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# Modulation of Ligand Binding by Alternative Splicing of the αPS2 Integrin Subunit

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**Abstract** The Drosophila αPS2 integrin subunit is found in two isoforms. αPS2C contains 25 residues not found in αPS2m8, encoded by the alternative eighth exon. Previously, it was shown that cells expressing αPS2C spread more effectively than αPS2m8 cells on fragments of the ECM protein Tiggrin, and that αPS2C-containing integrins are relatively insensitive to depletion of $Ca^{2+}$. Using a ligand mimetic probe for Tiggrin affinity (TWOW-1), we show that the affinity of αPS2CβPS for this ligand is much higher than that of αPS2m8βPS. However, the two isoforms become more similar in the presence of activating levels of $Mn^{2+}$. Modeling indicates that the exon 8-encoded residues replace the third β strand of the third blade of the α subunit β-propeller structure, and generate an exaggerated loop between this and the fourth strand. αPS2 subunits with the extra loop structure but with an m8-like third strand, or subunits with a C-like strand but an m8-like short loop, both fail to show αPS2C-like affinity for TWOW-1. Surprisingly, a single C > m8-like change at the third strand-loop transition point is sufficient to make αPS2C require $Ca^{2+}$ for function, despite the absence of any known cation binding site in this region. These data indicate that alternative splicing in integrin α subunit extracellular domains may affect ligand affinity via relatively subtle alterations in integrin conformation. These results may have relevance for vertebrate α6 and α7, which are alternatively spliced at the same site. J. Cell. Biochem. 102: 211–223, 2007.


**Key words:** cell adhesion; integrin structure–function; integrin alternative splicing; integrin α7

Integrins are the primary family of receptors through which metazoan cells interact with the extracellular matrix, although some integrins bind directly to other cell surface molecules [Hynes, 2002]. Integrins are heterodimers of α and β subunits, whose overall structures are highly conserved phylogenetically [Burke, 1999; Hynes and Zhao, 2000]. This conservation likely is a result of constraints that are related to the remarkable concerted conformational changes that characterize the cellular regulation of integrin function, and integrin signaling events that follow ligand binding. Changes in integrin shape were originally demonstrated by a number of conformation-specific antibodies. More recently X-ray data from fragments of integrins, along with electron microscopic characterizations of different conformations, have permitted more precise interrogation of the integrin structure–function relationships [Xiong et al., 2001, 2002, 2003; Beglova et al., 2002; Takagi et al., 2002; Luo et al., 2003; Mould and Humphries, 2004; Springer and Wang, 2004; Luo and Springer, 2006]. An overall picture is emerging of a globular headpiece, with the α and β components of the ligand binding site connected to the transmembrane domains by relatively long stalks. The stalks may be bent when the receptors are relatively inactive, and extended upon cellular activation. The headpieces also may sample relative high and low

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ligand affinity states (often referred to as "head open" and "head closed"), which are thought to be driven, at least in part, by changes in the tertiary structure of the β subunit head. Although molecular interactions are being uncovered that influence these transitions, it appears that integrin dynamics are dictated by a complex set of relatively weak interactions, and we are only beginning to understand these. Moreover, there is a growing appreciation that integrin "activation" may involve an increasingly complex set of conformations and affinity states [Jin et al., 2004; Mould and Humphries, 2004; Litvinov et al., 2005; Luo and Springer, 2006].

Integrin function may be regulated by a host of cellular proteins, depending on the cell type [Liu et al., 2000]. Structural variations also influence integrin function, as evidenced by the large families of α and β subunits in vertebrates. Within a single integrin gene, alternative splicing contributes to functional variety. However, alternative splicing affecting the extracellular domains is uncommon in vertebrates, with changes in a single region of α6 and α7 being the only well-characterized examples [de Melker and Sonnenberg, 1999]. Alternative splicing is more common in the fruit fly, *Drosophila melanogaster*, where the number of different subunit genes is much smaller than in vertebrates. Splicing variants altering extracellular domains have been found for each of the three well-characterized Drosophila α subunits, as well as the primary fly β subunit [Brower, 2003].

The Drosophila αPS2 subunit, with βPS, comprises the primary insect integrin of the "RGD binding" class. αPS2βPS is expressed on many tissues throughout fly development, and genetic and cell biological studies have defined numerous morphogenetic functions for the receptor, associated with both adhesive and probably signaling requirements [Brown et al., 2000]. αPS2 is found in two isoforms [Brown et al., 1989], a canonical ("C") form, and a version that is smaller by 25 amino acids, because it is missing exon 8 ("m8"). The ligand binding head of α subunits is comprised of a β propeller structure with seven blades, each comprised of four strands of β sheet [Xiong et al., 2001]. The top of the propeller associates with the β subunit, and the bottom contains four potential cation binding sites. The residues encoded by αPS2 exon 8 are inserted into the third β propeller blade in the primary sequence, which is the same location as the alternative splices in human α6 and α7 [Graner et al., 1998; de Melker and Sonnenberg, 1999]. This location has potential to affect ECM ligand contact sites directly, or could affect function indirectly by altering conformation.

No αPS2 isoform-specific antibodies have been developed; however the developmental distributions of the two forms were characterized by assaying the relative frequencies of cDNAs from different tissues [Brown et al., 1989]. In general, αPS2C is most common in morphogenetically stable tissues, while αPS2m8 predominates during dynamic developmental stages, such as during early embryogenesis and in larval imaginal disks. The cell biology of αPS2 variants has also been investigated in cell culture, using cell shape and adhesion on defined ECM proteins as an assay [Zavortink et al., 1993; Graner et al., 1998]. These studies show that αPS2C is superior to αPS2m8 on ECM ligands such as Tiggrin, which is found at the strong adhesions at muscle attachment sites, and Ten-m. However, αPS2m8 is somewhat better than αPS2C on some ligands, such as an RGD-containing fragment of the fly laminin α1, 2 chain. These studies suggested the possibility that exon 8-encoded residues may contribute to a specific ligand binding surface. However, the potential for more global structural differences were suggested by the observation that αPS2C and αPS2m8 display qualitatively different cation requirements, with the former supporting cell spreading in the complete absence of extracellular $Ca^{2+}$ [Zavortink et al., 1993].

Recently, we developed a ligand mimetic Fab for αPS2βPS integrins [Bunch et al., 2006]. TWOW-1 was generated by cloning 53 residues (including the RGD motif) of Tiggrin into the H-CDR3 of the αVβ3 ligand mimetic WOW-1 [Pampori et al., 1999]. TWOW-1 binding to αPS2βPS is rapid, reversible, inhibited by EDTA, dependent on the integrity of the ligand RGD motif, and sensitive to both activating and inactivating mutations in αPSβPS. Thus, TWOW-1 can be used to assay αPS2βPS affinity for Tiggrin. Here, we have re-examined the functional differences of αPS2C and αPS2m8, including a new series of mutants to further define regions important for PS2 integrin–Tiggrin interactions. These studies also make use of modeling based on more recent structural

data for integrin subunits, as well as phylogenetic comparisons to indicate critical sequence motifs.

## METHODS

### Structural Modeling

Homology models of αPS2m8 and αPS2C were generated by threading insect sequences onto the X-ray crystal structure coordinates of human αVβ3 (1JV2) [Xiong et al., 2001]. Various models were built including some comprising the whole protein, the α-subunit β-propeller domain alone, or this in combination with the β-subunit A/I domain. Modeller (version 7) was used for homology modeling of aligned sequences and then superimposed (homology aligned using Sybyl 7.2) on the original crystal structure and merged into a single coordinate file after removing the αV structural coordinates. The models were energy minimized using Sybyl 7.2. For αPS2C initial models of the extra loop structures were problematical, perhaps due to the high proline content in this region. To solve this, we first modeled αPS2C from the mosquito *Anopheles gambiae*, and subsequently used this as an initial template for *D. melanogaster* αPS2C. (Although no cDNA sequence was available for the mosquito, one was constructed from genomic sequences.)

The proline-rich exon 8 region insertion GQTYSIPPDAKFPFKPPLYQPFGTG was built by Modeller as a distorted α-helix. Since proline-rich regions are not α-helical we checked the phi–psi angles of the residues in this segment. To change the phi–psi angles, one residue on either side of each proline was extracted and the phi–psi angles were changed to match an ideal collagen-like polyproline helix. A local energy minimization was performed for a region of around 5 Å incorporating the four prolines in Sybyl 7.2. The final model was superimposed on the original model to obtain a root mean square deviation of $<1$ Å. For details, see the Supplementary Materials.

### PS Integrin Constructs

α and β subunits were expressed from pHSβPS4A, pHSαPS2C, pHSαPS2m8, and variants of these [Bunch and Brower, 1992]. Mutations of the αPS2 genes were introduced into pHSαPS2C and pHSαPS2m8 using standard PCR and molecular biology protocols. Regions mutated and those generated by PCR were sequenced to verify changes and to ensure that no unintended mutations had been introduced.

### Cell Culture and Transfection

Drosophila S2/M3 were cultured in Shields and Sang M3 medium supplemented with 12% heat-inactivated fetal calf serum as previously described [Bunch and Brower, 1992]. Cells were cotransfected with plasmids expressing a βPS subunit and an αPS2 subunit (either pHSαPS2m8, pHSαPS2C, or their variants), both under the regulation of the heat shock protein 70 promoter, and with the bacterial DHFR selectable marker (plasmid p8HCO) as described [Jannuzi et al., 2002]. For transient expression experiments, the day after transfection the medium was replaced with selection medium containing $2 \times 10^{-7}$ M methotrexate and the cells were allowed to grow for an additional 2 days prior to TWOW-1 binding assays.

### TWOW-1 Binding Assays

The TWOW-1 binding assay was done as initially described [Bunch et al., 2006] with the following modifications. To obtain more similar expression levels we have used transiently transfected cells. In this paradigm the cells express high levels of integrins in the first few days in the absence of heat shock; therefore, we did not heat shock the cells. Also, we did not protease-clear the cells as the transiently transfected cells do not quickly replace the cleared integrins following their removal. Because we examined only variants of the αPS2 subunit we did not remove endogenous βPS subunits by treating the cells with RNAi targeting the endogenous *myospheroid* gene. Our S2 cells express little, if any, αPS2 integrin subunits and bind no TWOW-1 in the absence of transfected αPS2 genes [Bunch et al., 2006]. Finally, the first wash of the EDTA-treated cells with BES-Tyrodes containing EDTA was found to be unnecessary. These cells therefore were simply washed in BES-Tyrodes prior to the TWOW-1 binding assay.

The TWOW-1 binding was assessed by flow cytometry as described [Bunch et al., 2006]. $1 \times 10^6$ cells were washed with BES-Tyrodes and resuspended in 30 μl BES-Tyrodes supplemented with 1 mg/ml (w/v) BSA, 1.66 mM

$MgCl_2$, and 16.6 μM $CaCl_2$. In some cases, 1.66 mM $MnCl_2$ was included in this buffer to activate the integrin. To assess the dependency of TWOW-1 binding on $Ca^{2+}$ we resuspended the cells in 1.66 mM $MgCl_2$ and 0.41 mM EGTA. To assess non-specific TWOW-1 binding, cells were resuspended in 30 μl BES-Tyrodes containing 1 mg/ml BSA and 16.6 mM EDTA. Binding assays were initiated by adding 20 μl of TWOW-1 at a concentration of 300 μg/ml (in BES-Tyrodes) for 10 min at room temperature. Fifty μl of 4% formaldehyde in BES-Tyrodes was added to fix the bound TWOW-1 to the cells, and after 5 min of fixation 1.5 ml BES-Tyrodes was added, cells were spun down and resuspended for 25 min on ice in 100 μl BES-Tyrodes (+1 mg/ml BSA) containing 20 μg/ml AlexaFluor488 goat anti-mouse IgG (Molecular Probes, A-11029) and 10 μg/ml R-Phycoerythrin labeled streptavidin (Molecular Probes, S-866). The R-Phycoerythrin-streptavidin was used to detect and exclude from analysis cells that had been disrupted. After incubation with secondary antibody, the cells were spun down and resuspended in 250 μl PBS followed by immediate addition of 250 μl of 4% formaldehyde in PBS to fix the secondary antibody; the cells were assayed in the flow cytometer in this solution.

Levels of αPS2 integrin on the surface of cells were determined by staining with the biotin labeled anti-αPS2 antibody CF.2C7 (which is not conformation specific) as described [Bunch et al., 2004]. $5 \times 10^5$ cells were incubated for 25 min at room temperature in 50 μl of biotinylated CF.2C7 in M3 + 12.5% FCS. Then R-Phycoerythrin-streptavidin diluted in M3 + FCS was added to a final concentration of 10 μg/ml and incubated for an additional 30 min on ice. Cells were fixed by the addition of 0.5 ml 2% formaldehyde in PBS. Other experiments determined that both antibody and streptavidin were present at saturating levels. Fluorescence levels for both TWOW-1 and αPS2 levels were analyzed by flow cytometry at the ARL-Biotechnology Cell Sorting Facility at the University of Arizona.

Specific TWOW-1 binding was measured as mean fluorescence intensity (MFI) minus the TWOW-1 MFI of the same cells in the presence of EDTA. For our analyses, this specific binding is expressed relative to surface integrin expression (TWOW-1 MFI/anti-αPS2 MFI).

### Cell Spreading

The ligand for cell spreading experiments was RBB-Tigg, a bacterial fusion protein that contains the same 53 amino acids of Tiggrin that are found in TWOW-1 (residues 1964–2016, including the RGD sequence of Tiggrin and 25 amino acids upstream and downstream), fused to a histidine tag, from the pTrcHisB vector [Bunch et al., 2004]. 96-well tissue culture plates were coated with 50 μl RBB-Tigg (0.5 μg/ml in PBS) overnight at 4°C, then blocked with 20% dried milk in PBS for 1 h at room temperature, and washed 3× with PBS.

Transiently transfected cells were rinsed in BES-Tyrodes and then diluted to $2 \times 10^5$/ml in BES-Tyrodes containing 1 mg/ml BSA (w/v), 1 mM $MgCl_2$, and 10 μM $CaCl_2$ or the same lacking $CaCl_2$ and containing 0.25 mM EGTA. One hundred μl of cells were allowed to spread for 1 h and then fixed by the addition of an equal volume of 4% formaldehyde in BES-Tyrodes. Photographs were obtained using a Nikon Coolpix 5000 camera attached to a Nikon phase-contrast microscope (Nikon Diaphot-TMD).

## RESULTS

### Modeling αPS2 Structures

At the time of the last investigation of αPS2 isoforms the best model for the structure of the integrin α subunit head was the seven bladed β-propeller, based on secondary structure predictions [Springer, 1997]. Sequence alignments predicted that the exon 8 residues specific to αPS2C inserted before the third β sheet strand of the third blade of the propeller [Graner et al., 1998]. One possibility is that the new residues form a novel structure between strands 2 and 3 of the blade. In this case the novel addition would be near regions thought important for ligand association, and also near the α/β interface. Alternatively, the exon 8-encoded amino acids could replace the third β sheet strand, and create an extended connection between strands 3 and 4 at the back of the β-propeller. Secondary structural predictions at the time were consistent with either possibility. The determination of high resolution X-ray data for the β-propeller region of human αV and αIIb [Xiong et al., 2001, 2002; Xiao et al., 2004] allowed us to build models for the β-propeller of αPS2 isoforms, using threading algorithms and energy minimization considerations. As expected from the

sequence, the models predict that for αPS2m8 the loop connecting the third and fourth β strands of blade 3 is short compared to that found in αV (Fig. 1A). When exon 8 is included, the models indicate that the MASS sequence motif that forms the third blade, third β sheet strand in αPS2m8 is replaced by QTYS encoded by the beginning of exon 8. The rest of the exon and the beginning of exon 9 then create a larger loop at the back of the β propeller before connecting to the fourth β sheet strand, which is unchanged. The larger loop includes a collagen-like polyproline helix at the top and back (away from the β subunit I domain) of the αPS2C β-propeller (Fig. 1B). Although some of our models have differed in the conformation of this extended loop, no predicted structure has resulted in which a new loop is created in αPS2C on the side facing the β subunit; in every case the first exon 8-encoded residues replace the third β strand of the third blade of the β-propeller.

The conclusion that exon 8 encodes a new strand of β sheet is further supported by sequence comparisons of αPS2 from other insects. In *D. melanogaster* the purported sequences of this strand are not highly similar (MASS in αPS2m8, QTYS in αPS2C). We searched the genomic sequence data for other insects, looking for potential "exon 8s" in their αPS2 genes (Fig. 2). The putative strand sequences are more similar in some other Drosophilids, and much more so in three other

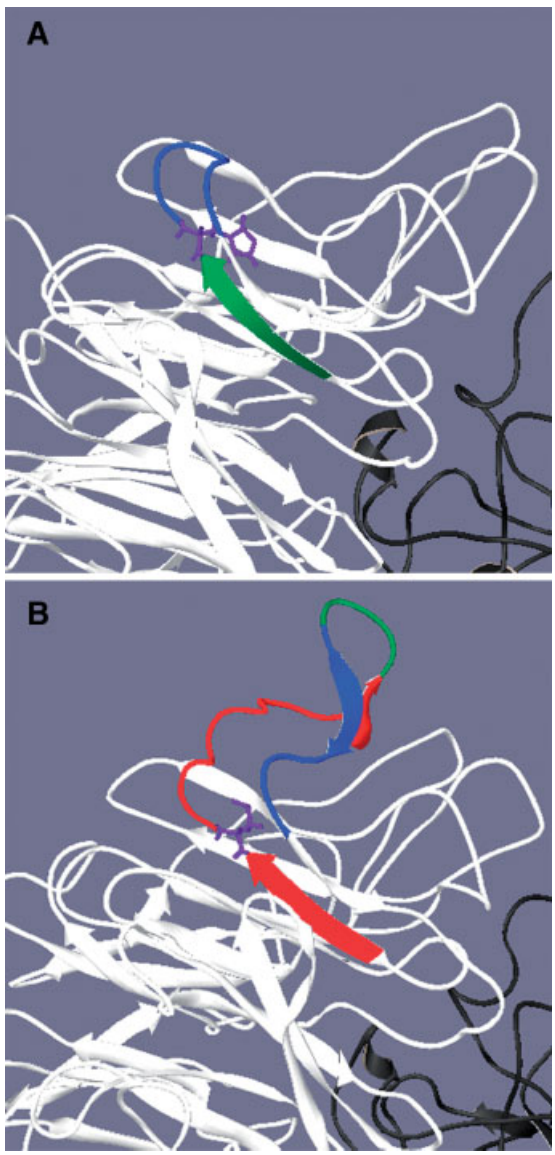


**Fig. 1.** Models of αPS2m8 (top) and αPS2C (bottom), generated primarily by threading sequences through the X-ray structure for αVβ3 [Xiong et al., 2001]. The sequences comprising the third β strand of the third blade of the αPS2 β-propeller in αPS2m8 are in green, with the loop connecting this to the fourth β strand in blue. Modeling consistently indicates that these residues are displaced in αPS2C, where exon 8-encoded residues (red) now comprise the third β strand, and contribute to an extended loop structure. In each panel, the residue at the end of the third strand is in violet (histidine in αPS2m8, isoleucine in αPS2C); see text for discussion of the importance of this residue. βPS structures are black.

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Dmel    GQTYSIPPDAKFPFKPPLYQP-FGTG GMASSHDVTRPENQVFST
Dsim    GQTYSIPPDAEFPFKPPLYQP-FGTG GMASSHDVTRPENQVFST
Dsech   GQIYSIPPDAMFPFKPPLYQP-FGTG GMASSHDVTRPENQVFST
Dyak    GQTYSIPPDAKFPFKPPLYQP-FGTG GMASSHDVTRPENQVFST
Dere    GQTYSIPPDAEFPFKPPLYQP-FGTG GMASSHDVTRPENQVFST
Dana    GQTYSIPPDAEFPFKPPYYQP-FGTG GQASSHDVTRPENQVFST
Dpers   GQTYSIPPDAEFPFKPPYYQP-FGTG GQASSHDVTKPENQVFST
Dpse    GQTYSIPPDAEFPFKPPYYQP-FGTG GQASSHDVTKPENQVFST
Dwil    GETYSIPPDASFPYKPPFYQP-FGTG GQASSQDVTKPENQVFST
Dvir    GQTYSIPPDAEFPFKPPYYQP-FGTG GQAISQDVTKPENQVFST
Dmoj    GQTYSIPPDAEFPFKPPYYQP-FGTG GQAISQDVTRPENQVFST
Dgri    GQTYSIPPDAEFPYKPPYYQP-FGTG GQAISQDVTKPENQVFST
Ccap    GQAYSISPDATFPYKPPFYQP-FGTG GQTYSYDVTRPENQVYST
Agam    GQMYSINTDVVFPYKPPRYGH-FGEG GQIYSFSLNNPKDKVYKT
Aaeg    GQVYSINTEAAFPYKPPRYGQ-FGEG GQIYSFSLNNPRDKVYST
Tcas    GQMYSVDAHATFEFKPTLFSATYGDE GQVHQQSLETRP-AVFAT
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**Fig. 2.** Sequences of αPS2 m8-C variants. Sequences of residues encoded by "exon 8" from various insects are on the left, with the corresponding "m8" residues on the right; an αPS2C subunit includes both. The proposed third and fourth β strand sequences of the third blade of the β-propeller are underlined for the m8 residues. In αPS2C the double underlining indicates residues that replace the m8 third strand residues in the structure. Note that the sequence divergence of these alternative strands is especially pronounced in *D. melanogaster* and its close relatives. Only some of these sequences have been confirmed by cDNAs, but genomic sequence is consistent with homologous alternative splicing in each of these species. *Drosophila* species in order are: *melanogaster, simulans, sechellia, yakuba, erecta, ananassae, persimilis, pseudoobscura, willistoni, virilis, mojavensis,* and *grimshaw*. *Ceratitis capitata* is the Mediterranean fruit fly, *Anopheles gambiae* and *Aedes aegypti* are mosquitoes, and *Tribolium castaneum* is a beetle.

dipterans. The difference appears to result from divergence in the αPS2m8 sequence (MASS) in *D. melanogaster* and its close relatives. For example, in two genera of mosquitoes the αPS2m8 strand (QIYS) is very similar to the proposed αPS2C strand, which is QXYS in all cases examined. Thus, the "exon 8" insertions into the translated polypeptide would be expected to contribute to the β strand structure as easily as the αPS2m8 sequence.

### Ligand Affinity of αPS2 Isoforms

Drosophila S2 cells expressing αPS2CβPS spread on tissue culture plates coated with a fragment of the fly ECM protein Tiggrin (RBB-Tigg). αPS2m8βPS also mediates cell spreading on Tiggrin and Tiggrin fragments, but less effectively than αPS2CβPS [Zavortink et al., 1993; Graner et al., 1998]. The Fab TWOW-1 facilitates direct measurements of ligand affinity of αPS2-containing integrins [Bunch et al., 2006]. Importantly for this study, TWOW-1 contains the same fragment of Tiggrin as RBB-Tigg, which mediates cell spreading as effectively as full-length Tiggrin on a molar basis, and displays similar αPS2 isoform preferences [Fogerty et al., 1994; Graner et al., 1998; Jannuzi et al., 2002]. Thus, we expect that TWOW-1 contains all of the sites that interact directly with αPS2βPS.

We quantitated TWOW-1 binding to cells expressing the two αPS2 isoforms with βPS. (In all cases, values are expressed as a ratio of TWOW-1 bound divided by total αPS2, determined by the binding of the monoclonal antibody CF.2C7. Previous work has shown that this ratio is not affected by integrin expression levels over a wide range.) αPS2CβPS binds TWOW-1 much more effectively than does αPS2m8βPS (Fig. 3). The difference in binding of soluble TWOW-1 between isoforms is greater than the difference seen in the ability of the αPS2 isoforms to mediate cell spreading on Tiggrin fusion proteins; in this latter assay, the effectiveness of αPS2m8 is at least 50% of the value for αPS2C at all concentrations of Tiggrin fusion proteins [Zavortink et al., 1993; Graner et al., 1998].

We next asked if the TWOW-1 binding capacities of the two isoforms are different, by assaying binding in the presence of activating concentrations of $Mn^{2+}$, a well-known activator of integrins generally, including αPS2CβPS [Bunch et al., 2006]. In the presence of $Mn^{2+}$,

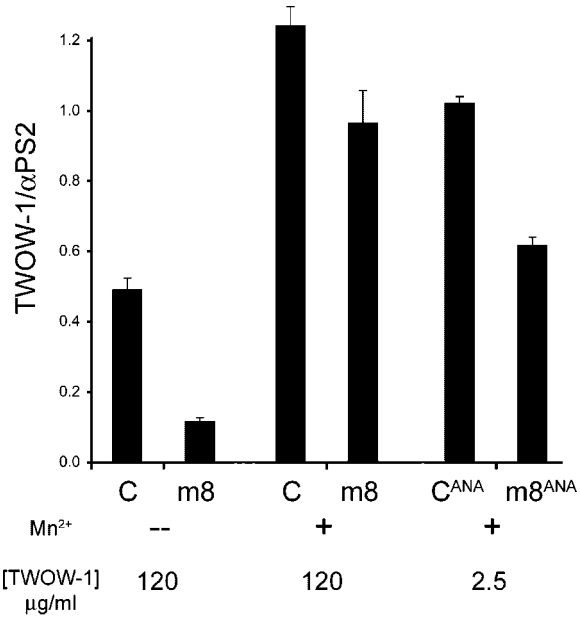


**Fig. 3.** Binding of the ligand mimetic TWOW-1 Fab/total αPS2 for αPS2m8βPS and αPS2m8βPS on S2 cells. αPS2C-containing integrins bind more than twice as well as αPS2m8 integrins. In the presence of 1mM activating $Mn^{2+}$ ions both variants show higher and more similar affinity for ligand at high concentrations of TWOW-1, which saturate for activated αPS2CβPS. αPS2C and αPS2m8 integrin isoforms that are activated by both $Mn^{2+}$ and a cytoplasmic mutation in αPS2 (GFFNR > GFANA) were also tested at very low TWOW-1 concentrations (2.5 µg/ml) that do not saturate for these high affinity receptors. Under these conditions, the apparent affinities of the isoforms are not equal, but they are more similar than for unactivated integrins. All values represent a minimum of three experiments; error bars equal the standard error.

TWOW-1 binding capacity increases for both αPS2m8- and αPS2C-containing integrins and the difference between the isoforms is greatly reduced (Fig. 3). At first glance this experiment might suggest that the differences between αPS2C and αPS2m8 result primarily from conformational changes; however the concentration of TWOW-1 used (120 µg/ml) is above saturation levels for αPS2CβPS in the presence of $Mn^{2+}$ [Bunch et al., 2006]. To get a better measure of the relative binding abilities of conformationally activated integrins, we tested receptors containing the activating cytoplasmic mutation GFFNR > GFANA in the presence of $Mn^{2+}$. In this experiment, we added TWOW-1 at 2.5 µg/ml, which is below saturation (the binding plateau) of these hyperactivated integrins. Under these conditions, TWOW-1 binding to αPS2CβPS is still greater than that of αPS2m8βPS, but the two isoforms are more

similar than under the original non-activating conditions ($\alpha$PS2C/$\alpha$PS2m8 = 1.7 vs 4.2 for normal cells at high TWOW-1; Fig. 3). Thus, much of the difference between PS2C and PS2m8 integrins' binding affinity for TWOW-1 can be removed as the integrins are activated to increasing degrees.

### Are Critical Residues of αPS2C in the β-Propeller Strand or the Extra Loop Structure?

The insertion of exon 8 adds a significant structure in the loop between two β sheet strands, and also changes the sequence in one of the strands. To ask if one or both of these alterations is important for the functional differences in αPS2 isoforms, we constructed a number of variants in this region (Fig. 4A; for all of the mutants, the initial m8 or C designation in the name refers to the form of the β strand at the beginning of the sequence). All of these were tested for TWOW-1 binding in the absence or presence of activating $Mn^{2+}$ ions. The fact that all of the variants can bind high levels of TWOW-1 in the presence of $Mn^{2+}$ (Fig. 4C) demonstrates that low affinity under normal conditions does not result from gross disruptions of the ligand binding capabilities of the mutants, for example, due to the elimination of a critical ligand interacting site. All of the mutants described here are expressed at levels similar to those of wild-type integrins.

We first eliminated most of the new loop residues with two largely overlapping deletions (C-del1 and C-del2), each of which leaves the αPS2C sequence in the third β sheet strand. Both deletions bind TWOW-1 in amounts more similar to that found for αPS2m8-containing integrins than αPS2C (Fig. 4B). TWOW-1 binding to C-del2 is better than to αPS2m8, but still less than half the value for αPS2C-containing integrins. Thus, the extra loop residues are required to fully confer αPS2C-like properties on integrin heterodimers. As can be seen from Figure 4A, the primary difference between the deletions is that C-del2 contains six residues at the beginning of the extra loop structure of αPS2C.

To address the importance of the extra loop residues relative to the strand sequence in a different way, we made an αPS2 with the extra residues of αPS2C, but with the potential β-propeller strand with the αPS2m8 sequence MASSHD. The TWOW-1 binding of integrins with this subunit (αPS2m8-C) is similar to that of receptors with the C-del2 variant of αPS2. That is, it is better than that seen for αPS2m8, but much less than found for αPS2C. Thus, the extra loop residues alone are not sufficient to confer αPS2C-like properties on integrin heterodimers.

### Detailed Analysis of the Proximal Loop Region

Comparison of the sequences and behaviors of the above mutants suggested that the sequence just after the end of the third strand of the third blade of the β-propeller might be a particularly critical region for distinguishing between αPS2m8 and αPS2C. This suggestion is consistent with phylogenetic sequence comparisons (Fig. 2). The first residue after the end of the strand in all of the exon 8-encoded αPS2C sequences is isoleucine (in the flies and mosquitoes) or valine (in the beetle). In αPS2m8 this residue is large and usually polar (histidine, glutamine, tyrosine, or in mosquitoes, phenyalanine). To examine this in more detail, we made a series of αPS2m8- and αPS2C-like subunits with changes in this region.

In the Drosophilids, the β-propeller strand of αPS2m8 extends to histidine or glutamine, followed by aspartate and valine; the corresponding sequence of αPS2C is isoleucine-proline–proline (HDV vs IPP). We made two variants of αPS2m8 (Fig. 4A). In one, most of the short loop between the third and fourth β-propeller strands was replaced with the corresponding αPS2C residues (m8-IPP+). In m8-IPP, only the HDV of αPS2m8 was replaced by the IPP sequence of αPS2C, and m8-I contains a single H > I replacement. All of these display TWOW-1 affinities similar to or even slightly worse than those of integrins containing intact αPS2m8 (Fig. 4B). We also made variants of αPS2C in which the IPP sequence was changed to HPP, IDP, or HDP (C-H, C-D, and C-HD, respectively). These mutants display TWOW-1 binding that is more similar to that found for αPS2C integrins (Fig. 4B). The two variants in which histidine replaces isoleucine bind TWOW-1 slightly less well, whereas the C-D subunit binds at least as well as intact αPS2C integrins. Thus, changing residues just after the end of the third strand of the third blade of the β-propeller can have relatively minor effects, but they are not sufficient to confer αPS2C-like TWOW-1 binding properties on αPS2m8, or vice versa.

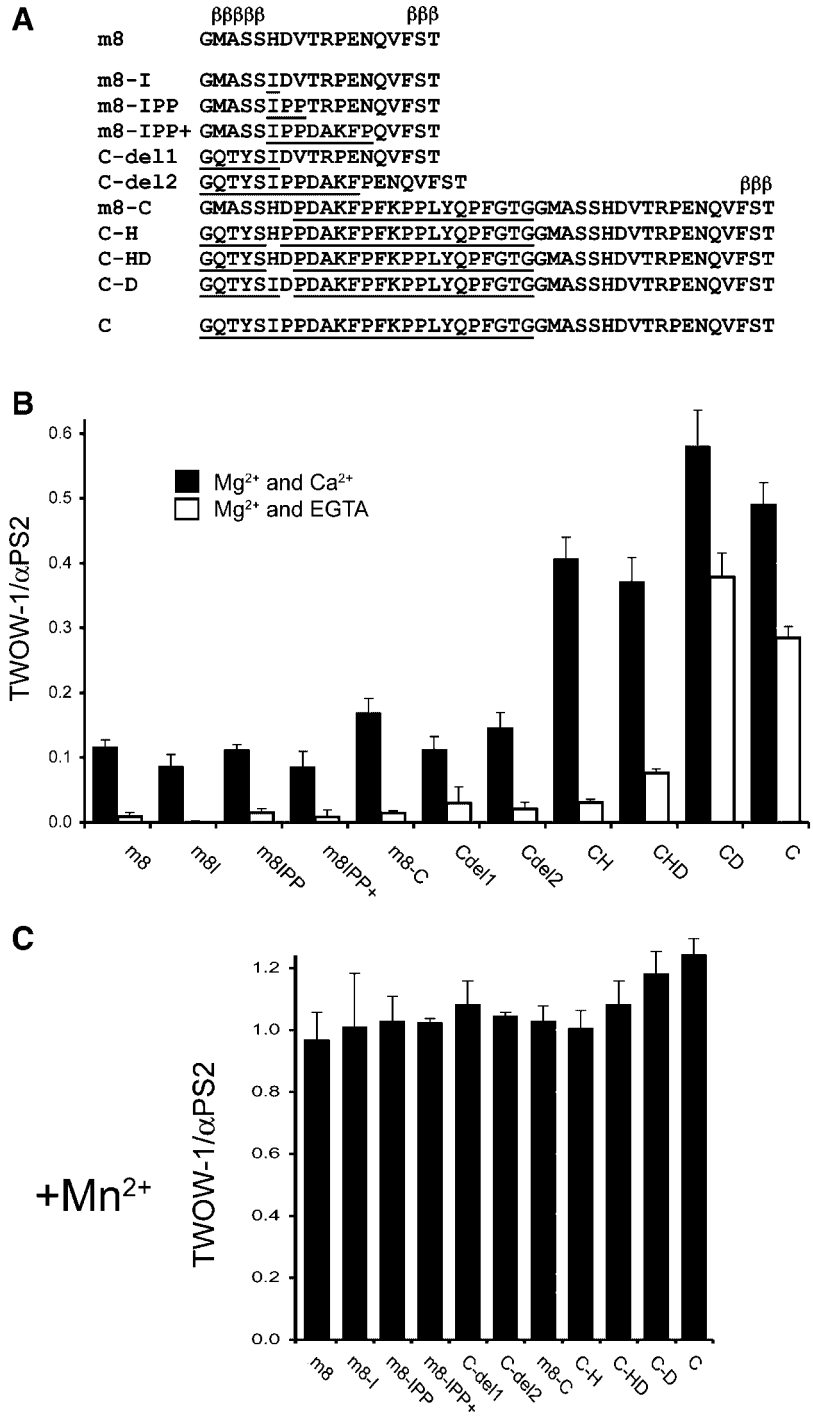


**Fig. 4.** **A**: Alternative sequences of αPS2m8 (top) and αPS2C (bottom), along with the variants constructed in this study. Residues typically encoded by exon 8 are underlined for each variant. As indicated by the ''β''s, modeling indicates that the sequences that contribute to the third β strand of the third blade of the αPS2 β-propeller structure immediately follow the first glycine, and the fourth β strand includes the FST motif. **B**: Binding of the ligand mimetic TWOW-1 (at 120 μg/ml) Fab/total αPS2 for αPS2βPS variants on S2 cells. For each variant, binding is shown in the presence of $Ca^{2+}$ and $Mg^{2+}$ (solid bars), and in $Mg^{2+}$ with saturating concentrations of the $Ca^{2+}$ chelator EGTA (open bars). **C**: All of the variants bind TWOW-1 in the presence of activating concentrations of $Mn^{2+}$ ions (1 mM), demonstrating that the mutations do not directly compromise the ligand interacting sites. Note that the Y-axis is different from panel B. All values represent a minimum of three experiments; error bars equal the standard error.

### $Ca^{2+}$ Requirements

In our earlier cell spreading assay, we found that cells expressing αPS2CβPS were able to spread efficiently on fragments of Tiggrin in the absence of $Ca^{2+}$ (i.e., in the presence of EGTA), but αPS2m8-containing integrins showed $Ca^{2+}$ ion dependence [Zavortink et al., 1993]. We assayed TWOW-1 binding for all of the mutants described here in the absence of $Ca^{2+}$ (i.e., in the presence of excess EGTA but with $Mg^{2+}$ present). As expected, αPS2C integrins bind significant amounts of TWOW-1 in the absence of $Ca^{2+}$, typically greater than 50% of the values in $Ca^{2+}$ and $Mg^{2+}$, while αPS2m8 shows a strong $Ca^{2+}$ requirement (Fig. 4B). Of the mutants, only C-D (αPS2C with IPP changed to IDP) shows a $Ca^{2+}$ independence similar to αPS2C, although for some others (C-del2, C-H, and C-HD) a very slight amount of TWOW-1 binding can be detected in the absence of $Ca^{2+}$. Most significantly, changing a single residue in αPS2C (the isoleucine immediately at the end of the third β sheet strand to the αPS2m8 histidine) is sufficient to confer a $Ca^{2+}$ requirement for efficient αPS2CβPS ligand binding.

$Ca^{2+}$ sensitivity can be somewhat difficult to assay with soluble ligand binding assays for some variants; because αPS2m8βPS binding to TWOW-1 is so low normally, measuring a decrease upon $Ca^{2+}$ depletion with EGTA might be unreliable. Therefore, we also examined $Ca^{2+}$ requirements in a cell spreading assay (Fig. 5, Table I), where wild-type αPS2m8 performs well in $Ca^{2+}$ and $Mg^{2+}$, but fails to support cell spreading in EGTA and $Mg^{2+}$ [Zavortink et al., 1993]. Cell spreading assays are influenced more by total integrin expression than is TWOW-1 binding (which is expressed as a ratio of TWOW-1 to total integrin), making quantitation less precise. However, even with this caveat, these experiments confirm the data for TWOW-1 binding; cells expressing integrins containing αPS2C or the C-D variant spread well in medium depleted of $Ca^{2+}$. All of the other variants display very slight spreading at most, including C-H, which contains the single residue replacement.

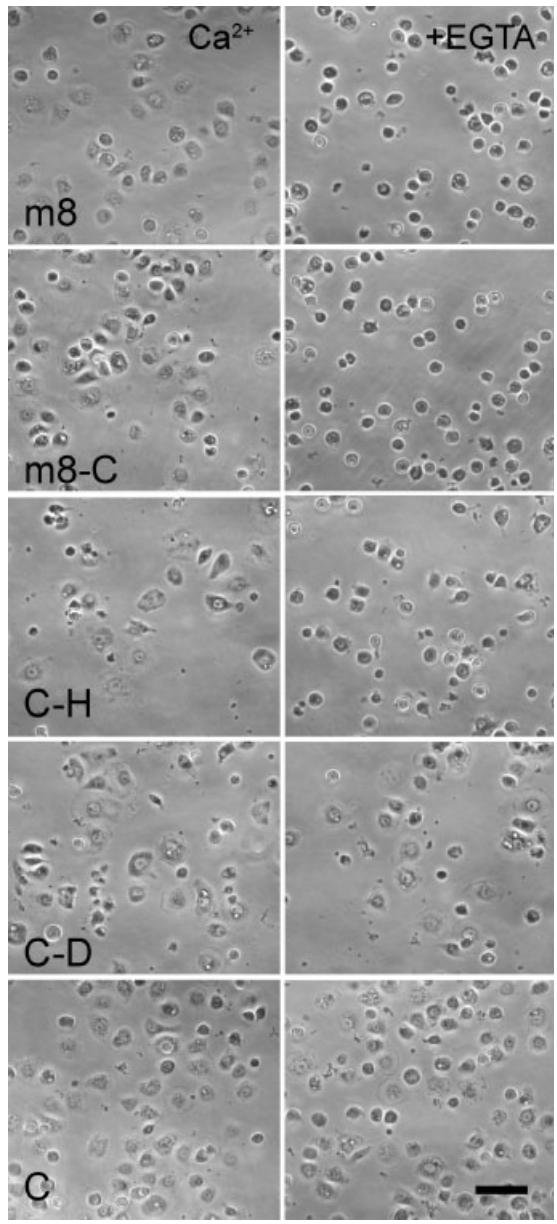


**Fig. 5.** Spreading on a fragment of the ECM protein Tiggrin by S2 cells expressing variants of αPS2 integrins. Integrins containing αPS2m8, αPS2C or any of the mutants tested will spread on plates coated with 0.5 μg/ml RBB-Tigg in the presence of $Ca^{2+}$ and $Mg^{2+}$ (left column). However, when $Ca^{2+}$ is removed by the addition of EGTA (right column), significant spreading is observed only for cells expressing αPS2C (bottom) or the αPS2C-D variant, which also show efficient TWOW-1 binding in EGTA. Notably, the single substitution of a histidine for isoleucine in αPS2C-H is sufficient to render these integrins $Ca^{2+}$ sensitive for spreading. Scale bar = 50 μm.

## DISCUSSION

Modeling strongly indicates that the new residues encoded by exon 8 replace the third β strand of the third blade of the αPS2 β-propeller, and lead to an exaggerated loop structure between the third and fourth β strands. Two lines of evidence provide confirmation of the model. First, examination of the homologous sequences from closely related species reveals

**TABLE I. Quantitation of Cell Spreading Data from Experiments Illustrated in Figure 5**

| Cell spreading on RBB-Tigg | | |
|---|---|---|
| | % Cells spread | |
| | $+Ca^{2+}$ | +EGTA |
| αPS2m8 | 61 ± 1 | 0 ± 0.2 |
| αPS2m8-C | 53 ± 6 | 1 ± 0.3 |
| αPS2C-H | 58 ± 6 | 1 ± 0.3 |
| αPS2C-D | 62 ± 5 | 50 ± 2 |
| αPS2C | 65 ± 3 | 65 ± 6 |

Spread cells were counted for three fields of each condition (minimum of 100 cells/field). Numbers represent means and standard error. The relatively high levels of spreading for αPS2m8 integrins in the presence of $Ca^{2+}$ (essentially identical to αPS2C integrins) are due to the high concentrations of ligand in these experiments. Even at these high RBB-Tigg levels, the $Ca^{2+}$ requirement of αPS2m8 and similar mutants is clear.

not only structural homology but also sequence similarity between the putative third β strands in αPS2C and αPS2m8. More directly, the C-del1 and C-del2 mutations produce functional integrins even though both remove the sequences of the third blade of the β-propeller found in the αPS2m8 integrin subunit. If the model is incorrect and exon 8 does not contain an alternative third β strand it is difficult to see how a functional integrin could be produced in these deletions.

The αPS2 residues encoded by exon 8 cause αPS2CβPS integrins to interact more effectively with Tiggrin, as assessed by cell spreading [Zavortink et al., 1993; Graner et al., 1998] and TWOW-1 affinity. This might suggest that exon 8 residues contribute to specific contacts with Tiggrin. However, the data showing that αPS2m8-containing integrins become more like αPS2C-containing integrins when activated by $Mn^{2+}$ ions and cytoplasmic domain mutations suggest an alternative hypothesis. The exon 8 sequences may function primarily to drive a conformational equilibrium to a state that is more conducive to Tiggrin association, without directly interacting with the ligand. Of course, these two hypotheses are not mutually exclusive. In any case, all of the exon 8 variants described here, when activated by $Mn^{2+}$, can bind similar amounts of TWOW-1 under saturating conditions, thus the changes do not eliminate structures that are required for ligand binding.

It is tempting to explain the m8-C difference in TWOW-1 binding as being due to the new extensive loop in αPS2C. However, the αPS2m8-C subunit contains virtually all of this loop, and its TWOW-1 binding is much less than that seen for αPS2C integrins. Another hypothesis is that the residues of the third β strand are critical. However, the two C-del variants each contain the αPS2C strand sequence GQTYSI, but result in relatively low TWOW-1 affinity compared to αPS2C. Thus, both the loop and strand appear to matter, but neither of these alone can fully or even mostly account for the m8-C difference. To summarize, for high levels of TWOW-1 binding the extra loop found in αPS2C appears to be necessary, but not sufficient. Conversely, the benefits of the αPS2C loop can largely be negated by incorporating the third β strand and immediately adjacent residues from αPS2m8 (as seen for αPS2m8-C).

Perhaps the most curious result derives from examination of $Ca^{2+}$ requirements for TWOW-1 binding and integrin-mediated cell spreading on Tiggrin fragments. αPS2C integrins perform well in both assays when $Ca^{2+}$ is depleted by saturating amounts of EGTA, but replacement of the αPS2C isoleucine at the end of the β strand with the αPS2m8 histidine (in C-H) is sufficient to restore most of the $Ca^{2+}$ requirement. Integrin α subunits contain at least five potential $Ca^{2+}$ binding sites, four along the base of the β-propeller and a fifth at a flex point (the "genu") in the stalk [Xiong et al., 2001]. None of these are obviously expected to be affected by exon 8 residues or the mutants described here, nor are there any data to indicate that these cation binding sites are important for integrin regulation. Much more work has been done to describe regulatory functions for the three cation binding sites of the β subunit A or I-like domain [Takagi et al., 2002; Xiong et al., 2002; Chen et al., 2003, 2004, 2006; Mould et al., 2003; Xiao et al., 2004; Arnaout et al., 2005; Pesho et al., 2006]. It is clear that the cation of the MIDAS domain interacts directly with the aspartate of RGD ligands, but there is no consensus opinion as to the roles of the flanking LIMBS and ADMIDAS sites, or for their cation binding specificities. What is clear is that cations in these sites can be important for modulating ligand binding and for propagating conformational changes through the integrin heterodimer.

The combined TWOW-1 affinity and $Ca^{2+}$ sensitivity data are most consistent with models in which the αPS2C loop effects conformational

changes via a mechanism that requires a specific structure at the C-terminal end of the third β sheet strand of the third blade of the propeller. Additional homology modeling does not immediately suggest that the I > H replacement in αPS2C-H will have significant effects on α subunit structure (K. Shakalya and D. Mahadevan, unpublished work). However, one must remember that the X-ray data upon which our modeling is based are snapshots of structures that are stabilized in crystals of integrin fragments. Although our understanding of the gross features of integrin structure and function has advanced significantly in recent years, we are only beginning to uncover finer structural, and often dynamic, nuances that lie behind the exquisite functional details that characterize integrin biology. These blades of the α subunit β-propeller are known to contain residues important in ligand binding [Irie et al., 1995, 1997; Kamata et al., 1996, 2001], and even minor alterations in the positioning of any residues in this region could alter binding.

Furthermore, it is not unreasonable to envision that changes in α and β subunits, brought about by αPS2 splicing variants and $Mn^{2+}$, respectively, would influence the α/β interface in ways that would affect affinity for specific ligands, such as Tiggrin/TWOW-1, and which might be difficult to capture in structures of crystals of integrin fragments. It is very possible that the $Ca^{2+}$ insensitivity of αPS2C results from a change in α/β contacts that can compensate for the requirement for cation binding to a β subunit site, and that an equilibrium involving these contacts can be influenced by a single residue that alters the conformation of the αPS2 β-propeller in subtle ways.

### Similarities to Human α Subunits

Alternative splicing in the extracellular domains of vertebrate integrins is uncommon, but one example bears a remarkable similarity to that seen for αPS2 [reviewed in de Melker and Sonnenberg, 1999]. In the identical position to the Drosophila exon 8, the human laminin receptor α6 and α7 genes have alternative exons denoted X1 and X2. α6 utilizes either X1 or both X1 and X2; no functional differences between these have been noted, although there appears to be a preference of the α6X1X2 isoform to associate with β4 instead of β1 [Delwel et al., 1995]. The largely muscle-specific α7β1 integrin has been studied more extensively. Unlike α6, α7 is found with X1 or X2-encoded residues, but not both [Ziober et al., 1993]. Like αPS2m8, α7X1 is most common where tissues are morphologically dynamic, whereas αPS2C and α7X2 are prevalent where stable cell-matrix adhesions are called for [Brown et al., 1989; Ziober et al., 1993; de Melker and Sonnenberg, 1999]. Cells expressing α7X1 or α7X2 show similar binding or motility properties on some laminins, but α7X2 is superior on laminin 1 [Ziober et al., 1997; Schöber et al., 2000; von der Mark et al., 2002]. However, α7X1β1 can become an efficient laminin 1 receptor if cells are incubated with activating β1 antibodies or when α7X1 is expressed with the muscle-specific β1D cytoplasmic splice variant [Ziober et al., 1997; Yeh et al., 2003]. These data indicate that like the inclusion of exon 8 in αPS2, the residues of the X1 and X2 exons of α7 do not define cell adhesion specificities only by making specific ligand contacts directly, but also possibly by more global effects on integrin conformation.

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