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Drosophila PS2 integrin mediates RGD-dependent cell-matrix interactions

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Summary

Integrins are a family of transmembrane glycoproteins that mediate cell-matrix and cell-cell interactions. We have transfected cultured *Drosophila* cells with genes that express the *Drosophila* PS2 integrin. We demonstrate that this integrin is expressed on the surface of the cells and can mediate cell spreading on an undefined component of fetal calf serum or on the purified vertebrate matrix molecules vitronectin and fibronectin. Additionally, PS2 integrin can cause cell spreading on RGD peptide. The spreading on matrix components or RGD peptide can be inhibited by soluble RGD peptide and is dependent on divalent cations.

Key words: PS2 integrin, vitronectin, fibronectin, RGD, *Drosophila.*

Introduction

The *Drosophila* PS1 and PS2 integrins were originally identified in monoclonal antibody screens for antigens showing an interesting Position Specific distribution (Wilcox et al., 1981; Brower et al., 1984). Biochemical and sequence analysis subsequently demonstrated that they are members of the integrin family of cell adhesion molecules (Leptin et al., 1987; Bogaert et al., 1987; MacKrell et al., 1988). Work in vertebrate systems has demonstrated that most integrins function as transmembrane molecules linking the cytoskeleton to the extracellular matrix (reviewed in Hynes, 1987). Integrins utilize a wide variety of extracellular matrix molecules as ligands (see Ruoslahti and Pierschbacher, 1987; Hynes, 1987 for reviews). At least one class of integrins is also responsible for direct cell-cell interactions (Springer, 1990).

In characterizing the regions of matrix molecules that are important for integrin binding, it has been recognized that the sequence RGD is found in many of the integrin binding sites and is important for integrin interaction. Moreover, the binding of integrins or cells containing integrins to matrix components can often be inhibited by soluble RGD-containing peptides. Matrix molecules containing the sequence RGD in their integrin recognition site include fibronectin, laminin, vitronectin, von Willebrand factor, fibrinogen, osteopontin, thrombospondin and collagens (see Ruoslahti and Pierschbacher, 1987 for a review).

There is strong evidence that the vertebrate integrins play roles beyond simply serving as cellular glue. They appear to be important in cell differentiation, signal transduction, cell migration and immune responses (e.g. Menko and Boettiger, 1987; Miyauchi et al., 1991; Fujimoto et al., 1991; Akiyama et al., 1989; Springer, 1990). To study some of these potential functions in vivo, we and others have undertaken genetic analyses in *Drosophila melanogaster* of the genes encoding the α and β subunits of the PS integrins (Brower and Jaffe, 1989; Wilcox et al., 1989; Leptin et al., 1989; Zusman et al., 1990; Volk et al., 1990; Wilcox, 1990). To complement these genetic studies, we have undertaken a study of the adhesive properties of PS2 integrins in cultured *Drosophila* cells.

We have introduced genes that express PS2 integrin cDNA, whose transcription is driven by a heat-shock promoter, into cultured *Drosophila* cells. Schneider's line 2 (S2) cells were chosen for these studies as they express very little endogenous integrin and grow as round, nonadherent, nonaggregating cells. These properties have made this cell line popular for many similar studies of *Drosophila* adhesion molecules (Snow et al., 1989; Barthalay et al., 1990; Elkins et al., 1990; Grenningloh et al., 1990; Keith and Gay, 1990; Krantz and Zipursky, 1990; Fehon et al., 1990; Rebay et al., 1991; for reviews see Hortsch and Goodman, 1991; Bunch and Brower, 1992). This approach is similar to the paradigm used by Takeichi and colleagues in their studies of vertebrate cadherins (e.g. Nagafuchi et al., 1987).

Two forms of PS2 integrin have been identified by analysis of cDNAs encoding the PS2α subunit. PS2(c), for canonical, differs from PS2(m8), for missing exon 8, by containing 25 additional amino acids. The 25 amino acids encoded by the alternative exon 8 are located between the conserved α repeats III and IV (Bogaert et al., 1987) near the putative divalent cation binding sites and ligand binding site (D'Souza et al., 1990). Thus the location of the alternative 25 amino acids is suggestive of a role for them in regulating ligand specificity or affinity (Brown et al., 1989). In the experiments reported here, we have used the PS2(m8) integrin but similarities with PS2(c) results will

be mentioned in the discussion. Using transformed cells expressing both the β subunit and PS2(m8)α subunit, we examined the ability of PS2 integrin to mediate cell spreading, its potential interaction with RGD sequences in the matrix molecules, and the divalent cation dependence of PS2-integrin-mediated cell spreading.

Materials and methods

Plasmids

The plasmid pHSPSβ contains the *Drosophila* HSP70 promoter controlling the transcription of the integrin β subunit cDNA, to which has been added the 3′ untranslated sequences and polyadenylation/cleavage site from the *Drosophila* tubulin α1 gene. This plasmid was constructed by replacing the *fushi-tarazu* sequences of plasmid F449 (kindly provided by Gary Struhl) with cDNA sequences from *myospheroid* (which encodes the *Drosophila* integrin β subunit; kindly provided by Y. Grinblat). Specifically, pHSPSβ contains 450 bp from the HSP70 gene beginning 260 bp upstream from the start of transcription and extending an additional 190 bp into the 5′ untranslated sequences of the HSP70 mRNA (Struhl, 1985). This HSP70 promoter is followed by *myospheroid* cDNA sequences that extend from the start of transcription to an *Spe*I site 36 bp 3′ from the end of translation. The 810 bp sequence from the tubulin gene that follows contains 230 bp of 3′ untranslated, transcribed sequences from the *Drosophila* tubulin α1 gene and an additional 580 bp beyond the polyadenylation/cleavage site (Lawrence et al., 1987). pUC18 (Yanisch-Perron et al., 1985) is the backbone of this plasmid and provides a bacterial origin of replication and confers ampicillin resistance to transformed bacteria.

The plasmid pHSPS2(m8) (kindly provided by M. Brabant, University of Arizona, Tucson, AZ) contains the PS2(m8) cDNA under the control of the same HSP70 promoter and has the same 3′ tubulin sequences as pHSPSβ. pHSPS2(m8) construction was similar to that for pHSPSβ.

The plasmid pH8CO has been previously described (Rebay et al., 1991). It contains the bacterial DHFR gene and is used to co-transfect the *Drosophila* cells, conferring methotrexate resistance.

Drosophila *cell culture and transformation*

S2/M3 cells (referred to in the text as S2 cells) are Schneider's line 2 cells (Schneider, 1972) adapted for growth in M3 medium + FCS (Lindquist et al., 1982). Growth of these cells and transformants has been previously described (Bunch et al., 1988). Cells were transformed using the calcium phosphate co-precipitate method (Wigler et al., 1979) as previously described (Bunch et al., 1988) except that N,N-bis(2-hydroxyethyl)-2-aminoethane sulfonic acid (Bes) was substituted for N-2-hydroxyethyopiperazine-N′-2-ethane sulfonic acid (Hepes) as suggested by Chen and Okayama (1987). We also purified the plasmid to be transfected twice on cesium chloride gradients with a phenol/chloroform extraction between these two gradients. In other experiments, we found that these modifications increase the percentage of cells expressing a reporter gene from <1% to >90% in a transient expression assay. Also the plasmid pHGCO (Bourouis and Jarry, 1983) was replaced by the plasmid pH8CO (Rebay et al., 1991) containing the same bacterial dihydrofolate reductase (DHFR) gene, allowing selection in methotrexate. The transformant used in this study was transformed with a 1:1:1 ratio of the plasmids pHSβ:pHSPS2(m8):pH8CO. Without selection for integrin expression, the transformed cells will slowly lose copies of the integrated plasmids (Moss, 1985). Taking advantage of the fact that the transformant shows increased adhesive properties under normal growth conditions, we have selected for those transformants expressing integrins by shaking the plate and replacing the medium and non-adherent cells with fresh medium each alternate transfer. The same treatment of S2 cells does not result in a population of adherent or spread cells.

Spreading assays

Spreading assays were done in 96-well plates that had been precoated with various substrata. The wells were coated overnight at 4°C with 50 μl of BSA (0.1 mg/ml; Sigma A4378), bovine vitronectin (0.01 mg/ml; Telios X029), bovine fibronectin (0.04 mg/ml; Sigma F1141), BSA-RGD (0.1 mg/ml BSA, 1 mg/ml GRGDSP) or BSA-RGE (0.1 mg/ml BSA, 1 mg/ml GRGESP) in PBS (4 mM Na_2HPO_4, 10 mM NaH_2PO_4, 100 mM NaCl, pH 7.4). Note that it is not necessary to precoat the wells with the BSA as uncoated wells give the same results as BSA coated wells (probably because there is 4 mg/ml BSA included in the medium during spreading, see below). The wells were washed once with Robb's saline (Robb, 1969) and once with M3 medium (M3 medium refers to the medium without fetal calf serum).

Cells were washed twice in Robb's saline prepared without calcium or magnesium (Ca^{2+}/Mg^{2+}-free Robb's saline), resuspended in typsin-EDTA (0.5 g/l trypsin, 0.2 g/l EDTA; Sigma T9395) in Ca^{2+}/Mg^{2+}-free Robb's saline at a concentration of 2×10^6 cells/ml and incubated, with rolling, at 23°C for 30 minutes. The trypsinized cells were then washed twice in 10 volumes of M3 medium and resuspended to 2×10^5 cells/ml in M3 medium + 4 mg/ml BSA. GRGDSP or GRGESP peptides (Telios P001, P005) or EDTA were added at this point. The cells were heat shocked at 37°C for 30 minutes in a waterbath. Cells were then plated in precoated wells in 96-well tissue culture plates (Corning 25860) and allowed to spread at 23°C for 4-6 hours. Following spreading, the cells were fixed by adding an equal volume of 6% formaldehyde (in Robb's saline) to the wells and incubated for 20 minutes at 23°C. The fixative was then replaced with Robb's saline and the plates stored at 4°C until the cells were photographed.

Cells were photographed with a Nikon Diaphot inverted microscope using phase-contrast optics. To ensure proper phase illumination, the wells were filled and then a glass coverslip was placed on top, creating a level surface with no liquid-gas interface. The pictures were printed and scored blind for either the percentage of phase-dark spread cells (phase-dark cells that were perfectly round and showed no signs of spreading were not counted) or sorted into morphology groups. At least two fields of cells were counted for each sample in each experiment. Three fields were counted in the divalent cation experiment. Each field contained at least 190 cells. The numbers reported represent the average of the fields counted.

Immunoblots

Cells were collected by centrifugation, washed once in Robb's saline, then resuspended in Laemmli sample buffer (Laemmli, 1970) at 90-100°C. The cells were vortexed, sonicated and heated at 90-100°C for 3-5 minutes, frozen in liquid nitrogen and stored at −80°C until they were run on SDS-polyacrylamide gels (Laemmli, 1970). Embryo extracts were prepared by collecting 20 ± 2 hour embryos (at 23°C) which were then dechorionated in 50% bleach for 5 minutes, rinsed with water and frozen at −80°C. The embryo pellets were resuspended in 10 volumes of Laemmli sample buffer (at 90-100°C), homogenized, sonicated and then heated to 90-100°C for 3-5 minutes. The extract was then cleared by centrifugation, frozen in liquid nitrogen and stored at −80°C until it was run on SDS-polyacrylamide gels. Following electrophoresis, the proteins were electroblotted to Immobilon-P membrane (Millipore P15552). Blocking of the membrane (at least 1 hour, 23°C), incubation with primary antibodies (overnight, 4°C), secondary antibodies (1 hour, 23°C), and washes (1 hour with four changes after primary and secondary antibody incubations) were

done in TBS-T20 (150 mM NaCl, 50 mM Tris-HCl, 0.05% Tween20, pH 7.5). The primary antibodies used were PShc/1 (Bogaert et al., 1987) for PS2α staining and DX.4C8 (Wilcox et al., 1984) for PSβ. Secondary antibodies were peroxidase-linked goat anti-rat IgG (Boehringer-Mannheim 605-190) and peroxidase-linked sheep anti-mouse IgG (Amersham NA931). The secondary antibodies were detected using an enhanced chemiluminescence kit (Amersham, RPN2106) according to the supplier's protocols. Relative molecular mass markers were from BioRad (161-0303).

Immunofluorescence

Cells were collected, resuspended in M3 medium at 1-2×10^6 cells/ml and allowed to settle and attach to glass slides for 5 minutes. The cells were rinsed in TBS (150 mM NaCl, 50 mM Tris-HCl, pH 7.5) and lightly fixed in 1% formaldehyde for 2 minutes (longer fixation times or higher concentrations of fixative prevent the primary antibody from recognizing PS2). The cells were washed in TBS (15 minutes with three changes) and then incubated in the anti-PS2 antibody CF.2C7 (Brower et al., 1984) diluted in TBS+10% FCS (20 minutes at 23ºC), washed in TBS (20 minutes with four changes), incubated in FITC-labeled goat anti-mouse secondary antibody (Antibodies Incorporated 2150) diluted in TBS+10% FCS, washed in TBS (20 minutes with four changes), fixed in 3% formaldehyde for 5 minutes, washed in TBS (20 minutes with four changes), mounted in 30% 0.1 M Tris-HCl (pH 9), 70% glycerol, to which was added 0.2% n-propyl galate, and photographed. The staining of the cells in Fig. 2 was done 4 months following the transfection of the S2/M3 cells with the integrin-expressing genes, and these staining levels have remained high for at least one year. Staining results were the same when both primary and secondary antibody incubations were done on unfixed cells.

Results

To study the adhesive properties of the *Drosophila* PS2 integrins, we have transfected S2 cells with two chimeric genes. cDNAs encoding PS2(m8)α subunit or the β subunit were inserted after the HSP70 promoter and 5′ untranslated leader sequence and before the tubulin 3′ untranslated sequences to generate the HSPS2α(m8) and HSPSβ genes. A stably transformed line [HSPS2(m8) cells] containing HSPSβ and HSPS2α(m8) genes was obtained.

Immunoblot analysis

To ascertain whether the transfected genes express protein, immunoblot analysis was performed (Fig. 1). Untransfected S2 cells and HSPS2(m8) cells were collected either before heat shock or at various time points after a 30 minute heat shock. The cells were resuspended in protein sample buffer, electrophoresed on SDS gels, blotted to nitrocellulose and probed with monoclonal antibodies specific for the PS2α subunit or the β subunit.

In untransformed S2 cells, there is a low amount of β expression but no detectable α expression as compared with embryos or transformed cells. The transformed cells express significant levels of α and β and this expression is greatly enhanced by the 30 minute heat shock. Neither protein appears to be stable, reaching peak expression at 2 hours following the heat shock, and returning to near basal levels by 24 hours. An original 160×10^3M_r PS2α is cleaved to

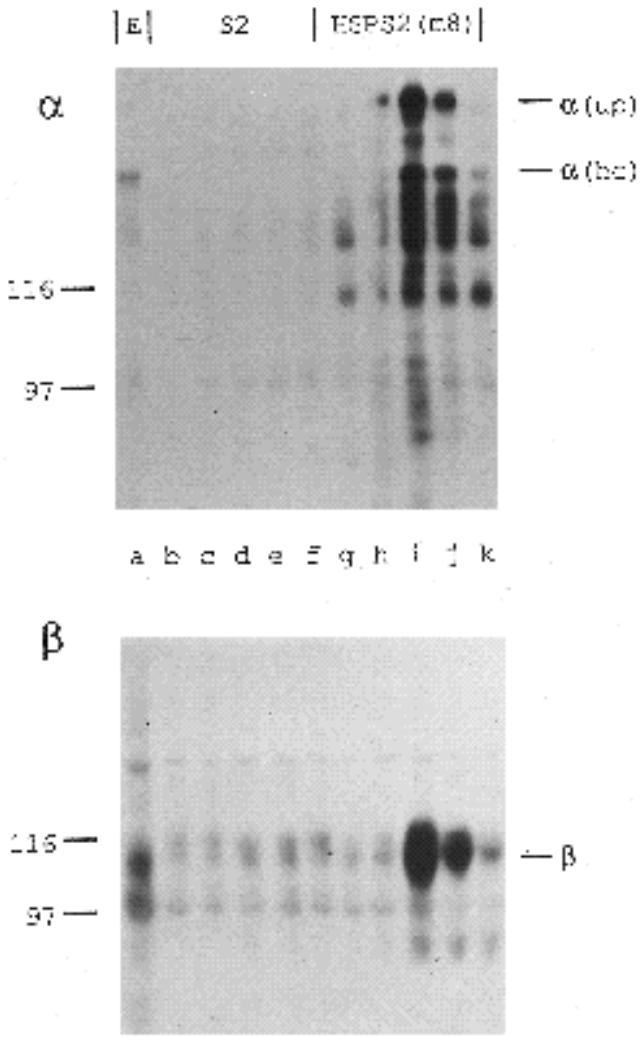

Fig. 1. Immunoblot analysis of PS2 expression in S2 and HSPS2(m8) cells. Extracts from embryos (lane a), S2 cells (lanes b-f), and HSPS2(m8) (lanes g-k) cells were electrophoresed, blotted and probed with either anti-PS2α (top blot) or anti-PSβ (lower blot) antibodies. The cells were either not heat shocked (lanes b and g), heat shocked for 30 minutes (lanes c and h), or heat shocked and then allowed to recover for 2 hours (lanes d and i), 6 hours (e and j), or 24 hours (lanes f and k). The 160×10^3M_r unprocessed PS2α form [α(up)], the processed PS2α heavy chain at 140×10^3M_r [α(hc)], and PSβ (β) are indicated.

generate a 140×10^3M_r heavy chain and a 20×10^3M_r light chain (Bogaert et al., 1987); both the 160×10^3M_r precusor and the 140×10^3M_r processed forms are seen in the cell extracts. The PS2α staining bands below 140×10^3M_r are breakdown products that are common in preparations from either embryos or cells (Bogaert et al., 1987; our observations). The increased ratio of breakdown products to intact PS2α at the later time points suggests that the degradation of PS2α is not simply a consequence of sample preparation. Whether from the endogenous *myospheroid* gene in the untransformed S2 cells or from the transformed gene constructs, β expressed in S2 cells runs with a slightly reduced mobility relative to β from embryos.

Immunofluorescence

To test if the PS2 integrin is being expressed on the cell surface, we stained the HSPS2(m8) with a monoclonal antibody that recognizes the PS2α subunit. Fig. 2 shows the phase-contrast and immunofluorescence images of the transformed and S2 cells. Though no immunofluorescence is seen in this exposure of the S2 cells, which is for twice as long as the HSPS2(m8) exposure, a low level of cell surface PS2 staining can be detected in longer exposures. By contrast, the transformed cells show intense staining. This result is in agreement with the immunoblot results indicating that integrin is being produced at high levels in the transformed cells, and it indicates that the integrin from the transfected genes is expressed on the cell surface. Cells that have been transfected with only the HSPS2α gene show a slight increase in surface staining (not shown) for PS2α, probably complexed with the endogenous β that was noted in the immunoblots. Cells transfected with only the HSPSβ gene show intense surface staining with an antibody that recognizes the β subunit, but no increase in PS1α or PS2α staining (not shown), indicating that β is either complexed with other endogenous proteins or is capable of being transported to the surface alone without an α subunit. Finally, it should be noted that staining of either of these cell lines with an anti-PS1 antibody shows an extremely low level of PS1 expression, lower than that seen for PS2 in untransformed cells.

Cell spreading

Preliminary experiments demonstrated that the transformed cells grown in 12% fetal calf serum accumulate a matrix on their surface that can mediate spreading on uncoated tissue culture dishes. The cells appear to require integrins for this spreading, as untransformed cells do not spread on uncoated plastic. If the HSPS2(m8) cells are allowed to spread on plastic in the absence of fetal calf serum and then removed by shear, they no longer spread on uncoated plastic. We believe this indicates that the HSPS2(m8) cells accumulate a matrix, from components in the fetal calf serum and/or matrix molecules that they themselves produce, which can mediate spreading on uncoated plastic. When the cells are allowed to spread and then removed by shear, we believe that they leave behind much of this matrix on the plastic dish and the matrix-free cells are now unable to spread on plastic. Some batches of fetal calf serum, pre-

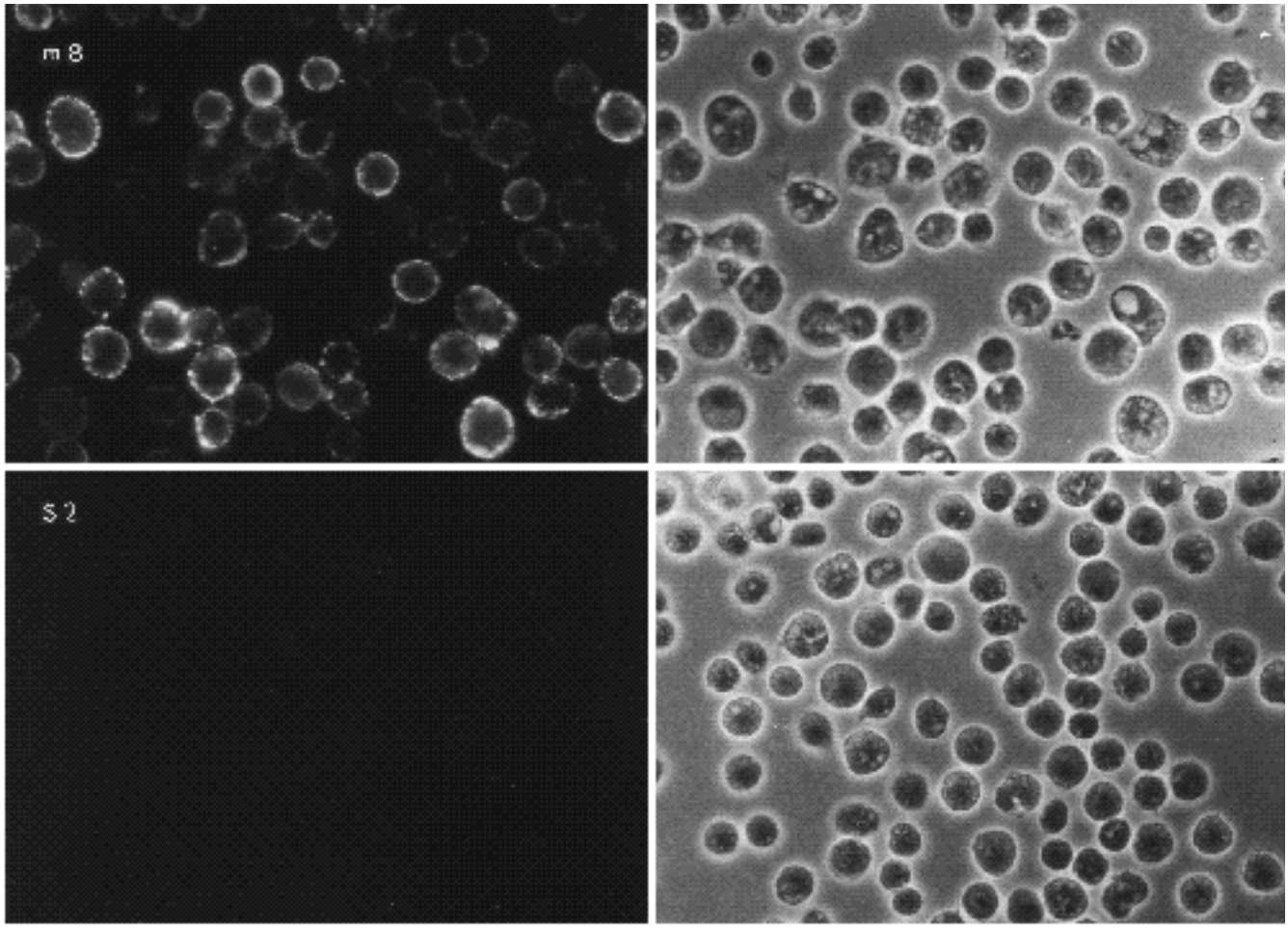

Fig. 2. PS2 expression in HSPS2(m8) cells. Shown are fluorescence (left) and phase contrast (right) images of HSPS2(m8) (top) and S2 (bottom) cells that have been stained with anti-PS2 antibodies and FITC-labeled secondary antibodies. The fluorescence exposure time for the S2 cells was twice as long as that for the transformants.

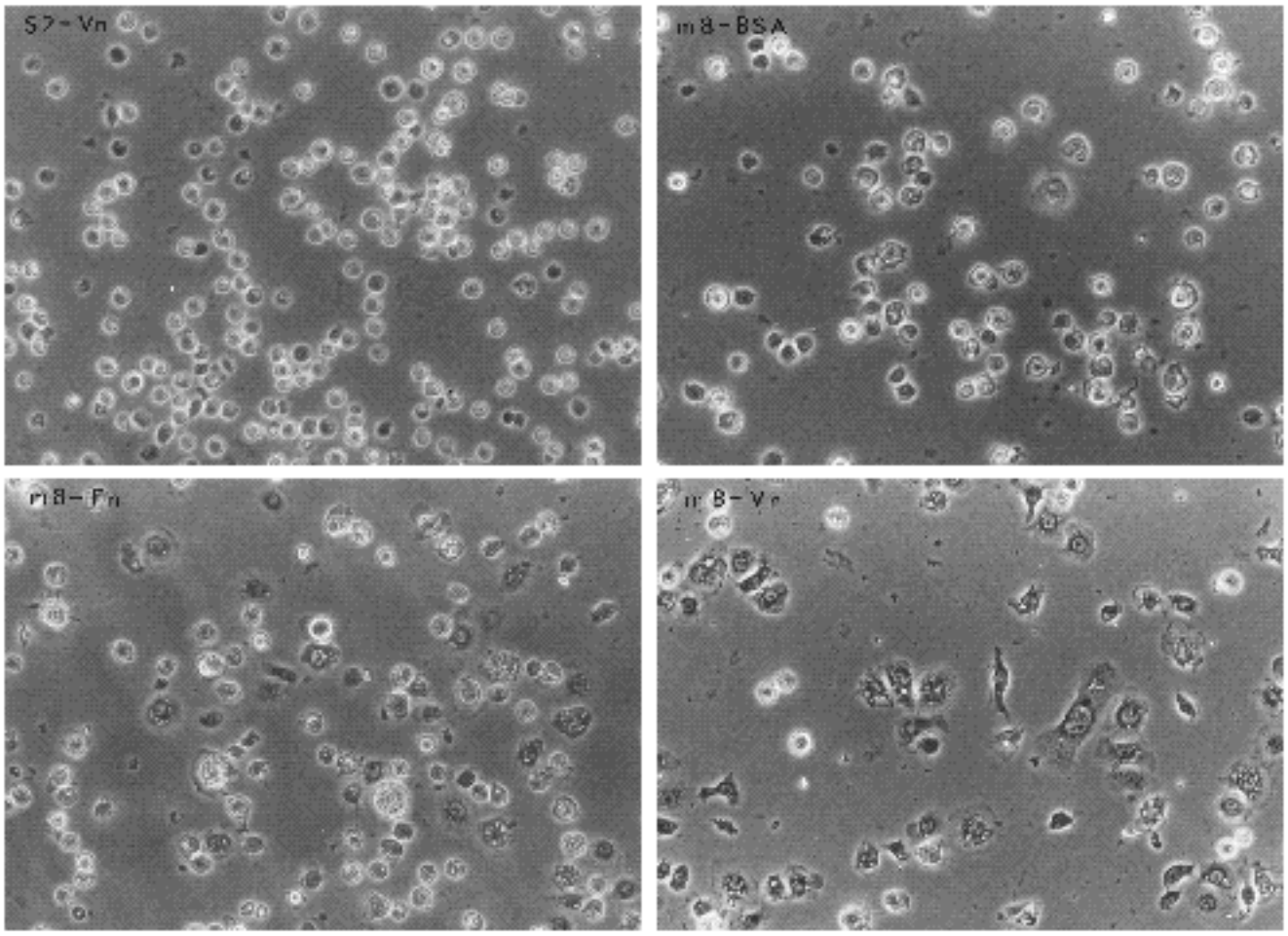

Fig. 3. Spreading of HSPS2(m8) cells on fibronectin and vitronectin. HSPS2(m8) cells were plated on BSA (m8-BSA), fibronectin (m8-Fn) and vitronectin (m8-Vn). Also shown are S2 cells plated on vitronectin (S2-Vn). The cells were trypsinized, heat shocked, plated in precoated wells, and allowed to spread for 4 hours. The wells were coated with 100 μg/ml BSA, 40 μg/ml fibronectin, or 40 μg/ml vitronectin.

sumably those containing sufficient concentrations of matrix components, could restore spreading of the matrix-free HSPS2(m8) cells in these preliminary experiments. The percentage of spread cells was increased 3-fold upon heat shock (15% to 48%) and was reduced to the level of untransformed cells (<1%) when 1 mg/ml of the peptide GRGDSP (referred to as RGD) was added to the medium but reduced only slightly (to 33%) when GRGESP (referred to as RGE) was added to the medium. This was the first indication that the *Drosophila* integrins expressed in the transformed cells could mediate cell-substratum interactions and, based on this preliminary work, we set out to examine PS-integrin-mediated cell spreading on defined matrix components.

To examine further the spreading of the transformed cells, we removed the matrix from the cells by treating them with trypsin and then plated them on either bovine serum albumin (BSA), vitronectin or fibronectin. The transformed cell line, HSPS2(m8), shows moderate cell spreading on fibronectin and extensive spreading and cell shape changes on vitronectin (Fig. 3). To quantitate this effect, the percentage of spread cells was determined after cells were plated on BSA, fibronectin or vitronectin (Fig. 4). The RGD or RGE peptides, at 1 or 2 mg/ml, were also added to the medium of identical samples to determine what effect they have on the PS2-integrin-dependent cell spreading. Plating HSPS2(m8) on fibronectin clearly results in an increased percentage of spread cells relative to plating cells on BSA. This effect is completely inhibited by RGD and much less so by RGE peptide in the medium. Vitronectin exhibits an even more dramatic increase in spread cells that is also sensitive to RGD peptide. Untransformed S2 cells exhibit no spreading (<1%) on any matrix that we have tested and are not affected by peptides in the medium.

As mentioned above, the morphology of the spread cells, as well as the percentage of spread cells, is affected by the matrix molecule that they attach to. Phase-dark cells either appear round when plated on BSA, essentially round and spread to varying degrees when plated on fibronectin, or show noticable shape changes to fibroblastic or bipolar morphologies on vitronectin (Fig. 3). The scoring of the cells by morphology (Table 1) illustrates the different effects that PS2-integrin-mediated interactions with vitronectin and fibronectin have on cell morphology in this in vitro system.

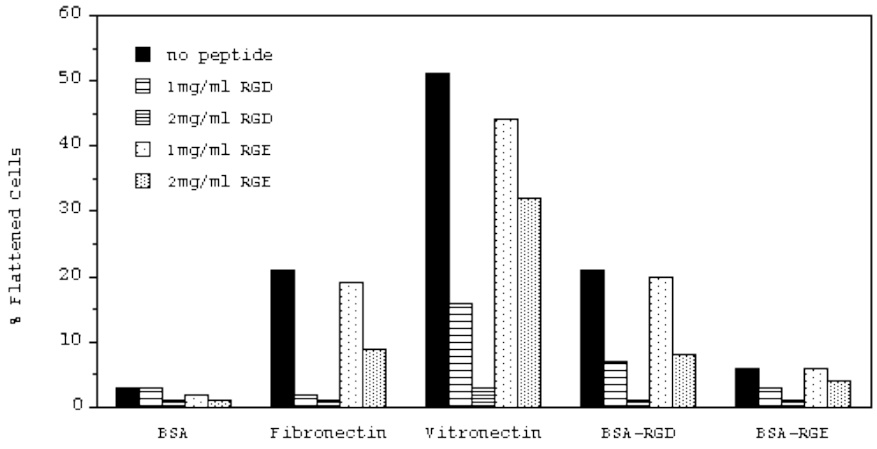

Fig. 4. Spreading of HSPS2(m8) cells on various substrata. The percentage of cells spread on BSA, fibronectin, vitronectin, and mixtures of BSA and RGD or RGE peptides is shown for HSPS2(m8) cells. Also shown are the effects of adding 1 mg/ml and 2 mg/ml RGD or RGE to the medium. The values given are the averages of three experiments. Untransformed S2 cells show less than 1% spread cells in all conditions tested.

We occasionally saw a small increase in spreading of transformed cells on BSA when RGD peptide was added to the medium, and we speculated that some of the peptide might be immobilized on the suface of the dish and serve as a ligand. To examine if RGD could mediate cell spreading, we coated wells with a mixture of RGD (or RGE) peptide and BSA and allowed cells to interact with these matrices (Fig. 4 and Table 1). Immobilized RGD does support spreading of the HSPS2(m8) cells to a level comparable with fibronectin, and this spreading is inhibited by soluble RGD peptide. The control RGE peptide was much less effective in promoting cell spreading.

Finally, we examined the requirement for divalent cations for the spreading of the HSPS2(m8) cells on vitronectin. The medium for these and the previous experiments contains approximately 7 mM Ca^{2+} and 18 mM Mg^{2+}. EDTA, which chelates both of these metals, completely inhibits cell spreading at a concentration of 20 mM. In this experiment, 36% of the cells in normal medium spread on vitronectin while less than 3% spread when EDTA was added to a concentration of 20 mM. Thus removing cations by EDTA is effective in preventing PS2-mediated cell spreading.

Table 1. *Morphology of HSPS2(m8) cells spread on various substrata*

Ligand	Peptide	Morphology
BSA	-	−
	RGD	−
	RGE	−
Fn	-	++
	RGD	−
	RGE	+
Vn	-	+++
	RGD	−
	RGE	++
BSA-RGD	-	++
	RGD	−
	RGE	+
BSA-RGE	-	+
	RGD	−
	RGE	+

Morphology scores for cells spread on BSA, fibronectin, vitronectin and BSA-peptides are shown along with the effects of 2 mg/ml RGD or RGE peptides in the medium. For morphology ratings, the photographs from three fields for each sample in a given experiment were given morphology values of − to +++ where: − means there were no phase-dark cells or those that were phase-dark exhibited no spreading (e.g. S2-Vn or m8-BSA in Fig. 3); + indicates that the phase-dark cells showed slight spreading; ++ indicates that the phase-dark cells were well spread with a few cells showing altered shapes (bipolar or fibroblastic rather than round; e.g. m8-Fn in Fig. 3); +++ indicates phase-dark cells were well spread with many cells showing altered shapes (e.g. m8-Vn in Fig. 3). The scores given represent averages from three experiments

Discussion

The distributions of the PS integrins during development suggest that they play key roles in morphogenesis (Wilcox et al., 1981; Brower et al., 1984; 1985; Bogaert et al., 1987; Leptin et al., 1989; Volk et al., 1990; Wilcox, 1990). This suggestion is confirmed by genetic studies using mutations in the genes encoding the PS2α and β subunits (Wright, 1960; Newman and Wright, 1981; Brower and Jaffe, 1989; Wilcox et al., 1989; Leptin et al., 1989; Zusman et al., 1990; Volk et al., 1990; Wilcox, 1990; see Bunch and Brower, 1992 for a review). Finally, the mutant phenotypes are consistent with the idea that the PS integrins interact with extracellular matrix components.

Sequencing of the *Drosophila* integrins shows that they are very similar to those found in vertebrates (MacKrell et al., 1988; Bogaert et al., 1987). The β subunit shares 41% sequence identity with the vertebrate β1 subunits with key domains showing 80-90% conservation. The PS2α gene, like many of its vertebrate counterparts, is synthesized as a precursor polypeptide that is cleaved to produce a transmembrane light chain linked, via covalent disulfide bonds, to an extracellular heavy chain. Like vertebrate α subunits, PS2α also contains 7 repeats in its amino terminus, three or four of which are potential divalent cation binding sites. Overall, the sequence identity of the PS2α subunit and the human fibronectin receptor is 35%.

We have tested the functional similarity of the *Drosophila* PS2 integrin to its vertebrate counterparts by asking three questions. First, can PS2 integrins expressed in transformed cells mediate cell-matrix interactions? Are RGD sequences in the matrix molecules likely to be involved in PS2 integrin binding? Is this interaction dependent on divalent cations? The answer to all three of these questions is yes and therefore the *Drosophila* PS2 integrin

shares many biochemical properties with the previously described vertebrate integrins.

Our preliminary experiments suggested that some component of fetal calf serum was responsible for the extensive spreading of cells transformed with PS2 integrin genes and that this spreading was inhibitable by RGD peptide. To eliminate the possibility of growth factors or other non-matrix components being responsible for the cell spreading, we tested two vertebrate matrix molecules, fibronectin and vitronectin, for their ability to support cell spreading in the absence of fetal calf serum. Cells plated on either of these matrix molecules show enhanced spreading with the greatest effect being seen on vitronectin. Therefore we conclude that the PS integrins are capable of mediating cell spreading on matrix molecules. As discussed below, a similar conclusion has also been reached by Hirano et al. (1991) who found that spreading of an established *Drosophila* cell line could be blocked by anti-PS integrin antibodies. We do not yet know what the *Drosophila* ligand(s) are, but experiments with our transformed cells plated on *Drosophila* collagen, laminin or glutactin (kindly provided by L. Fessler) failed to show any cell spreading (not shown). Although vitronectin has not yet been identified in *Drosophila*, an extracellular molecule similar to fibronectin has been reported to be present in haemolymph, but large amounts of this protein have not been purified and the gene encoding it has not been cloned (Gratecos et al., 1988; D. Gratecos personal communication; see Fessler and Fessler, 1989, for a review of matrix components in *Drosophila*).

The cells transformed with the PS2 integrin genes show extensive cell shape changes when plated on vitronectin. These cell shape changes suggest that the exogenously expressed PS2 integrin is not functioning simply as a cell-matrix adhesion molecule but rather is mediating interactions between the matrix and the cytoskeleton to produce bipolar or fibroblastic morphologies in cells that are normally round and not spread. This result also demonstrates that the *Drosophila* S2 cells, which are widely used for the expression of putative adhesion molecules, contain all of the cytoplasmic components necessary to mediate shape changes if they are provided with the appropriate matrix receptors.

Many vertebrate integrins bind to their ligands via an RGD sequence in the matrix molecule (see Ruoslahti and Pierschbacher, 1987 for a review), and the only integrin binding domain in vitronectin that has been characterized contains the sequence RGD (see Preissner, 1991, for a review of vitronectin and vertebrate integrins that bind vitronectin). As for the vertebrate vitronectin receptors, we find that PS2-mediated cell spreading is inhibited by soluble RGD peptide. While the concentrations of RGD required to inhibit cell spreading (1 and 2 mg/ml = 1.5 and 3.0 mM) are higher by a factor of 5-10 than that required to inhibit cell spreading or attachment mediated by some vertebrate integrins (Yamada and Kennedy, 1984; Hayman et al., 1985; Mould et al., 1991), they are similar to levels required in other vertebrate experiments (Pierschbacher and Ruoslahti, 1984a, 1984b; Hayman et al., 1985). Also the peptide RGE provides a very stringent control and in all cases (both those reported here and other observations, not shown), this RGE peptide was not as effective an inhibitor as RGD. Finally, immobilized RGD promotes cell spreading much more effectively than RGE. These results strongly suggest that the PS2(m8) integrin binds to the regions of vitronectin and fibronectin containing the RGD sequence. Though we have not identified the endogenous *Drosophila* ligands of the PS2 integrins, we would predict that they too will contain the sequence RGD or a closely related sequence in their integrin-binding domains.

Using an established *Drosophila* cell line with adhesive properties, Hirano et al. (1991) found that cell spreading on vitronectin and fibronectin could be partially inhibited by anti-PS integrin antibodies but not RGD peptide. The differences between these results and those presented here can easily be explained by the technical differences in the experimental methods and the particular cells used in each case. The cells used in the Hirano et al. (1991) study express both PS1 and PS2 integrins. It is possible that PS1 can mediate RGD-insensitive spreading on vitronectin. These cells may also have other receptors, perhaps RGD insensitive, that can bind vitronectin and fibronectin as perturbation of cell spreading by the anti-integrin antibodies was not complete, leaving about 15% of the cells still spread at saturating concentrations of antibody. In our system, less than 1% of the untransformed S2 cells show any spreading and we can therefore be confident that the spreading that we are seeing is due soley to PS2 integrin expressed from the transformed genes. Finally, and most importantly, the relative concentrations of RGD peptide and matrix differ in the two experiments. Hirano et al. (1991) used 0.2 mg/ml RGD peptide where we have used 1 and 2 mg/ml. Although we have not specifically tested 0.2 mg/ml RGD, a concentration of 0.1 mg/ml was not effective in preventing our cells from spreading on the vertebrate matrix molecules (not shown). Hirano et al. (1991) also coated the plates with vitronectin at a concentration of 50 μg/ml where we have used 10 μg/ml, and we find that the concentration of matrix used for coating also affects the efficacy of RGD inhibition.

The HSPS2(m8) cells require divalent cations for spreading on vitronectin. We cannot yet be certain whether removal of divalent cations has a direct effect on integrin PS2 function, as has been demonstrated in vertebrates (Fitzgerald and Phillips, 1985), or if there is an indirect effect of low divalent cation levels on general cell physiology. Experiments are currently underway to examine more carefully the divalent cation requirements of the PS2 integrins.

Although not detailed here, similar characterization of cells expressing the PS2(c) integrin show that this integrin will also promote cell spreading on vitronectin and fibronectin that is sensitve to RGD peptide and dependent on divalent cations. We have, however, noted some differences in the behaviors of the PS2(c)- and PS2(m8)-expressing cells, and we are working to determine if this variation reflects differences in the properties of the integrins or other, unrelated, differences in the cell lines.

Our initial characterization of the binding activity of the PS2 integrins shows that they do indeed share many properties with their vertebrate counterparts. This makes the PS integrins ideal for genetic and complementary cellular analyses to examine the structure-function rela-

tionships of integrins in complex processes such as morphogenesis.

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