Amino Acid Changes in Drosophila αPS2βPS Integrins That Affect Ligand Affinity

We developed a ligand-mimetic antibody Fab fragment specific for Drosophila αPS2βPS integrins to probe the ligand binding affinities of these invertebrate receptors. TWOW-1 was constructed by inserting a fragment of the extracellular matrix protein Tiggrin into the H-CDR3 of the αvβ3 ligand-mimetic antibody WOW-1. The specificity of αPS2βPS binding to TWOW-1 was demonstrated by numerous tests used for other integrin-ligand interactions. Binding was decreased in the presence of EDTA or RGD peptides and by mutation of the TWOW-1 RGD sequence or the βPS metal ion-dependent adhesion site (MIDAS) motif. TWOW-1 binding was increased by mutations in the αPS2 membrane-proximal cytoplasmic GFFNR sequence or by exposure to Mn2+. Although Mn2+ is sometimes assumed to promote maximal integrin activity, TWOW-1 binding in Mn2+ could be increased further by the αPS2 GFFNR → GFANA mutation. A mutation in the βPS I domain (βPS-b58; V409D) greatly increased ligand binding affinity, explaining the increased cell spreading mediated by αPS2βPS-b58. Further mutagenesis of this residue suggested that Val-409 normally stabilizes the closed head conformation. Mutations that potentially reduce interaction of the integrin β subunit plexin-semaphorin-integri (PSI) and stalk domains have been shown to have activating properties. We found that complete deletion of the βPS PSI domain enhanced TWOW-1 binding. Moreover the PSI domain is dispensable for at least some other integrin functions because βPS-APSI displayed an enhanced ability to mediate cell spreading. These studies establish a means to evaluate mechanisms and consequences of integrin affinity modulation in a tractable model genetic system.

Integrins are the primary family of receptors that connects cells to the extracellular matrix (ECM) (1). The cytoplasmic tails of integrin subunits associate with multiple intracellular components, which mediate both signaling functions and ECM-cytoskeleton connections (2). Cells can regulate the functions of integrins at least in part by inducing conformational changes in integrin structure that alter the affinity of the integrin heterodimer for ECM ligands. Similarly integrin binding to ligands can lead to outside-in signal propagation, which can regulate cellular behaviors such as growth, differentiation, and survival (3–7).

The integrin heterodimer is composed of α and β subunits that are nonhomologous to one another but strongly conserved structurally across the animal kingdom (8, 9). A long history of studies with conformation-sensitive antibodies indicates that integrins undergo large and concerted conformational changes as a result of cellular activation, and recent studies have begun to provide details of this structural switching (10–16). Inactive integrins are likely to be bent in the middle so that the headpiece faces toward the membrane-proximal part of the extracellular stalks. As a result of cellular activation, the integrin adopts an extended conformation with the headpiece facing away from the cell in optimal position to engage ECM proteins. The headpiece also can adopt two states, termed the open or closed conformations, corresponding to high and low affinity states for ligand binding. Binding of substrate ligands traps the integrin in the open conformation, and this shift in the equilibrium may then trigger outside-in signaling in many cases involving integrin clustering. This view of integrin dynamics is almost certainly simplified with other intermediate states probable (for example, bent integrins can also bind ligand (17)), but overall it can provide a consistent explanation of the structure-function relationships between cellular regulation and signaling.

Invertebrates such as Drosophila melanogaster provide a system for sophisticated genetic studies of integrin function that are not practical with vertebrates (18). Genetic studies with flies can also be complemented with cell biology experiments in cell culture, and this system has become especially attractive with the simplicity of RNA interference methods in Drosophila (19). However, one persistent drawback to studies of integrin structure and function in invertebrates has been a lack of probes for measuring affinity states of integrins directly. Even in cell culture, Drosophila experiments have relied on indirect assays such as cell adhesion or spreading to quantitate integrin activity. Although these methods have generally provided a consistent set of data, a tool for measuring ligand affinity directly could permit much more reliable and rapid assays that can complement genetic analyses of integrin function in Drosophila.

Here we describe the generation of TWOW-1, a novel monovalent antibody Fab fragment that functions as a ligand-mimetic affinity sensor for αPS2βPS. Using this probe, we show that previously described mutants in both the αPS2 and βPS subunits led to changes in affinity of the fly integrins. Finally we demonstrate that complete removal of the β subunit N-terminal PSI domain caused an increase in ligand affinity.

MATERIALS AND METHODS

Cell Culture—Drosophila S2/M3 were cultured in Shields and Seng M3 medium supplemented with 12% heat-inactivated fetal calf serum as described previously (20). Cells were co-transfected with plasmids expressing an αPS2C subunit and a βPS subunit, both under the regulational data.
lation of the heat shock protein 70 promoter, and with the bacterial dihydrofolate reductase selectable marker (plasmid p8HCO) as described previously (21).Transformed cells were selected in 2 × 10⁻⁷ M methotrexate.

Prior to performing TWOW-1 assays, cells were treated with double-stranded RNA targeting the 3’-untranslated sequence of myosinoid to remove expression of endogenous βPS integrin as described earlier (21); this 3’-untranslated sequence is not present in the βPS transgenes. A few modifications to our previous RNA interference protocol were made. The initial 30-min treatment of cells with double-stranded RNA in serum-free medium was done in M3 medium instead of HyQ-CCM3. Also instead of treating 1 × 10⁶ cells in 0.7 ml of serum-free medium in a 6-well plate, we treated 8 × 10⁶ cells in 1.4 ml of serum-free medium (containing 42 µg of myosinoid double-stranded RNA) in a 60-mm Petri dish for 30 min followed by the addition of 2 volumes of M3 medium containing 16.7% heat-inactivated serum. Preliminary experiments demonstrated that the RNA interference effect was not diminished by the higher concentration of cells and that Petri dishes instead of tissue culture plastic resulted in less disruption of the cells when they were removed prior to TWOW-1 binding experiments.

PS Integrin Constructs—pHSαPS2C (20) or the same construct containing the GFFNR → GFANA activating mutation (pHSαPS2C<sub>ANNA</sub>) express the α subunit (21). β subunits were expressed from pH5βPS4A (20) or its variants. The construct expressing βPS5-b5 has been described previously (22). The PSI deletion in βPS was introduced using standard PCR and molecular biology protocols and removed all of the amino acids that lie between but not including Thr-39 and Pro-78.

Construction and Purification of TWOW-1—TWOW-1 Fab was constructed by replacing the DNA fragment encoding the 50-amino acid penton base 5 in the heavy chain complementarity-determining region 3 (H-CDR3) of WOW-1 fd in pMThiBiP/V5-HisB (23) with a sequence encoding 53 amino acids found in Drosophila Tigrinn. These 53 amino acids contain the Tigrinn RGD sequence and 24 amino acids prior to and 26 amino acids following the RGD. As a negative control we also constructed LGA-TWOW-1, which is identical to TWOW-1 except for amino acids that lie between but not including Thr-39 and Pro-78.

Preliminary experiments gave the following results, which guided our design of the TWOW-1 assay. Phosphate or HEPES-buffered salines were not used for the TWOW-1 binding experiments as they result in precipitate formation when Mn²⁺ is added. Carbonate-buffered salines were avoided as their pH changes with time and exposure to CO₂ in the air. pH 7.5 gave slightly better TWOW-1 binding than pH 6.5 and pH 7.0. Simultaneous incubation of cells with TWOW-1 and secondary antibodies led to artificially high levels of binding. Varying the concentrations of the antibodies and TWOW-1 suggested that this resulted from aggregation of the primary and secondary antibodies prior to binding to the cells. Varying the TWOW-1 incubation time had relatively
little effect on the results. Specifically incubations of 1, 10, or 30 min were virtually indistinguishable. If cells were centrifuged out of the TWOW-1 solution and resuspended in fixative, specific binding was reduced ~20% relative to that seen when cells were diluted directly into fixative from the TWOW-1 incubation solution. Changing the BSA concentration between 0.2 and 5 mg/ml made no difference in TWOW-1 binding levels or background binding. This is not unexpected as the cells are incubated for 4 h in M3 + 2 mg/ml BSA prior to the TWOW-1 binding assay.

Transient Expression—To measure the surface expression of the activating mutants we performed transient expression experiments. To ensure uniformity of the different DNA constructs all were prepared at the same time. Cells were transformed with the indicated α and β subunits together with the selection plasmid pH8CO as for stable transformations. The β subunits were epitope-tagged (with Myc-EQKLISEEDL or hemagglutinin (HA)-YPYDVPDYA) in the serine-rich loop between Ser-137 and Gly-138 to allow immunostaining specifically for the transfected genes. One day after transformation the medium was replaced with medium containing methotrexate (to kill non-transfected cells), and the cells were transferred from 6-well tissue culture plates to 4-ml Petri dishes. Cells do not adhere to Petri dishes so potential artifacts because of differential adherence of cells were avoided. Three days later cells were collected for staining. For HA staining, cells were incubated at room temperature in 10 μg/ml AlexaFluor488-conjugated anti-hemagglutinin (Molecular Probes catalog number A-21287). For Myc staining, cells were first incubated at room temperature with 2 μg/ml biotin-conjugated anti-c-Myc (Sigma catalog number B-7554), then centrifuged, and resuspended in 10 μg/ml R-phycocerythrin-conjugated streptavidin (Molecular Probes catalog number S-866) for 25 min on ice. Antibody and streptavidin staining was done on 1 × 10⁶ cells in 100 μl of growth medium (M3 + 12% fetal calf serum). Following antibody incubation, cells were centrifuged and resuspended in 1 ml of PBS containing 2% formaldehyde, and then 10,000 cells were analyzed by flow cytometry. βPS-ΔPSI and βPS-ΔPSI-b58 double mutants were tagged with Myc, βPS-b58 was tagged with HA, and wild-type βPS controls were tagged with either Myc or HA. Expression levels are given as a percentage of the mean fluorescence of the wild-type βPS. The values given are the average and S.E. of three transfection experiments for each cell line.

Cell Spreading—Cell spreading assays were done as described previously (25). Briefly cells were protease-cleared as in the TWOW binding experiments and then plated on a Tigrin ligand (RBB-Tigg at a coating concentration of 32 ng/ml) for 4 h. Spread and round cells were visually scored. The values given are the average of two cell spreading assays. In each experiment more than 100 cells were scored from each of three separate fields.

Homology Modeling of Drosophila βPS—The human αvβ3 crystal structure coordinates (Protein Data Bank code 1Jv2) (10) were downloaded, and the structure was visualized in Sybyl 7.0 (Tripos). Drosophila βPS sequence was aligned with the human β3 chain (ClustalW), and the alignment was adjusted by eye to obtain an optimal template for homology modeling. The model was built based on the first 434 residues of the β3 structure as the template utilizing Modeler (version 7.0). The model was energy-minimized using the Powell method (1000 cycles, −1730 kcal/mol) (Sybyl 7.0). The validity of the model was confirmed using the Ramachandran plot, which was shown to be consistent with the β3 structure. Superposition of the backbone Ca atoms of βPS onto the β3 structure demonstrated a root mean square value of 0.75 Å confirming the correctness of the model. Subsequently the V409D mutation was incorporated into the βPS model and energy-minimized (100 cycles, −1700 kcal/mol) (Sybyl 7.0). Both βPS models were utilized to evaluate potential side chain interactions in the vicinity (5–10 Å) of the mutated residue.

RESULTS

Development of TWOW-1—A commonly used tool for assays of integrin affinity modulation is the monoclonal antibody PAC-1 or corresponding Fab fragment (26). PAC-1 is a ligand-mimetic for the platelet integrin αIIbβ3 and is used to quantify integrin affinity in soluble binding assays. Recently we demonstrated that PAC-1 could be modified by patch engineering to generate a new Fab (TWOW-1) specific for high affinity αvβ3 and αvβ5 (23). The integrin selectivities of PAC-1 and WOW-1 are due in large part to an RGD or RYD tract in H-CDR3, analogous to those in ECM proteins that function as integrin ligands (23, 27). The Drosophila ECM protein Tigrin is a natural ligand for the Drosophila αPS2βPS integrins, and integrin-Tigrin associations have been shown to require an intact RGD Tigrin sequence (28, 29). A 50-amino acid Tigrin fragment centered on the RGD motif also functions as a highly effective αPS2βPS ligand in a variety of cell biology assays (30). Therefore, we used the sequence of this Tigrin fragment to replace the RGD tract in the H-CDR3 of WOW-1 Fab to generate the TWOW-1 Fab, a novel tool for assessing structure-function relationships of the Drosophila αPS2βPS integrins.

TWOW-1 Fab showed dose-dependent binding to cells expressing αPS2βPS integrins (all experiments reported here use the αPS2C splice variant (31)) in the presence of Ca²⁺ and Mg²⁺ (Fig. 1A). This binding was inhibited by EDTA and RGD peptides and by mutations to the βPS MIDAS ligand binding site (supplemental data). Moreover TWOW-1 in which the RGD sequence was mutated to LGA also showed reduced binding (supplemental data). These controls indicate that binding of soluble monovalent TWOW-1 Fab is a valid indicator of αPS2βPS affinity for ligand.
Drosophila αPS2βPS Is Sensitive to Affinity Modulation by Mn2+ and αPS2 Cytoplasmic Domain Mutation—Cations form a critical part of the ligand binding region of the I domains of both α and β subunits, and changes in cation coordination are important mediators of the conformational changes that modulate integrin-ligand affinity (12, 14, 32, 33). Mn2+ is known to activate integrins generally and is believed to function primarily by binding to one or more sites on the integrin β subunit (12, 34). Indeed Mn2+ is often used to determine a “maximal” value for integrin-ligand affinity for comparisons with various experimental conditions. We tested TWOW-1 binding in various concentrations of Mn2+ (see supplemental data) and found maximal stimulation at 1 mM Mn2+, leading to saturable binding at lower concentrations of TWOW-1. For cells expressing wild-type integrins, the Mn2+-induced increase in TWOW-1 binding was greater than 2-fold at 15 μg/ml TWOW-1 (Fig. 1A). Expressed another way, ~8-fold more TWOW-1 was required to obtain similar levels of integrin binding in the absence of Mn2+. The half-maximal binding values yield apparent αPS2βPS-TOWOW-1 Kd values of ~1 and 0.12 μM in the absence or presence of Mn2+, respectively.

Mutations in the membrane-proximal region of the α subunit cytoplasmic domain have been shown to activate integrins, and these mutations are believed to at least partially mimic the inside-out signaling important in cellular regulation of integrin function (35–38). We have shown previously that the GFFNR → GFANA mutation of αPS2 increases the cell spreading and cell adhesion properties of αPS2βPS expressed in S2 cells (25). TWOW-1 binding to these cells was also increased dramatically, demonstrating that the mutation enhances integrin affinity as for vertebrate integrins (Fig. 1, A and B, compare “no Mn2+” curves).

Surprisingly the GFFNR → GFANA mutation also appeared to increase the affinity of αPS2βPS for TWOW-1 in the presence of Mn2+ (Fig. 1, A and B, compare Mn2+ curves), demonstrating that this cation does not fully activate the wild-type integrin. Affinity measurements of integrins and ligands are often measured as a ratio of binding under certain conditions relative to binding seen in the presence of optimal concentrations of Mn2+, which is assumed to be the maximal value. Our results indicate that this assumption is not necessarily true at least for αPS2βPS. Because binding of TWOW-1 to wild-type αPS2βPS in Mn2+ does not represent the maximal value, we chose throughout to express TWOW-1 binding relative to total integrin levels as measured by the αPS2-specific antibody CF.2C7.

**mys**<sup>586</sup> Increases Integrin Affinity for TWOW-1—In a screen for mutations in the Drosophila gene encoding the βPS subunit (myospheroid), we found an unusual mutant that alters integrin function. The mys<sup>586</sup> allele (V409D) has properties in developing animals that suggest it may enhance integrin activity, and cell spreading and adhesion experiments with mys<sup>586</sup> are consistent with this proposal (22). The V409D mutation lies at the top of the α7 helix of the β subunit I domain (Fig. 2A). This residue is highly conserved in integrin β subunits (but not completely so; see Ref. 22 for discussion of the exception), and in the low affinity “closed headpiece” conformation Val-409 forms part of a conserved hydrophobic interface between α7 and the neighboring α1 helix (Fig. 2B). It therefore seemed likely that insertion of an aspartate residue here might disrupt this interaction, and we proposed that mys<sup>586</sup> might specifically alter the equilibrium between the “open” and “closed” conformations of the β I domain (22).

We found that cells expressing αPS2βPS-b58 showed higher affinity for TWOW-1 than cells expressing wild-type integrin (Fig. 3), indicating that the phenotypic effects of mys<sup>586</sup> can be explained by increases in ligand binding activity. TWOW-1 binding to αPS2βPS-b58 was greater than TWOW-1 binding to GFANA-activated heterodimers and approached that obtained when wild-type cells were exposed to Mn2+. αPS2βPS-b58 binding was increased when cells were incubated with Mn2+, although the difference was small relative to Mn2+ effects on wild-type integrins. Thus, βPS-b58 is a strong activator of integrin-ligand affinity.

Models of βPS in the open or closed headpiece conformation suggest the possibility of a specific interaction between the b58 aspartate and a lysine residue in the α1 helix (Fig. 2). However, the models cannot directly answer the question of whether the increased ligand affinity of βPS-b58 integrins results from a loss of a closed conformation-stabilizing interaction dependent on Val-409 or a novel molecular interaction involving the new aspartate residue. To address this, we made αPS2βPS

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**FIGURE 2. Modeling of the βPS-b58 structure.** A, the I domain of βPS was modeled based on the x-ray structure of β3 in the “headpiece closed” conformation (10). The α7 helix, which shifts down in the open conformation, is colored violet, and side chains are colored as follows: Val-409, red; the two nearby leucines of the α1 helix, green; and the lysine (Lys-210) that potentially may interact with Asp-409 in the b58 mutant, blue. B, same structure rotated and with the colored residues filled, showing the close apposition of the α7 helix valine with the α1 helix leucines.
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integrins with different residues in position 409. Substitution of lysine, alanine, or phenylalanine for valine all led to increased TWOW-1 binding (Fig. 4). Although none of these new mutants bound as well as the original Asp-409 b58 mutant, they all gave values as least twice that of the wild type, and the Val → Ala mutation was almost as good as Val → Asp. This result indicates that the increased TWOW-1 binding of αPS2βPS-b58 results from the loss of a stabilizing interaction mediated by Val-409 and provides strong evidence that the conserved hydrophobic interface between the α1 and α7 helices helps to stabilize the closed headpiece conformation of integrin β subunits.

Deletion of the PS1 PSI Domain Increases Affinity for TWOW-1—The PSI domain connects the β subunit N-terminal region to the top of the β stalk (32, 39). Although recent data suggest that the ancestral “PSI domain” includes a small tract of primary sequence at the stalk-hybrid interface, we use the term PSI domain in the traditional sense to indicate only the domain encoded by contiguous primary sequence N-terminal to the hybrid domain. Specifically the PSI domain forms a disulfide bridge with the stalk at the beginning of the first epidermal growth factor-like repeat and also interacts with residues on the bottom of the hybrid domain. Mutations that disrupt the PSI stalk disulfide (in αIIbβ3) or potentially interfere with other PSI-stalk interactions (in αXβ2) have been reported to cause increased integrin activation (40, 41). On the other hand, a recent structural study of αIIbβ3 heads bound to small ligands found that the PSI domain remained firmly attached to the stalk and hybrid domains, and it was suggested that this connection may help to propagate structural movements of the hybrid domain to more membrane-proximal regions of the β subunit (32).

In a screen for β subunit lesions that affected integrin function, we isolated point mutations in the PSI domain (22), and preliminary experiments showed slightly increased cell spreading activity of the mutant integrins. This led us to question whether the PSI domain is required at all for integrin-ligand binding, and we constructed βPS subunits in which this domain was deleted completely. Cells expressing αPS2βPS-ΔPSI showed increased TWOW-1 binding relative to cells expressing wild-type integrins (Fig. 5A). Their affinity for TWOW-1 could be further increased by Mn2+. Fully functional integrins must do more than simply bind soluble ligands, so we also examined the ability of αPS2βPS-ΔPSI to mediate cell spreading on a Tigggin protein fragment. αPS2βPS-ΔPSI was superior to wild type in this assay as well (Fig. 5B).

If two activating mutations affect different molecular equilibria, one might expect that their effects on TWOW-1 binding would be at least partially additive. With this in mind, we asked whether the activating effects of PSI domain deletion are additive with the other two activating mutations described above. We attempted to test integrin heterodimers with both αPS2-GFANA and βPS-ΔPSI but were unable to detect this heterodimer on the cell surface. However, the double mutant αPS2βPS-ΔPSI-b58 integrins showed very high affinity for TWOW-1 similar to that seen with αPS2-GFANA/βPS-b58 (Fig. 6). This suggests that the αPS2βPS-ΔPSI-b58 double mutant can drive both of the major conformational changes proposed to alter integrin-ligand affinity (see “Discussion”).

Finally as shown below, the activating mutations often lead to reduced cell surface integrin expression. The TWOW-1 binding data shown in Fig. 6 were derived from various stably transformed cell lines

FIGURE 3. The ΔPS1 mutation (V409D) increases affinity for TWOW-1. Values are for binding at 15 µg/ml TWOW-1 (see Fig. 1). The ΔPS1 domain mutation increases affinity for TWOW-1, more so than the activating αPS2 cytoplasmic mutation. Binding is expressed as a ratio of specific TWOW-1 immunofluorescence over total integrin. wt, wild type.

FIGURE 4. Mutations at βPS 409 increase ligand binding. Binding of TWOW-1 (at 15 µg/ml) to αPS2βPS integrins in which Val-409 has been mutated to aspartate (as in βPS-b58), lysine, alanine, or phenylalanine is shown. Any change from valine leads to increased TWOW-1 binding with Ala-409 being almost as strong as aspartate. This indicates that the increased activity of ΔPS1-b58 is not due to new specific interactions involving Asp-409. Binding is expressed as a ratio of specific TWOW-1 immunofluorescence over total integrin. wt, wild type.

FIGURE 5. ΔPS1 increases integrin ligand affinity. A, complete removal of the N-terminal β subunit PSI domain (ΔPSI) greatly increases TWOW-1 binding. B, integrins without the PSI domain also show increased ability to mediate cell spreading on Tigggin fragments even though these cells express lower levels of surface integrin compared with wild type (see Fig. 7). Data in B are means from two experiments. Binding is expressed as a ratio of specific TWOW-1 immunofluorescence over total integrin. wt, wild type.

2 T. A. Bunch, unpublished results.
that may display significantly different levels of expression. However, the ratio of TWOW-1 binding/total integrin was similar for each mutant, regardless of integrin expression levels.

Effects of Activating Mutations on Integrin Expression—Deletion of the membrane-proximal GFNNR sequence of the αPS2 subunit cytoplasmic domain leads to reduced surface expression of integrin heterodimers in developing flies (42). Our transformed cell lines with GFNNR → GFANA mutations also displayed decreased integrin expression relative to those transformed with wild-type genes, and this phenomenon was also seen with βPS-ΔPSI transfomers. Various selected transformed lines can vary in their expression levels, so to better compare the effects of these activating mutations on surface expression we transiently transfected S2 cells with similar amounts of integrin DNA and assayed surface expression 4 days later. In each case, the transformed βPS genes contained an epitope tag (Myc or HA) in the serine-rich loop of the extracellular hybrid domain, allowing the detection of proteins expressed specifically from the transformed genes as opposed to endogenous βPS subunits.

Integrins with activating mutations were expressed at reduced levels in transient transfectants (Fig. 7). Although the reductions were not as severe as those generally seen in stable transformed lines, the relative expression levels of the various mutants were similar in both conditions. Most strikingly, increased ligand affinity did not necessarily correlate with reduced heterodimer expression at least quantitatively. This is illustrated by the fact that βPS-b58 reduced integrin expression relatively modestly despite the observation that this mutation greatly increased TWOW-1 binding. This is also consistent with observations of various stably transformed βPS-b58 lines where expression often is reduced slightly but may be at levels comparable to wild type. Interest-

FIGURE 6. βPS-ΔPSI-b58 is stronger than either individual mutation. Combining the two activating βPS mutations can lead to integrins with a very high affinity for TWOW-1 compared with βPS-ΔPSI or βPS-b58 alone. The upper panel shows binding at three different TWOW-1 concentrations in the absence of Mn$^{2+}$; the lower panel is in the presence of 1 mM Mn$^{2+}$. Binding is expressed as a ratio of specific TWOW-1 immunofluorescence over total integrin.

FIGURE 7. Activating mutations typically lead to reduced surface integrin expression. Total surface integrin (assayed by binding to βPS epitope tags) was measured for transiently transfected cell lines expressing wild type and the indicated mutants at 4 days following transfection. As typically seen in stable transfectants, surface expression follows the pattern wild type > βPS-b58 > αPS2-GFANA > βPS-ΔPSI. Double mutants are expressed at especially low levels. wt, wild type.

DISCUSSION

High resolution structural studies have provided a number of snapshots of integrin fragments in different conformations. This work suggested that there are at least two important equilibria that alter integrin-ligand interactions: that between the bent and extended heterodimers and that between the open (high affinity) and closed (low affinity) headpiece conformations (14–16, 43). However, other intermediates or unseen combinations are likely to exist under some conditions. For example, although the bent x-ray structure of αvβ3 is probably not compatible with a fully open head conformation, data indicate that soluble bent αvβ3 is competent to bind fragments of fibronectin (17). Is this because the x-ray structure samples only one of a number of possible bent conformations or because the open, high affinity state is not required for ligand binding under these experimental conditions? High resolution structural studies will continue to be critical in understanding integrin-ligand interactions, but to fully understand the dynamics of integrin heterodimers, the structural snapshots must be complemented with experiments that examine integrin function in situ in membranes of living cells.

Drosophila is useful as a model system because it is relatively easy to generate and study mutations in integrins or in cytoplasmic proteins that interact with integrins both in cell culture and whole developing animals. However, studies of invertebrate integrins have been hampered by a lack of probes that can directly assess integrin-ligand affinity. For example, using cell spreading or adhesion as an assay it was clear that the activity of mPy$^\text{vβg}$ mutant animals (or homozygous clones in heterozygous animals), we detected no difference in βPS surface expression in larval tissues (22). βPS-b58 did significantly exacerbate the expression reduction seen with αPS2-GFANA or βPS-ΔPSI in the transient transfectants (Fig. 7). This is similar to results from mutations expected to destabilize the βPS I domain (headpiece); these often show little or no reduction in cell surface expression in larval tissues on their own but lead to greatly diminished expression in combination with mutations in the αPS2 GFFNR sequence (22).
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sophia αPS2βPS integrin. In this study, we describe results from a variety of previously studied and new mutations in Drosophila αPS2 and βPS proteins. For the most part, the data reported here are in agreement with results from previous experiments: changes that increased cell spreading (αPS2 GFFNR → GFANA and βPS V409D) also increased integrin affinity for TWOW-1.

Mn$^{2+}$ and Affinity Modulation—As seen for vertebrate integrins, Mn$^{2+}$ increases the activity of αPS2βPS in cell spreading assays (44), and here we show that Mn$^{2+}$ increased the affinity of αPS2βPS for TWOW-1. However, TWOW-1 binding in Mn$^{2+}$ could be further increased by a GFFNR → GFANA mutation in the αPS2 subunit, which is expected to induce cytoplasmic tail separation (35–38), thus promoting an extended conformation of the integrin. In many published reports, integrin affinity is expressed as a fraction of the affinity seen in the presence of Mn$^{2+}$ with the implicit assumption that Mn$^{2+}$ ions induce maximal affinity. Our data indicate that this assumption is not always valid and suggest that in some cases measuring ligand binding relative to total integrin expression may be a more accurate reflection of overall affinity. Mn$^{2+}$ is a general activator of integrin affinity that most likely mediates its effects by occupying one or more of the β subunit cation binding sites (12, 32–34). Mn$^{2+}$ ions have been shown to alter integrin conformations, and this appears to be a direct effect, which can be detected in isolated proteins (Ref. 14, but see also Ref. 17). Interestingly Takagi et al. (14) saw that Mn$^{2+}$ affected the equilibrium between conformations but did not drive all of the molecules into one state. In any event, our results establish that “maximum” ligand affinity is an operationally defined entity and cannot simply be assumed to be the affinity measured in optimal Mn$^{2+}$.

ΔPS1 and Integrin Conformation—The PSI domain is in position to make contacts with the β subunit hybrid domain and stalk (32, 39). Thus, it is a candidate for transmitting structural changes in the β subunit head to the stalk and then to the cytoplasmic domains of the protein. Previous data indicated that mutations in mammalian integrin PSI domains can increase integrin activity (40, 41). We found that complete deletion of the PSI domain (βPS-ΔPS1) both increased ligand affinity and reduced integrin expression.

What is the likely mechanistic explanation for these βPS-ΔPS1 phenotypes? βPS-ΔPS1 is similar to GFFXR alleles in that affinity and expression are altered in comparable ways. GFFXR mutations most likely affect the equilibrium between bent and extended conformations, favoring the latter, by promoting cytoplasmic tail separation. It is thus tempting to hypothesize that the PSI domain-stalk interaction helps to stabilize the bent conformation and that its absence allows the integrin to extend. Consistent with this hypothesis, combination of βPS-ΔPS1 with βPS-b58 (which is expected to alter the equilibrium between the open and closed conformations of the βPS I domain; see below) caused further increases in affinity for ligand. Similar additive effects were also found when βPS-b58 and αPS2 GFFNR mutations were combined. We could not determine whether βPS-ΔPS1 and αPS2 GFFNR mutations were additive with respect to ligand affinity because these mutant subunits are too unstable to be expressed at detectable levels. Integrin conformations and stability appear generally to be the result of multiple weak interactions. It seems that either the associations of the transmembrane/cytoplasmic domains with one another (or other cytoplasmic proteins) or the presence of the PSI domain can provide enough heterodimer stability to result in some surface expression of integrins. However, compromising both of these stabilizing influences renders the proteins unable to be expressed on the cell surface.

βPS-b58 Increases Integrin-Ligand Affinity—Mutant phenotypes of mys$^{b58}$ (βPS V409D) flies and previous experiments in cell culture indicated that the mutant integrins are more biologically active than wild-type proteins (22). Here we show that βPS-b58 integrins had a much higher affinity for TWOW-1 than did wild-type integrins. Indeed this mutation had a greater affect on TWOW-1 affinity than the αPS2 cytoplasmic GFFNR → GFANA mutation and was almost equivalent to wild-type βPS activated by Mn$^{2+}$.

Modeling of βPS based on the derived structures of β3 suggested at least one possible novel molecular interaction that could result from the V409D change. However, substitution of three widely different residues into this site also increased TWOW-1 binding. The relatively large effect of the Val → Ala mutation indicates that the effects of βPS-b58 are mostly due to the loss of the valine at position 409 as opposed to the addition of the aspartate. It has been proposed that the closed conformation of the I domain of integrin α subunits is stabilized by hydrophobic interactions between the α7 and α1 helices of the domain (45). Although not exactly in a structurally homologous position, the effects described here (and the strong conservation of these hydrophobic residues in β subunits) suggest that similar forces may be at work in β subunit I domains.

Barton et al. (46) also investigated a hydrophobic linkage between the α1 and α7 helices of the human β1 subunit. They found three hydrophobic residues (one in the α1 helix and two in α7) that cause activation when mutated to alanine. They did not test the residue corresponding to βPS 409 but did mutate both of the neighboring conserved leucines in the α7 helix. Neither of these resulted in significant activation, although the double mutant, or changes to residues other than alanine, were not tested.

The weak effects of mys$^{b58}$ on fly viability are somewhat surprising in light of the high inherent ligand affinity of αPS2βPS integrins. αPS2 subunits that are deleted for the entire cytoplasmic domain (αPS2-Δcyt) or just the membrane-proximal GFFNR sequence (αPS2-ΔGFFNR) display increased integrin activity in situ as inferred by an expansion of muscle attachment sites in developing embryos and by phenotypes in developing wings (47). αPS2-ΔGFFNR can rescue most afflicted (αPS2) embryonic phenotypes; however, αPS2-Δcyt is less capable of supporting development through embryogenesis. It has been suggested that the inability of the αPS2 cytoplasmic mutants to support complete embryonic viability may be a consequence of a defect in inside-out signaling, resulting in an inappropriate increase in integrin-ligand affinity (47). mys$^{b58}$ also leads to a significant increase in αPS2βPS ligand affinity (although probably not as extreme as for αPS2-Δcyt), but mys$^{b58}$ has very little effect on viability, not only in embryos but for complete development of fertile adults (22). Thus one must use caution in assigning apparent loss-of-function phenotypes (such as decreased viability) in αPS2-ΔGFFNR and αPS2-Δcyt to loss of inside-out signaling; at least some phenotypes may result more directly from the deletion of required cytoplasmic residues.

The effects of βPS-b58 on surface expression of integrins is not as straightforward as for other activating mutants, which reliably cause significant reductions in expression both in S2 cells and, as shown for GFFXR mutations, in developing tissues. In contrast, mys$^{b58}$ has no discernable effect on integrin expression in developing larvae (22). In S2 cell transient transfections and stable transformants, βPS-b58 often shows reduced expression relative to wild-type βPS, but this reduction is not as pronounced or reliable as for αPS2-GFANA or βPS-ΔPS1. This difference may be because these mutations have primary effects on different aspects of integrin conformation. Both αPS2-GFANA and βPS-ΔPS1 are located in regions that might be expected to affect the bent ↔ extended switch, whereas the V409D mutation of βPS-b58 is positioned where one would expect it primarily to alter the head open ↔ closed.
equilibrium, so any effects on the bent ↔ extended equilibrium are likely to be indirect.

The results with known activators and inhibitors of integrin-ligand binding as well as with integrin mutations that affect ligand binding all point to the utility of TWOW-1 as a direct reporter of αPS2βPS ligand affinity. In addition to studies of integrin mutants, the development of TWOW-1 will open up the possibility for future genetic and other investigations of potential cytoplasmic regulators of Drosophila integrin activity.

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