amino acids for all \( \alpha \) and \( \beta \) subunits. These sequences, which are predicted to form a right handed alpha helix, make a single pass through the
hydrophobic core of the plasma membrane (Nermut et al., 1988). At this point, very little functional information is available about these short sequences; although a very high level of sequence conservation between all integrin transmembrane domains suggests functions beyond simply linking extracellular and intracellular domains. For example, a vertebrate fibronectin receptor, the $\alpha_5\beta_1$ integrin, is roughly one half identical to the evolutionarily distant *Drosophila* $\alphaPS_2\beta_FS$ integrin in this region. Specifically, the $\alpha_5$ and $\alphaPS_2$ subunits are 46% identical (12 of 27 amino acids) and the $\beta_1$ and $\beta_FS$ are 65% identical (17 of 26 amino acids) in their transmembrane domains (Bogaert et al., 1987; MacKrell et al., 1988). Most probably, these conserved residues are critical for specific interactions between the $\alpha$ and $\beta$ subunits or between these subunits and other, as yet, unidentified membrane components.

*The Intracellular Domain:* Relative to most transmembrane receptors, integrin intracellular sequences are quite short (typically less than 60 amino acids). Interestingly, integrin cytosolic domains do not have any apparent intrinsic enzymatic activity; rather they appear to interact directly with cytoskeletal proteins. Unfortunately, there is little known about these protein-protein interactions, although it seems likely that novel technical approaches, such as the "Two-Hybrid" or "Interaction-Trap" screens in yeast which are
designed to identify novel proteins involved in protein-protein interactions (Fields et al., 1994), may be useful for discovering which cytoplasmic proteins interact with integrins.

In addition to creating structural connections with the cytoskeleton, there is the intriguing finding that integrin cytoplasmic domains also influence regulatory proteins such as the recently discovered focal adhesion kinase (Schaller et al., 1992; Kornberg, et al., 1992). Along these lines, there appears to be a precedent for receptor induced, noncatalytic activation of protein kinases as the short intracellular domains of CD4 and CD5 (non-integrin receptors on T-cells) have been shown to associate with and activate a src-related protein kinase (reviewed by Weiss, 1993). If integrin cytoplasmic tails prove to activate protein kinases directly, then integrins would join a long list of receptors that transmit signals through changes in protein phosphorylation states.

Like the extracellular and transmembrane domains, \( \beta \) subunit intracellular domains from diverse species share striking structural similarity. Indeed, the \( \beta_1 \) subunit's cytoplasmic domain amino acid sequence (41 amino acids) is 100% identical in human, mouse, chicken and frog (DeSimone and Hynes, 1988). Remarkably, the evolutionarily distant Drosophila \( \beta_{ps} \) cytoplasmic sequence is also 76% identical (31 of 41 amino acids) to the vertebrate \( \beta_1 \) sequence in this
region (MacKrell et al., 1988). More importantly, the majority of vertebrate β subunits' intracellular domains share extensive regions of sequence identity with one another. For example, the β2, β3, β5, β6 and β7 subunit sequences are 30% to 53% identical to the β1 sequence in this region (Sastry and Horwitz, 1993). Such high levels of structural conservation indicate that most β subunits will prove to share significant levels of functional homology in this region.

In contrast, the structure of cytoplasmic domains from different α subunits have diverged to a much greater extent as they rarely share sequence motifs of greater than a few amino acids. However, it appears improbable that this lack of sequence conservation reflects a general lack of function for these domains as homologues of particular α subunits have been highly conserved between distantly related vertebrates. For example, the human and chicken α3, α5, and αv homologs have diverged little (84%, 100% and 94% identical respectively), indicating a high level of selective pressure to retain cytoplasmic domain structure (Sastry and Horwitz, 1993). Therefore, it seems likely that each α subunit homologue has diverged in this region in order to accommodate unique interactions with cytoplasmic components.

Even though the intracellular sequences from different α subunits have diverged considerably, there are several very
short sequence motifs common to many $\alpha$ subunits. By far the longest and best conserved motif is the 7 amino acid sequence KXGFFKR (where X is an uncharged amino acid) which is nearly perfectly retained in all known $\alpha$ subunits including invertebrates (e.g. see Werhli et al., 1993). Interestingly, this seven amino acid sequence is always located immediately adjacent to the hydrophobic transmembrane domain suggesting a critical role in membrane insertion, subunit association or interactions with other proteins localized to the inner surface of the plasma membrane.

**Integrin Research in Vertebrate Model Systems**

The differential expression by vertebrates of the 20 known integrins entails complex regulatory control of 22 different structural genes encoding 8 $\beta$ and 14 $\alpha$ subunits (Table 1.1). Although a few cell types appear to express only one integrin, the vast majority of vertebrate cells express multiple integrins indicating that a repertoire of integrins generally function in concert (reviewed by Albelda and Buck, 1988). To understand the role of integrins in vertebrate systems, a dissection of the functions specific to each integrin must be made.

Very often the first step in determining the function of a particular integrin has been to identify its adhesive properties. This process is usually complicated by two
related observations: first, most integrins have binding affinities for more than one ligand, and second, multiple integrins often share the capacity to bind a particular ligand. The most extreme case of a single receptor binding multiple ligands is the \( \alpha_v \beta_3 \) integrin which can bind vitronectin, fibrinogen, von Willebrand factor, thrombospondin, fibronectin, osteopontin and collagen (Table 1.1). Conversely, several extracellular matrix components, in particular laminin, collagen, fibronectin and vitronectin, are each recognized by multiple integrins. For example, laminin is a ligand for no less than five different integrins \( (\alpha_1 \beta_1, \alpha_2 \beta_1, \alpha_3 \beta_1, \alpha_6 \beta_1 \) and \( \alpha_7 \beta_1 ) \) (Table 1.1).

In light of these redundant binding properties, it is not surprising that integrin ligands often share some sequence identity. Of particular interest, the three amino acid sequence arginine-glycine-aspartic acid (RGD) has been found in numerous different extracellular matrix components recognized by integrins (Tables 1.1 & 1.2). This RGD sequence motif has been studied extensively and is known to form part of the site on ligands which many, but not all, integrins recognize (reviewed by Kuhn and Eble, 1994; and Tables 1.1 & 1.2). Presumably, the evolution of partially redundant receptors and ligands reflects the need for finely tuned communication between cells and their extracellular matrix environments.
Since most vertebrate cells express multiple integrins with moderately redundant ligand specificities, the function of integrins is often determined in relatively simple in vitro systems such as cell culture. With a homogeneous population of cultured vertebrate cells, cellular responses to purified extracellular components can be easily observed. Using such in vitro systems, the integrins which participate in specific cell differentiation events, like the differentiation of embryo myoblasts into contracting myotubes (Menko and Boettiger, 1988; Volk et al., 1990), can be readily identified. Importantly, the expression (or activity) levels of individual integrins can be manipulated rather easily in cultured cells using a variety of techniques. For example, transfection of genes (encoding integrin subunits) into cells can be used to increase expression levels of specific integrins, and alternatively "anti-integrin" antibodies or RGD peptides can be added to culture media to specifically block the ligand binding activity of integrins. Such in vitro approaches have been instrumental for characterizing integrin ligand binding properties and integrin mediated cellular responses.

Because integrins are thought to be required during tissue morphogenesis, in vivo systems are used to analyze the developmental requirements for integrins. Similar to blocking studies in tissue culture, integrins can also be inactivated in living animals by injecting "anti-integrin"
antibodies or RGD peptides directly into developing tissues. Even though these invasive types of experiments are prone to artifacts, when data from many different experiments are considered together, blocking studies strongly support the hypothesis that integrins play critical roles during vertebrate tissue morphogenesis (e.g. Bronner-Fraser, 1986; Jeffredo et al., 1988).

The cleanest way to address the role of integrins in whole animals is to examine the morphology of animals bearing mutations in the genes which encode integrin subunits. For example, humans with a rare inherited disease called "leukocyte adhesion deficiency" have been shown to express little or no functional \( \beta_2 \) subunit on their neutrophils (e.g. see Wardlaw et al., 1990). These patients have recurrent bacterial infections due to their neutrophil's inability to reach sites of acute inflammation. Therefore, one or more of the three \( \beta_2 \) integrins (\( \beta_2\alpha_L, \beta_2\alpha_M \) and \( \beta_2\alpha_X \)) are required for neutrophil adhesion to endothelial cells. Of course this type of clinical study in human adults requires that the specific integrin is not critically required during early development.

Strong mutations in critically required integrins are currently being sought in the more genetically tractable mouse model system using targeted gene disruption to inactivate specific integrin subunit genes (Stevens and Hynes, 1992), but the technical aspects of this approach are
quite tedious and time consuming (e.g., see Thomas and Capecchi, 1987). It is possible that in the next decade mouse strains carrying mutations in every integrin gene will be made, and analysis of the resultant mutant phenotypes will show the earliest requirements for each integrin during development. The next step beyond simply determining functional requirements is to explore the relationship between specific integrin structures and functions in vivo. To this end, an ideal genetic system would allow one to assay the function of artificially engineered integrin structures in vivo, an approach which is currently more feasible in Drosophila melanogaster.

**Discovery of Integrins in Drosophila**

The study of Drosophila integrins was initiated with the characterization of a set of "position-specific" (PS) antigens (Wilcox et al., 1981; Brower et al., 1984). PS antigens were discovered in a series of monoclonal antibody screens designed to identify developmentally important cell surface molecules on the larval tissues (imaginal discs) which differentiate during metamorphosis into specific adult tissues. Importantly, these screens identified two antigens (PS1 and PS2) which were designated "position-specific" because they are localized to unique areas (or positions) on the undifferentiated epithelium of mature wing discs. Immunostaining and immunoprecipitation experiments indicated
that PS1 and PS2 are expressed on the surface of imaginal disc cells in association with a third antigen (PS3). Thus PS antigens are expressed on cells as two distinct heterodimeric complexes: as either PS1/PS3 or PS2/PS3 heterodimers (Brower et al., 1984; Wilcox et al., 1984).

Molecular and biochemical characterization indicated that all three antigens were large cell surface glycoproteins displaying gross biochemical properties (e.g. size, charge and extensive disulphide bonding for PS3) similar to those described for vertebrate integrin subunits. In addition, N-terminal microsequencing of PS1 revealed a 12 amino acid sequence which displays significant sequence identity with the corresponding N-terminal sequences of several vertebrate integrin α subunits (Leptin et al., 1987). These data provided the first indication that the PS1/PS3 and PS2/PS3 complexes possess some level of structural identity with the integrin family of vertebrate receptors.

The deduced amino acid sequence from a cDNA clone of the myospheroid (mys) locus proved that the Drosophila genome actually encodes a protein homologous to vertebrate integrin β subunits (MacKrell et al., 1988), and the subsequent molecular analysis of mys mutants indicated that this gene actually encodes the PS3 antigen (Leptin et al., 1989). Likewise, the cloning and sequencing of cDNAs which encode the PS1 and PS2 antigens proved that these two glycoproteins share extensive sequence identity with vertebrate integrin α.
subunits (Bogaert et al., 1987; Wehrli et al., 1993). In fact, such sequence comparisons indicate that Drosophila PS antigens are about as closely related to vertebrate integrin subunits as vertebrate subunits are to one another. Importantly, these sequence analyses confirm the hypothesis that the two PS antigen heterodimer complexes are true integrin homologues. Moreover, these data indicate that studies which elucidate the relationship between integrin structure and function in Drosophila promise to be generally applicable to the field of integrin research.

To conform to the nomenclature of vertebrate integrins, the PS antigens (PS1, PS2 and PS3) are now referred to as the αPS1, αPS2 and βPS integrin subunits respectively (see Brown, 1993). The two PS integrins formed by the pairing of these subunits into αβ heterodimers are therefore designated as simply the PS1 (αPS1βPS) and PS2 (αPS2βPS) integrins.

Function of PS Integrins in Vitro

The functions of PS integrins have been (and continue to be) analyzed in vitro using several cell culture systems. In light of the high level of sequence identity shared between Drosophila and vertebrate integrins, it is not surprising that PS integrins display "cell adhesion" characteristics analogous to those previously described for other integrins in vertebrate cell culture systems. For example, PS integrins mediate cell adhesion and spreading on plastic
tissue culture dishes coated with purified vertebrate or Drosophila extracellular matrix molecules.

Indeed, two previously characterized vertebrate integrin ligands, vitronectin and fibronectin, both function as PS2 integrin ligands in cell spreading assays (Hirano et al., 1991; Bunch and Brower, 1992). Although homologues of vitronectin and fibronectin have not been identified in Drosophila, the recently discovered Drosophila extracellular matrix glycoprotein tiggrin appears to be an authentic PS2 integrin ligand since cells expressing PS2 spread very efficiently on tiggrin coated tissue culture dishes (Fogerty et al., 1994). Likewise, the in vitro characterization of PS1 expressing cells has shown that PS1 also promotes cell adhesion and spreading, but on Drosophila laminin (Gotwals et al., 1994).

Of particular interest, the three PS2 ligands identified in these experiments (tiggrin and vertebrate fibronectin and vitronectin) each contain an RGD amino acid sequence motif which is known to be a critical motif on many vertebrate integrin ligands (Table 1.1). Along these lines, it appears that the RGD motif constitutes a critical site on PS2 ligands as PS2-dependent cell spreading on these ligands is blocked when soluble RGD peptides are added to the media (Bunch and Brower, 1992; Zavortink et al., 1993; Fogerty et al., 1994). In contrast, PS1 dependent cell spreading on laminin is not inhibited by RGD peptides in the media (Tom Bunch, personal
communication), and this result is consistent with the fact that *Drosophila* laminin does not contain an RGD sequence. These data indicate that PS2 belongs to the class of integrins which binds ligands via an RGD sequence motif, while PS1 is a member of the class of integrins which recognizes other structural features on ligands (Tables 1.1 & 1.2).

Finally, the analysis of PS integrin function *in vitro* has revealed another characteristic which is displayed by most, if not all, vertebrate integrins: the level of PS2 mediated cell spreading is highly dependent on the concentrations of divalent cations (e.g. Mg$^{2+}$ and Ca$^{2+}$) in the media (Zavortink et al., 1993). Taken together, the data from *in vitro* adhesion assays strongly indicate that the functional properties of PS integrins are homologous to those of vertebrate integrins.

*Distribution of PS Integrins in Vivo*

To understand the *in vivo* functions of PS integrins, it is important to determine when and where these integrins are expressed during development. *Drosophila* development is characterized by two phases of extensive tissue formation: the embryonic phase, where positional information contained within the field of undifferentiated blastoderm cells orchestrates the morphogenesis of embryonic tissues, and the metamorphic phase, where positional information contained
within nested sets of undifferentiated epithelial cells (the imaginal discs) orchestrates the morphogenesis of most external adult tissues (legs, eyes, antennae, halteres, genitalia and wings). Data from immunolocalization and in situ hybridization studies indicate that PS integrins are expressed in many cell types during both of these developmental periods as outlined below.

**Embryogenesis:** Since the process of embryogenesis occurs over a period of about 24 hours, the onset of PS integrin expression at 4 hours 20 minutes (after blastoderm cellurization and germ band extension) is considered to be a relatively early event during embryonic tissue morphogenesis (Bogaert et al., 1987). The embryonic expression of PS2 integrins appears to be restricted to cells derived from the mesoderm, while PS1 is primarily expressed in endoderm and ectoderm cells. (The one known exception to this statement is PS1 expression on the cells of the mesodermally derived fat body.) The important observation is that in embryos, PS1 and PS2 are not generally found in the same cells, rather most cells primarily express just one of the two types of PS integrins.

Although PS1 and PS2 are expressed by distinct sets of cells, these cells are often situated within the embryo such that PS1 integrins are found in extremely close proximity to PS2 integrins. That is, PS integrins are typically
concentrated at sites where PS2 expressing mesoderm contacts PS1 expressing ectoderm or endoderm, and these types of cell-cell interfaces are typically filled with significant accumulations of extracellular matrix components. For example, somatic muscles express high levels of PS2 which is localized to the areas on each muscle cell that are in contact with PS1 expressing tendon cells; importantly, these muscle cell-tendon cell interfaces, known as muscle attachment sites, are sites where numerous extracellular matrix components are deposited, including the previously described PS integrin ligands laminin and tiggrin (Fessler et al., 1987; Fogerty et al., 1994). Taken together, these observations indicate that during embryogenesis PS1 and PS2 typically interact with the same extracellular matrix, but from opposing cellular surfaces.

Metamorphosis: During metamorphosis, which occurs within the confines of the puparium, each mature larval disc (~50,000 cells) differentiates into a particular adult structure. For example, one adult wing blade is created from one larval wing imaginal disc. As mentioned previously, the PS integrins were first identified because they are expressed at high levels on the basal surfaces of many larval imaginal discs including the leg, wing, haltere and eye-antennal discs (Brower et al., 1985).
Since the basal surfaces of imaginal discs are associated with an extensive extracellular matrix (the basal lamina), the subcellular localization of PS integrins to these surfaces indicates that PS integrins are intimately associated with extracellular matrix components throughout the larval period. Intriguingly, the PS1 and PS2 expressing wing disc cells become directly apposed during the first morphogenetic event (disc eversion) following pupariation; that is, PS1 and PS2 expressing disc cells become the dorsal and ventral epithelial surfaces of the wing blade during metamorphosis, and these closely apposed surfaces are separated by a thin extracellular matrix (see CHAPTER 2 for a comprehensive description of these morphogenetic events). Thus, it appears that in at least one tissue undergoing morphogenesis (the pupal wing), there exists a pattern of PS integrin expression which is analogous to the patterns observed during embryogenesis. That is, patterns which place PS1 and PS2 on opposing sides of an extracellular matrix during specific cell-cell adhesion events.

Function of PS Integrins in Vivo

To determine the in vivo functions of integrins, one must have the ability to manipulate integrin activities within the developing organism. Although often a substantial undertaking, the Drosophila genome can be experimentally altered to secure animals with altered levels of integrin
function. For example, the expression of PS integrins (on the previously described tissues) can be reduced or eliminated by creating mutations in the genes which encode PS integrin subunits. Importantly, characterization of any phenotypes associated with integrin mutations should reveal which tissues require integrin function. To this end, the genetics of PS integrins have been, and continue to be, an important focus of integrin research in *Drosophila*.

In contrast to vertebrate systems where the numerous genes (at least 22) encoding integrin subunits are largely uncharacterized, the three *Drosophila* genes encoding PS integrin subunits have been identified and mapped to specific positions within the genome using genetic recombination and *in situ* hybridization techniques (Figure 1.3). As mentioned previously, the *myospheroid* (*mys*) gene, which is located at 21.7 map units on the X chromosome (Wright, 1960), encodes β$_{PS}$ subunits (MacKrell et al., 1988; Leptin et al., 1989). In addition, the recently discovered *multiple edematous wings* gene (*mew*), which is located at about 42 map units on the X chromosome, has been shown to encode α$_{PS1}$ subunits (Brower et al., 1995). Finally, α$_{PS2}$ subunits have been shown to be encoded by the *inflated* (*if*) gene (Wilcox et al., 1989), and this locus is located at 55.7 map units on the X chromosome (Lindsley and Grimm, 1992; also see Chapter 6, page 196).

Null mutations in *mys* have been available for several decades. In fact, Ted Wright characterized the embryonic
defects associated with a lethal mys mutation in the late 1950's as part of his doctoral work, and this original allele (mys<sup>l</sup>) has recently been shown to belong to the "null" class of mys mutations as it deletes an integral part of the β<sub>PS</sub> coding sequence (Bunch et al., 1992). In retrospect, Wright's 1960 report, which gives a comprehensive description of the mys lethal phenotype, constitutes the
Genetic map: 21.7 42 55.7

Physical map: 7D1-5 11D7-E5 15A1-5

FIGURE 1.3 Genetic and physical map positions of genes encoding PS integrin subunits. *mys*, *mew* and *if* encode the $\beta_{PS}$, $\alpha_{PS1}$ and $\alpha_{PS2}$ subunits, respectively.
first paper to analyze the early developmental (embryonic) requirements for an integrin (Wright, 1960).

Since zygotes carrying null mutations are not able to transcribe functional $\beta_{PS}$ mRNA, $mys$ mutants undergo embryogenesis without the function of (zygotically synthesized) PS integrins. Along these lines, it should be noted that some maternally synthesized $\beta_{PS}$ mRNA is deposited in eggs during oogenesis, and maternally derived PS integrin function has been detected in $mys$ mutant embryos (Wieschaus and Noell, 1986; Leptin et al., 1989; Roote and Zusman, 1995). Specifically, removal of the maternal contribution of $\beta_{PS}$ from $mys$ mutant or wild-type embryos results in slightly irregular germ band movements during germ band elongation (Roote and Zusman, 1995). However, this maternal effect appears to be minor as the germ band undergoes retraction normally, and wild type zygotes lacking maternal $\beta_{PS}$ develop into phenotypically wild-type adults. Importantly, the major characteristics of the $mys$ lethal phenotype described below are observed regardless of whether the maternal contribution is eliminated or not.

PS integrin function is required for viability as $mys$ null mutants always die late in embryogenesis. Overall, $mys$ mutant embryos appear to develop fairly normally through the first half of embryogenesis (about 12 hours), but during the latter half of embryogenesis several tissues (including the midgut, muscles, hypoderm and ventral nerve cord) begin to
display morphological defects (Wright, 1960). For example, the entire somatic musculature becomes obviously defective following the onset of muscle contractions (at about 14 hours of development). Specifically, the body wall muscles appear to pull away from their hypoderm attachments, and after numerous contractions, all of these muscles have collapsed into the "myospheroidal bodies" for which this locus was named. Not surprisingly, mys embryos display relatively few body movements when compared to their wild type counterparts, and this lack of movement probably accounts for the fact that mys embryos never escape (hatch) from their vitelline and chorion membranes.

In recent years, PS integrins have also been shown to be required for the morphogenesis of adult tissues using an experimental technique which creates patches of mys mutant tissue in otherwise phenotypically wild type animals (a description of these "clonal analysis" experiments will be presented in CHAPTER 3). For example, this approach has shown that PS integrins are required during wing morphogenesis as wing imaginal disc cells bearing mys mutations always produce adult wing blades displaying a "blister" phenotype (Brower and Jaffe, 1989; Zusman et al., 1990).

Scope of Research Presented in this Dissertation
With the knowledge that PS integrins are critically required for tissue morphogenesis during embryogenesis and metamorphosis, the obvious question arises as to what are the normal functions of integrins in each tissue. To address this question, it is important to define the specific morphogenetic events which are compromised when integrins are removed. For example, the "myospheroid" muscle phenotype appears to be a direct result of inadequate adhesion between muscle and tendon cells. This observation, when taken together with the immunolocalization of PS integrins to the cellular surfaces comprising muscle attachment sites, indicates that integrins normally function to mediate strong adhesion between muscle and tendon cells.

In contrast, the process of wing morphogenesis involves a dynamic series of events which begin at pupariation with eversion of the larval wing disc and culminate (over 100 hours later) with the realization of the adult wing blade. At the time I undertook my research project, PS integrins had been immunolocalized to larval wing discs, but there were no data to indicate whether pupal wing cells continue to express PS integrins throughout the long metamorphic period. Since it was not obvious which wing morphogenetic events might require PS integrin function, I performed experiments to determine directly when PS integrin function is required during wing morphogenesis, and these experiments are presented in CHAPTER 2. Importantly, this line of inquiry
promised to provide critical insight into the role of integrins in a tissue (the wing) which has become a favorite model for studying morphogenesis of adult tissues from larval imaginal discs. Indeed, my data indicate which specific wing morphogenetic events require PS integrin function, and these data set the stage for additional integrin research in this model tissue (as will be seen in subsequent chapters).

With a basic understanding of the normal functions of PS integrins during specific morphogenetic events, my research focus turned to determining the individual roles of PS1 and PS2 during each of these events. Prior to my work, the developmental requirements for PS integrins had been determined through characterization of phenotypes associated with mys null mutations. As discussed previously, these studies have shown that PS integrins are required for morphogenesis of numerous tissues during the embryonic and metamorphic developmental periods, but they do not distinguish between requirements specific for PS1 and PS2. Because PS1 and PS2 are expressed in complementary patterns on many of these tissues, it was not obvious what the specific requirements for PS1 and PS2 would be.

Since if encodes \( \alpha_{PS2} \) subunits, tissues bearing if null mutations would be unable to express PS2, while their expression of PS1 should be unaffected. To determine the functional requirements specific for PS2 during development, I created lethal mutations in the if gene and then
characterized if mutant phenotypes as presented in CHAPTER 3. Importantly, I use the data from these experiments to construct two alternative models concerning the significance of PS1 and PS2 expression patterns during specific morphogenetic events.

Although further analysis of mew (αPS1), if (αPS2) and mys (βPS) mutants will undoubtedly unveil additional Drosophila tissues which require PS integrins, this experimental approach typically provides data which are limited to determining the normal roles of integrins during fly development. This limitation arises from the nature of mutations which can be easily recovered: mutations generally reduce gene function in the cells where the gene product is normally expressed, but rarely cause ectopic expression of the gene product. To directly evaluate the functional capacity of different integrin structures in vivo, genetic manipulations which facilitate the expression of alternative integrin structures in specific tissues undergoing morphogenesis would be invaluable, and such manipulations would provide one way to analyze the relationship between integrin structure and function.

Indeed I have successfully constructed the first αPS subunit transgenes with which to evaluate the in vivo function of alternative PS integrin structures during specific morphogenetic events as discussed in CHAPTER 4.
Importantly, I performed experiments with these transgenes to investigate two related areas: first, I examined whether the normal patterns of PS1 and PS2 expression are necessary for muscle and wing morphogenesis; second, I examined whether alternative integrin structures can substitute for one another during specific morphogenetic events. In CHAPTER 4, my findings using these transgenes are presented, and I also present a general discussion of how one could exploit this experimental approach to investigate numerous aspects of the relationship between integrin structure and function.

It is noted that the data presented in CHAPTERS 2 & 3 have been published previously in a report authored by my advisor Danny Brower and me (Brabant and Brower, 1993).
INTRODUCTION:

The Genetics of Wing Morphogenesis

The morphogenesis of the Drosophila wing is a long and complex process. Of all the adult structures, wing blade defects are one of the most commonly observed mutant phenotypes. Indeed, it is clear from the literature that mutations in a great number of seemingly unrelated genes can disrupt wing morphogenesis. Presumably, the morphogenesis of highly specialized structures, such as an aerodynamic wing blade, entails the concerted action of a wide variety of gene products. The identification of mutations which affect wing morphogenesis has been relatively easy for two reasons: first, wing blades are large translucent external appendages, thus minute changes in morphology are readily detected; second, flies do not require wings to survive in the laboratory, thus mutations which result in wing defects are easily recovered. These circumstances have made wing morphogenesis an ideal model in which to study the metamorphic requirements for developmentally important genes.

Mutations which affect wing morphology are found in genes encoding proteins of two general classes: those which
directly participate in morphogenetic events and those which do not. A classic example of a gene which indirectly affects wing morphogenesis is *rudimentary* (*r*); this gene is properly designated a "housekeeping" gene as it encodes the first enzyme in the pyrimidine biosynthetic pathway (Lindsley and Grimm, 1992). Interestingly, the only obvious phenotype displayed by *r* mutants is the shortening and broadening of adult wing blades for which the locus was named. Apparently, pyrimidine auxotrophy creates a generally sick animal and this weakened state simply affects wing morphogenesis more dramatically than other morphogenetic events. Other genes which indirectly affect morphogenetic events appear to encode prototypic regulatory proteins, like the putative transcription factor encoded by *apterous* (Bourgouin et al., 1992), which presumably controls the expression of downstream effector proteins. Of particular significance to my research project, one of the clearest examples of downstream effector proteins are the PS integrins which appear to directly participate in cell adhesion events during wing morphogenesis.
Wing Morphogenesis Requires PS Integrins

As mentioned in the previous chapter, the first clue that PS integrins might have a role in wing morphogenesis came from the discovery of PS integrins on the surface of the wing precursor tissue, the wing imaginal discs. Imaginal discs are created during embryogenesis and consist of just a few dozen epithelial cells when larvae hatch from their vitelline membranes. For example, the two first instar larval wing discs consist of only about 35-40 cells each (Madhavan and Schneiderman, 1977). During the four day larval growth period which includes two molts of the larval cuticle, disc cells undergo many mitotic divisions, ultimately producing imaginal discs with thousands of undifferentiated cells. By the end of the larval period (the late third instar stage) each wing disc has grown into a relatively large infolding of approximately 50,000 cells as shown in Figure 2.1A (Garcia-Bellido and Merriam, 1971).

During the larval growth period, these monolayers of epithelial cells develop a regular pattern of folds which allow different types of discs to be identified by their shape. For example, Figure 2.1B shows the topological features (folds) displayed by mature wing discs. Moreover, specific areas within the monolayer of each disc have been shown to create specific parts of the adult tissue which
FIGURE 2.1 Location and shape of mature wing discs. (A) Dorsal view of a mature (late 3rd instar) larva showing position of the two wing discs within the body cavity. (B) Higher resolution drawing of one wing disc showing typical pattern of folds (lines) and density of cells; i.e., the thick columnar cells comprising the "wing pouch" of the disc, which will become the adult wing blade (see Figure 2.2B), are indicated with darker shading.
allows "fate-maps" to be drawn for each type of disc. For example, Figure 2.2 shows the fate-map locations of adult wing structures on a diagram of the mature wing disc.

Importantly, immunostaining experiments show that second and third instar wing disc cells express high levels of PS integrins in provocative patterns (Brower et al., 1985). Indeed, the observations that the two major PS integrin subtypes (PS1 and PS2) are expressed in patterns on wing discs, and that these patterns correlate with areas on the fate-map, provided the first indication that PS integrins might function during wing morphogenesis. Specifically, on mature third instar wing discs (just prior to the beginning of metamorphosis), PS1 and PS2 expression is largely restricted to the presumptive dorsal and ventral surfaces of the wing disc respectively (Figure 2.3). Although this pattern will be discussed extensively in subsequent chapters, the important observation for this chapter is that high levels of PS integrins are expressed on the disc cells which differentiate into the dorsal and ventral surfaces of the adult wing blade.

The phenotypes associated with several mutations in the genes encoding PS integrins also provided evidence that PS integrins play a role in wing morphogenesis. Animals carrying any one of several hypomorphic (partial loss of function) mutations in mys and if, the genes encoding $\beta_{PS}$
FIGURE 2.2 Wing disc fate-map (after Bryant, 1975; Wilcox et al., 1981; Brower et al., 1981). (A) Wing disc showing anterior/posterior and dorsal/ventral lineage restrictions. (B) Fate-map locations of adult structures derived from disc. (C) Dorsal view of these adult structures. Abbr.: TG, tegula; CS, costa; TR, triple-row bristles; DR, double-row bristles; PR, posterior-row hairs; AL, alar lobe; AC, auxiliary cord; VH, ventral hinge; DV, dorsal hinge.
FIGURE 2.3 Patterns of PS integrins on mature wing disc. The highest levels of PS integrins are found within the "wing pouch", the cells destined to make the adult wing blade. PS1 and PS2 are expressed on opposite sides of the dorso-ventral lineage restriction (Figure 2.2A). That is, PS1 is found on cells that will make dorsal structures, while PS2 is found on cells destined to make ventral structures. Figure based on immunolocalization data (see e.g., Brower et al., 1985).
and $\alpha_{PS2}$ respectively, may display wing defects, albeit these defects are often observed at a low frequency or penetrance (Lindsley and Grimm, 1992; Wilcox et al., 1989). The predominant wing phenotype associated with integrin mutants is usually described as wing "blisters", although other descriptions (such as "inflated" wings) have been used to describe the same defect. In any case, this phenotype appears to result from poor adhesion between the two epithelial surfaces (one dorsal and one ventral) which comprise the developing wing blade as these surfaces are abnormally separated in mutant adults (Figure 2.4). Along these lines, it should be noted that integrin hypomorphs sometimes display abnormally thickened wing veins; since wing veins are areas on the flat wing blade where the two epithelial surfaces are not closely apposed, this defect is probably another manifestation of compromised adhesion between wing surfaces during morphogenesis.

This requirement for PS integrins during wing morphogenesis has been carefully analyzed using clonal analysis of flies with $mys$ null mutations. As mentioned previously, the $mys$ locus encodes the $\beta_{PS}$ subunit common to all PS integrins so that null mutations in this locus completely eliminate the ability to express PS integrins. Since zygotes carrying homozygous $mys$ null mutations always die during embryogenesis, clonal analysis experiments were
FIGURE 2.4 Light micrograph of typical blistered wing from an \( if^3 \) mutant fly. Scale bar = 1 mm.
undertaken to make patches of *mys* mutant tissue in otherwise phenotypically wild-type adult animals. In this way, the effect of eliminating PS integrins from specific cells during wing morphogenesis was determined.

The mechanism through which clonal analysis produces homozygous patches of *mys* cells in wing tissue is conceptually simple: mitotic recombination events are induced at a low frequency in *mys* heterozygous (phenotypically wild type) first instar larvae resulting in the creation of an individual *mys* homozygous cell within the immature wing disc; then the mutant cell undergoes mitotic divisions normally (throughout the remaining larval period of growth) eventually producing a contiguous patch of (clonally derived) *mys* mutant tissue in the otherwise phenotypically wild type mature wing disc (Figure 2.5). Because a recessive marker mutation has been placed on the *mys* mutant chromosomes used in these experiments, *mys* mutant cells are easily identified within the adult wing by scoring the phenotype associated with the marker mutation.

The important finding from these types of experiments is that large patches of *mys* mutant tissue always result in a wing displaying the blister phenotype. Specifically, areas on either the dorsal or ventral surface of the wing blade that are derived from *mys* mutant cells always appear to have separated from the other surface of the wing blade. These
Clonal analysis of *mys* mutant wing tissue.

A] Proliferating wing disc epithelium with one cell (center) depicted in G2 phase of cell division:

B] Artificial induction of recombination between chromatids:

C] Mitosis and cytokinesis produce one mutant cell (shaded):

D] Subsequent cell divisions result in clonal expansion:

E] The wing hair phenotype (curly hairs) caused by the *forked* mutation (*f*) allows localization of the (*mys*) clone within the adult wing blade:
data unambiguously show that PS integrins are critically required on both surfaces of the developing wing blade.

The severity of these wing blister defects appears to be directly related to the number of cells mutant for mys. For example, very small patches of mys mutant cells (<50 cells) result in minor separations of the wing blade surfaces while larger clones (>100 cells) cause much more severe blistering (Brower and Jaffe, 1989; Zusman et al., 1990). Generally, blisters are found to encompass significantly more surface area on the wing blade than the area occupied by the associated clones of mutant cells. Indeed, blisters are usually round in shape while the associated mutant patches of cells are usually long and thin (i.e. clones typically expand along the proximal-distal axis of the developing wing).

These data indicate that a localized loss of adhesion caused by the absence of PS integrins causes an excessive strain on neighboring wild-type cells.

The immunolocalization of PS integrins on wing discs, the observation that blisters are associated with PS integrin hypomorphic mutations, and especially the mys clonal analysis data, when taken together, strongly indicate that PS integrins function to facilitate or maintain close apposition of the two wing surfaces during morphogenesis. Since clonal analysis experiments remove PS integrins from cells throughout metamorphosis, and since the presence of PS integrins had only been confirmed prior to metamorphosis, the
temporal requirement for PS integrins during wing morphogenesis had yet to be addressed at the time I undertook this study. This issue is particularly pertinent because wing morphogenesis is a very dynamic and relatively long process as described below.

**Description of Wing Morphogenetic Events**

The complex metamorphic process of wing morphogenesis, which occurs within the confines of the puparium, clearly requires the coordinated activities of numerous effector proteins including the PS integrins. Waddington (1941), while investigating the relationship between various genes and their role in wing morphogenesis, reported the first description of the dynamic series of morphogenetic events that begin about the time of puparium formation with the eversion of the *Drosophila* wing imaginal disc, and culminate 4-5 days later (at 25°C) with the realization of the adult wing structure. Subsequent to this original description, higher resolution descriptions have been made of various stages of wing morphogenesis *in vitro* and *in vivo* (e.g., Milner and Muir, 1987; Johnson and Milner, 1987), and very recently the immunolocalization of PS integrins during these stages has been reported (Fristrom et al., 1993).

The major stages of wing morphogenesis are diagrammed in Figure 2.6 and described as follows. Just after pupariation, disc evagination brings the basal surfaces of the dorsal and
ventral wing epithelia into contact resulting in the tight apposition of the PS integrin expressing cells (Figures 2.6A,B & C). About 11 hours later, these two epithelia begin to separate as the wing swells into a balloon-shaped structure, presumably driven by pressure within the hemolymph which occupies the space between wing surfaces (Figures 2.6D & E). From 13 to 40 hours, the dorsal and ventral surfaces are again becoming tightly associated, and during this process of apposition the forming "wing veins" become clearly visible. By 60 hours, the two epithelia have again come apart, although the separation is not nearly so great as during the initial swollen phase. This separation persists through eclosion (escape from the puparium), by which time the dorsal and ventral epithelial cells are rapidly degenerating.

The developing wing therefore undergoes two periods of apposition and separation before the two cuticular surfaces finally become permanently fused shortly after eclosion. During the later period of separation, the dorsal and ventral epithelia remain connected by cytoplasmic extensions, characterized by a basal junction-like structure that is associated with extensive "trans-alar microtubular arrays"
FIGURE 2.6  Diagrams of transverse sections of the wing at successive stages of morphogenesis (after Waddington, 1941; Fristrom et al., 1993). Times shown are the number of hours after puparium formation. Apposition I and II are periods when the two wing blade surfaces are establishing contact. Adhesion I and II are the times of maximal apposition prior to Separation I and Separation II respectively. Expansion I and II are periods when cell shape changes cause expansion of the wing's surface area.
and accumulations of actin filaments (Tucker et al., 1986; Mogensen and Tucker, 1987; 1988; Milner and Muir, 1987). Basal connections have also been reported to connect the two wing surfaces during the earlier, more extensive separation \textit{in vitro} (Milner and Muir, 1987), although studies of wings \textit{in vivo} suggest that most of these connections are broken during the early phase of maximum separation (Waddington, 1941; Fristrom et al., 1993).

\textit{Scheme to Determine Critical Period for PS Integrin Function}

In light of the morphogenetic complexity described above, it was not at all obvious at what time during metamorphosis PS integrin function would be required for wing morphogenesis. Typically, the temporal requirement for a gene product during a morphogenetic process can be determined with temperature shift experiments using temperature-sensitive alleles. For example, shifts from permissive to restrictive temperatures at various times during the morphogenetic event should determine the end of the critical period for that gene product; conversely shifts from restrictive to permissive temperatures should determine the beginning of the critical period. Since tight temperature-sensitive alleles for the PS integrin genes have not been recovered, I executed a novel approach (with conceptual parallels to the traditional temperature shift experiments)
to determine the critical period for PS integrin expression during wing morphogenesis as described below.

The basic scheme involves the use of an inducible integrin transgene to put PS integrins back into mutant animals which have severely reduced integrin function in the cells of their wing discs. Importantly, these constructs were found to supply sufficient levels of PS integrin to substantially rescue the mutant animal's blister phenotype. Furthermore, by regulating when transgenic PS integrin expression occurred the minimal developmental interval in which expression is required to rescue blisters was determined, and this interval provides a measure of the critical period for PS integrin function during wing morphogenesis.

RESULTS:

Transgenic Integrin Expression Rescues Wing Phenotype

More than four days are required for morphogenesis of an adult wing from the undifferentiated cells of a larval imaginal disc, and mutations that alter integrin function during this period often result in wing phenotypes. An easily scored wing blister phenotype is associated with the viable $if^3$ mutation, but the penetrance of this phenotype is low (Brower and Jaffe, 1989, Wilcox et al., 1989). The frequency of wing blisters increases dramatically when $if^3$ is
combined with a second viable integrin mutation, $mys^{nj42}$ (Wilcox et al., 1989). From genetic and molecular analysis of these two hypomorphic alleles, it appears that $mys^{nj42}$ produces a $\beta_{PS}$ with compromised function and $if^3$ is a regulatory mutation that leads to reduced amounts of $\alpha_{PS2}$ in wing tissue (Brower and Jaffe, 1989; Wilcox et al., 1989). Therefore, $mys^{nj42}$ $if^3$ double mutants have a dramatically reduced level of PS integrin function in their wing tissue, and this reduction results in blistered wings.

To determine when PS integrin function is required during wing morphogenesis, I employed an inducible integrin transgene to replace much of the missing PS integrin function in $mys^{nj42}$ $if^3$ animals. Specifically, this transgene uses a heterologous promoter (HSP70) to drive the expression of wild type $\beta_{PS}$ subunits encoded by a $mys^+$ cDNA (Figure 2.7). When this transgene is expressed in $mys^{nj42}$ $if^3$ animals, the level of PS integrin function should increase as wild type (transgenic) $\beta_{PS}$ subunits begin to be incorporated into PS integrins (instead of the endogenous $mys^{nj42}$ mutant subunits).

I found that the wing blister phenotype of the $mys^{nj42}$ $if^3$ animals is rescued when wild type $\beta_{PS}$ subunits are transgenically expressed. Importantly, the level of $\beta_{PS}$ subunit expression from this particular $mys^+$ transgene construct can be easily manipulated (in vivo) because the
**FIGURE 2.7** Diagram of a *mys* transgene. This transgene expresses wild type $\beta_{PS}$ subunits under the control of the heat inducible HSP70 promoter (Bunch and Brower, 1992).
heat shock promoter (HSP70) driving transcription is activated when culture temperatures are elevated. My first experiments with the transgene utilized a heat shock protocol which was designed to elevate $\beta_{PS}$ subunit expression continuously throughout metamorphosis (i.e. one 30 minute heat shock was given every 7 hours). This induction protocol, when given to $mys^{nj42} if^3$ mutants carrying one copy of the transgene, dramatically reduces the penetrance of the $mys^{nj42} if^3$ wing blister phenotype from greater than 95% to less than 50% of the wings scored. Since more frequent heat shocks were found to have a detrimental effect on viability, this protocol was used for all blister rescue experiments described below; therefore, the reduction in $mys^{nj42} if^3$ mutants wing blister penetrance obtained with this protocol, from greater than 95% to less than 50%, is used as a benchmark for "maximal blister rescue" in the following experiments.

**Determination of the Critical Period for Integrin Function**

To examine the beginning of the critical period for PS integrin function during wing morphogenesis, transgene expression using the induction protocol was initiated at various times during larval and pupal development (i.e. the period of time in which the wing imaginal discs grow and differentiate into the adult wing structure). For technical reasons involving the accurate staging of animal age, all
timepoints are given in number of days prior to "eclosion" (or emergence of the adult fly from the pupal case). Using this eclosion reference point, control experiments show that all animals undergo pupariation between 4-5 days prior to eclosion; it should be noted that at 25°C pupariation occurs almost exactly 4 days prior to eclosion, while animals given the induction protocol (one 30 minute heat shock every 7 hours) during development have a slightly lengthened pupal period such that pupariation occurs up to 5 days prior to eclosion. Two additional technical points are important for interpreting my rescue data: first, since eclosed animals (adult flies) were collected and their wings scored for blisters once every 24 hours, all timepoints are given in one day increments plus or minus 12 hours (e.g. a 4.5 days before eclosion timepoint is a collection of animals which were induced from 4 to 5 days before eclosion); second, since these experiments were repeated numerous times, standard errors are included on all data points (see materials and methods for complete technical details).

To determine the start of the critical period for transgene induction, mutant animals were grown under conditions where the transgene is uninduced (standard temperature of 25°C), and at various times during development, the animals were transferred into an incubator programmed to deliver a continuous cycle of heat shocks (one 30 minute 37°C heat shock every 7 hours). Importantly, maximal blister
rescue was only achieved when the cycle of heat shocks was initiated 5.5 or more days prior to eclosion (Figure 2.8A). In fact, when heat shocks were initiated just slightly later, at 4.5 days prior to eclosion, the amount of blister rescue was reduced significantly, and initiating the induction protocol any later during the pupal period (e.g. 3.5 days before eclosion) provided no detectable rescue. Even allowing for the slight increase in pupal development caused by the heat shocks, this result indicates that the critical period for PS integrin function during wing morphogenesis begins at about the time of puparium formation.

To examine the end of the critical period for PS integrin function during wing morphogenesis, transgene expression using the induction protocol was initiated early in development (about 8 days prior to eclosion) and terminated at various times during larval and pupal development. For maximal blister rescue, the cycle of heat shocks must be maintained through 1.5 days before eclosion, or through roughly 66% of the pupal period (Figure 2.8B). Since the transgenically produced PS integrins probably remain active for many hours after the last heat pulse, it seems probable that PS integrins may be required significantly beyond the 1.5 days before eclosion timepoint.
FIGURE 2.8 Temporal requirements for integrin expression during wing morphogenesis. Using a transgene, $\beta_{PS}$ was expressed from a heat shock promoter to rescue the blister phenotype of $mys^{nj42 \ if^3}$ animals. Transgene induction was maintained by heat shocking animals for 30 minutes at 37°C every 7 hours. To examine the early requirement for integrins (A), the frequency of wild type wings was scored as a function of when $\beta_{PS}$ transgene expression was initiated. To examine the late requirement for integrins (B), the frequency of wild type wings was scored as a function of when $\beta_{PS}$ transgene expression was terminated. Control animals are $mys^{nj42 \ if^3}$ without the $\beta_{PS}$ transgene. The developmental timepoint when $\beta_{PS}$ transgene induction was initiated or terminated is expressed in days prior to eclosion of the $mys^{nj42 \ if^3}$ adults. Newly eclosed adults were collected daily, so each timepoint is ±12 hours. The indicated overlap in larval and pupal development reflects the fact that animals heat-shocked more times take longer to develop, as well as slight variation in the population generally. Error bars indicate the standard error. □, experimental (with integrin transgene); ○, control (no transgene).
A

INTEGRIN EXPRESSION INITIATED (days relative to eclosion)

B

INTEGRIN EXPRESSION TERMINATED (days relative to eclosion)
When both rescue experiments are considered together (Figures 2.8A & B), the data indicate that the critical period for PS integrin function begins very near the onset of pupariation and continues through most, if not all, of the pupal period (i.e., throughout most of the metamorphosis).

Curiously, cycles of heat shocks also resulted in a slight reduction in the frequency of blistered wings in control animals, without the mys+ transgene (Figure 2.8). This effect displayed a time dependence similar to that seen for rescue in experimental animals. One intriguing potential explanation for this phenomenon is that heat shock proteins may help to stabilize the mutant mys\textsuperscript{nj42} protein, either before or after its association with an αPS subunit to form a mature integrin (e.g. see Gething and Sambrook, 1992). Alternatively, the slightly increased developmental time caused by repetitive heat shocks presumably allows longer periods of time for cell-cell interactions, if so this might allow more connections to be created between the surfaces. Whatever the specific mechanism, these data also define the same critical period for integrin function during wing morphogenesis.
DISCUSSION:

Temporal Requirement for PS Integrins During Wing Morphogenesis

In light of the complex morphogenetic events which make up the process of wing morphogenesis, it was not at all obvious at what time during metamorphosis PS integrin function would be required for wing morphogenesis when this study began. I examined the temporal requirement for PS integrins by rescuing the blister phenotype of double mutants bearing hypomorphic alleles at the if and mys loci, using a heat shock inducible mys\textsuperscript{+} transgene. The if\textsuperscript{3} mutation causes wing blisters at a low penetrance, usually below 25\%, but this frequency is increased to greater than 95\% when combined with the mys\textsuperscript{nj42} mutation (Wilcox et al., 1989; and my observations). Periodic induction of the mys\textsuperscript{+} transgene throughout metamorphosis typically reduced the penetrance of this blister phenotype to below 50\%. By beginning or ending the heat shocks at different times, I asked which morphogenetic events require integrin function.

Maximal rescue of the blister phenotype of mys\textsuperscript{nj42} if\textsuperscript{3} animals requires induction of β\textsubscript{PS} subunit expression throughout most or all of the metamorphic period. Specifically, maximal rescue required that β\textsubscript{PS} be induced earlier than 4.5 days prior to eclosion, at or soon after wing disc evagination. Furthermore, the latest time that β\textsubscript{PS}
protein expression could be halted, and still give maximal rescue, was 1.5 days prior to eclosion. Of course, integrin may be required later than this time, since the protein products of the transgene are likely to function significantly longer than the period of the heat pulses. In this regard, I find that transgenically expressed PS2 integrin (from a similar heat shock-αPS2 transformant) ectopically expressed in the dorsal wing disc is still detectable (albeit at greatly reduced level) 6 hours after heat shock (as discussed in CHAPTER 4), and integrins that are incorporated into functional adhesion sites may be much more stable. Finally, it should be noted that after these data were published (Brabant and Brower, 1993), a subsequent report has confirmed the finding that PS integrins are required in wings throughout most of the metamorphic period (Zusman et al., 1993).

Thus, PS integrins are required from just after disc evagination through the beginning of the second period of epithelial separation, and may be functioning up to or even after eclosion. One question raised by this finding concerns the role of PS integrins during the initial period of separation, at 12-20 hours post-pupariation. Both Waddington (1941) and Fristrom et al. (1993) report that most or all of the basal connections between the dorsal and ventral epithelia are broken at this stage. These observations imply
that some integrin-dependent interaction is required before the separation, in order to reform the connections later and prevent the formation of a wing blister. If true, then PS integrins probably have functions in addition to simply mediating adhesion during the early apposition phase. For example, PS integrins might transmit signals to cells during the first period of apposition which make the cellular surfaces competent to reform connections during the later period of cell-cell apposition. Alternatively, some connections may be maintained even during maximum separation, but the extended (and presumably fragile) processes may not be preserved by fixation during this stage. In any event, it appears that if the integrin-mediated contacts between the two wing surfaces are broken during early metamorphosis, they can not be repaired by subsequent integrin expression.
myospheroid Mutants Reveal Requirements for PS Integrins

Through the characterization of phenotypes associated with mys mutations, a set of developmental events which require integrin function has been defined. The nature of integrin mutant phenotypes strongly suggests that PS integrins are involved in cell adhesion events. In most cases, these developmental defects appear to be the direct result of a localized loss of integrins (as opposed to a secondary effect), indicating that PS integrins are most likely functioning as cell adhesion molecules.

The early developmental requirements for PS integrins have been determined by analyzing the myospheroid (mys) lethal phenotype (Wright, 1960; Newman and Wright, 1981). Since mys encodes the $\beta_{PS}$ subunit common to both PS1 and PS2, zygotes bearing mys null mutations lack the ability to (zygotically) synthesize PS integrins. mys embryos die late in embryogenesis and display a number of characteristic defects. Specifically, morphogenesis of both the somatic and visceral musculature is disrupted in mys embryos. The multinucleate somatic muscles appear to form normally, but
upon contraction, they pull away from the body wall, rounding up into the myospheroid bodies for which the gene was named. The visceral musculature is also defective as it fails to condense around the midgut endothelium properly, so that the gut never forms into the long slender tube characteristic of wild type animals. In addition, the dorsal hypoderm suture, which is created when the lateral hypoderm extends dorsally to enclose the amnioserosa and internal tissues, ruptures in mys embryos producing a hole in the hypodermis through which some internal tissues herniate.

Later in development, PS integrins are again critically required for the morphogenesis of adult tissues. Clonal analysis experiments have shown that PS integrins are required for proper morphogenesis of adult eye and wing structures. As discussed in the previous chapter, patches of mys cells on either the dorsal or ventral surface of the developing wing blade result in localized wing blisters (Brower and Jaffe, 1989; Zusman et al., 1990). Likewise, mys clones in eye discs have been shown to result in photoreceptors which are disorganized and detached from their basement membrane (Zusman et al., 1990).

Both the imaginal discs and the mutant embryonic tissues normally express high levels of PS integrins. For example, immunolocalization studies detect exceptionally high levels of integrin expression at the sites of somatic muscle attachment to the body wall and also at the interface of gut
endoderm and mesoderm (Bogaert et al., 1987). Therefore, the 
mys embryonic gut and muscle phenotypes, like the adult eye
and wing phenotypes, are thought to be directly caused by the
localized loss of PS integrin expression in these tissues.

Since mys null mutations eliminate expression of all PS
integrins, these studies do not distinguish which mys mutant
defects might reflect a specific requirement for PS1 or PS2.
This is a particularly interesting issue since both integrins
are found on imaginal discs, on gut cells and at the sites of
muscle attachment to the body wall. Indeed, these tissues
express PS1 and PS2 in distinct patterns indicating that each
integrin may play different roles during development.

Inflated Mutant Analyses Reveal Requirements Specific for PS2

At the time I undertook this study, the inflated (if)
gene had recently been shown to encode the \( \alpha_{PS2} \) protein
(Wilcox et al., 1989), and it appeared that genetic studies
of this gene would probably reveal the requirements specific
for PS2. Specifically, if null mutants (by definition) would
be incapable of expressing PS2 integrins, but their
expression of PS1 should be unaffected. Previously, three
viable, partial loss of function (hypomorphic) alleles of if
were reported to exist (Lindsley and Zimm, 1992). Although
if\(^1\) and if\(^2\) have been lost, the if\(^3\) allele is still available
and has been examined in some detail. Of particular
interest, this mutation causes wing blisters at low frequency
(penetrance), and examination of \( \alpha_{PS2} \) expression indicates that \( if^3 \) probably represents a regulatory mutation (Brower and Jaffe, 1989; Wilcox et al., 1989). The phenotype of the single reported lethal \( if \) allele \( (if^{k27e}) \) has not been reported in detail, however, the lethal embryos have been described in passing as showing muscle and/or gut defects (Wilcox et al., 1989; Wilcox, 1990).

Here, I report the creation and characterization of two additional \( if \) lethal mutations and show that all three lethal alleles appear to represent a single "null for function" class of \( if \) mutations. Using these lethal \( if \) alleles, an analysis of the embryonic requirements specific for the PS2 integrin is presented. Using clonal analysis of animals with lethal \( if \) alleles, I also analyze the spatial requirements for PS2 integrin function during wing morphogenesis.

RESULTS:

Characterization of inflated Lethal Alleles

To create additional strong \( if \) alleles, I screened over 18,000 EMS-treated chromosomes for failure to complement the wing blister phenotype of \( if^3 \) (Figure 3.1), and recovered two
Select F1 females displaying wing blisters.

FIGURE 3.1 F1 screen to recover new strong alleles of inflated (if). Mutagenized chromosomes that fail to complement the if$^3$ allele are selected for analysis.
new lethal alleles ($iA^7$ and $iB^2$). These two mutations were determined to be alleles of $if$ by the following criteria. As expected from the nature of the screen, both alleles increase the penetrance of the wing blister phenotype when transheterozygous with $if^3$. That is, blisters are displayed at a higher frequency by $if^{lethal}/if^3$ transheterozygotes (where $if^{lethal}$ is $iA^7$, $iB^2$ or $iK^{27e}$) than by $if^3/if^3$ homozygotes. Additionally, transheterozygous combinations of the new alleles with each other or with $if^{k27e}$ are 100% lethal (e.g. $iA^7/iK^{27e}$), and in recombination experiments both map genetically very close (within 1 map unit) to the $if$ locus (located at 55.7 map units on the X chromosome).

A third genetic complementation test was also performed with the antimorphic $mys$ allele, $mys^{XR04}$. The "antimorphic" designation is given to alleles which appear stronger (e.g. in genetic tests) than the "amorphic" (null) class of alleles. In the case of $mys^{XR04}$, the antimorphic classification was partly based on the observation that $mys^{XR04}$ fails to complement $if^{k27e}$, in spite of the fact that $mys^{null}$ alleles complement $if^{k27e}$ (Wilcox, 1990; Bunch et al., 1992). That is, $mys^{null} +/- if^{k27e}$ zygotes develop into viable adults, while $mys^{XR04} +/- if^{k27e}$ zygotes almost always die prior to eclosion. I find that the new lethal $if$ alleles also fail to complement the antimorphic $mys^{XR04}$ allele as
zygotes with the $\text{mys}^{XR04}$ if\textit{lethal} double heterozygous genotype ($\text{mys}^{XR04} +/- \text{if\textit{lethal}}$) almost always die.

I had hoped that comparisons of the penetrance of the wing blister phenotype for $\text{if\textit{lethal}/if\textit{3}}$, or of the numbers of viable escapers among the $\text{mys}^{XR04} +/- \text{if\textit{lethal}}$ animals, would indicate whether certain alleles were stronger than others. However, these tests seemed to be quite sensitive to genetic background or minor variations in culture conditions, and I saw considerable variation in the quantitative results from experiment to experiment. Overall, none of the three lethal alleles was consistently stronger or weaker than the others. For the record, the penetrance of the $\text{if\textit{lethal}/if\textit{3}}$ wing blister phenotype ranged from 16-46%, and the frequency of viable $\text{mys}^{XR04} +/- \text{if\textit{lethal}}$ escapers varied from 0-14% of the expected number. For the latter test, two slightly different genetic approaches, which require alternative chromosomal duplications to be carried by parental males, were taken to create the $\text{mys}^{XR04} +/- \text{if\textit{lethal}}$ genotype (Figure 3.2).

Unexpectedly, $\text{mys}^{XR04} +/- \text{if\textit{lethal}}$ "escapers" (animals which survived to adulthood) were always found at a rate of at least 1% when the male parents were $\text{if\textit{lethal}; Dp(1,4)r}^+/-$, but were not observed in the one experiment in which the male parents were $\text{mys}^{XR04}; \text{Dp(1,2)sn}^+72d/+$. Whether this difference is related to some specific property of the
FIGURE 3.2 Alternative crosses used to create the mys\textsuperscript{XR04} +/- if\textsuperscript{lethal} genotype (mys\textsuperscript{XR04} if\textsuperscript{lethal} double heterozygotes). A) Parental mys\textsuperscript{XR04} males bear Dp(1,2)\textsuperscript{sn}72d-- this duplication contains the mys\textsuperscript{+} locus. B) Parental if\textsuperscript{lethal} males bear Dp(1,4)r\textsuperscript{+}-- this duplication contains the if\textsuperscript{+} locus.
duplications or to some other nonspecific genetic effect is unknown.

To compare the amount of $\alpha_{PS2}$ protein produced by the three lethal if mutations, I examined immunoblots from male embryos hemizygous for each allele with an anti-$\alpha_{PS2}$ monoclonal antibody, PS2hc/1 (Figure 3.3). The levels of $\alpha_{PS2}$ protein appear significantly reduced in $if^A7$ and $if^B2$ mutant embryos, and scanning densitometry of $\alpha_{PS2}$ and background control bands indicates that the amount of $\alpha_{PS2}$ is reduced about three-fold in $if^A7$ mutants and about nine-fold in $if^B2$ mutants relative to wild type. As previously reported by Wilcox et al. (1989), I also find that $\alpha_{PS2}$ protein is not detected in lysates of $if^{k27e}$ mutant embryos, and based on lanes with reduced loadings of wild type lysates, the sensitivity of my immunoblot experiments should allow detection of $\alpha_{PS2}$ protein levels 15 fold lower than wild type levels. Thus, if $if^{k27e}$ mutant embryos synthesize any $\alpha_{PS2}$ protein, the steady state level must be very low or the protein's antigenicity must be changed such that it is not recognized by the antibody. In this respect, it should be noted that immunostaining data using a different monoclonal antibody also indicate that $if^{k27e}$ embryos do not have detectable accumulations of PS2 integrin at muscle attachment sites (Hirano et al., 1991).
FIGURE 3.3 Immunoblot of wild type and if mutant embryo lysates probed with αPS2-specific monoclonal antibody. Lysates from 15 mutant or 5 wild-type embryos were loaded in the indicated lanes. Precursor αPS2 is normally proteolytically processed into a light chain and a heavy chain (Bogaert et al., 1987). Unprocessed αPS2 ($M_r = 160 \times 10^3$) and processed heavy chain $\alpha_{PS2}$ ($M_r = 140 \times 10^3$) are indicated. Scanning densitometry indicates that accumulation of $\alpha_{PS2}$ in $if^A7$ and $if^B2$ is reduced about three-fold and nine-fold, respectively, relative to wild type.
Description of the inflated Lethal Phenotype

In addition to their similarity in the complementation tests described above, I find that if\(^{A7}\), if\(^{B2}\) and if\(^{k27e}\) hemizygotes have similar embryonic phenotypes. The if mutants die as late embryos, and display somatic muscle and gut defects similar to those previously reported for mys null mutant embryos, which lack the PS integrin \(\beta\) subunit (\(\beta_{PS}\)) (Wright, 1960; Newman and Wright, 1981). Comparison of staged mys and if mutant embryos shows that these gut and muscle defects occur at the same time and to similar degrees in both classes of integrin mutants.

In stage 16 embryos (stages assigned as in Campos-Ortega and Hartenstein, 1985) the somatic musculature begins to undergo regular contractions (Poulson, 1950). In all three if mutant embryos, the somatic muscles begin to pull away from their attachment sites concomitant with the onset of these contractions (Figures 3.4A & B), and after several hours most, if not all, somatic muscles have collapsed into spheroidal masses of sarcomeric (and birefringent) material (Figures 3.4C & D). Not surprisingly, if mutant embryos display fewer and less dramatic body movements than wild type embryos.

During stages 15-16 in wild type embryos, the midgut develops into the slender, elongate tube characteristic of the first instar larva (Poulson, 1950). As in mys mutants,
**FIGURE 3.4** Light micrographs of wild type and \( if^{B2} \) mutant embryos. Embryonic stages according to Campos-Ortega and Hartenstein (1985). Anterior is left and dorsal up for all embryos. (A) and (B) Polarized light images of early stage 17 wild type (A) and \( if^{B2} \) (B) embryos. In these images, the birefringent somatic muscles appear black or white, depending on their orientation. In the mutant (B), the somatic muscles (arrows) have started to pull away from their attachment sites. These collapsing muscles are shorter than the wild type muscles shown in (A). (C) and (D) Polarized light images of a late stage 17 wild type embryo and an \( if^{B2} \) mutant embryo, respectively. Contrast the wild type musculature with the scattered spheroidal masses of muscle (arrows) in the \( if^{B2} \) mutant. (E) and (F) Nomarski DIC images of the same embryos shown in (C) and (D). Contrast the small diameter of the optical cross sections of the wild type midgut (mg) with the large yolk-filled midgut of the \( if \) mutant. Also note that the ventral nerve cord (nc) in the mutant has condensed less completely than the ventral nerve cord in the wild type embryo. Scale bar = 100\( \mu \)M.
the midgut in all three if mutant embryos fails to undergo this transformation into a tubular structure and remains as a large spherical tissue with a yolk-filled lumen (Figures 3.4E & F). At 22 hours of embryonic development (when wild type embryos hatch), an abnormally large amount of yolk remains in the mutants, and this excess yolk allows easy identification of if mutant embryos. These yolk proteins are slowly absorbed by the mutant embryos until the lumen of the midgut appears hollow by 28 hours.

It has been reported that the ventral nerve cord of mys embryos does not condense properly, and remains extended into the posterior abdominal segments (Wright, 1960; Newman and Wright, 1981). I find that the ventral nerve cord of if mutant embryos also fails to shorten completely (Figures 3.4E & F), although the precise degree of condensation appears variable in my preparations of both mys and if embryos.

During stages 13-15 of normal embryogenesis, the lateral hypoderm extends dorsally to enclose the amnioserosa and internal tissues (Campos-Ortega and Hartenstein, 1985). In mys embryos, the suture along the dorsal midline subsequently ruptures, resulting in herniation of neural and visceral tissue (Wright, 1960). I never observed herniation of tissue through the dorsal suture in any of the if mutant embryos; this was the single major difference observed between the mys and if lethal phenotypes.
Spatial Requirements for PS2 Function in Wings

Immunofluorescence of third instar wing imaginal discs shows that the expression of PS2 integrin is spatially restricted to cells comprising the presumptive ventral surface of the wing blade (Brower et al., 1984; 1985). As discussed in CHAPTER 2, PS integrin function is required long after (about 3 days) the last developmental stage (late third instar) in which this pattern has been observed. It should be noted that technical limitations (e.g. our anti-PS1 and anti-PS2 monoclonal antibodies do not react with integrins from fixed tissue sections) have prevented the immunolocalization of PS1 and PS2 integrins in wing tissue after pupariation. To examine this issue from a different perspective, I performed clonal analysis using the lethal \textit{ifB2} mutation to determine whether there is a restricted spatial requirement for PS2 integrin function in wing tissue during metamorphosis. Patches of \textit{ifB2} mutant tissue were located to the dorsal or ventral surface of the wing blade using the tightly linked wing hair marker \textit{f36a}.

Large \textit{ifB2} mutant patches located on the ventral surface of the wing blade were always associated with wing blisters (Figure 3.5A; Table 3.1), analogous to those previously described for \textit{mys} mutant patches lacking the PS integrin $\beta$ subunit (Brower and Jaffe, 1989; Zusman et al., 1990). Like
FIGURE 3.5 Light micrographs of wings with large $if^{B2}$ homozygous clones, indicated by the dotted outlines. (A) A typical wing with a ventral $if^{B2}$ mutant clone, which causes a large blister. (B) A typical wing with a large dorsal $if^{B2}$ clone. No morphological defects are observed associated with the mutant clone. Scale bar = 1 mm.
### TABLE 3.1 Phenotype of inflated Mutant Patches in Wings

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<th>$if^{B2} f^{36a}$ clones</th>
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290 wings were scored for $if^{B2} f^{36a}$ clones, 79 wings for control $f^{36a}$ clones.
the blisters caused by loss of $\beta_{PS}$ subunit, wing blisters caused by loss of $\alpha_{PS2}$ subunit were found to be generally larger and rounder than the associated $if^{B2}$ mutant patch. Furthermore, these wing blister defects did not affect differentiation of specialized wing structures such as wing margins, trichomes, bristles or thickened cuticle of veins. In contrast to results with $mys$ mutant patches, $if^{B2}$ mutant patches located on the dorsal surface of the wing blade were never associated with blisters (Figure 3.5B; Table 3.1). Thus the requirement for $\alpha_{PS2}$ protein function is spatially restricted to cells that make the ventral surface of the wing blade.

DISCUSSION:

Genetic studies with $mys$ mutants have shown PS integrins to be critically required for the morphogenesis of several larval and adult tissues. Since $mys$ mutants are unable to synthesize $\beta_{PS}$ subunits (and thus lack both PS1 and PS2), it is unclear whether $mys$-dependent morphogenetic events require both PS1 and PS2 or just one integrin subtype. This question is especially pertinent for processes such as muscle attachment, where PS1 and PS2 are expressed in discrete but closely associated spatial domains. In order to address these issues, I have generated and characterized new strong
alleles of the \textit{if} gene (which encodes the $\alpha_{PS2}$ integrin subunit). I have described the \textit{if} embryonic lethal phenotype, and using clonal analysis, examined the spatial requirements for the gene product during wing morphogenesis.

\textit{Generation and Characterization of New inflated Alleles}

Prior to this work, there were only two extant \textit{if} alleles, \textit{if$^3$} and \textit{if$^{k27e}$}. \textit{if$^3$} is a viable allele, and appears to be a regulatory mutation that alters expression in the wing and eye (Brower and Jaffe, 1989; Wilcox et al., 1989; Danny Brower, unpublished observations). The only obvious \textit{if$^3$} phenotype is the occurrence of wing blisters with a penetrance of 25\% or less (Brower and Jaffe, 1989; Wilcox et al., 1989). \textit{if$^{k27e}$} is a recessive lethal allele, and lysates from \textit{if$^{k27e}$} embryos contain no detectable $\alpha_{PS2}$ protein as determined by immunoblot experiments (Wilcox et al., 1989; and Figure 3.3). Using EMS as a mutagen, I created two more recessive lethal alleles, \textit{if$^{A7}$} and \textit{if$^{B2}$}. Both of these new alleles lead to synthesis of processed $\alpha_{PS2}$ heavy chains, judging by the location of immunopositive bands on western blots (Figure 3.3). Previous work has suggested that proper cleavage of the $\alpha_{PS2}$ precursor into heavy and light chains requires an association, or at least the presence, of $\beta_{PS}$ subunits (Leptin et al., 1989), and it is therefore important to ask whether the protein seen in the mutants is functional.
I find that \( if^{A7} \), \( if^{B2} \) and \( if^{k27e} \) mutant embryos display similar lethal phenotypes, and with the exception of dorsal herniation, this phenotype is similar to that observed for null mutations in \( mys \). Also, the three lethal alleles show similar properties in two genetic tests for gene function; when transheterozygous with \( if^3 \), all increase the penetrance of the wing blister phenotype, and all are typically lethal as double heterozygotes with \( mys^{XR04} \). Although the penetrance of the wing blister phenotype and the frequency of escapers in the \( mys^{XR04} \) test are variable, none of the alleles appears to be consistently stronger or weaker in these genetic tests. Finally, the immunological data for \( if^{k27e} \) indicate that this mutation fails to synthesize detectable amounts of \( \alpha_{PS2} \) (Wilcox et al., 1989; Hirano et al., 1991; and Figure 3.3). Taken together, these observations suggest that \( if^{A7} \) and \( if^{B2} \) retain very little if any wild type \( \alpha_{PS2} \) function.

The inflated Embryonic Phenotype

Using the original \( mys^1 \) mutation, which results from a deletion and makes no intact \( \beta_{PS} \) protein (Bunch et al., 1992), the \( mys \) lethal phenotype has been described in great detail (Wright, 1960; Newman and Wright, 1981). Prominent features of the phenotype that have been confirmed for other strong alleles (e.g., Leptin et al., 1989; and my observations)
include a failure of somatic muscle attachments, incomplete
gut morphogenesis and dorsal herniation of viscera. In my
analysis, I have concentrated on comparisons between mys
lethal embryos, which fail to express any PS integrins, and
if embryos, which should be missing only PS2 integrins.

In wild-type embryos undergoing organogenesis, PS1
integrin is expressed in tissues from all three germ layers,
with PS2 integrin found mainly in the mesodermal derivatives
(Bogaert et al., 1987; Leptin et al., 1989). Especially high
levels of PS integrin expression are seen at muscle
attachment sites, where the PS1 and PS2 integrins are seen in
tendon and muscle cells, respectively. At the onset of
muscular contractions in mys mutant embryos, the somatic
muscles begin to pull away from their tendon cell
attachments, which results in the contraction of most somatic
muscles into spheroidal bodies (Wright, 1960). PS2 integrins
are also expressed in the embryonic visceral musculature that
encompasses the PS1-expressing gut epithelium (Bogaert et
al., 1987; Leptin et al., 1989). In mys mutants, the gut
does not form its characteristic pattern of constrictions,
and the association between the musculature and the gut
epithelium does not form properly, leading to muscle clumps
and some regions that are devoid of visceral musculature
The *if* lethal phenotype includes failure of somatic muscle attachments and incomplete gut morphogenesis similar to the previously described phenotype for *mys* mutants. Indeed, when staged *if* and *mys* mutant embryos were compared, I could detect no differences in the expressivity of these muscle and gut phenotypes. Of course, this result does not exclude a function for other PS integrins in these processes. For example, based on the nonoverlapping expression patterns of PS1 and PS2 integrins at somatic muscle attachment sites, the simplest prediction is that loss of either integrin would lead to a "myospheroid" phenotype. This issue will be discussed in the next chapter as several groups have very recently isolated the first known mutations in the *multiple edematous wings* locus (*mew*) which encodes the $\alpha_{PS1}$ subunit (Brower et al., 1995).

Dorsal closure is normally accomplished during stages 13-15, prior to the first muscle contractions (Campos-Ortega and Hartenstein, 1985). In *mys* embryos, the dorsal suture in the hypoderm ruptures, leading to herniation of internal tissues (Wright, 1960), however, I never observed dorsal herniation in *if* mutant embryos. No specific accumulations of PS integrin have been noted in the region of the suture, and it is unclear whether PS integrins are directly involved in suture integrity, or if herniation results instead from some indirect effect of integrin loss. In any case, it appears that PS2 integrin is not required.
In principle, the failure to observe dorsal herniation in if embryos could also result from residual if+ function in the mutant embryos, if the three alleles examined are all hypomorphs, and if herniation is rescued especially easily by small amounts of PS2 integrin function. However, the herniation phenotype does not appear to be particularly insensitive to loss of integrins. For example, dorsal herniation is commonly observed in mysXN101 embryos, and this hypomorphic allele, while lethal, provides enough integrin function in muscles to generate significant internal movements, relative to those seen in null mys or if mutants (Bunch et al., 1992; and my observations). Alternatively, one could argue that the three lethal if alleles all specifically affect integrin expression or function in muscles and wings but not in the process required for maintaining dorsal closure, but it is highly unlikely that all of the three independently isolated alleles would have this property. Finally, it should be noted that the major features of the if phenotype, including a lack of dorsal herniation, have been confirmed in another study using an allele that is known to disrupt the if coding region (Brown, 1994).

One interpretation of my results, that dorsal herniation in mys embryos results from a loss of PS1 integrin function, is not supported by the recent characterization of the mew embryonic phenotype (Brower et al., 1995). Specifically,
neither mew mutant embryos nor if mew double mutant embryos display the dorsal herniation phenotype. Therefore, it appears that dorsal herniation in mys embryos results from loss of another (as yet uncharacterized) PS integrin. Along these lines, the identification of a third PS integrin α subunit was recently reported (Stark et al., 1994), and the dorsal herniation may result exclusively or primarily from a loss of this newly recognized αPS3βPS integrin. The final test of this prediction awaits the characterization of mutations which eliminate αPS3βPS function.

The inflated Wing Phenotype

Clonal analysis experiments using null mys alleles have shown that PS integrins are required in the epithelial cells that secrete both the ventral and dorsal sheets of cuticle that comprise the adult wing blade (Brower and Jaffe, 1989; Zusman et al., 1990). Specifically, clonally-derived patches of mys mutant cells on either wing surface are associated with blisters, indicating that PS integrins are directly required for tight apposition of the dorsal and ventral surfaces of the wing blade. In late third larval instar wing imaginal discs, just prior to puparium formation and disc eversion, PS1 is expressed primarily on the epithelial cells that will secrete the dorsal cuticle of the wing blade, while PS2 is expressed almost exclusively on the presumptive ventral cells (Brower et al., 1984; 1985). The recent
immunolocalization studies of Fristrom et al. (1993) indicate that integrins remain associated with regions of contact between the epithelia during metamorphosis, and are highly localized in the basal junctions during times of dorso-ventral epithelial separation. To date, there have been no localization studies performed in the pupa that would distinguish PS1 and PS2, so it is unknown whether the spatially restricted larval expression is conserved during metamorphosis. However, by examining wing clones mutant for if, I find that $\alpha_{PS2}$ is required for normal wing development in ventral, but not dorsal, wing cells. This functional restriction, combined with the temporal requirement for PS integrins discussed in the previous chapter, indicates that the dorso-ventral specificity of integrin expression observed in larvae is retained during at least the early period of wing morphogenesis.
CHAPTER 4
FUNCTION OF ALTERNATIVE PS INTEGRIN STRUCTURES IN VIVO

INTRODUCTION:

In CHAPTERS 2 and 3, data were presented which indicate that PS integrins are required for the morphogenesis of a variety of Drosophila tissues. In general, PS integrins appear to function as adhesive molecules which facilitate the close apposition of distinct sets of cells. The role of integrins in cell adhesion is exemplified most clearly during embryogenesis at the sites of somatic muscle attachment to epidermal tendon cells, and again during metamorphosis at the interface of the dorsal and ventral cellular surfaces of the maturing wing blade.

Like most integrins, PS integrins are thought to facilitate cell-cell adhesion by virtue of their ability to bind specific components of the extracellular matrix. Since differences in integrin subunit structure generally result in receptors possessing unique ligand binding specificities in vitro, each PS integrin structure is likely to function somewhat differently in vivo. In this chapter, I analyze the relationship between PS integrin structure and function in vivo by determining whether alternative PS integrin structures have the capacity to mediate specific cell-cell
adhesion events which occur during embryogenesis and metamorphosis.

**PS1 and PS2 Are the Two Major Classes of PS Integrins**

Similar to the integrins found in vertebrates, PS integrins comprise a related set of heterodimeric receptors created through the differential pairing of unique $\alpha$ and $\beta$ subunit structures. The PS1 and PS2 integrins, formed by pairing $\beta_5$ with either $\alpha_{PS1}$ or $\alpha_{PS2}$ respectively, represent the two major classes of PS integrins. The amino acid sequences of the $\alpha_{PS1}$ and $\alpha_{PS2}$ subunits have diverged significantly (<30% sequence identity) suggesting PS1 and PS2 have evolved to satisfy unique biological roles (Wehrli et al., 1993). Indeed, this hypothesis is supported by sequence comparisons between vertebrate $\alpha$ subunits and Drosophila $\alpha_{PS}$ subunits which strongly suggest that the genes encoding $\alpha_{PS1}$ and $\alpha_{PS2}$ subunits ($mew$ and $if$ respectively) had diverged prior to the separation of the protostome and deuterostome lineages (Wehrli et al., 1993; Brown, 1993).

Intriguingly, PS1 and PS2 are expressed on opposing cellular surfaces during several integrin dependent cell-cell adhesion events suggesting that these receptors may function cooperatively. As discussed in CHAPTER 3, embryos express PS1 on tendon cells which adhere to PS2 expressing muscle cells; similarly when wing imaginal discs undergo eversion, PS1 expressing larval disc cells destined to make the dorsal
wing surface become attached to ventral cells expressing PS2 (see CHAPTER 2). In addition, an analogous PS1/PS2 expression pattern is observed in the embryonic midgut tissue where PS1 expressing endodermal cells adhere to the surrounding visceral musculature which expresses PS2 (Bogaert et al., 1987; Leptin et al., 1989). Although the functional significance of placing PS1 and PS2 on opposing sides of cell-cell junctions is unclear, the fact that this restricted distribution is observed in at least three independent tissues indicates these patterns may be important for facilitating cell-cell adhesion.

**Variations in \( \alpha_{PS2} \) Subunit Structure Result in Two PS2 Integrin Subtypes: PS2m8 and PS2c**

In addition to the major structural differences between PS1 and PS2 integrins, alternative splicing of the \( if \) primary transcript results in two slightly different \( \alpha_{PS2} \) subunit structures. Specifically, the \( if \) gene contains a 75 base pair exon which is either spliced out of the mRNA producing \( \alpha_{PS2m8} \) subunits (m8 for "missing exon 8") or retained within mRNA producing \( \alpha_{PS2c} \) subunits (c for "canonical", or perhaps "complete" would be a more descriptive designation) (Brown et al., 1989). Therefore, PS2m8 and PS2c integrins are identical except that an additional 25 amino acids are found in the extracellular domain of PS2c. This 25 amino acid sequence is situated within the putative ligand binding
domain indicating that PS2m8 and PS2c integrins probably differ in their affinity for ligands (Brown et al., 1989). This hypothesis is supported by in vitro cell adhesion experiments which show that PS2m8 and PS2c integrins differ in their ability to mediate cell spreading on plastic tissue culture dishes coated with purified extracellular matrix components (Bunch and Brower, 1992; Zavortink et al., 1993; Fogerty et al., 1994).

The expression of the two alternative PS2 mRNA subtypes appears to be developmentally regulated. That is, the ratio of PS2m8 to PS2c mRNA transcripts has been shown to differ between tissues and developmental stages (Brown et al., 1989), but to date there are no data to indicate which \( \alpha_{PS2} \) subtypes are actually expressed on embryonic muscle cells or ventral wing cells. Although the functional significance of expressing alternative PS2 structures is not known, the eighth exon of the \( if \) homologue from a distantly related Mediterranean fruit fly (Ceratitus capitata) encodes a strikingly similar 25 amino acid sequence suggesting this small domain has an evolutionarily conserved function (Brown et al., 1989).

Requirements for \( PS1, PS2m8 \) and \( PS2c \) During Muscle Attachment and Wing Morphogenesis

Since the analysis of \( mys \) mutant phenotypes has demonstrated that PS integrins are required for muscle
attachment and wing morphogenesis, the question arises as to which integrin structures actually facilitate adhesion in these tissues. Below I briefly review the available data and conclude that all three integrins (PS1, PS2c and PS2m8) could potentially function during both of these morphogenetic events.

Previous analyses have clearly shown that PS integrins are required for muscle attachment site integrity as virtually every adhesion site fails when PS integrin function is eliminated during embryogenesis. Additionally, my characterization of the if lethal phenotype shows that PS2 integrins are specifically required for muscle attachment site integrity, and this result correlates well with the immunolocalization of PS2 integrins on muscle cells (see CHAPTER 3). Since my experiments used if mutations which eliminate all PS2 integrin expression, just one PS2 subtype or both subtypes may fulfill the requirement for if+ function in muscle cells.

From the observation that high levels of PS1 are expressed on tendon cells during muscle attachment, it is tempting to speculate that PS1 might also be required for muscle attachment site integrity. Surprisingly, recent data indicate that mewnull embryos that develop without PS1 typically display significantly more muscle function than embryos carrying strong mys or if mutations. Specifically, mew embryos are not only able to escape (hatch) from their
vitelline membranes and chorion shells, but after hatching mew first instar larvae display considerable mobility as they are able to crawl about on petri dishes; indeed their somatic musculature appears wild type when viewed with polarized light optics. (Brower et al., 1995). Since these mew mutants are unable to express PS1 on tendon cells, this integrin is not necessary for adhesion of muscle cells to their epidermal tendon cell attachments. One interpretation of this result is that tendon cells express a redundant adhesion molecule in addition to PS1. Regardless of whether PS1 is critically required for muscle attachment or not, the available data leave open the possibility that all three integrins participate in adhesion (albeit PS1 mediated adhesion may be redundant) at the sites of muscle attachment.

The available data are also consistent with a role for all three PS integrins during wing morphogenesis. Clonal analysis with mys mutants has clearly shown that both surfaces of the maturing wing blade require PS integrin function. Because I have shown in CHAPTER 3 that the requirement for PS2 function is restricted to ventral cells, PS2 is not the integrin structure which is required on the dorsal surface of the maturing wing blade. Since immunolocalization studies place PS1 on the imaginal disc cells which become the dorsal surface of the wing blade (Brower et al. 1984; 1985), the logical conclusion is that the integrin which functions on the dorsal surface during
wing morphogenesis is PS1. In fact, very recent clonal analysis experiments with mew mutations confirms this hypothesis (Brower et al., 1995). Thus both PS1 and PS2 integrins are critically required on opposite surfaces of the wing blade to facilitate adhesion during wing morphogenesis. Importantly, the requirement for PS2 integrins in ventral wing cells was also determined using if null mutants, thus it is not obvious whether both PS2m8 and PS2c integrins are required or not. Therefore, the available data are consistent with a requirement for PS1 and one PS2 subtype, or alternatively, all three integrins may be required to facilitate adhesion between wing surfaces.

Modeling the Function of PS1, PS2m8 and PS2c in Embryonic Muscle and Pupal Wing Tissues

Several models can be envisioned to explain the role of PS1, PS2m8 and PS2c integrins during muscle attachment and wing morphogenesis. Below I propose two general hypotheses which are consistent with the available data:

1) The PS1 and PS2 (PS2m8 and/or PS2c) integrins are functionally equivalent at mediating adhesion between tendon, muscle or wing cells and the extracellular matrix that separates them. For example, these integrins could all act as generic extracellular matrix glues, perhaps recognizing ligands common to most extracellular matrices. If this hypothesis is true, then these unique PS integrin structures
should be functionally interchangeable during muscle attachment and wing morphogenesis.

2) The PS1 and PS2 (PS2m8 and/or PS2c) integrins must be expressed on opposing sides of extracellular matrix in order to facilitate adhesion during these two morphogenetic events. For example, PS1 and PS2 integrins might bind ligands which must be oriented in a polarized manner in order to effect adhesion. If this hypothesis is true, then disruption of wild type PS1/PS2 expression patterns should compromise adhesion during muscle attachment and wing morphogenesis.

Further refinement of these two hypotheses can be made with respect to the two forms of PS2. For example, a particular PS2 mediated adhesion event (e.g. muscle attachment) might require the combined function of both PS2m8 and PS2c, the function of either PS2 subtype alone might be sufficient, or the function of just one subtype might be specifically required.

Testing Models Using Alternate PS Integrin Structures

The Drosophila system allows in vivo tests of these hypotheses. In general, genetic manipulations that alter the normal PS integrin expression patterns in vivo are potentially useful for examining the functions of alternative integrin structures during specific morphogenetic events. One way to achieve ectopic gene expression is through the creation of chimeric transgenes containing regulatory
sequences from one gene and coding sequences from another. As an idealized example, a transgene created with if regulatory sequences and mew coding sequences could be used to express PS1 integrins in tissues normally requiring PS2 integrins (e.g. PS1 in embryonic muscle cells and ventral wing cells). Although a general lack of knowledge concerning Drosophila promoters (not to mention the fact that \( \alpha_{PS1} \) coding sequences were unavailable at the time I conducted this research) made such an elegant construct strictly an imaginative exercise, there existed sufficiently well characterized sequences to allow the construction of integrin transgenes. Indeed I successfully constructed the first \( \alpha_{PS} \) subunit transgenes with which to evaluate the \textit{in vivo} function of alternative PS integrin structures during specific morphogenetic events as discussed below.

\textit{Creation of Transgenic Lines to Express PS2 Ectopically}

During the last decade, the heterologous promoter most often used in \textit{Drosophila} transgene construction has been the heat shock inducible HSP70 promoter. The HSP70 promoter has proven to be a good choice for many applications because of the following properties: first, this relatively short promoter sequence minimizes transgene size, increasing the odds of successful transformation; second, in the uninduced state this promoter is quite weak, reducing the chances that
a transgenic product will kill transformants; third, most cells respond to heat shock induction of transcription from this promoter, allowing high levels of transgenic expression in virtually any tissue. Since these properties also seemed to be desirable for transgenes designed to express integrin subunits in vivo, the HSP70 promoter was incorporated into my integrin transgene constructions.

At the time I conducted this study, two integrin subunit coding sequences had been cloned and sequenced. Specifically, full length cDNAs encoding the two alternatively spliced if gene products, αPS2m8 and αPS2c subunits, were available to our lab for transgene construction. Splicing the HSP70 promoter to either of these cDNAs produced two different chimeric genes: a HS-αPS2m8 and a HS-αPS2c gene.

In the short term, I believed these transgenes would be useful for examining the functional significance of the wild type PS1/PS2 expression patterns at muscle attachment sites and on the maturing wing blade surfaces. Specifically, the HSP70 promoter should drive αPS2 expression in virtually every cell including tendon cells and the cells comprising the dorsal surface of the wing blade. Thus after heat shock, ectopic αPS2 subunit expression should result in PS2 expression on both surfaces of the muscle/tendon cell-cell interface as well as on both surfaces of the maturing wing blade. If the normal patterns of PS1/PS2 displayed on these
tissues are necessary for adhesion, then such disruptions of the PS1/PS2 patterns should compromise adhesion resulting in spheroidal muscle and blistered wing phenotypes. Obviously, such a result would indicate that PS1 and PS2 integrins are not functionally equivalent. This result has been partially borne out in my experiments with $\alpha_{PS2mS}$ and $\alpha_{PS2c}$ expressing transgenes. I discuss two alternative interpretations of my data and provide an experimental approach which should allow future investigators to determine which interpretation is correct.

In the long term, I had hoped to use this approach to distinguish functional differences between the $\alpha_{PS1}$, $\alpha_{PS2mS}$ and $\alpha_{PS2c}$ subunits by determining which $\alpha_{PS}$ structures are able to mediate cell-cell adhesion during specific morphogenetic events in vivo. For example, if mutant phenotypes, such as spheroidal muscles and blistered wings, might be fully rescued by transgenic expression of some $\alpha_{PS}$ subunit structures (e.g. $\alpha_{PS2mS}$), but not other subunits (e.g. $\alpha_{PS1}$). Using such an approach, structural differences could be correlated with functional differences. Unfortunately, the $\alpha_{PS1}$ cDNA coding sequence, which had been cloned by another lab (Wehrli et al., 1993), did not become available for my use in transgene construction during this study. Nevertheless, I was able to rescue the if muscle phenotype using both forms of transgenically expressed $\alpha_{PS2}$. I discuss
the significance of these results and provide direction for future structure/function studies using this approach.

RESULTS:

Creation of Transgenes to Express Alternate PS Integrins

Transgenes capable of \( \alpha_{PS2mB} \) or \( \alpha_{PS2c} \) subunit expression were first constructed in \( E. coli \) by fusing the heat inducible HSP70 promoter to if cDNA coding sequences. To facilitate high levels of translation following heat shock induction of transcription, I engineered these chimeric transgenes to transcribe \( \alpha_{PS2} \) mRNA with HSP70 5' and tubulin 3' untranslated sequences in place of the authentic if 5' and 3' untranslated sequences. Presumably, the HSP70 5' untranslated sequences should permit efficient translation under heat shock conditions and the tubulin 3' untranslated sequences should create a relatively stable polyadenylated mRNA.

My HS-\( \alpha_{PS2c} \) and HS-\( \alpha_{PS2mB} \) constructions are identical except the HS-\( \alpha_{PS2mB} \) construct lacks the 75 base pair eighth exon which encodes 25 amino acids near the ligand binding domain (Figures 4.1A & B). Before proceeding to making transgenic animals, both gene constructs were successfully tested for PS2 expression by transiently transfecting \( Drosophila \) tissue culture cells with plasmid DNA containing either HS-\( \alpha_{PS2} \) fusion gene. Two hours following heat shock,
transfected cells display high levels of $\alpha_{PS2}$ subunits on their plasma membrane surface as detected by immunostaining with an anti-$\alpha_{PS2}$ monoclonal antibody, and the transfected cells spread on PS2 ligand-coated plastic (Bunch and Brower, 1992; Zavortink et al., 1993).

With the knowledge that these fusion genes are active in vitro, each construct was then introduced into the germ line of animals using P-element transfection technology. Specifically, each HS-$\alpha_{PS2}$ gene construct was inserted into a P-element transformation vector that contains P-element inverted repeats for transposition, and an independent marker gene ($ry^+$) to facilitate the selection of transgenic animals (Figure 4.1C). Microinjection of these transformation
FIGURE 4.1 Diagram of transgenes used to express if+ function in animals. (A) (B) HS-αPS2m and HS-αPS2c are if cDNA gene constructs designed to express alternative forms of αPS2 under the control of a heat inducible promoter (HSP 70). (C) These HS-αPS2 gene constructs were inserted into transformation vectors containing the selectable marker gene ry+ and P-element inverted repeat sequences (hatched triangles). See Materials and Methods for description of cloning and transformation procedures.
plasmids (along with DNA containing the P-element transposase gene) into the posterior end of ry embryos resulted in the random insertion of each transgene into pole cell (germ line) chromosomes.

Two transformants, one HS-α_{PS2m8} (P[A]) and one HS-α_{PS2c} (P[1]), were recovered by selecting ry\textsuperscript{*} animals from the progeny of several hundred microinjected ry embryos. By following the ry\textsuperscript{*} marker through a series of genetic crosses, the P[A] transgene was determined to have inserted into the second chromosome, while P[1] had inserted into the third chromosome. Since both transgenes had caused recessive lethal mutations at their sites of insertion, stable transgenic lines bearing P[A] or P[1] were made by placing the transgenic chromosomes over balancer chromosomes (i.e. P[A]/CyO and P[1]/TM3; see Material and Methods for details).

Previous reports have shown that the chromosomal site at which a transgene inserts can influence the level of transgene expression (for discussion see Kellum and Schell, 1991). To eliminate the possibility that my transgenes may have inserted into a chromosomal location which would adversely effect their usefulness in future experiments, P[A] and P[1] were mobilized to new chromosomal positions. By crossing the P[A] or P[1] transgenic lines with animals carrying the P-element transposase gene, several additional insertions of each transgene were recovered. These genetic
crosses are diagrammed in Figure 4.2A and a pedigree showing the origin of the new transgenic lines is outlined in Figure 4.2B. From this series of experiments, I have created four stable HS-\(\alpha_{PS2m8}\) transgenic lines \((P[A], P[B], P[C] \& P[D])\) and two HS-\(\alpha_{PS2c}\) transgenic lines \((P[1] \& P[2])\) with which to manipulate in vivo levels of PS2m8 and PS2c integrins respectively.

Transgenic Animals Express High Levels of Ectopic PS2

The \(P[A]\) and \(P[1]\) transgenic lines were examined for \(\alpha_{PS2}\) synthesis after heat shock by running Western blots of embryo lysates. Specifically, mature 20 hour old (stage 17) wild-type and transgenic embryos were given a one hour heat pulse (at 37°C) and allowed a one half hour recovery (at 25°C) to translate induced mRNAs. Elevated levels of \(\alpha_{PS2m8}\) or the slightly larger \(\alpha_{PS2c}\) were detected on Western blots in lanes loaded with embryo lysates made from \(P[A]\) or \(P[1]\) lines respectively (Figure 4.3). From these data it is clear that both transgene constructions, when induced with heat shocks, express high levels of \(\alpha_{PS2m8}\) or \(\alpha_{PS2c}\) subunits in animals. Since previous studies with HSP70 driven transgenes indicate that this promoter is active in most (if not all) embryonic cell types (e.g. see Edgar and O'Farrell, 1990), the excess \(\alpha_{PS2}\) detected in my Western analysis probably reflects \(\alpha_{PS2}\)
**A)** Cross to mobilize transgenes to new locations:

![Diagram of transgenesis process]

**B)** Results of hopping HS-αPS2 transgenes:

<table>
<thead>
<tr>
<th>Original insertion</th>
<th>HS-αPS2m8 transgenes</th>
<th>HS-αPS2c transgenes</th>
</tr>
</thead>
<tbody>
<tr>
<td>P[A]</td>
<td></td>
<td>P[1]</td>
</tr>
</tbody>
</table>

Subsequent insertions:
- P[B]
- P[C]
- P[D]
- P[2]

**FIGURE 4.2** Mobilization of transgenes to additional sites within the genome. (A) "Jump-start" males were produced by crossing transgene into genetic background encoding P-element transposase. (B) After transposition, copies of both original transgenes (HS-αPS2m8 and HS-αPS2c) were successfully recovered at novel chromosomal locations. Chromosomal locations: P[A], P[B], P[C] & P[2] occupy genetically separable sites on the second chromosome, while P[D] & P[1] reside at unique sites on the third chromosome.
FIGURE 4.3  Immunoblot of wild type and transgenic embryo lysates probed with $\alpha_{PS2}$-specific monoclonal antibody. Lysates made from transgenic or wild-type embryos (2 embryos/lysate) were loaded in lanes 1-3 as indicated. A more concentrated wild-type embryo lysate (15 embryos/lysate) was loaded in lane 4. Precursor $\alpha_{PS2}$ is normally proteolytically processed into a light chain and a heavy chain (Bogaert et al., 1987). Unprocessed $\alpha_{PS2}$ ($M_r = 160 \times 10^3$) and processed heavy chain $\alpha_{PS2}$ ($M_r = 140 \times 10^3$) are indicated. The excess $\alpha_{PS2}$ detected in the transgenic lysates (lanes 2 & 3) is largely unprocessed (160K). The difference between $\alpha_{PS2c}$ and $\alpha_{PS2m8}$ subunits (25 amino acids) results in a slight difference in electrophoretic mobility (compare lanes 2 & 3).
synthesis not only in cells which normally express if gene products, but also in other cells which are normally devoid of \( \alpha_{PS2} \) subunits.

Since there are data which indicate \( \alpha_{PS} \) subunits must first pair with \( \beta_{PS} \) in order to be transported to the cell surface (Leptin et al., 1989), transgenic expression of PS2 integrins should be restricted to cells which normally express significant levels of (endogenous) \( \beta_{PS} \). To directly test this hypothesis, I used immunofluorescence techniques to determine which larval tissues display transgenic PS2 integrins on their surface. Third instar transgenic larvae (and wild type controls) were heat shocked for one hour (at 37°C) and their heads (which contain most imaginal discs as well as numerous other tissues) were harvested after a two hour recovery period (at 25°C). Using an anti-\( \alpha_{PS2} \) monoclonal antibody to locate PS2 expression on transgenic head tissues, PS2 was found on the basal surface of virtually all imaginal disc epithelial cells (which normally express PS integrins), but little or no staining was detected on tissues normally lacking significant levels of PS integrins (e.g. the brain). These observations indicate that transgenic \( \alpha_{PS2} \) must associate with endogenous \( \beta_{PS} \) in order to be expressed on the surface of cells.

In this regard, it should be noted that I also have examined the pattern of (endogenous) PS1 expression on identically processed transgenic head tissues (using an anti-
αPS1 monoclonal antibody) and find the pattern of PS1 expression to be indistinguishable from wild type expression patterns. Furthermore, the intensity of PS1 staining detected on transgenic discs appears to be similar to the intensity of PS1 staining on identically treated wild type discs. Although quantitation of antigen levels through immunostaining intensity is imprecise at best, these data indicate that transgenic αPS2 subunits do not drastically compromise (e.g. through competition for βPS) endogenous PS1 expression levels. Taken together, these results indicate that the level of endogenous βPS within imaginal disc cells is sufficient to promote the concurrent expression of endogenous and transgenic PS integrins.

Most importantly, these immunofluorescence experiments show that the wild type patterns of PS integrin expression are dramatically changed in transgenic animals following heat shock. These changes are exemplified most clearly on transgenic wing discs where (after heat shock) transgenic PS2 is ectopically expressed on the surface of disc cells destined to make the dorsal wing surface, cells which normally express only PS1 integrins (Figure 4.4). Thus the wild type pattern of PS integrins (where PS1 and PS2 are restricted to cells destined to make the dorsal and ventral wing surfaces respectively) is replaced in transgenic animals
FIGURE 4.4  Pattern of PS2 expression on transgenic and wild type wing discs following heat shock. A) A wild type disc displaying PS2 integrins on cells destined to make the ventral wing epithelium, but PS2 is absent from the cells destined to make the dorsal epithelium (i.e. wild type pattern). B) P[HS-aPS2c]2 transgenic disc displaying PS2 on virtually every disc cell, including ectopic PS2 on cells destined to make the dorsal wing epithelium.
(after heat shock) by a novel pattern in which PS2 is expressed on both surfaces.

**Transgenes Express Functional PS2 Integrins**

With the ability to induce ectopic PS2 expression in otherwise wild type animals, I wanted to determine whether the normal patterns of PS1 and PS2 integrins are critical for mediating the cell-cell adhesion events which occur during muscle attachment and wing morphogenesis. Presumably, transgenic expression of $\alpha_{PS2}$ subunits in tendon and dorsal wing cells (which normally express only PS1) causes some level of ectopic PS2 function on these cell types. If PS2 functions differently than PS1 during these events, then it seemed plausible that PS integrin mediated adhesion in muscle and wing tissues might be compromised. That is, ectopic PS2 expression in tendon cells or wing cells might lead to familiar integrin mutant phenotypes such as spheroidal muscles or blistered wings respectively.

Surprisingly, very early on it became apparent that the transgenic lines were quite healthy despite the induction of significant levels of ectopic $\alpha_{PS2}$ subunits. For example, transgenic embryos induced to express the high levels of ectopic $\alpha_{PS2}$ depicted in Figure 4.3 are 100% viable when compared to their nontransgenic siblings (i.e. a negative control group). In fact, experiments designed to induce (with one heat shock at 37°C for every 3 hours of
embryogenesis) transgenic $\alpha_{PS2}$ expression in embryos throughout the processes of myogenesis, muscle attachment, and muscle contraction had no detectable effect on muscle attachment site integrity or on any other critical tissue, as these embryos develop into phenotypically wild type adults. Similarly, transgenic larvae with wing imaginal discs expressing the high levels of ectopic PS2 on their dorsal cells depicted in Figure 4.4 also developed into phenotypically wild-type adults; even close examination of their wings failed to reveal any abnormalities.

Like most negative results, these findings were disconcerting because they could be interpreted in several ways. For example, one could postulate that disruption of the normal PS1/PS2 patterns would never cause any phenotype because PS integrins may be functionally interchangeable. A more problematic possibility became immediately apparent: perhaps the level of transgenic PS2 activity had been insufficient to cause a phenotype. Moreover, since my immunoblot and immunostaining data do not address the question of whether the transgenically expressed $\alpha_{PS2}$ subunits are functional, one could argue that the PS2 integrins expressed by these transgenes might be mutant (e.g. a cloning artifact during transgene construction could have created a missense mutation in the $\alpha_{PS2}$ coding sequence).

To directly address these concerns, my immediate focus turned to proving that these transgenes express functional
PS2 integrins. Basically, the experimental approach was to use the transgenes as tools to rescue if mutant phenotypes. A positive result would clearly show that the transgenes were capable of supplying if+ function in animals. In order to exhaustively pursue this experimental approach, I set up experiments to attempt the rescue of three different if mutant phenotypes with both αPS2 transgenic constructs. Experiments designed to rescue the two best characterized if phenotypes, spheroidal muscles and blistered wings, will be discussed later in the results. Below I focus on a third if phenotype which provided the first conclusive evidence that the transgenes can contribute if+ function in animals.

As discussed in the CHAPTER 3, the mysXRO4 if doubly heterozygous (mysXRO4 +/- if or mysXRO4 if/+ +) genotype almost always results in lethality. Presumably, the antimorphic mysXRO4 allele encodes a mutant form of βPS that has "dominant negative activity" when placed in an if heterozygous background. For example, the mutant βPS subunits might compete with wild-type βPS subunits for the limited pool of free αPS2 subunits, thereby lowering the effective concentration of PS2 integrins below some critical threshold.

Interestingly, this genotype appears to be "on the edge" for PS integrin function as a small number of animals escape lethality (i.e. roughly 1% of animals bearing the mysXRO4 if/+ + genotype survive to adulthood). Therefore, it seems probable that only a small increase in the amount of
functional PS2 would be needed to rescue the lethality associated with this genotype. For this reason, the $\text{mys}^{XR04}$ $if/+ +$ genetic background was considered to be the most attractive for an initial determination of whether the transgenes express functional $\alpha_{PS2}$ subunits.

Before crossing transgenes into the $\text{mys}^{XR04}$ $if/+ +$ genetic background, I attempted to work out conditions which would lead to maximal expression of transgenic PS2. Specifically, I determined that after heat shock induction (one hour at 37°C) of transgene transcription, maximal expression of ectopic PS2 on the dorsal surface of transgenic wing discs was observed when animals were allowed a two hour recovery period (at 25°C). Importantly, the level of ectopic PS2 expression diminished to low (but still detectable) levels after animals were allowed to recover for six hours. Since the delivery of heat pulses at a frequency greater than once every 7 hours diminishes the viability of wild type and transgenic animals (as mentioned in CHAPTER 2), I considered this to be the maximal induction protocol that could be used in experiments designed to look at adult viability.

Therefore, after crossing transgenes into the $\text{mys}^{XR04}$ $if/+ +$ background (Figure 4.5), transgene expression was induced throughout development by administering a single 30 minute heat pulse at 37°C for every 7 hours of development. In addition to the experimental groups which consisted of $\text{mys}^{XR04}$ $if/+ +$ animals bearing either $P[D]$ ($\text{HS}^\text{apS2m8}$) or $P[1]$
(HS-\(\alpha_{PS2c}\)), negative control (\(mys^{XR04} if/+\) animals without a transgene) and positive control experiments were also performed. The positive control consisted of \(mys^{XR04} if/+\) animals carrying a HS-\(\beta_{PS}\) transgene that is known to express functional \(\beta_{PS}\) subunits (see CHAPTER 2 and Figure 2.7). Theoretically, the positive control transgene should also increase in vivo titers of functional PS2 by increasing the ratio of wild type \(\beta_{PS}\) subunits relative to mutant \(\beta_{PS(XR04)}\) subunits.

The results presented in Table 4.1 indicate that \(\alpha_{PS2}\) expression from either transgene rescues the lethality associated with the \(mys^{XR04} if/+\) genotype. Specifically, mutant animals bearing the \(\alpha_{PS2m8}\) or \(\alpha_{PS2c}\) expressing transgenes typically developed into viable adult flies, while "no transgene" negative control mutants never survived to adulthood. As expected, mutants bearing the \(\beta_{PS}\) transgene (the positive control group) were also rescued. These data support the hypothesis that the transgenes provide functional protein.
A) Cross to place HS-αPS2 transgenes into a lethal (mysXR04 if/+ +) genetic background:

\[ \text{M} \\ \text{H} \times \text{F} \rightarrow \text{H} \]

B) Genotypes of zygotes resulting from this cross:

\[ \text{M} \\ \text{H} \times \text{F} \rightarrow \text{H} \]

FIGURE 4.5 Scheme to rescue mysXR04 if/+ + lethality with αPS2 expressing transgenes. A) Male flies bearing transgenes are crossed with mysXR04 if/+ + female flies—note that these females are viable because of the if+ duplication on their 4th chromosome. B) List of zygotes resulting from this cross. The circled class of progeny are the experimental group: mysXR04 if/+ + females bearing an αPS2 expressing transgene. (The 2 classes of mysXR04 hemizogotes (males) are crossed out to indicate that they die during embryogenesis.)
TABLE 4.1  Rescue of $mys^{XR04}$ ifB2/+ lethality with transgenic integrin subunits.

<table>
<thead>
<tr>
<th>Genotype</th>
<th>RESCUED?</th>
<th>(# of flies/# expected)</th>
</tr>
</thead>
<tbody>
<tr>
<td>$mys^{XR04}$ ifB2/+ with $P[HS-\alpha_{PS2m8}]$</td>
<td>YES*</td>
<td>(37/70)</td>
</tr>
<tr>
<td>$mys^{XR04}$ ifB2/+ with $P[HS-\alpha_{PS2c}]$</td>
<td>YES</td>
<td>(54/45)</td>
</tr>
<tr>
<td>$mys^{XR04}$ ifB2/+ without transgene</td>
<td>NO</td>
<td>(0/167)</td>
</tr>
<tr>
<td>(negative control)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>$mys^{XR04}$ ifB2/+ with $P[HS-\beta_{PS}]$</td>
<td>YES</td>
<td>(15/50)</td>
</tr>
<tr>
<td>(positive control)</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Note. All larvae were grown under heat shock conditions (30 minutes @ 37°C every 7 hours) until eclosion to induce transgenic expression. The number of flies expected (# expected) is the number of flies with the specified genotype plus $Dp(1;4)\tau^+(if^*)$.

* In addition to rescuing the $mys^{XR04}$ ifB2/+ class of progeny, $P[HS-\alpha_{PS2m8}]$ expression caused wing blisters in all classes of progeny including wild type (see Figure 4.5).
Although this experimental approach provided the first evidence that both transgenes produce functional α<sub>PS2</sub> subunits, it is unclear which tissues were rescued because the specific defect(s) that causes lethality in <i>mys<sup>XR04</sup> if/+ +</i> animals is unknown. The lethal period for <i>mys<sup>XR04</sup> if/+ +</i> animals has been reported to be during pupariation (Wilcox, 1990). Therefore, <i>mys<sup>XR04</sup> if/+ +</i> zygotes must have enough PS integrin function to complete embryogenesis and hatch as viable larvae; this observation obviously implies that <i>mys<sup>XR04</sup> if/+ +</i> mutant embryos do not have substantial defects in their somatic musculature. Consequently, these data do not address the question of whether the transgenes express significant levels of α<sub>PS2</sub> in embryonic muscle cells. The only conclusion that can be made is that the transgenes provide functional PS2 integrins in some undetermined tissue(s).

On the other hand, an unexpected result concerning the function of PS2 integrins during wing morphogenesis emerged from these experiments. Specifically, all classes of progeny bearing the <i>P[D]</i> transgene (HS-α<sub>PS2m8</sub>) displayed a significant frequency of blistered wings. In contrast, all the other viable F1 genotypes produced in these crosses, including those bearing <i>P[1]</i> (HS-α<sub>PS2c</sub>) or <i>P[mys+]</i> (HS-β<sub>PS</sub>), developed into animals with phenotypically wild-type wings. Therefore, transgenically expressed α<sub>PS2m8</sub> subunits appeared to
dominantly disrupt wing morphogenesis, and this phenomenon was examined further as described in the following sections.

Wing Phenotype Associated With Transgenic PS2m8 Expression

In contrast to my initial observation that wing morphogenesis is unaffected when the normal PS1/PS2 pattern on larval discs is disrupted (Figure 4.4), it was now apparent that induction of transgenically expressed αPS2m8 subunits throughout the pupal period causes wing blistering. Since the transgene induction protocol had been lengthened considerably for the mysXR04 if/+ mutant rescue experiments (where heat shocks were administered continuously throughout the larval and pupal periods), it seemed likely that the levels of PS2m8 had been insufficient to cause blisters in my original experiment (where heat shocks were restricted to the late third instar larval period).

To test this hypothesis directly, animals bearing a HS-αPS2m8 or a HS-αPS2c transgene were heat shocked during development using two different transgene induction protocols, and after eclosion the adult flies were examined for wing blisters. Specifically, mature transgenic larvae were either given a continuous cycle of 30 minute heat pulses (37°C) every 7 hours to induce αPS2 expression throughout the metamorphic period, or they were given a very limited period (7 hrs total) of heat shock induction just prior to puparium formation. As expected from the previous experiments,
animals bearing HS-\(\alpha_{PS2m8}\) transgenes displayed significant levels of wing blistering (10-33\% of wings blistered) when heat shocked every 7 hours (Table 4.2), while the more restricted induction protocol failed to cause blistering. Furthermore, identical experimental conditions with animals bearing HS-\(\alpha_{PS2c}\) transgenes failed to elicit blistering above the level (~1\%) displayed by similarly treated control animals (Table 4.2). Therefore, the induction of transgenic \(\alpha_{PS2m8}\) expression throughout metamorphosis dominantly disrupts wing morphogenesis, but transgenic \(\alpha_{PS2c}\) expression during the same period fails to induce the phenotype.

Although other mechanisms can be imagined, there are two relatively straightforward mechanisms through which transgenic \(\alpha_{PS2m8}\) subunits might disrupt wing morphogenesis. Since no affect is seen when high levels of \(\alpha_{PS2c}\) subunits are expressed, transgenic \(\alpha_{PS2m8}\) might compete with endogenous \(\alpha_{PS2c}\) function on the ventral surface of the maturing wing blade. Alternatively, transgenic \(\alpha_{PS2m8}\) subunits might compete with \(\alpha_{PS1}\) function on the dorsal surface of the wing. If the latter hypothesis is true, then (to account for the observation that only \(\alpha_{PS2m8}\) subunits cause blisters) the transgenic \(\alpha_{PS2m8}\) and \(\alpha_{PS2c}\) subunits must have qualitative differences in their ability to compete for \(\alpha_{PS1}\) function on dorsal cells, or the transgenic \(\alpha_{PS2c}\) subunits must be
**TABLE 4.2** Frequency of blistering in animals bearing a single $P[HS-\alpha_{PS2}]$ integrin transgene.

<table>
<thead>
<tr>
<th>genotype</th>
<th>blister frequency</th>
<th>(# blistered/total # wings)</th>
</tr>
</thead>
<tbody>
<tr>
<td>+/+</td>
<td>1.3%</td>
<td>(9/718)</td>
</tr>
<tr>
<td>$P[1]/+$</td>
<td>1.2%</td>
<td>(5/408)</td>
</tr>
<tr>
<td>$P[2]/+$</td>
<td>1.5%</td>
<td>(7/462)</td>
</tr>
<tr>
<td>$P[A]/+$</td>
<td>11%</td>
<td>(57/540)</td>
</tr>
<tr>
<td>$P[B]/+$</td>
<td>10%</td>
<td>(38/400)</td>
</tr>
<tr>
<td>$P[C]/+$</td>
<td>33%</td>
<td>(133/400)</td>
</tr>
<tr>
<td>$P[D]/+$</td>
<td>14%</td>
<td>(57/400)</td>
</tr>
</tbody>
</table>

**Note.** All animals were reared at 25°C until the third larval instar stage, then the animals were heat shocked for 30 minutes (37°C) every 7 hours until eclosion. $P[1]$ & $P[1]$ are $P[HS-\alpha_{PS2c}]$ transposons. $P[A], P[B], P[C]$ & $P[D]$ are $P[HS-\alpha_{PS2m8}]$ transposons.
expressed at a lower level than is achieved for $\alpha_{PS2m8}$ subunits.

**Experiments to Modify Dominant Effect of Transgenic $\alpha_{PS2m8}$**

I pursued experiments to determine whether transgenic $\alpha_{PS2m8}$ subunits disrupt endogenous PS2c function on ventral cells, or whether they disrupt endogenous PS1 function on dorsal cells. One experimental approach was to use genetic backgrounds that should alter the levels of endogenous PS2c on ventral cells or PS1 on dorsal cells. By placing the blister-causing $\alpha_{PS2m8}$ transgene into such genetic backgrounds, I asked whether reductions in endogenous PS2c or PS1 levels enhance the blister penetrance, and whether additions to the endogenous levels of PS2c or PS1 suppress this phenotype.

These types of experiments are genetically feasible in *Drosophila* as gene copy number typically influences the ultimate expression level of the gene product. For example, adding an extra copy of a locus (e.g. with a duplication) generally increases expression of that gene product to roughly 150% of wild type levels, while removal of one copy of a locus reduces gene expression to about 50% of wild type levels (e.g. see the ry locus in Lindsley and Zimm, 1992). Such observations have shown that the regulation of gene expression in *Drosophila* is often exclusively controlled at
the level of transcription, although there are a few well-documented cases where post-transcriptional regulatory mechanisms do dramatically influence expression levels.

Here are two idealized sets of results I might have obtained using gene copy number to manipulate the levels of endogenous PS integrins:

Scenario I) If reduced endogenous $\alpha_{PS2c}$ expression ($if/+$) enhances the blistering (caused by HS-$\alpha_{PS2m8}$ transgenes) while increased endogenous $\alpha_{PS2c}$ expression (+/+;$Dpif^+$) suppresses the phenotype, then these results would support the hypothesis that transgenic $\alpha_{PS2m8}$ disrupts PS2c function in dorsal cells.

Scenario II) If reduced endogenous $\alpha_{PS1}$ expression ($mew/+)$ enhances blistering while increased endogenous $\alpha_{PS1}$ expression (+/+;$Dpmew^+$) suppresses the phenotype, then these results would support the hypothesis that transgenic $\alpha_{PS2m8}$ disrupts PS1 function in dorsal cells.

Figure 4.6A outlines the genetic scheme I used to examine the frequency of blistering (caused by $\alpha_{PS2m8}$ expression from $P[C]$) in genetic backgrounds with reduced mew or if gene copy numbers. The results presented in Table 4.3 indicate that the integrin deficient genetic backgrounds ($mew/+ & if/+)$ do not enhance blister frequencies (23-35% wings blistered) above the levels displayed by animals bearing wild type $mew^+$ and $if^+$ copy numbers (17-48% wings
FIGURE 4.6 Schemes to alter integrin gene copy number in P[HS-\(\alpha_{PS2m8}\)]C transgenic animals. (A) P1 cross produces F1 progeny bearing the HS-\(\alpha_{PS2m8}\) transgene in genetic backgrounds of 1 or 2 mew\(^+\) copies. Similarly, cross (B) produces transgenic progeny with different numbers of if\(^+\) loci. Note: only the experimentally relevant (female) F1 progeny are illustrated for cross A.
blistered). In fact, the genotypes giving the highest (48%) and lowest (17%) blister frequencies had wild type integrin backgrounds (i.e. +/FM7c and y w f FRT/y w f FRT respectively), and the genetically identical +/FM7c progeny produced in four different trials displayed a wider range of blister frequencies (26-48% wings blistered) than was observed between the integrin deficient genotypes (Table 4.3). These data indicate that the blistering associated with transgenic αPS2m8 expression is modestly influenced by culture conditions and genetic background generally, but not by reductions in integrin gene copy number.

To reduce affects caused by minor differences in culture conditions, a second cross (Figure 4.6B) was devised to examine the affect of altered if+ gene copy number on blistering levels using animals that were propagated from identical parents and reared in the same vials. At first glance, the results from this cross (Table 4.4) appear to indicate that genetic backgrounds bearing an if+ duplication modestly suppress blistering, while if-/if+ backgrounds enhance the phenotype. Specifically, the blister frequency in the y w if+ f/Y ("wild-type") background (2.5% of wings blistered) is enhanced (to 27% of wings blistered) in the if heterozygotic background (g ifK27e f/y w f), and adding the if+ duplication to either of these backgrounds appears to modestly suppress the phenotype to 0.6% (y w if+ f/Y;Dpr+/+)
TABLE 4.3  Frequency of $P[HS-\alpha_{PS2m8}]$ blistering in if or mew heterozygotic backgrounds.

<table>
<thead>
<tr>
<th>Exp 1)</th>
<th>genotype of viable F1 progeny</th>
<th>blister frequency</th>
<th>(# blistered/total # wings)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>ifA7/+ ; $P[C]/+$</td>
<td>35%</td>
<td>(72/206)</td>
</tr>
<tr>
<td></td>
<td>FM7c/+ ; $P[C]/+$</td>
<td>48%</td>
<td>(75/156)</td>
</tr>
<tr>
<td></td>
<td>FM7c/Y ; $P[C]/+$</td>
<td>*</td>
<td>(4/6)</td>
</tr>
<tr>
<td>Exp 2)</td>
<td>ifB2/+ ; $P[C]/+$</td>
<td>27%</td>
<td>(79/298)</td>
</tr>
<tr>
<td></td>
<td>FM7c/+ ; $P[C]/+$</td>
<td>41%</td>
<td>(111/272)</td>
</tr>
<tr>
<td></td>
<td>FM7c/Y ; $P[C]/+$</td>
<td>*</td>
<td>(22/44)</td>
</tr>
<tr>
<td>Exp 3)</td>
<td>mewH7/+ ; $P[C]/+$</td>
<td>23%</td>
<td>(123/528)</td>
</tr>
<tr>
<td></td>
<td>FM7c/+ ; $P[C]/+$</td>
<td>28%</td>
<td>(101/364)</td>
</tr>
<tr>
<td></td>
<td>FM7c/Y ; $P[C]/+$</td>
<td>*</td>
<td>(0/2)</td>
</tr>
<tr>
<td>Exp 4)</td>
<td>mewM6/+ ; $P[C]/+$</td>
<td>24%</td>
<td>(75/310)</td>
</tr>
<tr>
<td></td>
<td>FM7c/+ ; $P[C]/+$</td>
<td>26%</td>
<td>(54/214)</td>
</tr>
<tr>
<td></td>
<td>FM7c/Y ; $P[C]/+$</td>
<td>*</td>
<td>(0/0)</td>
</tr>
<tr>
<td>(control)</td>
<td>$+1/+ ; P[C]/+$</td>
<td>17%</td>
<td>(51/292)</td>
</tr>
<tr>
<td></td>
<td>$+1/Y ; P[C]/+$</td>
<td>(4%)*</td>
<td>(3/78)</td>
</tr>
</tbody>
</table>

Note. All progeny were reared at 28°C. $P[C]$ is a $P[HS-\alpha_{PS2m8}]$ transposon on the second chromosome. FM7c, $+1$ and $+$ chromosomes are wild type with respect to integrin loci. Several chromosomes also bear multiple marker mutations as follows: ifA7, y w svb sn f; ifB2, g f; mewH7, y f FRT; mewM6, y f FRT; $+1$, y f FRT; FM7c, y w sn v g B.

* The blister frequencies for these under represented classes of progeny are not statistically relevant. Apparently, these genotypes are semi-lethal at 28°C.
### TABLE 4.4 Frequency of \( P[HS-\alpha PS2m8] \) blistering in genetic backgrounds with altered \( if \) gene copy number.

<table>
<thead>
<tr>
<th>genotypes of F1 progeny</th>
<th>blister frequency</th>
<th>(# blistered/total # wings)</th>
</tr>
</thead>
<tbody>
<tr>
<td>( if^{K27e}/+1 ); ( P[C]/+ ); (+/+ )</td>
<td>27%</td>
<td>(103/380)</td>
</tr>
<tr>
<td>( if^{K27e}/+1 ); ( P[C]/+ ); (+/Dpr^+ )</td>
<td>13%</td>
<td>(52/396)</td>
</tr>
<tr>
<td>(+1/Y ); ( P[C]/+ ); (+/+ )</td>
<td>2.5%</td>
<td>(8/322)</td>
</tr>
<tr>
<td>(+1/Y ); ( P[C]/+ ); (+/Dpr^+ )</td>
<td>0.6%*</td>
<td>(1/174)</td>
</tr>
</tbody>
</table>

**Note.** All progeny were reared at 28°C.

\( Dpr^+ \) contains the \( if^+ \) locus.

\( P[C] \) is a \( P[HS-\alpha PS2m8] \) transposon on the second chromosome.

\(+1\) and \(+\) chromosomes are both wild type with respect to integrin loci.

\(+1\) and \( if^{K27e} \) chromosomes also bear multiple marker mutations as follows: \(+1\), \( y w f \) and \( if^{K27e}, g f \).

* The blister frequency for this class of progeny may not be statistically relevant as more than half the expected progeny are missing. Apparently, the genotype is semi-lethal at 28°C.
or 13% \((g if^{K27e} f/y w f;Dpr^{+}/+\)) respectively. When these data are considered in light of control experiments, the levels of enhancement and suppression may be attributed as easily to non-integrin genetic background differences between experimental groups. For example, the control in Table 4.3 indicates that \(y^{f} FRT/Y;P[C]/Y\) males display significantly lower rates of blistering (4% wings blistered) than their identically marked \(y^{f} FRT/y^{f} FRT;P[C]/+\) female siblings (17% wings blistered) indicating that the sex of \(P[C]\) animals influences the expressivity of the HS-\(\alpha_{PS2m8}\) blister phenotype. This experimental approach was not refined further because an expedient method for ameliorating the affects of non-integrin genetic background differences between experimental groups does not exist. For example, the \(mew\) and \(if\) duplications and mutations currently reside on genetically dissimilar chromosomes and the process of making all of these chromosomes isogenic is not trivial.

To circumvent many of these non-integrin background affects, a related approach was devised using (essentially isogenic) chromosomes that bear combinations of integrin transgenes. Specifically, animals bearing multiple copies of integrin transgenes were used to examine whether transgenically expressed \(\alpha_{PS2c}\) can rescue the blisters associated with ectopic \(\alpha_{PS2m8}\). If the blistering associated with HS-\(\alpha_{PS2m8}\) transgenes is caused by reduced PS2c expression
on the ventral surface of wings (e.g., transgenic \( \alpha_{\text{PS2m8}} \) might compete with endogenous \( \alpha_{\text{PS2c}} \) for \( \beta_{\text{PS}} \)), then transgenic supplementations to the endogenous pool of \( \alpha_{\text{PS2c}} \) should rescue the wing phenotype. Mature larvae bearing one copy of HS-\( \alpha_{\text{PS2m8}} \) and one copy of HS-\( \alpha_{\text{PS2c}} \) were heat shocked (30 min at 37°C every 7 hrs) throughout metamorphosis, and then scored for blisters after eclosion. As shown in Table 4.5, no significant differences were observed in the frequency of blisters between animals bearing both transgenes (17% of \( \text{P}[\text{D}, \text{P}[\text{I}]/+ \) wings were blistered) and animals bearing only the HS-\( \alpha_{\text{PS2m8}} \) transgene (14% of \( \text{P}[\text{D}]/+ \) wings were blistered). These data indicate that adding \( \alpha_{\text{PS2c}} \) does not suppress the blister phenotype associated with ectopic \( \alpha_{\text{PS2m8}} \).

Along these lines, I also attempted to suppress the blister phenotype by adding the HS-\( \beta_{\text{PS}} \) transgene (\( \text{P}[\text{mys}^+]\text{B1} \)) to animals bearing chromosomes with \( \text{P}[\text{D}] \& \text{P}[\text{I}] \) or just \( \text{P}[\text{D}] \). Specifically, mature larvae bearing a third chromosome with \( \text{P}[\text{D}], \text{P}[\text{I}] \& \text{P}[\text{mys}^+]\text{B1} \) or \( \text{P}[\text{D}] \& \text{P}[\text{mys}^+]\text{B1} \) were heat shocked (30 min at 37°C every 7 hrs) until adult flies eclosed, and adult wings were scored for blisters. Again, the results in Table 4.5 show that no significant changes were observed in blistering rates between animals bearing the additional HS-\( \beta_{\text{PS}} \) transgene (26% or 18% blistered for flies bearing a \( \text{P}[\text{D}], \text{P}[\text{mys}^+]\text{B1}/+ \) or \( \text{P}[\text{D}], \text{P}[\text{mys}^+]\text{B1}/+ \) genotype, respectively) and animals bearing only the HS-\( \alpha_{\text{PS2}} \) transgenes.
**TABLE 4.5** Frequency of blistering in animals bearing multiple integrin transgenes.

<table>
<thead>
<tr>
<th>genotypes of flies</th>
<th>blister frequency</th>
<th>(# blistered/total # wings)</th>
</tr>
</thead>
<tbody>
<tr>
<td>P[D]/+</td>
<td>14%</td>
<td>(57/400)</td>
</tr>
<tr>
<td>P[D] P[1]/+</td>
<td>17%</td>
<td>(67/400)</td>
</tr>
<tr>
<td>P[D] P[mys⁺]B1/+</td>
<td>18%</td>
<td>(70/400)</td>
</tr>
<tr>
<td>P[D] P[1] P[mys⁺]B1/+</td>
<td>26%</td>
<td>(103/400)</td>
</tr>
</tbody>
</table>

Note. All animals were reared at 25°C until the third larval instar stage, then the animals were heat shocked for 30 minutes (37°C) every 7 hours until eclosion.

*P[D]* is a *P[HS-αPS₂m₈]* transposon on the third chromosome. *P[1]* is a *P[HS-αPS₂c]* transposon on the third chromosome. *P[mys⁺]B1* is a *P[HS-βPS]* transposon on the third chromosome.
These data indicate that adding $\beta_{PS}$ does not suppress the blister phenotype associated with ectopic $\alpha_{PS2m8}$. Taken together, these data indicate that the dominant blister phenotype associated with HS-$\alpha_{PS2m8}$ transgenes is not caused by loss of endogenous PS integrin function.

One final experiment was attempted to specifically address the hypothesis that PS2c is the integrin which normally functions on ventral cells during wing morphogenesis. Basically, $\alpha_{PS2c}$ expressing transgenes were tested for their ability to rescue wing blisters caused by if mutations. Theoretically, if PS2c is the PS2 subtype normally expressed by ventral wing cells, then adding transgenic $\alpha_{PS2c}$ subunits should rescue wing blisters caused by compromised if gene function.

To compromise if gene function in wings, clonal analysis was performed using if mutations. As discussed in CHAPTER 3, X-ray induced mitotic recombination in first instar if heterozygotes (if/+)) produces a low frequency of mutant clones (if/if) which are always associated with wing blisters (when the if clones are located on the ventral surface of the wing blade). Using the more efficient "heat shock flippase" technique to induce mitotic recombination (Xu and Rubin, 1993), I found that clones and thus blistered wings in if heterozygotes could be created at a level (roughly 50% of wings blistered) appropriate for examining changes in the
frequency of blistering. After crossing $\alpha_{PS2c}$ transgenes into an if/+ genetic background as diagrammed in Figure 4.7, mitotic recombination was induced (by heat shocking first instar larvae) to initiate the creation of mutant if/if patches of cells in wings. As these larvae entered the critical period for PS integrin function during wing morphogenesis (Chapter 2 discusses this critical period), one heat shock every 7 hours was administered to induce transgenic $\alpha_{PS2c}$ expression. Unfortunately, this protocol dramatically reduces the viability of the experimental genotypes. Presumably, the repetitive heat shocks, which are required to induce transgenic $\alpha_{PS2}$ expression, also induce the expression of large quantities of the "flippase" enzyme during metamorphosis, and this enzyme is apparently responsible for the reduced viability. In any case, this experimental approach was not pursued as there are better tools and techniques to address these questions. A discussion of an alternative approach, which promises to efficiently determine where the ectopic $\alpha_{PS2m8}$ functions during wing morphogenesis, will be presented later in the discussion section of this chapter.
A) F1 cross:

\[
\text{if}^{-} \quad \text{flippase} \quad \text{HS-} \alpha_{PS2c} \quad \text{if}^{-} \quad \text{flippase}
\]

B) F1 zygotic genotype of interest:

\[
\text{if}^{-} \quad \text{flippase} \quad \text{HS-} \alpha_{PS2c} \quad \text{if}^{-} \quad \text{flippase}
\]

C) Genotype of clones (after mitotic recombination):

\[
\text{if}^{-} \quad \text{flippase} \quad \text{HS-} \alpha_{PS2c} \quad \text{if}^{-} \quad \text{flippase}
\]

D) Score wings with clones for blisters:

Can HS-\( \alpha_{PS2c} \) transgene rescue if clones in the wing?

FIGURE 4.7 Scheme to rescue the if wing phenotype with transgenic PS2c. (A) Cross to produce if heterozygotes bearing flippase and HS-\( \alpha_{PS2c} \) transgenes. When flippase expression is induced in these heterozygous larvae (B), mitotic recombination occurs resulting in patches of if mutant wing tissue (C). Then transgenic \( \alpha_{PS2c} \) expression is induced during metamorphosis to attempt rescue of the if blister phenotype (D).
Rescue of the inflated Muscle Phenotype with PS2m8 or PS2c

At this point in my research, mysXR04 if/+ rescue experiments had indicated that both transgenes were capable of expressing if+ function in animals, but transgenic αPS2m8 subunits had displayed dominant negative function when expressed in wild type wing tissue during metamorphosis. Since it had become clear that experiments designed to express positive (if+) function in wings were becoming increasing problematic using these transgene constructs (and because an alternative approach promises to address the situation in wings more quickly and definitively), I focused on experiments to determine whether transgenically expressed PS2m8 or PS2c integrins could function during muscle attachment in embryos.

To address this issue, I set up experiments to rescue if embryo's spheroidal muscle phenotype with transgenic αPS2 subunit expression. The previously discussed mysXR04 if/+ and if wing blister rescue experiments required a relatively mild transgene induction protocol (one heat pulse every 7 hours) during metamorphosis to ensure the survival of adult flies for scoring rescue frequency. In contrast, experiments designed to rescue if muscle phenotypes only require that the induction protocol does not disrupt muscle morphogenesis. In fact, a very aggressive induction protocol, one heat shock (20 min at 37°C) delivered every 50 minutes, can be used throughout embryogenesis without reducing viability of
transgenic or wild type animals (Edgar and O'Farrell, 1990; and my observations), but the same protocol delivered during metamorphosis is lethal. Therefore, this "embryonic" induction protocol, which should induce higher transgene expression levels than previous protocols, was used for inducing transgenic $\alpha_{PS2}$ expression in if embryos.

Muscle rescue experiments were initiated by crossing males homozygous for either the HS-$\alpha_{PS2m8}$ or the HS-$\alpha_{PS2c}$ transgene on the second chromosome (+/Y; $P[C]/P[C]$ or +/Y; $P[2]/P[2]$ males) to females heterozygous for both shaven baby (svb) and if (svb if/++; +/+ females) as shown in Figure 4.8. Note that the recessive X-linked svb mutation was placed on the if chromosome to provide an independent embryonic marker as will be discussed later. All the progeny from these crosses carry one copy of the HS-$\alpha_{PS2m8}$ or the HS-$\alpha_{PS2c}$ transgene. To induce expression of $\alpha_{PS2}$ subunits throughout the period of muscle morphogenesis, the transgenic progeny were given heat pulses from about the cellular blastoderm stage (three hours after eggs were laid) until the end of the embryonic period (24 hours after eggs were laid). At 24 hours, the mature embryos were fixed and mounted on microscope slides for further analysis.

Since the if mutations are X-linked recessive, only 25% of the progeny from these crosses are if mutants; that is,
A) F1 cross to place transgene in if background:

\[ \text{svb} \text{ if} \]

\[ \text{HS-} \alpha_{\text{Ps2}} \]

B) F1 zygotic genotypes with the experimental class of animals circled (i.e. \text{svb if}/Y; \text{P[HS-} \alpha_{\text{Ps2}}]/+) :

Display \text{svb} phenotype?

Yes, the hairs (denticles) on this embryo's cuticle are shortened or missing.

No, denticles are wild type.

No, denticles are wild type.

No, denticles are wild type.

C) Examine \text{svb if} embryo's somatic muscle morphology to determine whether transgene rescued if phenotype.

\textbf{FIGURE 4.8} Scheme to rescue the if muscle phenotype with transgenic \( \alpha_{\text{Ps2m8}} \) or \( \alpha_{\text{Ps2c}} \) subunits. (A) F1 cross places one copy of the HS-\( \alpha_{\text{Ps2m8}} \) (or HS-\( \alpha_{\text{Ps2c}} \)) transgene into all classes of F1 progeny. (B) 25% of these progeny are the experimental animals -- the \text{svb if} mutants (circled class). (C) Muscle phenotype of the \text{svb} mutant embryos is assayed. Note: recombination between the \text{svb} and if loci does not occur in this cross because maternal \text{svb if} chromosome is balanced over FM7c (not shown).
all progeny except the male if/Y hemizygotes carry an if+ bearing chromosome (Figure 4.8). Therefore, these male if embryos were identified by the genetically linked recessive mutation svb which causes an easily scored cuticle phenotype. Specifically, svb reduces the number and length of hairlike processes called denticles (Lindsley and Zimm, 1992). A control experiment using a similar cross ("no transgene" control) indicated that the svb phenotype is 100% penetrant as all animals displaying the if lethal phenotype (e.g. spheroidal muscles) also have missing and shortened denticles, while phenotypically if+ embryos always display wild type denticles.

To determine whether the if muscle phenotype is rescued with transgenic αPS2m8 or αPS2c function, somatic muscles from the experimental groups (if svb embryos bearing an αPS2 transgene) were examined under a microscope using polarized light. Remarkably, the somatic musculature of if mutant animals appears to be efficiently rescued when αPS2c (Figure 4.9A & B) or αPS2m8 subunits (Figures 4.9C & D) are transgenically expressed during embryogenesis. Specifically, embryos carrying the if mutation and either αPS2 transgene display muscles which appear to have normal morphology, while identically treated svb if control animals (with no transgene) never display muscles with normal morphology (Figure 4.9E & F).
FIGURE 4.9  Polarized light micrographs of if embryos and showing that the if muscle phenotype is rescued with transgenic PS2m8 or PS2c. (A and B) Two ventral views of an if embryo bearing a HS-αPS2c transgene. (C and D) Two ventral views of an if embryo bearing a HS-αPS2m8 transgene. (E and F) Two ventral views of an if mutant embryo (no transgene control). The somatic muscles in the if mutant (E and F) have collapsed into birefringent spheroidal masses (arrows) In contrast, no muscle detachment is displayed in if mutants bearing HS-αPS2 transgenes typically display wild type morphology. Moreover, the morphology of these transgenically rescued muscles appears wild type (see striated muscles in A, B, C, and D).
Not only do the rescued muscles appear to have wild type morphology in fixed preparations, but living if embryos (with rescued muscles) typically display coordinated body movements like those of similarly staged wild type embryos. Since these if mutant embryos also carry the svb mutation which causes lethality shortly after hatching (Lindsley and Zimm, 1992), rescued animals are obviously unable to develop beyond the early first instar larval stage. Even without the svb marker, if first instar larvae with transgenically rescued musculature are probably incapable of further development because of other requirements for if+ function or because of possible dominant effects caused by ectopic αPS2 subunits. Along these lines, it should be noted that if mutants often displayed gross defects in other tissues (e.g. gut and brain) despite the fact that their musculature had been rescued.

From these results, it is clear that either PS2m8 or PS2c expression is sufficient to replace the PS2 function missing in if mutants muscle cells. Reflecting on the dominant negative effect αPS2m8 subunits impart on wing morphogenesis in a wild-type background, I decided to address the question of whether transgenic αPS2 expression affects muscle attachment in wild-type embryos. This was easily accomplished by carefully looking at several hundred svb+ progeny from the previous experiment as their somatic musculature is phenotypically wild type (i.e. if+/Y, if+/if+ or if*/if; see Figure 4.8). No mutant muscle phenotypes
(e.g. spheroidal muscles) were observed in any of these animals. This negative result is not particularly surprising as expression of these subunits rescues the if muscle phenotype.

DISCUSSION:

Creation of Transgenic Lines That Express $\alpha_{PS2}$ Subunits

At the time I set out to make transgenes capable of ectopic PS integrin expression, two integrin subunit coding sequences had been cloned and sequenced. Specifically, full length cDNAs encoding the two alternatively spliced if gene products, $\alpha_{PS2m8}$ and $\alpha_{PS2c}$ subunits, were available to our lab for transgene construction. Using recombinant DNA techniques, I spliced the well characterized HSP70 promoter to either of these cDNAs to create two different chimeric genes: a HS-$\alpha_{PS2m8}$ and a HS-$\alpha_{PS2c}$ gene.

Since these cDNA coding sequences are devoid of introns, each chimeric gene is capable of expressing only one of the two alternative if gene products. Importantly, the cell specific splicing machinery (which normally determines where each alternative $\alpha_{PS2}$ structure is expressed) becomes irrelevant with respect to expression of cDNA encoded gene products. Thus the HS-$\alpha_{PS2c}$ cDNA construct should cause ectopic expression of PS2c subunits in cells where the eighth exon would normally be spliced out, while the HS-$\alpha_{PS2m8}$ cDNA
construct should express ectopic PS2m8 subunits in cells
which normally express PS2c subunits.

After constructing HS-α_{PS2m8} and HS-α_{PS2c} chimeric genes
in vitro, each gene construct was successfully transferred
into flies using P-element transfection vectors. Two
independent transgenic lines were selected: P[A] contains the
transgene designed to express α_{PS2m8} subunits, and P[1]
contains the transgene designed to express α_{PS2c} subunits. To
control for possible site specific influences on transgene
expression, six additional lines were recovered by jumping
the P[A] and P[1] transgenic inserts to additional
chromosomal locations. In all, I have created eight
different transgenic lines which contain a single copy of
either HS-α_{PS2m8} or HS-α_{PS2c} stably inserted into an autosomal
chromosome.

PS Integrin Expression in Transgenic Animals

Transgenic embryos were shown to express elevated levels
of α_{PS2} subunits following a single heat shock induction as
determined by Western blot analysis (Figure 4.3). Since the
HSP70 promoter is typically active in most cell types,
transgenic α_{PS2} synthesis is probably induced in virtually
every tissue. Importantly, my analysis of transgenic larval
head tissues indicates that transgenic α_{PS2} subunits are only
detected on the surface of cells which normally express PS
integrins. This observation strongly supports previous
reports which indicate that $\alpha_{PS}$ must first pair with $\beta_{PS}$ in order to be expressed on the cell surface (Leptin et al., 1989). Therefore, it appears that transgenic PS2 function is largely restricted to tissues which normally express PS integrins.

The wild type patterns of PS integrins on tissues were found to be dramatically changed in transgenic animals following heat shock induction. For example, transgenic wing imaginal discs display PS2 integrins on cells destined to make both surfaces of the wing blade, while wild type discs only express PS2 on cells destined to make the ventral surface (Figure 4.4). Since I find that the wild type patterns of PS1 persists on tissues despite transgenic PS2 expression, it appears that the level of $\beta_{PS}$ in cells is sufficient to promote the expression of both transgenic PS2 and endogenous PS1 integrins.

Taken together, these data indicate that transgenic tissues which would normally express only PS1, such as embryonic tendon cells and cells comprising the dorsal surface of the pupal wing blade, express both PS1 and PS2m8 or PS2c following heat shock induction. When these data are extrapolated to cells which normally express PS2 integrins, it appears that the normal complement of PS2 integrins is supplemented with a specific PS2 subtype (either PS2m8 or PS2c). For example, if wild type somatic muscle cells normally express the PS2c subtype, then HS-$\alpha_{PS2m8}$ transgenic
embryos likely express PS2m8 and PS2c on their muscles after heat shock induction. Thus, transgenic animals appear to superimpose a field of PS2m8 or PS2c integrins onto the wild type patterns of PS integrin expression.

**Transgenic PS2 Integrins Have Wild Type Function**

The transgenes were shown to express significant levels of functional PS2 using phenotype rescue experiments. Specifically, either transgene, when induced with repetitive heat shocks, confers enough if+ function to efficiently rescue the lethal phenotype associated with an integrin deficient genotype (i.e. mysXr04 if/+). This result confirms that the quality and quantity of transgenically expressed αPS2 subunits is sufficient to produce detectable levels of PS2 function in vivo. Taken together, the phenotype rescue and transgenic expression data strongly suggest that these relatively large transgenes, which were constructed in an involved multistep process (see materials and methods), are capable of functioning as they were designed to.

Along these lines, it should be noted that my HS-αPS2 constructs also function in *Drosophila* tissue culture cells. Transfecting my HS-αPS2 chimeric gene constructs into PS integrin deficient host cells dramatically changes cell behavior in "cell adhesion" assays (Bunch and Brower, 1992; Zavortink et al., 1993; Fogerty et al., 1994). Transformed cells display several properties consistent with the idea
that these constructs express functional integrin subunits: first, transformed cells acquire the ability to adhere, flatten and spread out on petri dishes coated with some purified extracellular matrix components (e.g. vitronectin); second, this transgene mediated cell adhesion is disrupted when soluble RGD peptides are added to the media; and third, the efficacy of cell adhesion is dramatically influenced when divalent cation concentrations in the media are manipulated (Bunch and Brower, 1992; Zavortink et al., 1993). All of these properties are very similar to properties displayed by vertebrate cells expressing wild-type integrin structures.

In addition to supporting the idea that the transgenes express functional PS2 integrins, experiments with these transformed cell lines have revealed the first functional differences between PS2m8 and PS2c integrins. That is, transformed cells display different ligand binding specificities depending on which PS2 integrin they express. For example, PS2c expressing cells adhere better to vitronectin than fibronectin while PS2m8 expressing cells bind to fibronectin more efficiently than to vitronectin (Zavortink et al., 1993). These data support the hypothesis that each PS2 subtype performs unique functions during development.

In Vivo Structure/Function Experiments Using Transgenes
With these transgenic tools, two experimental situations can be created in vivo: first, ectopic PS2m8 or PS2c integrins can be expressed in an otherwise wild-type genetic background to change the normal patterns of PS integrin expression; second, transgenic PS2m8 or PS2c integrins can be expressed in if mutant backgrounds to replace the missing if\(^+\) gene product(s). Careful analysis of integrin mediated morphogenic events under these experimental conditions should address issues concerning the function of different integrin structures during development. Below I discuss how muscle and wing morphogenetic events are influenced when transgenic manipulations cause alternative PS integrin structures to be expressed in these tissues.

Function of Alternative PS Integrin Structures in Muscles

The adhesion of somatic muscle cells to their tendon cell attachments is critically dependent on PS integrin function. As first described several decades ago, mys mutant embryos display dramatic defects in their somatic musculature (Wright, 1960). Prior to the first muscle contractions, muscles in mys mutants appear to be fully extended and properly inserted into their tendon cell attachments. The "myospheroid" muscle phenotype only becomes evident when muscles undergo contractions and pull away from their attachment sites. After several hours of contractions, almost all of the sarcomeric material has separated from the
body wall attachments and is localized within compact spheroidal bodies.

In wild type embryos, the highest concentrations of PS integrins are located at muscle attachment sites, i.e. the muscle/tendon cell interface. Since mys mutant embryos fail to express PS integrins on muscle or tendon cells, the mys muscle phenotype is apparently caused by the localized loss of PS integrins at muscle attachment sites (Leptin et al., 1989). Presumably, PS integrins facilitate muscle cell/tendon cell adhesion by binding one or more of the numerous extracellular matrix molecules which accumulate at the interface of these two cell types.

Interestingly, tendon cells express PS1 while muscle cells express PS2 integrins which results in a polarized distribution of PS integrin structures at muscle attachment sites. To determine the individual roles of PS1 and PS2 during muscle attachment, embryos carrying strong mutations in if or mew have been examined for somatic muscle defects. I have shown that PS2 integrins are necessary for the integrity of muscle attachment to tendon cells as if mutants display a muscle phenotype analogous to the muscle phenotype found in mys mutants. In contrast, results with strong mew mutants indicate that the PS1 on muscle cells is not required for muscle attachment as mew mutants have considerable muscle function relative to if or mys mutant embryos (Brower et al., 1995). One interpretation of this observation is that PS1 is
just one of multiple redundant adhesion molecules expressed by tendon cells.

From these mutant analyses, PS2 integrins on muscle cells clearly play a critical role in creating or maintaining adhesion between muscle and tendon cells. Since alternative splicing can create two different if gene products, the requirement for PS2 integrins on muscle cells may reflect a requirement for one or both PS2 integrin structures. To date, there are no data available to suggest which form(s) of PS2 integrins are expressed by muscle cells during embryogenesis.

One way of directly determining which PS2 structure is capable of functioning at muscle attachment sites is to rescue the if muscle phenotype with transgenically expressed PS2m8 or PS2c integrins. Using this experimental approach, I found that transgenic expression of either PS2m8 or PS2c is sufficient to rescue the if spheroidal muscles phenotype (Figure 4.8). In fact, the somatic musculature in rescued animals appears to be morphologically and functionally normal. Surprisingly, the small structural domain which distinguishes the PS2m8 and PS2c integrins (i.e. 25 amino acids near the presumptive ligand binding domain), does not discernibly influence the rescuing function of PS2 integrins during muscle attachment, although the possibility remains that subtle functional differences between the two forms may have been missed in my assay. For example, minor differences
in the morphology of muscles rescued by PS2m8 and PS2c may have been overlooked as my analysis used a light microscope to view whole mount preparations (instead of looking at sectioned muscles with an electron microscope).

In addition to the critical role of mediating adhesion at the interface of muscle and tendon cells, there is evidence that PS2 integrins play a role in the morphogenesis of sarcomere structure within somatic muscle cells. Primary cultures of myoblasts (from gastrula stage *Drosophila* embryos) can be induced to differentiate *in vitro* when cultured in serum containing fibronectin (Seecof et al. 1971; Gratecos et al., 1988). This *in vitro* differentiation process produces large contractile (twitching) multinucleated myotubules which display striations characteristic of authentic sarcomere formation. In contrast, myoblasts prepared from *mys* embryos (which lack PS integrin expression) fail to form these sarcomeric striations indicating that PS integrin function is necessary for creating these structures (Donady and Seecoff, 1972; Volk et al., 1990).

Interestingly, wild type muscle cells formed *in vitro* or *in vivo* express PS2 integrins at positions along their plasma membranes coincident with the location of Z-bands (Volk et al., 1990). These observations have led to the hypothesis that PS2 integrins specifically facilitate Z band formation, presumably by connecting intracellular Z-disk proteins to the extracellular matrix. The Z-disks in *if* muscles rescued with
either PS2m8 or PS2c, although not viewed directly, are probably formed normally as these muscles display striations characteristic of well anchored actin thin filaments, even after repeated contractions (Figure 4.9). Therefore, these data indicate that either form of PS2 can facilitate the creation of sarcomeric cytoarchitecture.

The role of PS1 in tendon cells remains obscure as mew mutant embryos have significant muscle attachment site integrity. Since my transgenes should be efficiently expressed in tendon cells, I looked for muscle attachment phenotypes which might be created by ectopic PS2 function in otherwise wild type tendon cells. For example, ectopic PS2 integrins might have a dominant negative effect on the function of the normal complement of adhesion receptors presumably expressed by tendon cells. No muscle defects were observed in wild type animals when transgenic \( \alpha_{PS2} \) subunits were induced to high levels. This negative result can be interpreted as supporting the idea that ectopic PS2 on tendon cells does not disrupt muscle attachment, but one caveat is that the presence of transgenic PS2 on tendon cells has not been confirmed directly.

Using all the available data, a working model emerges for the function of PS integrins during myogenesis in embryos. PS1 probably functions as an adhesion molecule promoting connections between tendon cells and muscle cells. Since PS1 expression does not appear to be critical for
muscle attachment site integrity, there are likely to be other undiscovered adhesion molecules expressed on tendon cells. Such a role for PS1 would not be particularly novel, as there are other examples in Drosophila of redundant adhesion molecules being expressed simultaneously during critical morphogenetic events (reviewed in Bunch and Brower, 1993). If PS1 does function during muscle attachment in concert with redundant adhesion molecules, this function should be revealed by finding mutations which compromise the function of the redundant gene product(s) and placing them in a mew genetic background.

PS2 integrins on muscle cells clearly function during muscle attachment and probably have an additional role in creating sarcomere cytoarchitecture. From the muscle rescue data, it seems plausible that wild type muscle cells may express only one of the two alternative forms of PS2. However, if future experiments find that significant levels of both PS2 structures are expressed on wild type muscle cells, then it would seem that PS2m8 and PS2c represent another situation where redundant adhesion molecules are used by Drosophila to effectuate a morphogenic event. Perhaps subtle differences between the function of PS2m8 and PS2c in muscle cells (not detected in my analysis) do exist, and these combined functions result in increased fitness by creating muscles more efficiently (or by creating muscles of higher quality) than a single integrin could alone.
The technique of rescuing if muscle defects with $\alpha_{PS}$ transgenes could be exploited much further to determine which $\alpha_{PS}$ sequences are critical for facilitating muscle attachment. For example, if muscle rescue experiments using transgenes expressing $\alpha_{PS1}$ subunits might show that $\alpha_{PS1}$ possesses considerable functional homology with $\alpha_{PS2}$ (such transgenes are now feasible as the cloning and sequencing of the $\alpha_{PS1}$ cDNA has been recently reported by Wehrli et al., 1993). Probably, the relatively divergent $\alpha_{PS1}$ structure will be unable to rescue if muscles, but then transgenes with chimeric $\alpha_{PS1}/\alpha_{PS2}$ coding sequences (e.g. $\alpha_{PS1}$ intracellular domain fused to $\alpha_{PS2}$ extracellular domain) could be engineered to ask which $\alpha_{PS1}$ domains can function in place of the corresponding $\alpha_{PS2}$ domains during muscle attachment. Similarly, the sequences within $\alpha_{PS2}$ subunits which are critical for function in muscle cells could be determined by directly mutating my transgenes (e.g. small deletions) and testing the rescuing capacity of the mutant $\alpha_{PS2}$ subunits. This line of inquiry promises to differentiate between sequences that are critical for specific functions (e.g. that interact with specific ligands at muscle attachment sites) and those that perform more universal integrin functions (e.g. divalent cation binding).

Function of Alternative PS Integrin Structures in Wings
PS integrins are required during metamorphosis to facilitate adhesion between the two epithelial layers that comprise the maturing wing blade. When PS integrin function is compromised on either epithelial surface, loss of adhesion is manifested in the adult wing blade as a "blister" phenotype (Jaffe and Brower, 1989; Zusman et al. 1990). As discussed in CHAPTER 2, the dorsal and ventral surfaces of the wing blade first become closely apposed at the onset of pupariation (upon disc evagination), and some cytoplasmic contacts are maintained throughout most, if not all, of the metamorphic period (Waddington, 1941; Milner and Muir, 1987; Fristrom et al., 1993). Upon eclosion, these intercellular connections apparently hold the wing surfaces together as the hydrostatic pressure within the hemolymph causes the folded wing surfaces to unfurl into a flat blade. The elimination of PS integrins from either surface probably prevents these cytoplasmic connections from forming properly, and blisters result when the unattached surfaces are pushed apart by pressurized hemolymph.

Intriguingly, PS1 function is only required on dorsal cells, while an equivalent requirement for PS2 function is restricted to ventral cells. Since both epithelial layers undergo essentially the same differentiation program, PS1 and PS2 would seem to perform indistinguishable functions as they mediate adhesion between seemingly identical cell types. Thus the functional significance of employing different PS
integrin structures during wing morphogenesis is not at all obvious.

To determine whether the normal distribution of PS integrin structures on maturing wings is necessary for effectuating adhesion between wing surfaces, I performed experiments to disrupt the wild type pattern of PS integrins during wing morphogenesis. Using my HS-αPS2 transgenes, wing imaginal discs could be induced to express high levels of ectopic PS2 on their presumptive dorsal surface (Figure 4.4). Importantly, transgenic expression of ectopic PS2 on dorsal cells does not appear to reduce greatly the normal complement of endogenous PS1 on these cells as both PS1 and PS2 are readily detected on the dorsal surface of the maturing wing blade after transgene induction. Extrapolating these observations to the other surface suggests that transgenic expression of αPS2m8 or αPS2c subunits probably creates an abnormal distribution of PS2 subtypes on ventral cells. For example, if ventral cells normally express PS2c, then transgenic expression of αPS2m8 subunits should cause these ventral cells to express significant levels of both PS2c and PS2m8.

Animals induced to express transgenic αPS2m8 subunits during wing morphogenesis were found to display significant levels (10-33%) of wing blistering (Table 4.2); but surprisingly, identical experiments with transgenic lines expressing αPS2c result in wild type wing morphology.
Apparently, ectopic $\alpha_{PS2m8}$ subunits act dominantly to disrupt adhesion between the two epithelial surfaces of the wing blade, while ectopic $\alpha_{PS2c}$ subunits do not affect wing morphogenesis.

In fact, the hypothesis that transgenically expressed $\alpha_{PS2m8}$ subunits might disrupt wing morphogenesis had been considered during the experiment which mobilized the $\alpha_{PS2m8}$ transgene from its original chromosomal position in the $P[A]$ line to new chromosomal locations (Figure 4.2B). One of the new insertion events produced the $P[C]$ line which displays blisters without heat shock induction (~15% of wings display blisters at 25°C), and a higher level of blistering when heat shocked (~35% of wings display blisters when animals were heat shocked every 7 hours). At that time, I concluded that this blister phenotype could be caused by one of several mechanisms:

1) $P[C]$ may be located near an endogenous enhancer of transcription that promotes high levels of transgenic $\alpha_{PS2m8}$ expression in wing tissue without heat shock induction. For a discussion of these types of enhancer/transgene interactions, see O'Kane and Gehring (1987).

2) During mobilization, the $\alpha_{PS2m8}$ coding sequence within the $P[C]$ transposon could have been mutated (e.g. truncated) producing a defective $\alpha_{PS2m8}$ subunit with a dominant negative activity in wings.
3) $P[C]$ may have landed in a gene disrupting the gene's wild type function, and this novel mutation could be responsible for the dominant blister phenotype.

Under my guidance, Andreas Reindl performed experiments to distinguish among these hypotheses. The results from this analysis definitively rule out the second hypothesis, and although the data do not prove the first hypothesis, they are entirely consistent with the idea that the wing blisters associated with $P[C]$ are caused by transgenically expressed $\alpha_{PS2m8}$ subunits (Andreas Reindl, 1993 Diplom Thesis). Taken together with my analysis of $P[C]$ function in rescue assays (e.g. $P[C]$ rescues the $mys^{KRO4}$ if/++ genotype), the available data make a strong argument that $P[C]$ inserted near an enhancer element that promotes $\alpha_{PS2m8}$ expression in wing tissue during metamorphosis in the absence of heat shock induction (and even higher levels of expression are achieved with heat shock induction).

After communicating my observation that ectopic $\alpha_{PS2m8}$ expression disrupts wing morphogenesis to Nicholas Brown at the University of Cambridge (UK), we were informed that he had obtained similar results using an analogous experimental approach. Specifically, Nick Brown had found wing blisters in every animal (100% penetrance) bearing a transgene that expresses $\alpha_{PS2m8}$ subunits in the developing wing (unpublished data). Presumably, the quantitative difference between our data (Brown's $\alpha_{PS2m8}$ transgenic line caused significantly
higher levels of blistering) reflects a difference in the level of $\alpha_{PS2m8}$ expression achieved in each experiment. That is, the HSP70 promoter in my constructs likely produces lower steady state levels of $\alpha_{PS2m8}$ subunits in wing cells (after heat shock induction) than the promoter elements used in Brown's experiment. Importantly, both sets of results support the same conclusion: the expression of transgenic $\alpha_{PS2m8}$ subunits in wing tissue effectively disrupts adhesion between the two epithelial surfaces.

Since all of the transgenes in these experiments were designed to express PS2 on both surfaces of the maturing wing blade, the dominant wing blister phenotype could result from $\alpha_{PS2m8}$ function in one epithelial layer or in both layers. For example, these three possibilities exist: 1) $\alpha_{PS2m8}$ subunits may compromise the function of adhesion molecules expressed on dorsal cells. 2) $\alpha_{PS2m8}$ subunits may compromise the function of adhesion molecules expressed on ventral cells. 3) $\alpha_{PS2m8}$ subunits may compromise the function of adhesion molecules on both cell types. It is easy to imagine relatively simple mechanisms through which ectopic $\alpha_{PS2m8}$ subunits might disrupt adhesion on dorsal or ventral cells. For example, ectopic $\alpha_{PS2m8}$ might compete with $\alpha_{PS1}$ in dorsal cells for components necessary for PS1 function (e.g. extracellular ligands or intracellular cytoskeletal components), thus reducing the degree of PS1 mediated
adhesion. Alternatively, if PS2c is the normal integrin structure expressed by ventral cells, then ectopic \( \alpha_{PS2mB} \) subunits may compete with \( \alpha_{PS2c} \) subunits for molecular components necessary for PS2c function on this surface.

Using genetic tools available in our lab, experiments were undertaken to determine whether the function of PS1 (on dorsal cells) or PS2c (on ventral cells) is compromised in animals expressing transgenic \( \alpha_{PS2mB} \) subunits. My first experimental approach was designed to determine whether the blister phenotype caused by transgenic \( \alpha_{PS2mB} \) could be enhanced or suppressed by manipulating the levels of endogenous \( \alpha_{PS1} \) in dorsal cells or \( \alpha_{PS2} \) in ventral cells. For example, if ectopic \( \alpha_{PS2mB} \) causes blisters by interfering with PS1 function on ventral cells, then reducing PS1 levels in transgenic animals might be expected to increase blister frequency, while increasing PS1 levels might reduce blister frequency.

To specifically change the quantity of \( \alpha_{PS1} \) or \( \alpha_{PS2} \) in animals bearing HS-\( \alpha_{PS2mB} \) transgenes, the level of \textit{mew}^+ or \textit{if}^+ function was manipulated through changes in gene copy number (Figure 4.6). My data indicate that genetic backgrounds with reduced \textit{mew}^+ or \textit{if}^+ gene copy do not modify the dominant blister phenotype associated with HS-\( \alpha_{PS2mB} \) (Table 4.3). Likewise, genetic backgrounds with increased \textit{if}^+ copy number did not appear to significantly modify the HS-\( \alpha_{PS2mB} \) blister phenotype (Table 4.4). One interpretation of these data is
that the predicted reductions of PS1 and PS2 function (attributed to these genetic backgrounds) may not have been realized, or if realized, may not be expected to dramatically modify the blister phenotype. These arguments are not easily supported as there are considerable genetic data to indicate that animals with reduced integrin gene copy number express significantly reduced levels of PS integrin function. For example, the frequency of blistering associated with the if3 hypomorphic mutation is significantly enhanced when the mutation is placed in a mysnull/+ genetic background, and the antimorphic mysXR04 mutation is lethal when placed in a ifnull/+ genetic background indicating that the if/+ genetic background dramatically lowers PS2 function. Likewise, one copy of the duplication Dp(1;4)r+(if+) has been shown to be sufficient to completely rescue blister phenotypes in if mutant flies. Considering these data, it seems improbable that the failure to modify the HS-αPS2m8 dominant blister phenotype with altered integrin gene copy number represents a failure to reduce (or supplement) endogenous PS1 or PS2 levels enough to dramatically increase (or decrease) blistering.

Similar experiments were also performed using HS-αPS2c (and HS-βPS) to augment endogenous PS integrin levels in animals bearing the blister causing HS-αPS2m8 transgene. For example, these experimental conditions could be expected to
rescue the blister phenotype if blistering is caused by competition between transgenic $\alpha_{PS2m8}$ subunits and endogenous $\alpha_{PS2c}$ subunits. My results indicate that supplementing endogenous integrin subunit pools with $\alpha_{PS2c}$ and/or $\beta_{PS}$ subunits does not suppress blistering in HS-$\alpha_{PS2m8}$ animals.

When both approaches are considered together, the data indicate that the HS-$\alpha_{PS2m8}$ dominant blister phenotype is not readily modified by altered levels of if* or mew* function in wings. These results appear to make a strong argument against the hypothesis that transgenic $\alpha_{PS2m8}$ subunits cause blistering by lowering (e.g. through competition for a limited pool of free $\beta_{PS}$ or unbound integrin ligands) the wildtype levels of PS integrin function in wings. Therefore, it is probably the activity of ectopic PS2m8 integrin that is directly responsible for the events leading to the blister phenotype. Perhaps ectopic PS2m8 expression in wings causes an ectopic signaling event to occur that prevents wing cells (dorsal, ventral or both) from expressing their normal complement of adhesion molecules.

Since animals bearing $P[HS-\alpha_{PS2c}]$ transgenes do not display wing phenotypes, the activity of transgenic PS2c integrin does not appear to disrupt wing morphogenesis. Although the activity level of transgenic PS2c was not measured directly, there are several observations to indicate that substantial levels of transgenic PS2c function were
probably realized in these experiments. For example, other transgenes containing the identical HSP70 promoter (HS-βPS and HS-αPS2m8) have been shown to express detectable levels of integrin function in wings when induced with heat shocks. That is, the \( P[mys^+]B1 \) transgene provided enough integrin to rescue blistering associated with the \( mys^{nj42} if^3 \) genotype (Chapter 2), and the \( P[HS-\alpha_{PS2m8}] \) transgenes provided enough integrin to cause blistering in wild type genetic backgrounds. Furthermore, I have shown that the \( P[HS-\alpha_{PS2c}] \) transgenes are capable of expressing significant levels of integrin function (after heat shock) as they rescue both the \( if \) embryonic muscle phenotype and the \( mys^{XR04} if/^+ \) lethal phenotype. These data support the hypothesis that ectopic PS2c function on the dorsal surface of the developing wing blade does not disrupt wing morphogenesis.

Previous experiments have shown that PS2 integrins are not expressed in imaginal disc cells destined to make the dorsal wing surface, and that PS2 integrin function is not required on this surface (Chapter 3). Since my data indicate that transgenic PS2c expression on dorsal wing cells is compatible with normal wing morphogenesis, it appears that the wild type pattern of PS2 integrin expression does not reflect a critical requirement to exclude PS2c integrins from dorsal cells. The functional significance surrounding the wild type patterns of PS1 and PS2 integrins in wings (and
also in other tissues) remains one of the major unanswered questions; at the end of this section, I propose a concise set of experiments that should determine which specific integrins (PS1, PS2c and PS2m8) can rescue \textit{if}\textsuperscript{-} ventral cells and \textit{mew}\textsuperscript{-} dorsal cells. For example, the proposed experiments should determine whether ectopic PS2c expressed in \textit{mew}\textsuperscript{-} dorsal cells can function in place of PS1.

Taken together, the data from transgenic expression of the two $\alpha_{PS2}$ subtypes appears to support the idea that PS2c is the integrin that normally satisfies the requirement for \textit{if}\textsuperscript{+} function on the ventral surface of the wing. To directly test this hypothesis, I attempted to rescue blisters (caused by loss of \textit{if}\textsuperscript{+} function) with transgenically expressed $\alpha_{PS2c}$. Experiments were set up, but my approach failed to provide interpretable results for uninteresting reasons; i.e. transgene expression requires a heat shock induction protocol that proved to be incompatible with the best method for creating \textit{if}\textsuperscript{-} wing tissue. Because this experiment does not promise to produce positive results expediently, I propose the creation of a constitutively expressed (in wings) $\alpha_{PS2c}$ transgene to execute an almost identical approach to the problem without detrimental heat shocks. In addition, the new $\alpha_{PS2c}$ (and $\alpha_{PS2m8}$) transgene constructs could also be designed to express $\alpha_{PS2}$ subunits in a ventral or dorsal compartment specific pattern; experiments with such constructs could be used to quickly and clearly determine
which wing surface is affected by the dominant action of transgenic PS2m8 integrin, and they could also be used in blister rescue experiments to determine whether PS2m8 or PS2c integrins can function in place of mew\textsuperscript{+} function in dorsal cells and if\textsuperscript{+} in ventral cells.

To construct novel transgenes with these special expression properties, one would need to utilize enhancer elements capable of dorsal or ventral specific expression in wings. Theoretically, such enhancers will become available when the mew and if promoters are fully characterized. In fact, an if\textsuperscript{+} transgene has already been created using genomic if promoter sequences (Nick Brown, personal communication), and excision of the eighth exon from this transgene (using recombinant DNA techniques) should provide a construct which expresses PS2m8 specifically on the ventral wing surface. Likewise, dorsal specific \( \alpha_{PS2} \) transgenes could be created by splicing cDNAs encoding \( \alpha_{PS2} \) to the apterous promoter as this promoter has been shown to express heterologous gene products in the dorsal compartment of wing discs (Danny Brower, personal communication). If ventral specific PS2 or dorsal specific PS1 transgenes are shown to rescue blisters associated with if\textsuperscript{-} or mew\textsuperscript{-} clones in wings, then the function of different \( \alpha_{PS} \) subunits in each compartment could be determined using transgenes constructed with the reciprocal promoter. For example, \( \alpha_{PS2c} \) expressed in dorsal wing cells might rescue blisters associated with mew\textsuperscript{-} clones. Finally,
chimeric $\alpha_{PS}$ (e.g. $\alpha_{PS1}/\alpha_{PS2c}$) and mutant $\alpha_{PS}$ (e.g. small deletions) transgenes could be used to examine numerous aspects of the relationship between $\alpha_{PS}$ structure and PS integrin function during wing morphogenesis.
During the past decade, research has revealed that the integrin family of homologous receptors mediate most interactions between animal cells and the extracellular matrix. As the primary receptors for the extracellular matrix components, integrins execute two vital roles in cell biology: first, integrins regulate cell adhesion to the extracellular matrix; second, integrins facilitate bidirectional signalling between the cell and the extracellular environment. Integrins are thought to perform essential roles in a wide variety of biological phenomena such as the differentiation of cells, the formation of tissues, the regulation of cell-mediated immune responses, the clotting of blood, the fertilization of eggs, and the metastasis of cancer cells. Although many crucial questions regarding the mechanisms through which these complex biological processes are accomplished still remain unanswered, basic research describing the mechanisms of integrin function promises to provide important insights into each of these biological phenomena.

Currently, research efforts often focus on the relationship between integrin structure and function. Using molecular cloning and sequencing techniques, the primary
structural features shared by all members of the integrin family, as well as the sequences that distinguish distinct integrins from one another, are readily recognizable. The immediate challenge has been to clearly define the specific cell biological events that are mediated by integrins. When this knowledge is at hand, a dissection of the relationship between integrin structure and function can be undertaken.

This dissertation has presented an examination of PS integrin function during *Drosophila* development. Previous studies have shown that this class of integrins are critically required for the morphogenesis of a variety of larval and adult tissues and organs. The general conclusion from these studies is that PS integrins function as adhesion molecules that mediate specific adhesion events between different cell types or epithelial layers. My analyses expand our knowledge of integrin function during *Drosophila* tissue morphogenesis in three major ways: first, I found that, in order to properly form the connections that hold the two surfaces of the adult wing blade together, integrins must function during two distinct periods of cell-cell apposition in the developing pupal wing (Chapter 2); second, I found that the PS2 integrin subtype is critically required for adhesion between cells that express complementary patterns of PS1 and PS2 (Chapter 3); third, I found that disruption of the normal, complementary pattern of PS1 and PS2 expression in one model tissue, the wing, is incompatible with adhesion
(Chapter 4). As discussed below, all of these data indicate that the roles PS integrins play during Drosophila tissue morphogenesis are far greater than simply mediating attachment of cells to the extracellular matrix; I conclude that PS integrins probably have signalling activities in addition to their role as cell adhesion molecules.

Integrins Required in Wings During Two Apposition Events

Previous studies have shown that PS integrins are clearly necessary to effectuate adhesion between the two surfaces of the adult wing blade. Upon disc eversion, the two wing epethilia become closely apposed, then the epethilial surfaces separate considerably before becoming closely apposed for a second time. I have found that integrin function is required during both the first and second apposition events for adhesion to be realized between the adult wing surfaces. Intriguingly, these data indicate that integrin function is required during the first apposition period to "prepare" the epethilial surfaces for the second apposition event. Since integrins often transduce signals to cells upon binding ligand, one interpretation of my results is that PS integrins could transmit a signal to the epethelia during the first apposition event to elicit specific changes in cell biology, changes that are required for the second apposition event to occur. For example, integrin signalling during the first apposition event might
induce the expression of adhesion molecules necessary for the second apposition event. Whatever the specific mechanism, my data indicate that an integrin-dependent cellular event occurs during the first period of apposition that enables the epethelia to come together and form basal connections during the latter period of apposition. Therefore, some cellular transformation appears to occur around the time when integrins are first required in wing morphogenesis, and this transformation may result directly from a signal generated when integrins bind ligands.

**PS2 Function Required in Tissues Expressing Both PS1 and PS2**

I found that PS2 integrins are critically required for adhesion in tissues (midgut, muscle attachments & wing) that express both PS1 and PS2. Intriguingly, I have shown that PS2 is critically required on only one surface of the developing wing blade (the ventral epethelium). Furthermore, my data, when considered in the light of the restricted patterns of PS2 expression within embryonic tissues, strongly suggest that PS2 integrins are critically required on only one surface of the developing midgut (the sheet of visceral musculature), and one surface of muscle attachments (the muscle cell). Since PS1 integrin has been shown to be expressed on and/or required on the opposing surface in all of these tissues (wings, muscle attachments, and midgut), adhesion between these surfaces appears to be accomplished
with distinct integrin structures that are known to possess unique functional properties in vitro. If the sole function of PS integrins is to hold cells together by binding to the extracellular matrix that separates them, then it is not obvious why distinct integrins are employed on either side of the same matrix. This point is especially relevant in light of the fact that there are no data to suggest that these matrices have an intrinsic polarity. One interpretation of these data is that PS1 and PS2 have additional roles beyond simple cell adhesion in these tissues, and the distinct integrin structures are employed to execute cell-type specific signalling activities. For example, PS2 may be employed in visceral and somatic muscle cells to promote muscle-specific changes in the cytoskeletal architecture upon ligand binding, while PS1 ligand binding may transduce another type of signal to midgut epithelium and tendon cells.

The Complementary Pattern of PS1 and PS2 in Wings Is Required

Since the two surfaces of the wing appear to undergo nearly identical differentiation programs, the biological significance behind the complementary requirements for PS1 (on dorsal surface) and PS2 (on ventral surface) was not obvious, and thus it seemed plausible that PS1 and PS2 might prove to be functionally interchangeable on these surfaces. However, I found that disrupting the wild-type pattern of PS integrin expression in wings by adding additional PS2m8
results in loss of adhesion between the two surfaces. Since my data are entirely consistent with the hypothesis that PS2m8 activity directly causes the blister phenotype, I conclude that different PS integrins are not functionally equivalent in wings, and the blister phenotype is probably caused by ectopic PS2m8 function on the dorsal surface of the developing wing blade. If true, then the pattern of PS integrins must have some critically important biological function.

Because my data indicate that adding more of a known adhesion molecule (PS2m8) to wings causes loss of adhesion, it would appear that PS integrins in wings have activities in addition to simple adhesion, and these activities may necessitate the restricted pattern of PS1 and PS2 expression in wing discs. Since I have shown that PS integrins are required in wings during two distinct periods of apposition, one interpretation of the biological significance of the spatially restricted requirements for PS1 and PS2 is as follows: during the first apposition event, PS1 and PS2 may send distinct signals to the dorsal and ventral epethilia, respectively, and these signals would then prepare each epethilial surface for the second apposition event. For example, PS1 signalling might induce the expression of a cell adhesion protein (CAM#1) in dorsal cells, while PS2 signalling might induce the expression of another cell adhesion protein (CAM#2); then after these epethilia
separate, the surfaces could be brought together or aligned during the second phase of apposition by heterophilic binding of CAM#1 and CAM#2.

Very recently, Danny Brower (University of Arizona) has performed additional experiments supporting the hypothesis that the pattern of PS integrin expression is critical during the early requirement for integrins, and less so during the latter requirement for integrins in wings (Brower et al. 1995; and D. Brower, unpublished observations). Therefore, our current working hypothesis is that PS integrins are important for adhesion and dorso-ventral specific signalling during the initial phase of tight apposition between the two epidermal surfaces, and during the latter phase of tight apposition, integrins are again required for adhesion and the construction of the basal connections that will hold these surfaces together.

General Significance to the Field of Integrin Research

My research has advanced our understanding of the requirements for PS integrins in Drosophila development, and significantly, my results are the first to suggest that signalling occurs during in vivo adhesion events mediated by PS integrins. Since the integrin class of receptors are known to have the intrinsic ability to transduce signals from the extracellular matrix to the cytoplasm, the most straightforward interpretation of my observations is that PS
integrins mediate signalling in addition to their role as cell adhesion molecules. If this hypothesis is correct, then further examination of PS integrin function in Drosophila tissues should reveal general mechanisms through which integrins perform their adhesion and signalling activities. Since the Drosophila model organism is particularly amenable to classical genetic, molecular genetic, and biochemical experimentation, this system remains one of the best choices to examine basic aspects of integrin function in vivo.

The immediate challenge is to directly test the hypothesis that the spatially restricted requirements for PS1 and PS2 in the developing wing actually reflect an absolute requirement for PS1 on the dorsal surface and PS2 on the ventral surface, and I presented an experimental approach to accomplish this goal in Chapter 4. The next step will be to prove the hypothesis that these distinct PS integrins are mediating dorso-ventral signalling during wing morphogenesis, and this hypothesis can be examined in several ways. One approach would be to set up a genetic screen to identify proteins that physically interact with the cytoplasmic domains of PS integrins (e.g. the "Two-Hybrid" screen discussed in Chapter 1). This approach should identify the cytoplasmic proteins that integrins interact with, and if these integrins do mediate signalling events in the pupal wing, it seems likely that a classical signalling protein (e.g. a protein kinase) might be found using this type of
approach. Alternatively, genetic complementation tests could be set up to look for specific interactions between integrin mutations and mutations in loci known to encode proteins participating in various intracellular signalling pathways (e.g. focal adhesion kinase, FAK). For example, if the wing blister phenotype associated with partial loss-of-function integrin mutations is enhanced when FAK activity is reduced below wild-type levels and suppressed when FAK activity is increased above wild-type levels, then these data would strongly support the hypothesis that PS integrins mediate signalling in wings through the FAK pathway.

Additional steps would be required to follow up such promising results including determining the sequences within the integrin's intracellular domain that activate the signalling pathway, determining the ultimate cellular event that is achieved through the signalling pathway, determining how the cellular event is translated into a specific morphogenetic event, determining the mechanism through which the integrins transduce signals through the plasma membrane, determining which PS integrins are able to activate this signalling pathway, and finally determining whether other tissues in Drosophila (e.g. the midgut & somatic muscles) or other animals employ the same type of integrin mediated signalling pathway. Although such a project will require an enormous effort, the knowledge gained from this type of
research will undoubtedly provide key insights into many aspects of animal biology.
CHAPTER 6
MATERIALS AND METHODS

Drosophila Strains

Culture Conditions

All flies were reared in plastic vials (ca. 3cm diameter x 10cm) containing a layer of food at the bottom and a foam stopper at the top. The basic food medium consisted of a mixture of Carolina Biological Formula 4-24 Instant Drosophila Medium (63 g/l), Quaker Quick Oatmeal (45 g/l), agar (0.5% w/v), and proprionic acid (0.1% v/v) in water; this recipe is essentially as previously described (Condie and Brower, 1989), except that proprionic acid has been substituted for another antifungal agent. Food was prepared by heating the ingredients until the agar granules went into solution, then small aliquots (~20 ml) of the hot slurry were poured into the bottom of vials. The hot aliquots of food were then allowed to solidify by cooling vials to room temperature (about 20°C). At this stage of preparation, food vials were either kept at 20±3°C for use within several days or stored at 4°C (to keep fresh) for use within several weeks.

Just prior to placing adult flies (parents) into prepared vials, dry baker's yeast (~0.5 g) was sprinkled into each vial to enrich the basic food (as females prefer to lay
eggs on food rich in protein). The food in vials of growing larvae was also supplemented with yeast in order to optimize larval growth rates. Similarly, vials with larvae competing for feeding space were typically split into several fresh vials to reduce the detrimental affect of overcrowding on larval growth rates. Most flies were raised in high humidity incubators at a constant temperature of 25°C (standard growth temperature), although several experiments required different temperatures as will be noted in the appropriate section.

**Marker Mutations**

Mutant chromosomes typically carried some combination of genetic markers including $yw\ svb\ sn^3\ v\ g^2\ r^{36}$ or $f$ on X-chromosomes (aka first chromosomes), $cn$ or $Sp$ on second chromosomes, and $ry$ or $Sb$ on third chromosomes (Lindsley and Zimm, 1992). These marker mutations produce phenotypes which allowed identification of animals bearing specific mutant chromosomes.

**Balancer Chromosomes**

Special "balancer" chromosomes, which have been engineered (through multiple rearrangements) to effectively eliminate meiotic recombination (see e.g. Ashburner, 1989), were used to create stable stocks of animals bearing chromosomes with recessive lethal mutations. X-chromosomes with lethal mutations were balanced over the $FM7c$ chromosome
(Lindsley and Zimm, 1992). Second and third chromosomes (autosomes) bearing recessive lethal mutations were placed over CyO or TM3 balancer chromosomes (Lindsley and Zimm, 1992), respectively.

Duplications

Several experiments required crosses with males bearing an X-linked lethal mutation (i.e., males bearing a mys or if lethal allele). To create viable mys\textsuperscript{lethal} or if\textsuperscript{lethal} males, I employed autosomes with X-chromosome duplications (\textit{Dp}) that contain either the \textit{mys}\textsuperscript{+} or the \textit{if}\textsuperscript{+} locus, respectively. Specifically, males carrying these X-chromosome lethals were rescued by \textit{Dp}(1;2)\textit{sn}\textsuperscript{+}72\textit{d}, which is \textit{sn}\textsuperscript{+} and \textit{mys}\textsuperscript{+}, or \textit{Dp}(1;4)\textit{r}\textsuperscript{+}, which is \textit{if}\textsuperscript{+} and \textit{f}\textsuperscript{+} (Lindsley and Zimm, 1992); in each case, all relevant chromosomes in the cross were marked with \textit{sn} or \textit{f} to follow the duplication in the progeny. For example, to examine the viability of \textit{mys}\textsuperscript{XR04} +/+ if\textsuperscript{lethal} animals (Chapter 3), \textit{mys}\textsuperscript{XR04} \textit{f36a}/FM7c females were crossed to if\textsuperscript{lethal} \textit{f36a}; \textit{Dp}(1;4)\textit{r}\textsuperscript{+} males, and the \textit{f36a} to \textit{f}\textsuperscript{+} ratio among the \textit{mys}\textsuperscript{XR04} +/+ if\textsuperscript{lethal} progeny was determined. The reciprocal cross, using \textit{Dp}(1;2)\textit{sn}\textsuperscript{+}72\textit{d} to rescue the \textit{mys}\textsuperscript{XR04} males, was also performed, except that all chromosomes were marked with \textit{sn}\textsuperscript{3}. These crosses are presented in Figure 3.2, but both the specific duplication names (\textit{Dp}(1;2)\textit{sn}\textsuperscript{+}72\textit{d} & \textit{Dp}(1;4)\textit{r}\textsuperscript{+}) and the marker mutations (\textit{sn} & \textit{f}) have been omitted for clarity.
Construction of Integrin Transgenes

The transgenes $P[HS-\alpha_{PS2m8}]$ and $P[HS-\alpha_{PS2c}]$ contain the Drosophila HSP70 promoter controlling the transcription of the integrin $\alpha_{PS2m8}$ and $\alpha_{PS2c}$ cDNAs, respectively. These constructs eliminate much of the authentic if mRNA's 3' untranslated sequences, and replace them with 3' untranslated sequences (including the polyadenylation site) from the Drosophila tubulin $\alpha1$ gene. Two HSP70/if cDNA/tubulin fusion genes (e.g. see fusion gene "cassette" in Figure 6.1) were constructed by replacing the fushi-tarazu sequences of plasmid F449 (kindly provided by Gary Struhl) with alternatively spliced cDNA sequences from the inflated (if) gene (Bogaert et al., 1987). Specifically, the HS-$\alpha_{PS2m8}$ and HS-$\alpha_{PS2c}$ constructs contain 450 bp from the HSP70 gene beginning 260 bp upstream from the start of transcription and extending an additional 190 bp into the 5' untranslated sequences of the HSP70 mRNA (Struhl, 1985). This HSP70 promoter is followed by if cDNA sequences that extend from 8 bp upstream of the translation start codon (Sal I site) to 158 bp downstream (Sac I site) from the translation stop.
FIGURE 6.1 Recombinant DNA techniques used to construct pCHSα2+, a plasmid containing the \( P[HS-\alpha_{PS2c}] \) transposon. Note that pCHSα2- (containing \( P[HS-\alpha_{PS2ms}] \)) was created using an identical protocol, except that \( if \) cDNA encoding \( \alpha_{PS2ms} \) (75 bp shorter) was used. Enzyme abbr.: Sal, Sal I; Sac, Sac I; Apa, Apa I; H3, Hind III; Xba, Xba I.
codon. The 810 bp from the tubulin gene that follows the cDNA contains 230 bp of 3' untranslated, transcribed sequences from the Drosophila tubulin α1 gene and an additional 580 bp beyond the polyadenylation/cleavage site (Lawrence et al., 1987).

The only difference between the \( P[\text{HS-}\alpha_{\text{PS2m8}}] \) and \( P[\text{HS-}\alpha_{\text{PS2c}}] \) transgenes is that \( P[\text{HS-}\alpha_{\text{PS2m8}}] \) lacks the 75 bp encoding exon #8. The final step in the construction of these transgenes was to place the alternatively spliced HS-\( \alpha_{\text{PS2}} \) fusion genes into pCarnegie20NX transformation plasmid vectors as shown in Figure 6.1; thereby producing two transformation competent plasmid vectors pCHSa2+ and pCHSa2-, respectively. The plasmid pCarnegie20NX (kindly supplied by Gary Struhl) is identical to the \( ry^+ \) P-element-based transformation vector pCarnegie20 (Rubin and Spradling, 1983), except that novel Not I and Xba I restriction sites have been engineered into the multiple cloning site in place of the original Hpa I and Sal I sites respectively (Gary Struhl, personal communication). All pCarnegie based plasmids (e.g. pCHSa2+ and pCHSa2-) contain sequences necessary for P-element-based transposition (P-element inverted repeats) that flank both the gene of interest (e.g. HS-\( \alpha_{\text{PS2m8}} \) and HS-\( \alpha_{\text{PS2c}} \)) and the marker gene \( ry^+ \) (to facilitate selection of transformants) as shown in Figure 4.1C. In addition, all the plasmids used in this study contain the
same origin of replication and ampicillin resistance gene found in pUC18 (Yanisch-Perron et al., 1985).

The \( P[\text{mys}^+] \) transgene, which expresses \( \beta_{PS} \) subunits under the control of the HSP70 promoter (Figure 2.7), was derived from the previously described plasmid pCHSPS\( \beta \) (Bunch et al., 1992). Briefly, Tom Bunch constructed this heat shock inducible transgene by fusing the coding region of a \( \text{mys}^+ \) cDNA to the same HSP70 promoter and tubulin 3' untranslated sequences I had employed during the construction of \( P[\text{HS-}\alpha_{PS2m8}] \) and \( P[\text{HS-}\alpha_{PS2c}] \). Unlike \( P[\text{HS-}\alpha_{PS2m8}] \) and \( P[\text{HS-}\alpha_{PS2c}] \), which were placed into pCarnegie20NX (Figure 6.1), the \( P[\text{mys}^+] \) transgene was placed into the pCasper (Klemenz et al., 1987) transformation vector. Therefore, the \( P[\text{HS-}\alpha_{PS2m8}] \) and \( P[\text{HS-}\alpha_{PS2c}] \) transgenes reside on transposable elements with the \( \text{ry}^+ \) gene (a selectable marker), while the \( P[\text{mys}^+] \) transgene resides on a transposable element with a different selectable marker, the mini-white\( ^+ \) gene \( (w^+) \).

Insertion of Transgenes Into Flies

\( P \)-element-based transposition of the \( P[\text{HS-}\alpha_{PS2m8} \text{ry}^+] \), \( P[\text{HS-}\alpha_{PS2c} \text{ry}^+] \) and \( P[\text{mys}^+ \text{w}^+] \) transposons from their position on plasmids (described above) into the genome of flies was accomplished using Gary Struhl's protocol which is based on previously described techniques (reviewed by Ashburner, 1989). The large transposon bearing plasmids (e.g. pCHS\( \alpha_2^+ \))
were isolated from bacteria using a gentle lysis technique, two rounds of CsCl centrifugation, an ethanol precipitation and then resuspended in 1mM Tris-Cl pH 8 (Maniatis et al., 1982). Two "Wings Clipped" helper plasmids WC-pBR and WC-pUC (kindly provided by Gary Struhl) were similarly prepared. These plasmids contain a P-element encoding the transposase that catalyzes the insertion of the transgenes into chromosomes, but they do not contain a functional (mobile) transposon, as the P-element's inverted repeats have been excised; thus the "wings clipped" designation. A cocktail of plasmids (final plasmid DNA 200µg/ml) was prepared for injections by mixing both of these helper plasmids (50 µg/ml each) with plasmid DNA (100 µg/ml) containing the integrin transgene to be transposed (e.g. pCHSα2+). (Theoretically, the employment of either helper plasmid should have been adequate, but there was no compelling reason to spend the effort to determine this empirically.)

Just prior to using the DNA for embryo injection, 1/20 volume of McCormick's green food dye was added to the DNA solution (to enhance the observation of DNA flow into embryos). Finally, the DNA solution was centrifuged in an eppendorf microfuge (~20 seconds), and the solution (minus particulates) backloaded into a microinjection needle using a three inch 23 gauge hypodermic needle.
One hour old embryos bearing deletions of the appropriate selectable marker gene, \( r_y^{506}/r_y^{506} \) for pCarnegie20NX based transposons and \( D_f(1)w^{67c2}/D_f(1)w^{67c2} \) for pCasper based transposons (Lindsley and Zimm, 1992), were collected for injection with plasmid DNA; embryos were harvested from yeasted grape juice agar petri dishes (Ashburner, 1989) which had been placed over the opening of a cage (an inverted 600ml plastic beaker with ventilation holes) containing several hundred adult flies. Each embryo's chorion membrane was removed manually, and about 30 dechorionated embryos were lined up on double stick tape (Scotch brand) coated microscope slide such that their posterior poles were all ~1mm from an edge of the slide. The embryos were then slightly dehydrated by incubating the slide in a reduced humidity container (a box with desiccant) for 5 to 20 minutes; the exact desiccation time was determined empirically each day that injections were done. Desiccated embryos were covered with Halocarbon oil (Ashburner, 1989), viewed with a low power (~50X) inverted microscope, and then injected (into their posterior pole) with the DNA solution. The injection procedure was considered successful when the DNA solution (colored green) could be observed flowing from the needle's tip into the embryo without loss of cytoplasm after needle removal. Failure to observe the flow of DNA solution and/or loss of cytoplasmic fluid indicated that the embryos had not desiccated sufficiently, and overdesiccation
was evident when the embryos were extensively flaccid (i.e. the vitelline membrane was obviously folded and wrinkled).

After injection, slides with embryos were placed in high humidity incubators (15°C) and allowed to complete embryogenesis (~36 hours). Hatched larvae (typically displaying a green tinge) were transferred to regular food vials. After pupation and eclosion, adult flies were single paired mated with animals of identical genotypes (ry<sup>506</sup>/ry<sup>506</sup> for pCarnegie20NX based transposons and Df(1)67c2w/Df(1)67c2w for pCasper based transposons) to recover germ line chromosomes (which may now bear transposons).

**Selection of Chromosomes Bearing Transposons**

To recover any (germline) chromosomes bearing transposons, F1 progeny from each injected animal were scored for the ry<sup>+</sup> (or w<sup>+</sup>) selectable marker. For example, 300 embryos were injected with pCHSa2+, 26 of these embryos developed into viable adults, 17 of these adults produced F1 progeny after crossing with ry<sup>506</sup>/ry<sup>506</sup> animals, and one of these adults produced F1 progeny displaying the ry<sup>+</sup> phenotype (3/21 progeny were ry<sup>+</sup>). To determine which chromosome(s) contained the ry<sup>+</sup> transposon, the three ry<sup>+</sup> animals were single pair mated to flies bearing marked and balanced second and third chromosomes (Sp/CyO;ry Sb/ry, TM2). Following the segregation of the ry<sup>+</sup> chromosome relative to these marked chromosomes indicated that the ry<sup>+</sup> transposon had inserted
into the third chromosome in all three animals. Mapping the ry+ marker relative to Sb indicated that three transposons shared similar genetic map positions. Since these chromosomes were derived from the same injected animal, it seemed probable that all three chromosomes were derived from a single insertion event; thus one ry+ chromosome was chosen for future experiments and made into the balanced stock ry506 P[HS-αPS2c ry+]1/ry506,TM2 (aka P[1]). The integrity of the transposon's integrin gene (i.e. P[HS-αPS2c]) was determined using a variety of molecular biological techniques including assaying transgenic expression of PS2 antigen in embryos and larval imaginal discs following heat shock.

Similar results were obtained with animals injected with pCHSα2-, except that one insertion of P[HS-αPS2m8 ry+] was identified on the second chromosome. Specifically, of more than 500 embryos injected with pCHSα2-, 56 developed into viable adults, and one of these adults produced several ry+ progeny. Since these ry+ progeny appeared to bear identical insertions of the ry+ transposon, a single ry+ second chromosome was selected for future experiments and made into the balanced stock P[HS-αPS2m8 ry+]A/CyO;ry506/ry506 (aka P[A]). Likewise, Danny Brower selected w+ chromosomes from the progeny of Df(1)67c2w/Df(1)67c2w embryos that we (Danny Brower and I) had injected with Tom Bunch's pCasper plasmid bearing the P[mys+ w+] transposon, and he found several unique
insertions of this transposon including the \( P[mys^+] \)B1 (aka \( P[mys^+] \)) insertion on the third chromosome used in this work.

Mobilization of \( P[HS-\alpha_{PS2} \, ry^+] \) Transposons

To recover additional copies of the two alternatively spliced \( P[HS-\alpha_{PS2} \, ry^+] \) transposons (\( P[1] \) & \( P[A] \)), each transposon was mobilized to additional sites within the genome as shown in Figure 4.2. The transposase gene used in these "hopping" experiments was an immobile insertion of a modified P-element on the third chromosome (\( P[\Delta2-3]99B \)); this P-element constitutively expresses transposase in both somatic and germline tissues (Robertson et al., 1988). From crosses with 86 individually mated "jump-start" males bearing \( P[1] \) (Figure 4.2), one novel insertion on the second chromosome (\( P[2] \)) was recovered. Similarly, with 72 "jump-start" males bearing \( P[A] \), two new insertions were recovered on the second chromosome (\( P[B] \) & \( P[C] \)) as well as one insertion on the third chromosome (\( P[D] \)). Each chromosome bearing a new insertion was made into a balanced stock. To verify the integrity of the \( HS-\alpha_{PS2} \) transgene following transposition, several wing discs from each stock were examined for ectopic PS2 expression (see immunoflourescencce below).

Chromosomes and strains bearing multiple copies of the integrin transgenes were made using standard genetic techniques. For example, a chromosome bearing both \( P[1] \) and
was created by genetic recombination, and then animals bearing this chromosome were made into the balanced stock \( P[1] \ P[D]/TM3 \). Since both transposons are nearly identical and bear the same selectable marker \((ry^*)\), the presence of both alternatively spliced \( P[HS-\alpha_{PS2}] \) transgenes was confirmed using a technique developed to monitor the segregation of unmarked P-elements (Engels et al., 1990). Specifically, polymerase chain reaction (PCR) was performed on DNA from single \( P[1] \ P[D]/TM3 \) flies using primers that flank the 75 bp alternatively spliced eighth exon. Specifically, the primer in exon seven is 5'TCGTGTCAGGCTGGATTCAGT3' (Brower lab #3652-1) and the primer in exon nine is 5'TCCGAAGTGGAGAACACTTGG3' (Brower lab #3624-1). The predicted sizes of PCR products using these primers on different if DNA templates are as follows: if genomic DNA should produce a fragment of \( >3000 \) bp because of large introns (Brown et al., 1989), if cDNA containing the eighth exon (e.g. \( P[1] \)) should produce a 217 bp fragment, and if cDNA missing the eighth exon (e.g. \( P[A] \)) should produce a 142 bp fragment. As expected, PCR of template DNA from \( P[1] \ P[D]/TM3 \) flies always produced both of the smaller fragments, while DNA from \( P[1]/TM3 \) or \( P[D]/TM3 \) flies only produced the 217 bp or the 142 bp fragment, respectively. Considering the very short ramp time (50 sec) between annealing (57°C) and denaturing (95°C) temperatures, it was not surprising that my PCR experiments failed to synthesize significant quantities (typically a very faint
band) of the larger PCR fragment predicted for if genomic DNA templates.

Creation of New inflated Mutations

if$^{A7}$ and if$^{B2}$ were created by EMS mutagenesis using a standard protocol (Grigliatti, 1986). Briefly, $g^2 f^{36a}$ males were fed 0.026 or 0.013M EMS in 1% sucrose and mated with homozygous y w if$^3$ virgin females. F1 females were screened for wing blisters. About 75 of more than 18,000 mutagenized chromosomes were selected for further analysis. Two chromosomes were found to have lethal mutations (if$^{A7}$ and if$^{B2}$) which mapped genetically to the if locus (i.e. 1.0 map unit distal to forked), and which failed to complement previously known if alleles. The if$^{A7}$ and if$^{B2}$ chromosomes were cleaned of other lethal mutations; that is, the distal portion of the chromosome was replaced three times through recombination with garnet (g). Both chromosomes are rescued by $Dp(1;4)r^+$ indicating that no lethals exist outside of the area covered by this duplication.

Examination of Mutant Tissues

inflated Embryonic Phenotype

Balanced females heterozygous for the three lethal inflated mutations were allowed to lay eggs for short (1 to 3
hour) intervals at 25°C in cages made by taping a food filled petri dishes over the top of 600 ml plastic beakers. At the desired developmental timepoint, the embryos were dechorionated with bleach, fixed with a 50:50 mixture of 22.5% glutaraldehyde in PBS pH 7 and heptane, devitellinized in a 50:50 mixture of cold heptane and methanol, transferred to 100% ethanol and mounted in Gary's magic mountant (Ashburner, 1989). Embryos were examined using a Zeiss Universal microscope, using Nomarski DIC and polarized light optics.

inflated Wing Phenotype

For clonal analysis, y w ifB2 f36a/FM7c or y w ifB2/FM7c females were mated to Oregon-R-C males, and the progeny irradiated with X-rays (2000 rads) at 48±4 hours after egg laying. Wings from y w ifB2 f36a/+ flies were stored in ethanol-glycerol (7:3), dehydrated in ethanol, mounted in Euparal (ASCO Labs) for microscopy, and then scored for f36a mutant patches on each wing surface. Although systematic scoring without regard to blister phenotype was done only for ifB2, ifA7 was also found to cause wing blisters in clones.

Analysis of PS Integrin Expression

Immunoblots
Protein gel samples were prepared as described in Leptin et al. (1989) from wild-type, transgenic or individually selected hemizygous if embryos. For analysis of $\alpha_{PS2}$ subunit levels in mutant and wild-type embryos (Figure 3.3), embryos were harvested at 22 ± 2 hours after egg laying at 25°C. The mutant embryos (if hemizygotes) from balanced stocks were distinguished from their if+ siblings by their gut phenotype (see Chapter 3). For analysis of $\alpha_{PS2}$ subunit levels in transgenic and wild-type embryos under heat shock conditions (Figure 4.3), embryos were heat shocked for 30 minutes at 37°C after 19 ± 1 hours of development (at 25°C), and then allowed a 30 minute recovery (at 25°C) before harvesting. After collection, embryos were placed in the cap (cut off and inverted) of an 0.5 ml eppendorf tube with 1μl of 4X SDS PAGE sample buffer. The embryos were flattened (squished) using a piece of glass cover slip and a dissection needle. 4 μl of 1x sample buffer were added on top of the glass within the cap, and then the cap was returned to the eppendorf tube. The sealed tube was totally immersed in boiling water for several seconds, and then spun in a eppendorf microfuge for 1 minute. After centrifugation, the tube was again immersed in boiling water for 3 minutes, and then frozen on dry ice. Samples were stored at -80°C until run on SDS polyacrylamide gels (Laemmli, 1970).

Following electrophoresis, the proteins were electroblotted to Immobilon-P membrane (Millipore P15552).
Blocking of the membrane (at least 1 hour, room temperature), incubation with primary antibody (overnight, 4°C), secondary antibody (1 hour, room temperature), and washes (1 hour with 4 changes after primary and secondary antibody incubations) were done in TBS-T20 (150 mM NaCl, 50 mM Tris-Cl, 0.05% Tween20, pH 7.5). The primary antibody was the anti-αPS2 monoclonal PShc/1 (Bogaert et al., 1987), and the secondary antibody was peroxidase-linked goat anti-rat IgG (Boehringer-Mannheim 605-190). The secondary antibody was detected using an enhanced chemiluminescence kit (Amersham, RPN2106) according to the supplier's protocols. Molecular weight markers were from BioRad (161-0303).

Immunofluorescence

The patterns of PS1 and PS2 expression on wild-type and transgenic wing discs were determined using previously described techniques and monoclonal antibodies (see Brower et al., 1984). Late third instar larvae were decapitated using small surgical scissors in Ringer's (recipe in Ashburner, 1989) and their heads inverted with dissection needles to expose imaginal discs to reagents. The inverted heads were immediately incubated in primary antibody (for 30 min), washed twice (for 5 & 10 min), incubated in secondary antibody (for 30 min), washed twice (for 5 & 10 min), fixed with 2% formaldehyde in Ringer's (for 10 minutes, washed in Ringer's (for 5 minutes), and then mounted on a slide in a
solution of 70% (v/v) glycerol/30% (v/v) 0.1 M. Tris-Cl pH 9 to which was added 2% (w/v) n-propyl gallate. All incubations, washes and fixes were done in microtiter dishes at room temperature with frequent stirring. On the microscope slide, wing discs were dissected away from the other head tissues using dissection needles and then viewed under epi-illumination on a Zeiss Universal photomicroscope.

Anti-PS1 (#DK.1A4) or anti-PS2 (#CF.2C7) primary antibodies (mouse) were diluted (1:100 or 1:500 respectively) in Drosophila M3 cell culture media supplemented with 10% Fetal Calf Serum (M3+FCS). The secondary antibody was affinity purified, fluorescein-conjugated goat anti-mouse IgG (Antibodies Inc.) which was diluted 1:400 in M3+FCS. M3+FCS was also used for the washes between antibody incubations.

Experiments Using Transgenes to Rescue Integrin Mutant Phenotypes

Wing Blister Rescue

Male animals homozygous for the P[mys+]B1 transgene on the third chromosome (i.e. y Df(1)w67c2/Y; P[mys+]B1) were crossed to homozygous mysnj42 if3 females to generate mysnj42 if3/Y; P[mys+]B1/+ flies to be scored. Control animals were
made by an almost identical cross, in which the males lacked the $P[\text{mys}^+]B1$ transgene.

The progeny from the above crosses were incubated at 25°C except during periods of transgene induction. Larvae were grown in thin-walled plastic vials (ca. 3cm diameter X 10cm length), and were split into additional vials as needed to prevent overcrowding. The $\text{mys}^+$ transgene was induced by incubation in a Biosycler oven (BIOS Corporation, New Haven, CT) which was programmed to provide a 30 minute, 37°C heat pulse every 7 hours. These heat pulses did not significantly reduce the viability of either experimental or control animals, although the period of time required from pupariation to eclosion was increased by about one-half day for animals that were heat pulsed throughout this period. All newly eclosed adults, including animals stuck in the food, were collected daily and their wing phenotypes scored. All time points therefore have a range of ±12 hours. Each data point represents an average of several experiments (2 to 14 per time point) with 20 to 380 wings assayed per experiment.

*Lethality Rescue*

Male animals heterozygous for $P[\text{HS-}\alpha_{\text{PS}2}\text{m8}]$ or $P[\text{HS-}\alpha_{\text{PS}2}\text{c}]$ on the third chromosome ($y\ w\ f/Y;\ ry\ [\text{HS-}\alpha_{\text{PS}2}\text{m8}^{\text{ry}^+}]/y^+\ \text{Ser, TM3}$ or $y\ w\ f/Y;\ [\text{HS-}\alpha_{\text{PS}2}\text{c}^{\text{ry}^+}]/y^+\ \text{Ser, TM3}$) were crossed to
heterozygous $\text{mys}^{XR04} \text{ifB2}$ females ($y\ w\ \text{mys}^{XR04}\ \text{ifB2}$ $f/FM7c;\text{Dp}(1;4)r^+$) generating $\text{mys}^{XR04}\ \text{ifB2}/++; [\text{HS-}\alpha_{PS2c}]/+$ or $\text{mys}^{XR04}\ \text{ifB2}/++; [\text{HS-}\alpha_{PS2m8}]/+$ zygotes. The viability of this class of zygotes (the experimental group) determines whether the transgenes provide positive integrin function. Negative control animals were made by an almost identical cross, in which the males lacked transgenes ($y\ w\ f/Y$ males). Positive control animals were again made by an almost identical cross, in which the males were homozygous for the $P[\text{mys}^+]\text{Bl}$ transgene on the third chromosome (i.e. $y\ w\ f/Y;\ P[\text{mys}^+]\text{Bl}$). The basic cross and expected classes of $F1$ progeny are shown in Figure 4.5, although marker mutations and balancer chromosomes ($\text{FM7c}$ and $\text{TM3}$) have been omitted for clarity; (e.g. inclusion of $\text{TM3}$ would have required listing another eight $F1$ genotypes that are irrelevant to the experiment).

Animals were cultured and transgenes induced in the Biosycler as before. All newly eclosed adults, including flies stuck in the food, were collected daily (as adults do not survive long under these heat shock conditions) and their genotypes scored. After all progeny had eclosed, the number of $y\ w\ \text{mys}^{XR04}\ \text{ifB2}\ f/++$ flies bearing an integrin transgene (non-balancer females, displaying forked bristles) was compared to the number of $y\ w\ \text{mys}^{XR04}\ \text{ifB2}\ f/++$ flies bearing both a transgene and the $\text{if}^+$ duplication $\text{Dp}(1;4)r^+$ (non-balancer females displaying wild-type bristles). Three
trials of each cross were performed, and greater than 100 animals of the rescued class were recovered each time.

**Muscle Rescue**

Male animals homozygous for $P[HS-\alpha_{PS2m8}]$ or $P[HS-\alpha_{PS2cl}]$ on the second chromosome ($P[HS-\alpha_{PS2m8} \, ry^+]/P[HS-\alpha_{PS2m8} \, ry^+]$; $ry^{506}/ry^{506}$ or $P[HS-\alpha_{PS2m8} \, ry^+]/P[HS-\alpha_{PS2m8} \, ry^+]$; $ry^{506}/ry^{506}$) were crossed to inflated (if) shaven baby (svb) double heterozygotes ($yw\, svb\, sn\, if^A7\, f/FM7c$ females) generating progeny that includes the class of embryos (svb if/Y males bearing one copy of $P[HS-\alpha_{PS2}]$) to be scored for muscle phenotype. Control animals were made by an almost identical cross, in which the males lacked the $P[HS-\alpha_{PS2}]$ transgene.

Eggs from the above crosses were incubated at 25°C except during periods of transgene induction. Aged embryos (3±1 hours old) were collected from cages on grape juice agar plates supplemented with a layer of yeast paste. The $P[HS-\alpha_{PS2}]$ transgenes were induced by incubation in a Biosyclyer oven (BIOS Corporation, New Haven, CT) which was programmed to provide a 20 minute, 37°C heat pulse every 50 minutes. These heat pulses did not significantly reduce viability as >95% of wild type embryos reared under these conditions hatch into larvae, and these first instar larvae will continue to develop into apparently healthy adult flies when incubated at 25°C.
After 21 hours of incubation in the Biosycler oven, the embryos (24±1 hours old) were dechorionated with bleach, fixed with a 50:50 mixture of 22.5% glutaraldehyde in PBS and heptane, devitellinized in a 50:50 mixture of cold heptane and methanol, transferred to 100% ethanol and mounted in Gary's magic mountant (Ashburner, 1989). Embryos were examined using a Zeiss Universal microscope, using Nomarski DIC and polarized light optics.

The denticle band phenotype was examined for those embryos that could be viewed ventrally with Nomarski DIC light optics to determine the embryos genotype. Because the svb phenotype is difficult to score except when embryos were mounted with their ventral cuticle pointed up, only those animals that had been mounted in this orientation were scored for muscle phenotype. That is, once a determination of svb or svb+ genotype was confirmed, then polarized light optics were used to view muscle morphology. I typically have more difficulty imaging muscles within a single plane with ventral views than other views (e.g. the lateral images in Figure 3.4); presumably, this difference is due to the larger number of overlapping muscles that reside under the ventral cuticle. By manipulating the focal plane and the orientation of the polarized light as it passes through these ventral muscles, I found that the morphology of individual muscles could be examined. For example, Figure 4.9 provides two ventral images of each transgenically rescued embryo, and within each
image different muscles are revealed. These images are indistinguishable from ventral views of wild-type embryos grown at 25°C.

Analysis of Wings After Induction of Transgenic PS2mB or PS2c

Larvae containing single or multiple copies of the P[HS-\(\alpha_{PS2mB}\)], P[HS-\(\alpha_{PS2c}\)] and P[HS-\(\beta_{PS}\)] transgenes (P[A], P[B], P[C], P[D], P[1], P[2] & P[mys\(^+\)B1]) were given heat shocks, and then the resultant flies were scored for wing blisters. In each case, balancer chromosomes had been crossed out so transgenes existed in wild type genetic backgrounds. Larvae were either heat shocked once (1 hour at 37°C) at the end of the larval period and allowed to pupate at 25°C or heat shocked throughout the pupal period (1 hour heat shock every 7 hours). At least two trials (with qualitatively identical results) were performed for each genotype and heat shock condition, and at least 200 wings were scored per trial.


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