Genetic Interaction Between Integrins and *moleskin*, a Gene Encoding a Drosophila Homolog of Importin-7

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ABSTRACT

The Drosophila PS1 and PS2 integrins are required to maintain the connection between the dorsal and ventral wing epithelia. If αPS subunits are inappropriately expressed during early pupariation, the epithelia separate, causing a wing blister. Two lines of evidence indicate that this apparent loss-of-function phenotype is not a dominant negative effect, but is due to inappropriate expression of functional integrins: wing blisters are not generated efficiently by misexpression of loss-of-function αPS2 subunits with mutations that inhibit ligand binding, and gain-of-function, hyperactivated mutant αPS2 proteins cause blistering at expression levels well below those required by wild-type proteins. A genetic screen for dominant suppressors of wing blisters generated null alleles of a gene named *moleskin*, which encodes the protein DIM-7. DIM-7, a Drosophila homolog of vertebrate importin-7, has recently been shown to bind the SHP-2 tyrosine phosphatase homolog Corkscrew and to be important in the nuclear translocation of activated D-ERK. Consistent with this latter finding, homozygous mutant clones of *moleskin* fail to grow in the wing. Genetic tests suggest that the *moleskin* suppression of wing blisters is not directly related to inhibition of D-ERK nuclear import. These data are discussed with respect to the possible regulation of integrin function by cytoplasmic ERK.

INTEGRINS are a strongly conserved family of cell surface receptors (Hynes 1992), and genes for α- and β-subunits of integrin heterodimers have been found in the most primitive metazoans. Most integrins bind components of the extracellular matrix (ECM), although some integrins in vertebrates recognize other cell surface proteins. Typically, integrins make strong connections between the ECM and the actin cytoskeleton (Yamada and Miyamoto 1995; Dedhar and Hannigan 1996). Integrins are also signaling proteins, and integrin ligand binding can have a multitude of effects in regulating cellular events (Dedhar and Hannigan 1996; Howe *et al.* 1998). Moreover, cells can often regulate the function of their integrins in what is referred to as "inside out" signaling (Fernandez *et al.* 1998; Hughes and Pfaff 1998).

Drosophila genetics has been instrumental in the identification and analysis of an extraordinary number of genes encoding proteins important for developmental and cell biological processes. The genetic study of integrin function in Drosophila has included a combination of classical forward and reverse genetics approaches (Gotwals *et al.* 1994; Brown *et al.* 2000). The gene encoding the βPS subunit (*myospheroid*) was originally identified by mutation and analyzed extensively by genetics before it was discovered that it encoded an integrin subunit (Wright 1960; Newman and Wright 1981; MacKrell *et al.* 1988). The αPS1, αPS2, and αPS3 proteins were all identified biochemically as integrins, and gene localization subsequently was used to identify the corresponding genes, *mew* (Wehrli *et al.* 1993; Brower *et al.* 1995), *inflated* (Bogaert *et al.* 1987; Wilcox *et al.* 1989), and *scab* (Stark *et al.* 1997), respectively (see also Grotewiel *et al.* 1998, who came upon the αPS3 gene independently in a forward genetic screen). The αPS4, αPS5, and βν genes have yet to be extensively analyzed genetically.

As in vertebrates, studies of integrin function in Drosophila are moving toward analyses of components that work in conjunction with the αβ-heterodimers. The elucidation of the fly genome makes it relatively straightforward to generate and study mutations in proteins previously associated with integrins from other systems. Also, forward genetic screens can identify novel cellular components involved in integrin function. The PS1 and PS2 integrins are required to maintain the connection between dorsal and ventral wing surfaces (reviewed by Brown *et al.* 2000), and this phenotype has been used

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to devise relatively efficient strategies for identification of integrin-related genes that are likely to be recessive lethals when mutated. For example, the generation of homozygous clones of mutant cells has been used in screens to find integrin and related mutants that cause the wing surfaces to separate (Brower *et al.* 1995; Prout *et al.* 1997; Walsh and Brown 1998).

Another tactic for identification of interacting components is to look for dominant enhancers or suppressors of weak integrin phenotypes (*e.g.*, Wilcox 1990). Screens for suppressors of a phenotype, where a defect must be repaired by the mutation, typically are more reliable than enhancer screens in identifying components that are functionally associated with the initial mutation. Since most mutations are due to loss of gene function, suppressor screens often begin with a gain-of-function phenotype to be suppressed.

During the late larval and early pupal development of the Drosophila wing, the PS1 and PS2 integrins show a predominantly dorsal and ventral, respectively, restriction in their expression (Brower *et al.* 1985). Soon after pupariation, the wing pouch evaginates and folds along the nascent wing margin, bringing together the basal surfaces of the dorsal and ventral cells. After ~10–12 hr, the dorsal and ventral epithelia separate as the wing epithelium expands. Approximately 10–12 hr later, the two sides reappose and remain attached until the adult fly ecloses from the pupal case (Waddington 1941; Fristrom *et al.* 1993).

If integrin α-subunits are inappropriately expressed in the developing wing, the epithelia do not reappose and wing blisters result (Brabant *et al.* 1996; N. Brown, personal communication). It is not clear which is more important for blister formation, expression of an α-subunit on the wrong surface or the unusually high level of expression typically required (see also Brown *et al.* 2000). What is known is that the critical time for this phenotype is during the initial apposition of the dorsal and ventral epithelia; high level or spatially incorrect integrin expression later is without effect (Brabant *et al.* 1996). It appears that if some necessary event does not take place at the initial apposition, reapposition is prevented, even if integrin expression is returned to normal. Whether the defect results from interference with a specific intracellular signal or simply from the disruption of dorsoventral connections necessary for reapposition is unclear.

Early gene dosage experiments suggested that the wing blistering due to misappropriate integrin expression results from a gain-of-function effect (Brabant *et al.* 1998), although this had not been demonstrated convincingly. If so, the phenotype can be a useful starting point to look for suppressor mutations that might function downstream of the integrin misexpression. Here we show that the wing blistering is indeed a gain-of-function phenotype and describe a screen for suppressors that has identified mutations in a gene involved in the nuclear import of the phosphorylated form of the mitogen-activated protein (MAP) kinase, D-ERK.

MATERIALS AND METHODS

Fly husbandry and stocks: For all crosses, animals were grown on the food described by Condie and Brower (1989). For crosses in which phenotypes were to be assayed, all flies from a vial were scored to guard against potential developmental rate variations.

Misexpression of αPS2 in the wing was generated in most cases using the GAL4 UAS system (Brand *et al.* 1994). The GAL4 enhancer traps *684* (wing pouch) and *337* (most tissues) are described by Manseau *et al.* (1997); these *P* elements are inserts into the third chromosome and are marked with w^+. *Blistermaker* is a third chromosome (homozygous viable) containing both the *684* enhancer trap and a *P* element with *UAS-αPS2m8* (this particular insertion is designated *m8K6*), also marked with w^+.

The *tubulinα1-αPS2m8* flies were generated by first creating an inserted *P* element (*P[tubα1-promoter FRT* y^+ *FRT αPS2m8]* on chromosome 3) with a cassette for making αPS2-expressing clones using the FRT FLP system (Struhl and Basler 1993). The y^+ sequence was recombined out by expression of FLPase in the germ line, and the *tub-αPS2*-containing chromosome was balanced over *TM3, Ser.* Other stocks used in crosses to assay suppression of blistering or vein formation are: *y* csw^{OP} *w* sev^{d2} *f car/FM7* (from Mike Simon); y $Draf^{C110}$ *sn/Binsc* and *y w spl sn* $Dsor^{r2}$*/Binsc* (from Yasuyoshi Nishida); *y w; HS-rho*^{27B}*/TM3, Sb* and *y w; HS-rho*^{30A}*/TM3, Sb* (from Ethan Bier); *en-GAL4* enhancer trap (from Ruth Palmer); nw^D pu^2 $Egfr^{E1}$ Pin^{Yt}*/SM1* (from the Bloomington Stock Center); $Draf^{HM7}$*;* rl^{Sem}*/CyO*; and rl^1. The *UAS-msk* chromosome is described in Lorenzen *et al.* (2001).

Mutant αPS2 experiments: The *αPS2-LOF* (222-224 YWQ> AWA) and *αPS2-GOF* (deletion of the cytoplasmic CGFFN) mutations were made by PCR mutagenesis, confirmed by sequencing, and inserted into pUASPS2m8 or pUASPS2C for fly transformation (Brabant *et al.* 1996) or into pHSPS2m8 or pHSPS2C for transformation into S2 cells (Bunch and Brower 1992; Zavortink *et al.* 1993). Numerous independent chromosomal insertions of the mutant αPS2 subunits were generated by embryo injection. All fly transformants were of the "m8" isoform of αPS2 (Brown *et al.* 1989). LOF inserts were designated *H* and *K* on chromosome 2 and *B*, *L*, and *P* on chromosome 3. GOF inserts that showed some blistering activity were *G* on chromosome 2 and *A*, *C*, and *O* on chromosome 3. The wild-type cDNA insert was *m8Z4* on the X chromosome. S2 cell transformants were made as described (Bunch and Brower 1992). Both αPS2m8 and αPS2C isoforms were generated; the data shown in Figure 1 derive from the "c" isoform.

Spreading of transformed S2 cells was performed as previously described (Jannuzi *et al.* 2002). Briefly, cells were cleared with protease, heat shocked, allowed to spread for 3–4 hr on a recombinant fragment of the matrix ligand Tiggrin, and scored by direct observation.

To score wing blistering and examine expression in wing discs, animals bearing inserts of mutant or wild-type *UAS-αPS2* genes were crossed to animals bearing the *337* enhancer trap. For most experiments, including those for which discs were stained, homozygous stocks of all chromosomes were crossed, so that the animals to be examined were heterozygous for both the enhancer trap and the *UAS-αPS2* transgene. For experiments to score interactions with *myospheroid* mutants, we made stocks with either a LOF (insert *B*) or a GOF (insert *C*) *UAS-αPS2* recombined onto a chromosome with the *337* enhancer trap, balanced over *TM3, Sb.* These stocks were then

crossed to *mys/FM7c* females (*myospheroid* alleles *M2*, *G4*, and *G1*; see Jannuzi *et al.* 2002) to score blistering and viability. The *337 UAS-αPS2* mutant animals displayed reduced viability; to minimize this, eggs were generally laid and progeny were allowed to develop for 2–3 days (through embryogenesis) at 22°, and then raised to the temperature at which blistering was to be scored. Immunostaining of integrins in wing imaginal discs was performed as described (Brower *et al.* 1984) using the monoclonal antibodies DK.1A4 (αPS1) and CF.2C7 (αPS2).

Suppressors of *Blistermaker*: Oregon-R (wild-type) males were mutagenized with EMS (Grigliatti 1986) and crossed to *w; Blistermaker/Blistermaker* virgin females at 28°. Progeny with two wild-type wings were kept; retesting and mapping were initiated by crossing these flies to *w; Blistermaker Sb/TM3, Ser*. Although subsequent mapping and balancing should have theoretically isolated suppressors on any of the three large chromosomes, the screen turned out to be strongly biased toward the third chromosome. This was likely because identification of other suppressors relied on suppression of *Blistermaker/TM3* animals during mapping, and *TM3* turned out to be a general enhancer of blistering. Chromosomes with suppressing activity all contained recessive lethal mutations, and these were tested against one another for complementation. Only the *moleskin* complementation group contained multiple alleles, and the *moleskin* recessive lethals were mapped by recombination to a region of chromosome 3L. The locus was fine mapped using a set of deficiencies; *moleskin* alleles failed to complement *Df(3L)pbl-X1* and *Df(3L)66C-G28* as well as a smaller deficiency that was generated in an attempt to hop a nearby *P* element into the locus. The locus was finally identified molecularly by sequencing candidate genes within the deficiencies from the *moleskin* mutant chromosomes.

DNA sequencing: Genomic DNA from *moleskin* alleles balanced over *TM3* was prepared using a QIAGEN (Valencia, CA) QIAamp tissue kit. Using the Drosophila genome sequence, PCR primers were designed to amplify the potential coding exons. The products of amplification were prepared using QIAGEN's QiaQuick PCR purification kit and sequenced directly by the University of Arizona LMSE Automated DNA Sequencing Service. All mutants were confirmed by sequencing of both strands.

Generation of *moleskin* clones: Males of the genotype *y w; en-GAL4 47m1UAS-DIM-7/+; mwh msk*^{5} *P[w*$^+$*]70C P[FRT]80B/+* were crossed to *y w hsFLP; P[y*$^+$ *FRT]80B/TM3, Sb* at 22°, and the progeny were given 60-min heat shocks (37°) at various times during larval development to induce recombination at the *FRT* sites. Wings were mounted in Euparol and clone sizes were scored using the *multiple wing hairs* marker.

RESULTS

***Blistermaker* results from a gain of function:** The wing blister phenotype caused by inappropriate integrin subunit expression resembles the loss-of-function phenotype, as evidenced by viable integrin mutants or wing clones homozygous for null integrin alleles. This might suggest that the *Blistermaker* chromosome (containing an αPS2 gene driven by the wing pouch enhancer trap, *684*; Brabant *et al.* 1996; Manseau *et al.* 1997) causes a dominant negative situation. For example, since both PS1 and PS2 integrins share a common βPS subunit, flooding dorsal wing cells with αPS2 subunits might reduce dorsal αPS1βPS dimer expression below a critical level. Alternatively, the extra PS2 integrins might directly lead to wing blistering through some gain-of-function event, such as the activation of an unknown regulatory pathway or an inappropriate adhesion. Gene dosage studies tended to support the gain-of-function proposal (Brabant *et al.* 1998; D. L. Brower, unpublished data). For example, reducing βPS expression via heterozygosity for null mutations in the βPS-encoding *myospheroid* gene does not have the enhancing effects that would be predicted by the dominant negative scenarios, and increasing βPS expression with transgenes does not suppress the effects of αPS2 overexpression.

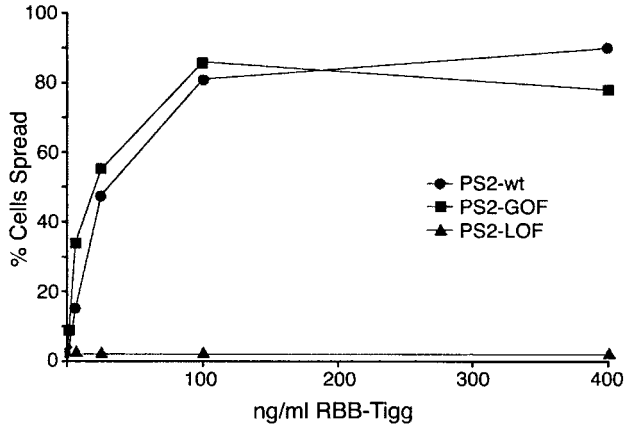

Figure 1.—Cell spreading mediated by αPS2 mutants. S2 cells transformed with the indicated αPS2 and wild-type βPS subunit genes were allowed to spread on various concentrations of a fragment of the ECM protein Tiggrin. Only the loss-of-function mutant does not support spreading in this assay. Flow cytometry of these cells indicates that the wild-type and LOF mutants are expressed on the cell surface at similar levels, while the GOF mutant is expressed at 5- to 10-fold lower levels.

We sought a more direct demonstration that *Blistermaker* does indeed result from a gain of function. We transformed flies with one of two mutant αPS2 genes, under the control of the GAL4 UAS (Brand *et al.* 1994). In one gene (α*PS2 loss of function* or α*PS2-LOF*), residues 222–224 (YWQ) of the extracellular domain are changed to AWA. This alteration is expected to inhibit extracellular ligand binding (*e.g.*, Irie *et al.* 1995), and indeed we find that in Drosophila S2 cells transfected with α*PS2-LOF*, cell spreading is severely inhibited relative to wild type (Figure 1). The other mutant is a deletion of the cytoplasmic, membrane proximal CGFFN sequence (residues 1366–1370 for αPS2C), which is expected to lead to activation of integrin heterodimers (*e.g.*, O'Toole *et al.* 1994). This expectation is supported by observations of S2 cells transfected with this mutant gene (α*PS2-GOF*). These cells spread very efficiently on PS2 ligands, even at very low levels of integrin expression (Figure 1).

To drive mutant integrin expression in developing flies, we used a GAL4 enhancer trap (*337*; Manseau *et al.* 1997) that is expressed fairly ubiquitously, so that relative expression levels could be discerned easily in the dorsal proximal wing disc (outside of the wing pouch), where PS2 expression is normally close to zero. Numerous chromosomal inserts of each gene were generated

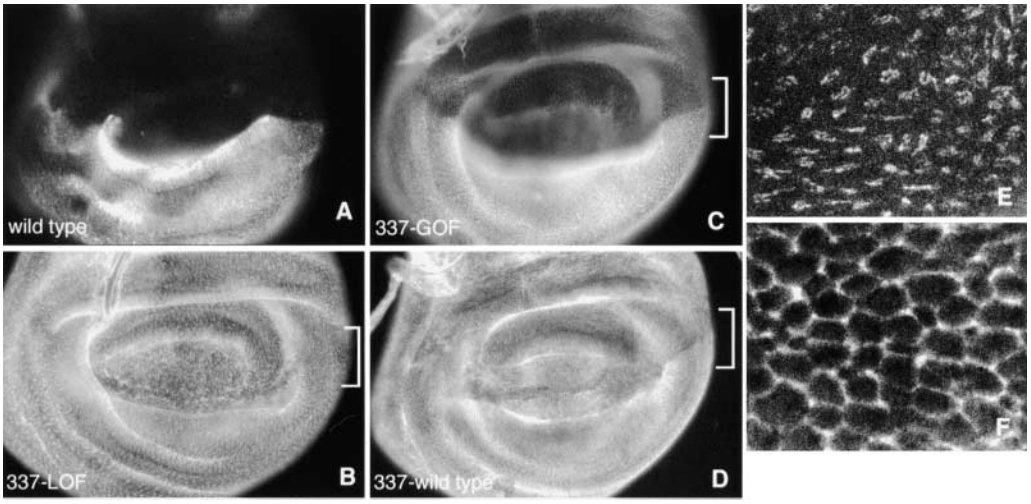

FIGURE 2.—Expression of mutant integrins on the cell surface in late third instar (just prior to pupariation) wing imaginal discs. (A) Wild-type αPS2 is primarily restricted to ventral cells. (B) Using the *337* enhancer trap, a *UAS-αPS2-LOF* (deficient for ligand binding) transformant (line "*L*") is expressed throughout the wing at high levels. Adult wings from these animals rarely blister. (C) A *UAS-αPS2-GOF* (activated) transformant (line "*G*") is expressed dorsally at low levels. Adult wings from this cross blister at >50% frequency. (D) A *UAS-αPS2* wild-type transformant (line *m8Z4*). Dorsal αPS2 is expressed at a level below that of the loss-of-function mutant in B, but expression is much greater than that for the activated mutant in C. (To assess ectopic dorsal expression relative to ventral expression, compare the dorso-ventral boundaries at the posterior margins, indicated by the brackets.) About 2% of the wings from this cross are blistered, more than for the loss-of-function cross in B but less than for the gain-of-function cross in C. (E and F) High magnification of dorsal folds from *αPS2-GOF* discs, stained with antibody against αPS2 (E) or αPS1 (F). The activated αPS2-containing integrins are clustered in basal plaques on each cell, but the αPS1-containing heterodimers are not.

and tested at various temperatures (wing blistering due to *Blistermaker* is greater at higher temperatures). It should be noted that both sets of mutant animals often show significant lethality, especially at 28°, and for the experiments at higher temperatures the flies are allowed to transit embryogenesis at 22° to increase viability.

Examination of PS2 expression in wing imaginal discs from larvae bearing the *αPS2-LOF* mutant shows that it can be expressed at high levels (Figure 2B), similar to those seen when wild-type αPS2 is driven from the same enhancer trap. However, even with this high level of surface expression, adults have a relatively low frequency of wing blisters. For example, the disc illustrated in Figure 2B is typical of expression levels from three different transgenes, but in the 25° crosses done for disc staining, virtually no blisters were observed in adults expressing these transgenes. Overall, a number of *αPS2-LOF* inserts cause significant (>50%) blistering at 28°, but even for the strongest inserts, the frequency of defects falls abruptly at 25°, typically to <5%. Using the same enhancer trap, a wild-type *αPS2* insert typically blisters at close to 100% levels at 25°.

The activated αPS2-GOF integrins are expressed at low levels in imaginal discs (Figure 2C), just as they are in S2 cells in culture (not shown). This is consistent with findings from integrins *in situ* bearing similar mutations (MARTIN-BERMUDO *et al.* 1998). The αPS2-GOF-containing integrins are typically clustered in a plaque on the basal surface of each imaginal disc cell (Figure 2E); this is similar to the plaques of wild-type integrins observed during pupal stages by BRABANT *et al.* (1996). The dorsal PS1 integrins in the same cells are not clustered by the activated PS2 dimers (Figure 2F). The mutant subunits are quite capable of generating wing blisters, even though expressed at levels that, for wild-type αPS2, would never make blisters. For example, wings from adults grown (at 25°) in the same vials as the larval disc shown in Figure 2C had a blister frequency of 65%. By contrast, Figure 2D shows a disc expressing wild-type αPS2m8 driven by the same enhancer trap (at 18°); although ectopic PS2 expression is much greater than that for the activated mutants, adult flies from this cross have a blister frequency of ~2%. (At 25°, the same wild-type-expressing cross shows dorsal PS2 expression similar to that seen for the αPS2-LOF in Figure 2B and a blister frequency of close to 100%.) Overall, four of eight *αPS2-GOF* inserts cause blisters as heterozygotes, although others begin to do so when homozygous. The *αPS2-GOF* inserts display a temperature sensitivity similar to that of wild-type *Blistermaker*, with the penetrance of blistering at 25° typically being at least 50% or more of the 28° frequency.

In summary, nonfunctional αPS2 subunits blister wings poorly, and activated subunits blister wings more efficiently than do wild type; these experiments demonstrate that the *Blistermaker* phenotype results from a gain of integrin function.

Finally, some additional observations indicate not only that the αPS2-LOF subunits are less effective at making wing blisters than are the wild-type or activated proteins, but also that the loss-of-function mutants may have their effects through a different mechanism as well. The inability of reduced βPS expression to enhance the penetrance of the wild-type *Blistermaker* was one of the reasons for originally thinking that this was a gain-of-function phenotype (BRABANT *et al.* 1998). By contrast, het-

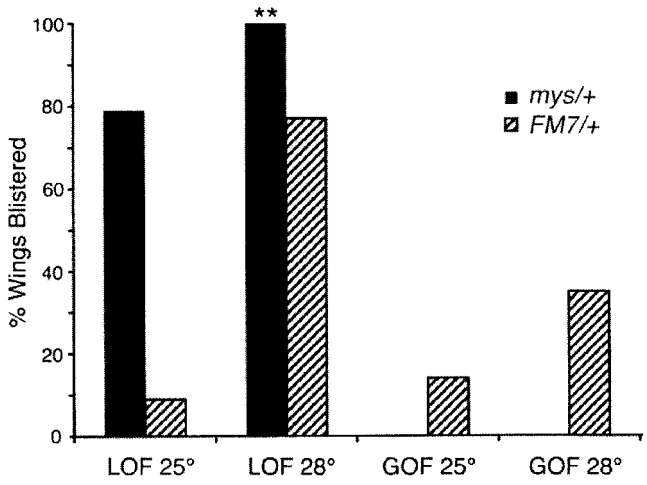

FIGURE 3.—Wing blistering of α*PS2-LOF* but not α*PS2-GOF* mutants is enhanced by reducing the dosage of βPS (*myospheroid*). Males with *UAS-αPS2* transgenes and the *337-GAL4* enhancer trap were crossed at 22° to females heterozygous for a strong *myospheroid* mutation over the *FM7* balancer chromosome, and progeny went through late larval and pupal development at 25° or 28°. Heterozygosity for *myospheroid* increased wing blisters induced by the loss-of-function αPS2; for the gain-of-function mutant, blistering was reduced to zero in *myospheroid* heterozygotes. The asterisks above the LOF 28° bar indicate that this value is based on only two wings, due to the greatly reduced viability of *myospheroid* heterozygotes that express αPS2-LOF; however, examinations of pharate adults trapped in pupal cases is consistent with the notion that this genotype results in completely nonapposed wings. For this experiment, α*PS2-LOF* line "*B*" and α*PS2-GOF* line "*C*" were used.

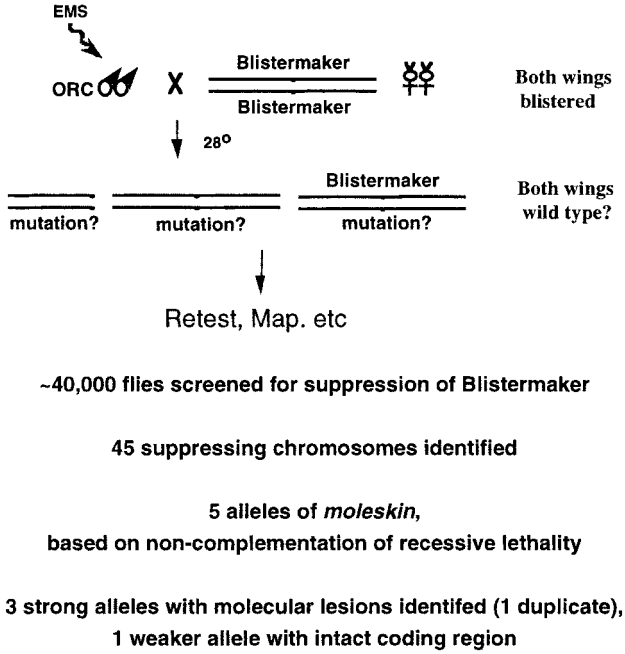

FIGURE 4.— Screen for suppressors of *Blistermaker*.

erozygosity for mutations in *myospheroid* (βPS) strongly enhances the wing blistering of α*PS2-LOF*-expressing animals (Figure 3). Additional evidence that the α*PS2-LOF* behaves as a dominant negative comes from its synthetic lethality with *myospheroid* (βPS) null mutations. That is, *myospheroid* mutants are typically recessive, and heterozygotes are completely viable and wild type. However, if *myospheroid* heterozygotes also express αPS2-LOF subunits, they are killed by high temperatures (28°). Even if embryogenesis (which is the most sensitive stage) is allowed to proceed at low temperature (22°), a postembryonic shift to 28° reduced adult viability to ~2% in one experiment (which included data from three different *myospheroid* alleles).

Second-site suppressors of *Blistermaker*: Since the *Blistermaker* phenotype results from an inappropriate integrin-related process, one might hope to suppress the phenotype by reducing the activity of related functions. We had found previously that the *Blistermaker* phenotype could be suppressed by heterozygosity for various mutant chromosomes (*e.g.*, BRABANT *et al.* 1998). Unfortunately, further analyses indicated that the phenotype was very sensitive to genetic background; that is, chromosomes from different wild-type strains could also have large effects on the penetrance of wing blistering. When testing for interactions by crossing *Blistermaker* to a stock with a particular mutant chromosome, the control and experimental classes of progeny will be genetically different at almost 20% (for the X chromosome) to 40% (for chromosomes 2 and 3) of the genome. We found that for most mutant chromosomes tested by crossing to different stocks, it was not possible to map all of the suppressing activity to a single mutant locus on the chromosome.

To circumvent the genetic background problem as well as to find potential unanticipated suppressing loci, we performed a genetic screen for suppressors of *Blistermaker*. The screen, illustrated in Figure 4, asks for mutations that will suppress *Blistermaker* in a dominant manner; the mutated chromosomes may or may not be recessive lethals. Because the beginning strains are isogenic, the genetic background is uniform and any changes in activity should result from mutations created by the EMS. Complementation between the different suppressing chromosomes was examined, using recessive lethality as an assay, and we found that one complementation group on the third chromosome was represented five times (although two of these alleles subsequently proved to be duplicates). Because this locus was identified as a suppressor of blistering, we named the corresponding gene *moleskin* (*msk*).

Before proceeding further, it was important to show that *moleskin*-mediated suppression is due to an effect independent of integrin expression. For example, we had found in earlier work (before the screen was undertaken) that some of our *Blistermaker*-suppressing chromosomes acted by reducing expression through the *684-GAL4* enhancer trap. We examined integrin expression

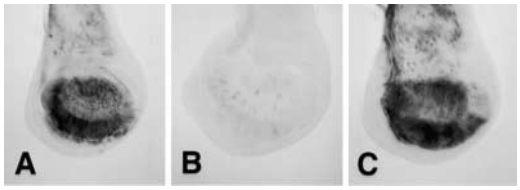

FIGURE 5.—β-Galactosidase expression in the wing pouch of late third instar imaginal discs, resulting from a *UAS-lacZ* transgene and the *684-GAL4* enhancer trap. (A) Wild type. (B) Heterozygote for the $Draf^{C110}$ chromosome, which suppresses *Blistermaker* apparently by reducing expression from the *684-GAL4* system. (C) Heterozygote for msk^5; there is no clear reduction in β-galactosidase expression.

directly in *moleskin/Blistermaker* animals and detected no obvious differences relative to *Blistermaker* heterozygotes alone (not shown). We also looked in detail at β-galactosidase expression in wing discs from animals bearing the *684* enhancer trap and a *UAS-lacZ* insert, with and without *moleskin* mutations (Figure 5). Finally, we asked if *moleskin* heterozygosity could suppress blistering in flies in which integrin expression is driven by a completely different set of regulators. These test animals contain an α*PS2* transgene driven directly by a *tubulin* promoter, with no enhancer trap or GAL4 intermediate. As shown in Figure 6, wing blistering is suppressed by *moleskin* regardless of the mode of expression. Chromosomes known to suppress the *Blistermaker* chromosome via reduction of expression from the enhancer trap are completely ineffective in suppressing the *tubulin-*α*PS2* animals.

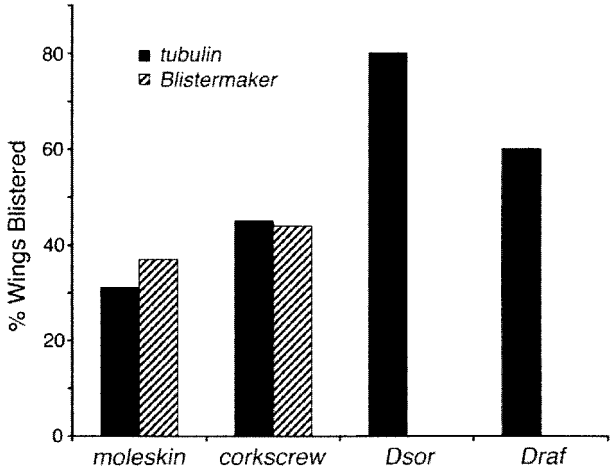

FIGURE 6.—*moleskin* suppression is independent of the GAL4-UAS expression system. Wing blistering suppression is similar for the msk^4 and csw^{eOP} heterozygotes whether integrin expression is driven by the *684* enhancer trap and a *UAS-*α*PS2* gene (*Blistermaker*) or directly from a transgene with a tubulin promoter. The $Dsor^{r2}$ and $Draf^{C110}$ chromosomes suppress *Blistermaker* completely (0% wings blistered), but have little effect on the tubulin-regulated blisters. For all crosses, blistering in the controls with the relevant balancer chromosomes (*TM3* for *moleskin*, *FM7* for *corkscrew*, and *Binsc* for *Dsor* and *Draf*) is 83–100%.

TABLE 1

Locations of *moleskin* mutations

Allele	Nucleotide change[a]	Protein change
msk^1	None in coding sequence	None
msk^2, msk^3	C inserted after A559	Frameshift after L184[b]
msk^4	C940 > T	R314 > stop
msk^5	G4 > T	E2 > stop

[a] Where nucleotide 1 is the A of initiating AUG.
[b] Leads to stop codon after residue 235.

***moleskin* encodes Drosophila importin-7:** To determine the molecular nature of the *moleskin* gene, the suppressing activity was genetically mapped to a region on the left arm of the third chromosome. Using the recessive lethality and a series of deficiencies, the gene was further localized to a small region within polytene bands 66B8–10. Molecular determination of deficiency breakpoints and comparison to the Drosophila genome defined a set of potential open reading frames, and one was selected for further analysis on the basis of the finding that its encoded protein, Drosophila importin-7 (DIM-7), was found to bind to the cytoplasmic tyrosine phosphatase Corkscrew in a two-hybrid screen (LORENZEN *et al.* 2001). The "Corkscrew connection" seemed potentially relevant since we had earlier found that a chromosome containing an antimorphic allele of *corkscrew* (csw^{eOP}) was a dominant suppressor of *Blistermaker*, although this finding has not been verified to be independent of other genetic background effects. Since the identification of *moleskin* as DIM-7, we have found that the csw^{eOP} mutant chromosome also suppresses blistering from the *tubulin-*α*PS2* insert (Figure 6). Dominant *Blistermaker* suppression by *corkscrew* appears to be dependent on the dominant negative properties of the csw^{eOP} mutation (ALLARD *et al.* 1996), since two chromosomes containing other strong alleles of *corkscrew* fail to suppress at similar levels (not shown). Sequencing of the *moleskin* chromosomes revealed that four of the five alleles contain mutations expected to truncate the encoded DIM-7 protein (Table 1); all of these alleles terminate translation in the first one-third of the predicted coding sequence of 1049 amino acids. These alleles have a similar lethal recessive phenotype (death of late embryos or early larvae, with a normal-looking cuticle) and, considering that the msk^5 allele contains a stop in the second codon, we believe that this is likely to be a protein null allele. The animals most likely survive embryogenesis as a result of a significant maternal contribution of wild-type activity (LORENZEN *et al.* 2001), which we have not been able to eliminate genetically (see below). We found no lesions in the coding region of the msk^1 chromosome. This allele has a weaker lethal phenotype (most homozygotes die as pupae), and it

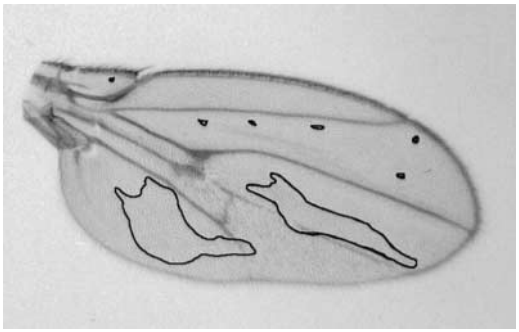

FIGURE 7.—Wing with homozygous clones of *msk*^{5} cells (outlines), induced during larval development. The posterior (lower) wing expresses wild-type DIM-7 protein, under the control of an *engrailed* enhancer trap. The wild-type DIM-7 rescues the growth inhibition phenotype of the posterior clones, which can grow to hundreds of cells. Anterior clones are typically eight or fewer cells.

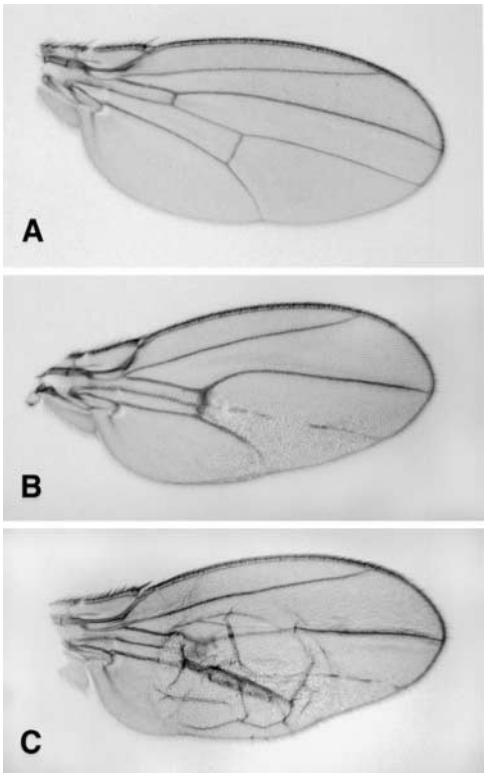

FIGURE 8.—Wild-type wing (A) and wings expressing DIM-7 under the control of an *engrailed-GAL4* enhancer trap (B and C), which is specific for the posterior wing. High levels of DIM-7 can cause the cross-veins to move closer (as in Figure 7) or to actually fuse into one long cross-vein (B). In some cases, blisters can result (C).

therefore probably results from a regulatory mutation. The DIM-7 protein is a member of the β-importin family of nuclear import proteins (LORENZEN *et al.* 2001). It is a close homolog of human importin-7 (also known as Ran binding protein 7, or RanBP7), being 53% identical in sequence, with the homology extending throughout the sequences. Recently, we showed that DIM-7 was important for the nuclear import of the activated MAP kinase D-ERK in response to signaling from receptor tyrosine kinases (LORENZEN *et al.* 2001).

***moleskin* function is required for growth:** To examine the phenotype of cells lacking wild-type DIM-7 in the wing, we generated clones of cells homozygous for the *msk*^{5} allele via somatic recombination in heterozygous animals (XU and RUBIN 1993). Induction of clones (identified by the cell marker *multiple wing hairs*) at various times during development fails to yield *moleskin* mutant clones >4–8 cells. Developmentally early induction often yields no clones at all, suggesting that the small clones depend on perdurance of wild-type gene product from the heterozygous clone precursor cell.

Since the entire left arm of the mutant chromosome 3 is made homozygous by this procedure, we wanted to make certain that the lack of clone growth was due to the *moleskin* mutation, and not to some other lesion. To do this, we made *msk*^{5} clones in wings in which wild-type DIM-7 was expressed in the posterior compartment, under the control of an *engrailed-GAL4* enhancer trap. In these wings, posterior *msk*^{5} homozygous clones grow to typical sizes of >100 cells, while anterior clones of >8 cells are not observed (Figure 7). Thus, DIM-7 function is required for growth of cells in the wing epithelium. This requirement for cell growth is likely to be fairly general, as we also failed to generate *moleskin* mutant clones in the female germ line, in an attempt to produce embryos missing the strong maternal component of DIM-7.

In the course of the above experiments, we noticed that abnormal expression of DIM-7 affects the patterning of the adult wings. With many *GAL4* enhancer traps to drive *UAS-DIM-7* we found that the animals are killed, but the *engrailed-GAL4* trap yields a variety of wing abnormalities, in part depending on temperature (the *GAL4* system often expresses at greater levels at higher temperatures). Comparing a series of wings of varying severity, we find that the two cross-veins move closer with increasing DIM-7, until they line up into one large vein (Figure 8). Also, in a small number of wings, blisters are formed.

***Blistermaker* suppression and D-ERK nuclear import:** Integrins have been shown in numerous systems to interact with growth factor receptors in regulating ERK activity (GIANCOTTI and RUOSLAHTI 1999; ASSOIAN and SCHWARTZ 2001), and so it seemed possible that *Blistermaker* suppression by *moleskin* mutants could indicate an integrin activation of the nuclear import of activated D-ERK. While D-EGFR (Drosophila epidermal growth factor receptor) and D-ERK are important throughout the wing epithelium for normal growth, increased function of each is also necessary in a complex series of steps to induce and support the differentiation of veins in the wing (STURTEVANT and BIER 1995; GUICHARD *et al.* 1999; MARTIN-BLANCO *et al.* 1999). We examined the

effects of heterozygosity for *moleskin* mutations in various mutants that affect vein formation. In each case we assayed the effects of the *moleskin*-containing chromosome relative to the control *TM3* balancer chromosome. These are the same *moleskin/TM3* stocks that have been used for *Blistermaker* crosses, where *moleskin* does suppress relative to *TM3*. Thus, if *Blistermaker* suppression is via D-ERK import, we might expect to see similar relative effects on vein formation.

The *rhomboid* gene encodes a protein that is believed to be involved in processing an extracellular activator of D-EGFR (Klämbt 2000), and *rhomboid* activity is specifically upregulated in the vein-forming regions of the wing (Sturtevant and Bier 1995; Guichard *et al.* 1999). We used two lines of flies that contain an inserted *rhomboid* transgene under the control of a heat-shock promoter (*HS-rhomboid*). Both inserts tested express low levels of *rhomboid* throughout the wing, resulting in ectopic veins (*HS-rhomboid*^{27B}) or at higher expression levels, wing blisters (*HS-rhomboid*^{30A}), without the need for inducing heat shocks. As shown in Figure 9, the *msk*^{4} chromosome does not suppress wing blisters generated by *HS-rhomboid*^{30A} nor does it suppress the extra veins of *HS-rhomboid*^{27B}. Indeed, the phenotypes are typically stronger in the *moleskin*-containing animals than in those bearing the *TM3* balancer chromosome. There also is no obvious suppression by the antimorphic *csw*^{eOP} chromosome or by two chromosomes that suppress *Blistermaker* by repressing the *684* enhancer trap (not shown).

Wing vein formation requires a complex scenario of D-ERK regulation in space and time (Martin-Blanco *et al.* 1999), and so we examined the ability of *moleskin* chromosomes to suppress other activating mutants. *Ellipse* (*Egfr*^{E1}) is a gain-of-function mutation of the gene encoding D-EGFR (Baker and Rubin 1989), and *Sevenmaker* (*rl*^{Sem}) is a dominant gain-of-function mutation in the gene (*rolled*) encoding D-ERK (Brunner *et al.* 1994). Both alleles tested here cause ectopic vein formation. As for the *HS-rhomboid* phenotypes above, neither the *Ellipse* nor the *Sevenmaker* wing phenotype is suppressed by the *msk*^{4} or *msk*^{5} chromosomes (not shown).

We also examined the effects of our *Blistermaker* suppressor stocks on a viable hypomorphic mutation in the D-ERK-encoding gene, *rolled*^{1} (*rl*^{1}). Reduced D-ERK function in homozygous *rl*^{1} flies often results in gaps in wing vein 4. The *msk*^{4} and *msk*^{5} chromosomes showed no clear enhancement of the *rl*^{1} wing gaps (Figure 10); in fact, relative to the *TM3* balancer, these chromosomes enhanced the phenotype.

In summary, we have crossed *moleskin* mutant stocks to a variety of mutations that alter the D-EGFR → D-ERK signaling pathway and gene expression. When comparing the effects of the same *moleskin* and control *TM3* chromosomes in each case, we see no positive correlation between the ability of a *moleskin* mutant chromosome to suppress integrin-induced wing blistering and its ability to suppress (in the case of gain-of-function mutations) or enhance (for loss-of-function mutations) phenotypes dependent on D-ERK-regulated gene expression (Table 2).

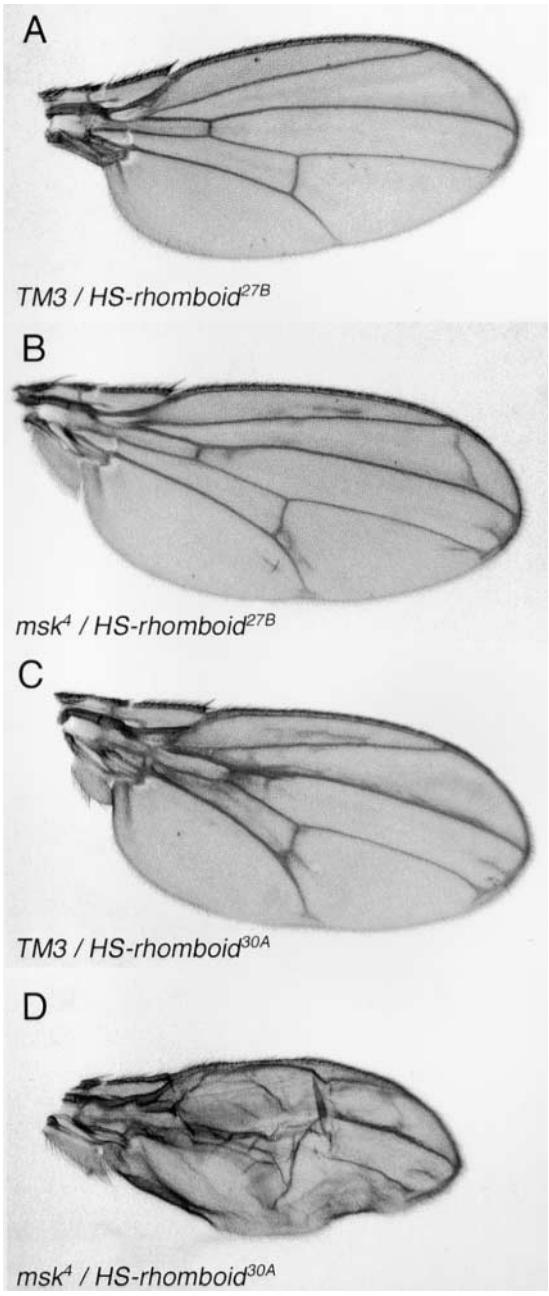

Figure 9.—Wings from cross of *msk*^{4}*/TM3* to flies with *HS-rhomboid* inserts that are expressed constitutively at relatively low (*HS-rhomboid*^{27B}; A and B) or high (*HS-rhomboid*^{30A}; C and D) levels. The inappropriate expression of *rhomboid* induces extra vein material (B and C) or, at high levels, wing blisters (D). In both cases, the phenotype is more severe in the animals with the *msk*^{4} chromosome than in those with the *TM3* balancer; this is in contrast to the relative effects of these chromosomes on the *Blistermaker* phenotype.

DISCUSSION

***Blistermaker* is a gain of function:** The *Blistermaker* phenotype is similar to the wing blistering that results

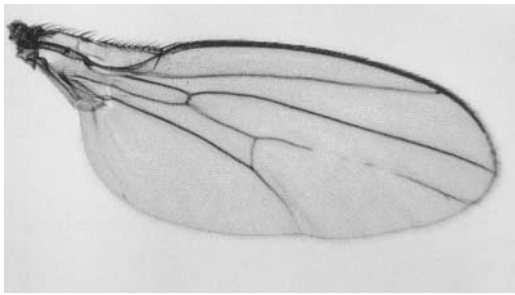

FIGURE 10.—Wing from *rolled*[1]*; msk*[4]/+ animal, showing breaks in vein 4. This phenotype is not significantly enhanced by *msk* relative to the *TM3* control chromosome (see also Table 2).

TABLE 2

Genetic interactions of *msk*[4] and D-EGFR or D-ERK mutations

	% of wings with phenotype	
msk[4]*/TM3* crossed to	*msk*[4]/+	*TM3*/+
Blistermaker; integrin gain of function (blisters)	14	99
HS-rhomboid[30A]; D-EGFR gain of function (blisters)	100	1
HS-rhomboid[27B]; D-EGFR gain of function (extra veins)	64	0
rolled[1]; D-ERK reduced function (break in vein)	0	38

from a loss of integrin function, either in integrin weak alleles or in clones of strong mutants (BROWN *et al.* 2000). However, the results reported here support the general idea of an active role for integrins in blistering. Especially striking is the fact that, although the ligand-binding-deficient subunits behave as enhancers of other loss-of-function phenotypes [for example, expression of α*PS2-LOF* can make *myospheroid* (βPS) heterozygotes lethal], they are poor inducers of wing blistering on their own. Moreover, the wing blistering that α*PS2-LOF* does induce is enhanced by *myospheroid* heterozygosity, suggesting that it results from a different mechanism from that of *Blistermaker*. The complementary finding that hyperactivated αPS2 subunits can create wing blisters when expressed at relatively low levels, which have no significant effect on endogenous integrin expression, demonstrates convincingly that *Blistermaker* is indeed a gain-of-function phenotype.

***Blistermaker* and D-ERK-dependent gene expression:** The *Blistermaker* phenotype is suppressed when expression of the β-importin DIM-7 is reduced in heterozygous *moleskin* mutant flies. The β-importins comprise a large family, responsible for the nuclear import of a wide variety of proteins (GÖRLICH and KUTAY 1999). The vertebrate homolog of DIM-7, importin-7, has been shown to translocate ribosomal proteins and histones (JÄKEL and GÖRLICH 1998; JÄKEL *et al.* 1999). Most interesting, though, is the demonstrated function of DIM-7 in the nuclear import of the activated MAP kinase D-ERK (LORENZEN *et al.* 2001), especially in light of the extensive literature connecting integrins and ERK signaling (GIANCOTTI and RUOSLAHTI 1999; ASSOIAN and SCHWARTZ 2001). Although the direct functional connection between DIM-7 and D-ERK has been demonstrated primarily in embryos, the inability of homozygous *moleskin* mutant clones to grow in the wing epithelium is consistent with a requirement downstream of growth factor receptor signaling in this tissue as well (*e.g.*, DIAZ-BENJUMEA and GARCIA-BELLIDO 1990; DIAZ-BENJUMEA and HAFEN 1994).

However, it seems unlikely that *moleskin* mutants dominantly suppress *Blistermaker* by reducing expression of genes that depend on the nuclear translocation of activated D-ERK. We say this because wing vein formation, which depends on a series of specific growth factor-initiated D-ERK signals (STURTEVANT and BIER 1995; GUICHARD *et al.* 1999; MARTIN-BLANCO *et al.* 1999), and *Blistermaker* display different relative sensitivities to the *moleskin* and *TM3* chromosomes.

At first glance, it might appear paradoxical that *moleskin* chromosomes do not seem to suppress (and even enhance) events known to require nuclear import of activated D-ERK or, conversely, that *moleskin* chromosomes do not seem to enhance the effects of *rolled* (D-ERK) loss-of-function alleles. Indeed, we previously reported data that appear to contradict the current findings (LORENZEN *et al.* 2001). However, it must be remembered that in each cross we are comparing a chromosome with a *moleskin* allele to another chromosome, in this case a third chromosome balancer, *TM3*. Thus, ~40% of the genomes are different between the experimental and control classes, and comparisons with other crosses (in which the control is a first chromosome balancer) suggest that our *TM3, Sb* chromosome is something of a suppressor of the activated D-ERK phenotypes. So, in each case here we are assaying the effects of the *moleskin*-containing chromosome relative to a chromosome that also may be a suppressor of vein formation; we are not asking if *moleskin* is a suppressor or enhancer in an absolute sense. What is important is that these same stocks are those that have been used for *Blistermaker* crosses, in which *moleskin* does suppress relative to *TM3*. Thus, if *Blistermaker* suppression is via D-ERK import, we would expect to see similar relative effects on vein formation, and we do not.

Although DIM-7 immunoprecipitates activated D-ERK (LORENZEN *et al.* 2001), we have no direct evidence that *moleskin* mutants mediate *Blistermaker* suppression through D-ERK. There almost certainly are other cargos for DIM-7, and we have no data concerning the sensitivities of these potential nuclear transport or other events to *moleskin* dosage. However, for simplicity, we will focus the following discussion of mechanistic possibilities on ERK, since we know that it can both associate with DIM-7

and regulate integrin function. Formally, the arguments presented could be applicable to other, currently unknown regulators as well.

Does *moleskin* suppress *Blistermaker* by increasing cytoplasmic D-ERK? H-Ras can be a suppressor of integrin activation, and data suggest that it may act via activated ERK in a transcription-independent manner (Hughes *et al.* 1997). These findings lead to possible models for *Blistermaker* suppression by *moleskin* heterozygosity, via the negative regulatory activity on integrins of activated cytoplasmic D-ERK. For example, reducing DIM-7 by 50% in a *moleskin* heterozygote would be expected to reduce nuclear import of activated D-ERK, although since DIM-7 is not necessarily the major rate-limiting step in D-ERK signaling, the reduction in activated D-ERK in the nucleus would likely be <50%. But because the amount of activated D-ERK that transits to the nucleus is typically much greater than that which travels to the cell periphery, a modest reduction in nuclear import can result in a relatively large increase in cytoplasmic D-ERK activity. Thus, compared to their effects on gene expression, *moleskin* heterozygotes might have a greater capacity to affect integrin activation directly, suppressing the effects of *Blistermaker*.

Two observations might seem to argue against the notion that DIM-7 and D-ERK regulate integrin function in the wing, but in fact they do not. The first is that *moleskin* chromosomes appear to be able to suppress blistering from the α*PS2-GOF* transgenes (our unpublished data), which should not easily be regulated by cytoplasmic events. This suppression is less dramatic than that for *Blistermaker*, and its interpretation is subject to some of the genetic background difficulties described earlier. Most importantly, however, it must be remembered that blistering may depend on some amount of active αPS1-containing integrins in the same dorsal cells, and these will still be susceptible to D-ERK-mediated regulation.

A second potential difficulty is that this model predicts that activated D-ERK generally should be a *Blistermaker* suppressor. In apparent contradiction, the *Sevenmaker* mutation of the D-ERK-encoding gene *rolled*, which leads to elevated levels of phosphorylated D-ERK, is an enhancer of *Blistermaker*, not a suppressor. However, the *Sevenmaker* mutation is in a docking domain of D-ERK that alters the ability of the protein to interact with downstream effectors as well as with D-ERK-regulating kinases and phosphatases (Tanoue *et al.* 2000). Thus, although *Sevenmaker* enhances some phenotypes that require D-ERK-activated gene expression, it is difficult to predict its effects on a particular event *a priori*.

A function for cortical DIM-7? The β-importin family of proteins is principally linked with nuclear import of protein cargos. However, recently other functions have been associated with members of the importin superfamily. For example, importin-β, in some cases with importin-α, functions in vertebrates to sequester microtubule polymerization factors early in mitosis (Gruss *et al.* 2001; Nachury *et al.* 2001; Wiese *et al.* 2001). Mitotic microtubule formation can be triggered by the release of the polymerization regulators by RanGTP, just as RanGTP binding to importin-β leads to release of cargos inside the nucleus.

DIM-7 protein can be detected immunologically at the cell cortex, both in early Drosophila embryos (Lorenzen *et al.* 2001) and in S2 cells in culture (our unpublished results). It thus seems reasonable to consider a more direct connection between the peripheral DIM-7 and integrin regulation. Additionally, it appears that a mutation in *corkscrew*, the Drosophila SHP-2 homolog (Perkins *et al.* 1992, 1996), can also suppress *Blistermaker* and that Corkscrew protein binds directly to DIM-7 (Lorenzen *et al.* 2001). Although Corkscrew has been implicated primarily in signaling events downstream of receptor tyrosine kinases (Perkins *et al.* 1992, 1996; Allard *et al.* 1996; Herbst *et al.* 1996; Cleghon *et al.* 1998; Johnson Hamlet and Perkins 2001), vertebrate SHP-2 has been implicated in signaling via a host of growth factor receptors, cytokines, hormones, and antigens (reviewed by Feng 1999). Most relevant to our discussion, SHP-2, often in association with the membrane glycoproteins PECAM-1 or SHPS-1, has been shown to be involved in many integrin-dependent signaling events and also to be important in regulating integrin-mediated cell adhesion, spreading, or migration (Jackson *et al.* 1997; Sagawa *et al.* 1997; Tsuda *et al.* 1998; Yu *et al.* 1998; DeMali *et al.* 1999; Mañes *et al.* 1999; Oh *et al.* 1999; Inagaki *et al.* 2000; Schoenwaelder *et al.* 2000; Lacalle *et al.* 2002). While SHP-2 is a cytoplasmic tyrosine phosphatase, some experiments suggest that it can serve as a scaffolding protein at or near the plasma membrane. For example, a Corkscrew protein mutated in the phosphatase domain retains significant wild-type activity *in situ*, and this activity is increased if the protein is targeted to the plasma membrane (Allard *et al.* 1998).

It is likely therefore that cell surface receptors mediate a localized Corkscrew/SHP-2 activation of cortical DIM-7. This active DIM-7, in combination with associated factors such as D-ERK, could then function more directly in integrin regulation. A more direct connection between DIM-7 and integrin function is also consistent with the fact that *moleskin* mutations were especially common among the suppressors isolated in the screen. A key question for future work, therefore, will be defining the subcellular location at which DIM-7 functions with respect to integrin-related phenotypes.

Integrin regulation of nuclear import: Recently, evidence has begun to appear that integrin engagement with the ECM can regulate nuclear import of regulatory molecules. For example, Bianchi *et al.* (2000) found an association between αLβ2 and the c-Jun coactivator JAB1 and suggest that this connection regulates the nuclear localization of JAB1. More directly relevant to

our results, Aplin *et al.* (2001) reported that ERK nuclear translocation in fibroblasts is dependent on an integrin-mediated event, also involving the actin cytoskeleton. Also, Hirsch *et al.* (2002) found that primary mouse embryo fibroblasts with a β1 integrin cytoplasmic mutant show reduced nuclear translocation of phosphorylated ERK. Regardless of the importance of nuclear transport in *Blistermaker* suppression, our genetic data indicate a functional connection between integrins and a specific importin-β that can transport activated ERK and suggest another potential molecular mechanism whereby integrin and growth factor signals can be integrated by the cell.

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