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Fibronectin peptide DRVPHSRNSIT and fibronectin receptor peptide DLYYLMDL arrest gastrulation of *Rana pipiens*

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Abstract. Gastrulation is characterized by dramatic cell migration which is thought to require the interaction of cell adhesion molecules with extracellular molecules. We have tested two novel peptides, a fibronectin peptide and a fibronectin receptor peptide, for their effects on gastrulation of the leopard frog *Rana pipiens*. The fibronectin peptide DRVPHSRNSIT corresponds to residues 1373–1383 of the cell-binding domain of fibronectin; the receptor peptide DLYYLMDL corresponds to residues 124–131 of $\beta 1$ subunit of a variety of integrins including $\alpha 5\beta 1$. Either of these peptides significantly inhibited gastrulation after being microinjected into mid-blastulae. These results indicate that these sequences may correspond to the ligand/receptor interaction sites of fibronectin and its receptor(s).
Key words. Fibronectin; fibronectin receptor; peptides; gastrulation; *Rana pipiens*; embryo.

Gastrulation is the process of highly integrated cell and tissue migrations whereby the cells of blastula are dramatically rearranged to produce the primary germ layers. A large body of evidence indicates that interaction of fibronectin with its receptors plays an important role in amphibian gastrulation. Immunofluorescence methods have demonstrated fibronectin fibrils in the blastocoel roof of the amphibians *Pleurodeles* and *Xenopus*[1,2]. The extracellular fibronectin fibrils formed on the blastocoel roof are oriented parallel to the animal pole-blastopore axis[3] and thus located optimally for interaction with gastrulating cells. Presumptive mesoderm cells from *Xenopus* gastrulae attached to and migrated on substrata coated with fibronectin in vitro[4–6]. Furthermore, cells cultured on fibronectin substrate could be detached by the peptide GRGDSP which corresponds to a sequence in the 10th type III repeat of fibronectin[7,8]. In addition, microinjection of anti-fibronectin antibodies or peptide GRGDSP into the blastocoel of *Pleurodeles waltlii*[9,10] and *Rana pipiens*[11] at the early blastula stage blocked normal gastrulation.

The receptor binding sites on fibronectin and the ligand binding site(s) on the fibronectin receptor have been widely studied in a number of systems. The cell-binding domain of fibronectin is composed of type III modules 7–10 (ref. 12). The RGD sequence in the 10th type III module was postulated to be the principal cell adhesion site of fibronectin. However, subsequent work[13,14] found that a region preceding the RGD sequence was also required for the full adhesive function of the fibronectin cell-binding domain. This synergistic region is in the 9th type III module which is adjacent to RGD-containing module. The region includes a sequence of 26 residues, namely $_{1360}$RHHPEHFSGRPREDRVPHSRNSITLT$_{1385}$. A peptide, DRVPHSRNSIT, corresponding to residues 1373–1383 from this region has been found to inhibit the binding of fibronectin to the integrin $\alpha IIb\beta 3$[15].

A study using antibodies provided evidence that the fibronectin receptor subunit $\beta 1$ plays an important role in the gastrulation of *Rana pipiens* embryos[11]. The results of Takada et al.[16] suggested to us that a peptide corresponding to residues 124–131 of $\beta 1$ (DLYYLMDL) might have a specific effect on gastrulation.

In the experiments presented here, the physiological importance of residues 1373–1383 of fibronectin (i.e., DRVPHSRNSIT) and residues 124–131 of the $\beta 1$ subunit of the fibronectin receptor (i.e., DLYYLMDL) were investigated by observing the effects of corresponding peptides on gastrulation of *Rana pipiens* embryos.

Materials and methods

Animals. Adult leopard frogs (*Rana pipiens*) were obtained from a commercial supplier (Hazen Inc., Alberg, VT, USA) and were maintained in artificial hibernation (4 °C, total darkness). Animals were held in trays containing 10% Ringer's solution, which was changed three times per week. Before use, males and females were warmed by incubation at 25 °C overnight and given an intraperitoneal priming dose of gonadotropin (3 pituitary equivalents) in the morning. After 24 h, the female was given a second injection of 6 pituitary equivalents

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and 1 mg progesterone; ovulation occurred approximately 24 h later. Eggs were extruded manually from the females into dry 100 × 20 mm petri dishes. Primed males were anesthetized in tricaine methane sulfonate, decapitated, pithed and the testes minced in 10% Ringer's saline. Sperm suspensions were monitored with an inverted microscope to ascertain maximal motility. Sperm suspensions were poured over the eggs to produce fertilization.

Embryo microinjection. Micropipets were pulled from 1 mm (od) borosilicate capillary glass on a Narishige PP83 microelectrode puller; the tips were beveled manually to approximately 5 μm. The micropipets were fitted into an MM33 micromanipulator and microinjection syringe assembly (Stoelting, Chicago, IL, USA). Embryos were prepared for microinjection by removal of the jelly coat with watchmaker forceps. The dejellied blastula were transferred into wells formed in 2% agar-lined dishes filled with 10% Ringer's saline. Approximately 100 nl of peptide solutions or 10% Ringer's saline were injected into the blastocoel of midblastula stage embryos.

Gastrulation assay. After microinjection the embryos, including uninjected controls, were incubated at 20 °C in the dark for up to 24 h. The embryos were scored for gastrulation by visualizing the vegetal pole using a Nikon TMS inverted microscope fitted with a fiber optics lamp to give reflected light illumination of the opaque embryos. Normal gastrulation was denoted and thus scored by the formation of yolk plug due to epibolic movement of animal pole cells over the vegetal pole.

Peptide preparation. Peptides GRGDSP, GRGESP, DLYYLMDL, and MYDLDYLL were synthesized using a PSS-80 automated peptide synthesizer (Applied Protein Technologies, Cambridge, MA, USA) and were cleaved from resins with trifluoromethane sulfonic acid, purified, the amino acid compositions, and sequences confirmed, as previously described[17]. The concentration of the peptide in each solution was determined by quantitative amino acid analysis. Stock solutions were made in 10% Ringer's saline and the pH adjusted to neutrality with HEPES. The peptides DRVPHSRNSIT and VHPDRNTISRS were generously donated by Dr. R. Bowditch, Scripps Research Institute, La Jolla, CA, USA.

Results

Microinjection of approximately 100 nl of 20 mM DRVPHSRNSIT, or DLYYLMDL into the blastocoels of mid-blastula stage embryos of *Rana pipiens* resulted in significant inhibition of gastrulation compared to 10% Ringer's-injected controls (table 1). The inhibited embryos (figs 1 and 2 respectively) had a

Table 1. Effects of microinjected peptides on gastrulation of *Rana pipiens*.

Microinjected solution	Total embryos injected	Number of gastrulae	Percent gastrulation
No. injection	556	541	97.3^{ih}
Ringer's	797	683	85.7^{gf}
GRGDSP 20 mM	356	52	14.6^{a}
GRGDSP 1 mM	184	111	60.3^{d}
GRGESP 20 mM	314	247	78.7^{e}
GRGESP 1 mM	39	33	84.6^{fe}
DRVPHSRNSIT 20 mM	68	28	41.2^{cb}
VHPDRNTISRS 20 mM	42	42	100.0^{ih}
DLYYLMDL 20 mM	76	24	31.6^{b}
MYDLDYLL 20 mM	35	34	97.1^{h}

Percentage values with dissimilar superscripts (i.e., no matching single letters) are significantly different at $p = 0.05$.

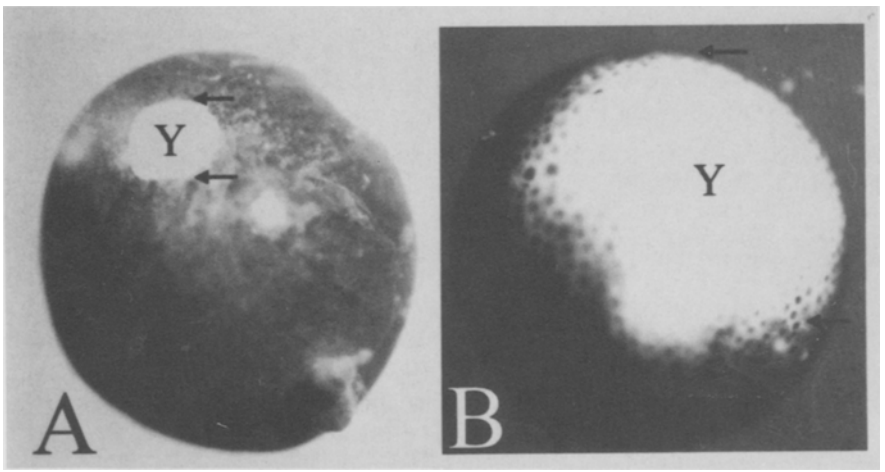

Figure 1. *Rana pipiens* embryos.
A) GDGRHDLLVGAPL injected embryo showing normal gastrulation with small yolk plug (Y); arrows indicate extent of yolk plug.
B) DRVPHSRNSIT injected embryo with abnormal gastrulation and large yolk plug (Y); arrows indicate extent of yolk plug, note that animal pole cells (dark spots) are intermingled with light colored yolk plug cells.

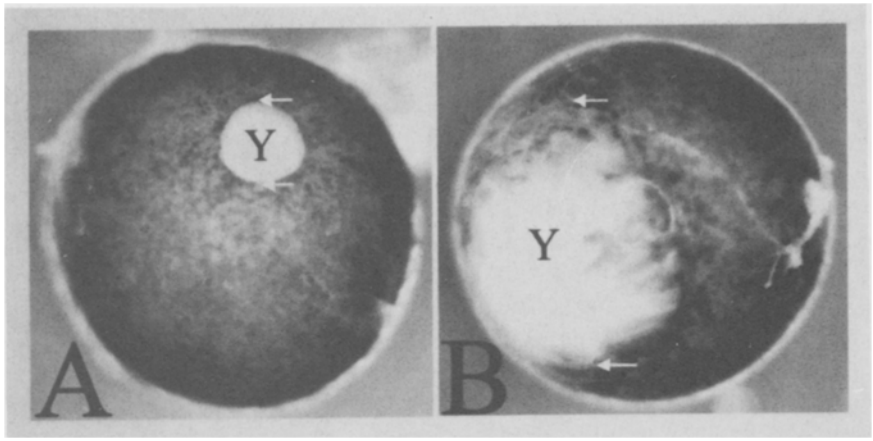

Figure 2. *Rana pipiens* embryos.
A) MYDLDYLL injected embryo, this is a scrambled control version of the active peptide (compare with *B*); the yolk plug (Y) is small and indicated by arrows.
B) DLYYLMDL injected embryo has an abnormally large yolk plug (Y) indicated by arrows.

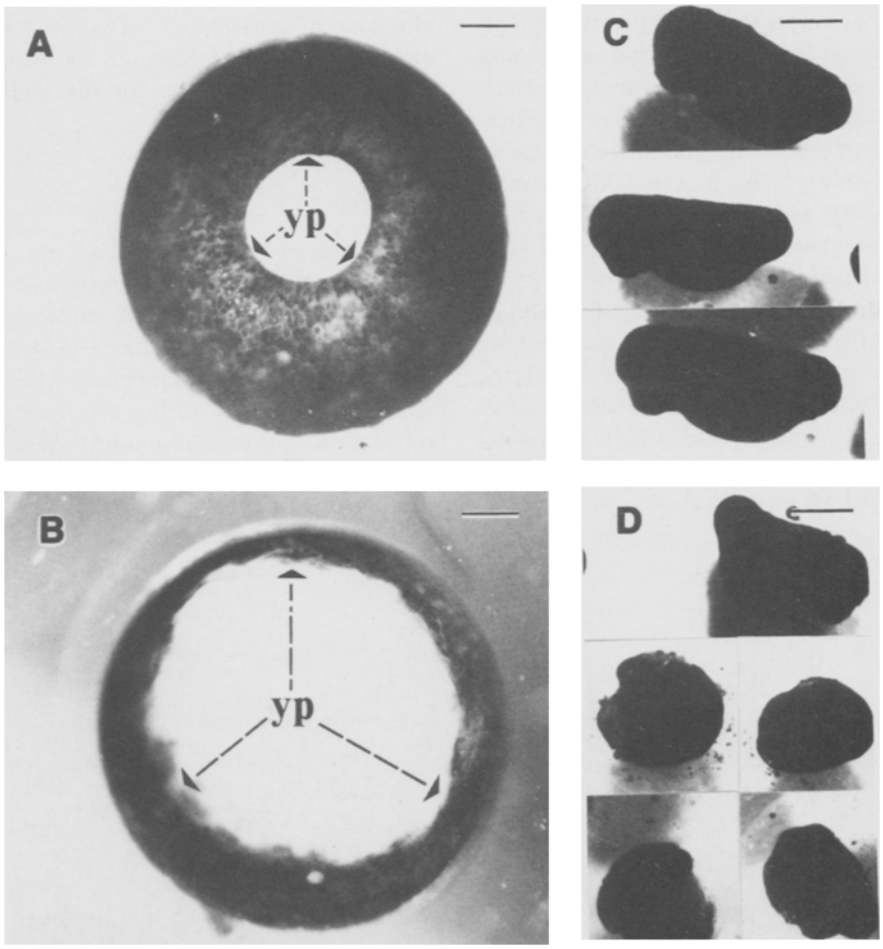

Figure 3. *Rana pipiens* embryos.
A) Control 10% Ringer's injected embryo showing normal gastrulation and small yolk plug (yp); arrows indicate extent of yolk plug. Bar equals 0.25 mm.
B) Embryo injected with 20 mM of GRGDSP with abnormal arrested gastrulation, the majority of the vegetal pole cells remain visible without evidence of epibolic movement of the dark pigmented cells. Thus yolk plug (yp) formation was incomplete; arrows indicate extent of abnormal yolk plug. Bar equals 0.25 mm.
C) Montage of 3 normal tailbud embryos after blastula injection with Ringer's. Bar equals 1 mm.
D) Montage of 5 (20 mM GRGDSP) GRGDSP-injected embryos at same incubation time as *C*). Note dissociated cells within perivitelline space of some of the latter embryos. Bar equals 1 mm.

characteristic rugose animal pole, and apparent arrest of the epibolic movement of the animal pole cells at the marginal region; this resulted in an abnormally large yolk-plug compared to the co-incubated controls which had small yolk-plugs characteristic of Witschi stage 11 embryos[18].

Several peptides have also been used as controls. The peptide GRGDSP is a positive control (fig. 3). Johnson et al.[11] reported that microinjection of this peptide into the blastocoel of *Rana pipiens* blocked normal gastrulation. Among the peptides tested here, GRGDSP was the most potent inhibitor of gastrulation. As little as 1 mM GRGDSP caused significant inhibition of gastrulation (table 1). In a few cases, embryos were subsequently incubated until the controls reached Witschi stages 17–20 (tailbud stages)[18]. This extended incubation resulted in GRGDSP-injected embryos with a further exaggeration of the characteristic 'mushroom cap' appearance (fig. 3). The results of the long term incubation study suggest that gastrulation was arrested rather than simply delayed. The abnormal embryos had blastomeres with distinct cell outlines and nuclei or mitotic spindles clearly visible upon examination, although some dissociated cells were often observed in the perivitelline space of GRGDSP-injected embryos (fig. 3). GRGESP, an inactive form of GRGDSP which contains a single amino acid difference, was used as a negative control. The peptide GRGESP had weak inhibitory activity at 20 mM but was without effect at 1 mM (table 1). The peptide GDGRHDLLVGAPL[19] was also used as a negative control for RGD-containing peptides since it has the retromer sequence DGR. This peptide was without effect on gastrulation (fig. 1). The peptides VHPDRNTISRS and MYDLDYLL are scrambled versions of DRVPHSRNSIT and DLYYLMDL, respectively. Both scrambled peptides had no significant effect on gastrulation of *Rana pipiens* (table 1).

Dose response assays were performed for all three inhibitory peptides. Peptide concentration was directly related to the inhibitory effect of each peptide (fig. 4). The IC50s estimated from figure 1 are 1.8 mM, 15.8 mM, and 6.3 mM for GRGDSP, DRVPHSRNSIT, and DLYYLMDL, respectively. It should be noted that the delivered solution would be diluted by fluid already present in the blastocoel of the recipient embryos. The blastocoel volume is about 400 nl as estimated from published median sections[18]. Thus, the calculated value of IC50s may be as much as a 5-fold underestimate of the inhibitory potency of those peptides, since only 100 nl of peptide solution were injected into each embryo.

In contrast to the finding of Aota et al.[14] that the DRVPHSRNSIT-containing region synergizes with RGD site for full activity of fibronectin in cell spreading assay, the data in table 2 reveal that the peptides DRVPHSRNSIT and GRGDSP were not synergistic as inhibitors of gastrulation. That is, the simultaneous injection of both peptides into the blastocoels did not result in an enhancement of the inhibitory effect of GRGDSP alone.

Discussion

The process of morphoregulation, e.g. development of form and pattern in animal embryos, has been hypothesized to involve cell adhesion molecules (CAMs) and substratum adhesion molecules (SAMs)[20,21]. A particularly important stage in amphibian embryonic development is gastrulation, during which the principal germ layers, especially the presumptive mesoderm cells, are positioned with the embryo[5]. The importance of the SAM fibronectin in amphibian gastrulation was demonstrated first in *Pleurodeles waltlii* embryos[9]. In that study, the residues RGD were demonstrated to play an

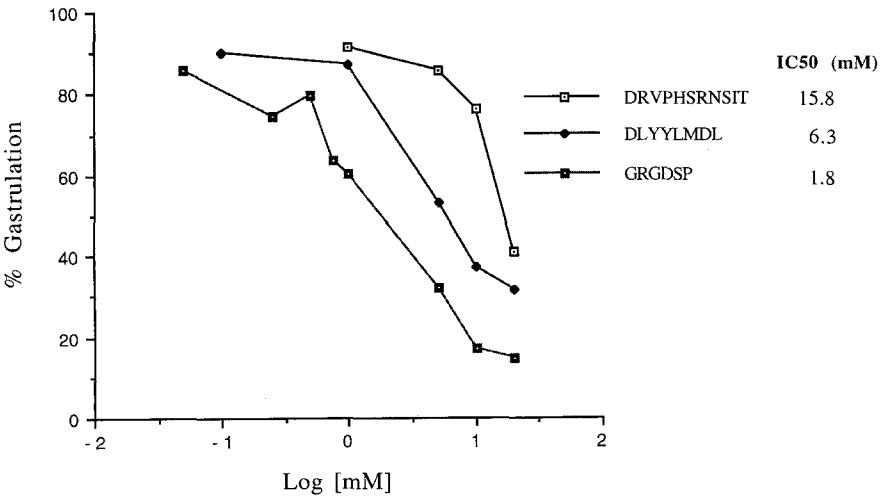

Figure 4. Dose-response of *Rana pipiens* gastrulation to peptides GRGDSP, DRVPHSRNSIT and DLYYLMDL microinjected into the mid-blastula stage embryos (N = 801, 317, 269, respectively). IC50 = the inhibitory concentration yielding 50% abnormal gastrula.

Table 2. The inter-peptide effects of peptides GRGDSP and DRVPHSRNSIT on gastrulation of *Rana pipiens* embryos.

Microinjected solution	Total embryos injected	Number of gastrulae	% inhibition of gastrulation
Ringer's	797	683	14.3[a]
GRGDSP 1 mM	184	111	39.7[dc]
DRVPHSRNSIT 10 mM	93	71	23.7[b]
GRGDSP 1 mM + DRVPHSRNSIT 10 mM	53	34	35.8[cb]

Percentage values with dissimilar superscripts (i.e., no matching single letters) are significantly different at $p = 0.05$.

important role in the function of fibronectin in embryonic development. A role for fibronectin and the CAMβ1 in the gastrulation of *Rana pipiens* embryos was demonstrated later[11]. Our work demonstrates that in addition to RGD residues, the fibronectin residues DRVPHSRNSIT appear to play an important role in gastrulation. The data presented here also corroborate a role for the receptor subunit β1 in gastrulation of *Rana* embryos and demonstrate the importance of β1 residues DLYYLMDL in this process.

The results from our in vivo assays of the peptides DRVPHSRNSIT and DLYYLMDL support the conclusion that these regions mediate ligand receptor interactions required for cell migration during gastrulation. Specifically, the peptide DRVPHSRNSIT may bind to fibronectin receptors and thereby inhibit fibronectin binding to its receptor. Likewise, the inhibitory effect of peptide DLYYLMDL on gastrulation is consistent with finding of Takada et al.[16] that a point mutation affecting aspartic acid residue 130 on β1 blocked the binding of $\alpha 5\beta 1$ to fibronectin. Thus the peptide DLYYLMDL may interact with fibronectin and block the interaction of fibronectin with its receptors. Although the DLYYLMDL sequence may be involved in adhesive recognition of fibronectin by $\alpha 5\beta 1$, our data do not exclude an effect of this peptide on other integrin β subunits since this peptide corresponds to a highly conserved region of other integrin β subunits[16,22]. For example, the sequence DIYYLMDL was found in approximately the same location in the β3 subunit of *Xenopus*[23] as the DLYYLMDL sequence of the human β1 subunit. Furthermore, the mRNAs for both the β1 and β3 subunits were expressed in the blastula and gastrula *Xenopus* embryos. So, further work is required to determine which receptor(s) the peptide DLYYLMDL mimics during gastrulation of *Rana* embryos.

Since a DRVPHSRNSIT-containing region on fibronectin synergizes with RGD region in cell spreading assay, we studied the inter-peptide effects of two corresponding peptides, DRVPHSRNSIT and GRGDSP. Injection of 1 mM GRGDSP, alone, into embryos inhibited about 40% of gastrulation, injection of 10 mM DRVPHSRNSIT, alone, inhibited about 24% of gastrulation. However, the simultaneous injection into embryos of both peptides at these submaximal concentrations resulted in an inhibition of only 36%, which indicated that the peptide DRVPHSRNSIT did not synergize with peptide GRGDSP in its inhibition of embryo gastrulation. Thus it appears that the presence of GRGDSP precluded the action of DRVPHSRNSIT. These results may mean that the peptides do not bind to identical or overlapping sites of the receptor, nor does their binding appear to be subject to mutually exclusive allosteric regulation. That is, if the peptides bound to the same or overlapping sites or if their binding were subject to mutually exclusive allosteric regulation, their effects should have been additive at submaximal doses[17].

Our data do not support the suggestion that binding of the peptide DRVPHSRNSIT is specific for αIIbβ3[15], however they do support the suggestion that the peptide DRVPHSRNSIT may bind to $\alpha 5\beta 1$[15].

Bowditch et al.[15] reported that the peptides DRVPHSRNSIT and GRGDSP correspond to mutually exclusive αIIbβ3 binding sites on fibronectin. Similarly, the peptide LGGAKQAGDV[24], which corresponds to residues 402–411 of the γ chain of fibrinogen, and the peptide RGDS correspond to mutually exclusive αIIbβ3 binding sites on fibrinogen[17,25,26]. Thus the peptide LGGAKQAGDV is analogous to the peptide DRVPHSRNSIT with regard to binding to αIIbβ3. Also, neither of these peptides inhibit the binding of RGD containing ligands to the closely related integrin $\alpha v\beta 3$[27,28]. The peptide LGGAKQAGDV binds to αIIbβ3[25] and thereby inhibits the binding of fibrinogen and fibronectin to that receptor. Because of this similarity in behavior of LGGAKQAGDV and DRVPHSRNSIT, LGGAKQAGDV was tested in another set of gastrulation assays. LGGAKQAGDV did not inhibit gastrulation (table 3). This result indicates that the

Table 3. Effects of a fibrinogen peptide and an αIIbβ3 peptide on gastrulation of *Rana pipiens*.

Microinjected solution	Total embryos injected	Number of gastrulae	Percent gastrulation
Ringer's	331	258	78.0[ab]
LGGAKQAGDV 20 mM	109	79	72.5[a]
GDGRHDLLVGAPL 20 mM	97	82	84.5[b]

Percentage values with dissimilar superscripts (i.e., no matching single letters) are significantly different at $p = 0.05$.

receptor $\alpha IIb\beta 3$ does not play a role in the gastrulation of *Rana pipiens* embryos. The result that peptide GDGRHDLLVGAPL[19] (table 3) did not inhibit gastrulation also supports this conclusion. This peptide corresponds to residues 300–312 of αIIb[19]. It inhibits the $\alpha IIb\beta 3$ mediated adhesion of platelets to fibrinogen and fibronectin, but does not inhibit the $\alpha 5\beta 1$ mediated adhesion of platelets to fibronectin[19]. If interaction of $\alpha IIb\beta 3$ with fibronectin is important in gastrulation, the peptide should have had inhibitory effects on gastrulation. Taken together, these observations mean that the ability of the peptide DRVPHSRNSIT to inhibit gastrulation results from it inhibiting the function of another receptor, probably $\alpha 5\beta 1$.

Thus, the results presented here demonstrate that the peptides DRVPHSRNSIT and DLYYLMDL can inhibit the gastrulation of *Rana pipiens* embryos. These results support the conclusion that the corresponding regions of fibronectin and $\beta 1$, respectively, mediate an important function(s) during gastrulation. Finally, the use of other peptides demonstrate that the peptide DRVPHSRNSIT can bind to a receptor(s) other than $\alpha II\beta 3$, probably $\alpha 5\beta 1$.

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