

Integrin-dependent adhesive activity is spatially controlled by inductive signals at gastrulation

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SUMMARY

Integrins mediate cell-ECM interactions essential for morphogenesis, however, the extent to which integrin adhesive activities are regulated in the embryo has not been addressed. We report that integrin-dependent cell adhesion to the Arg-Gly-Asp (RGD) containing central cell-binding domain of fibronectin is required for gastrulation in *Xenopus*. Although all cells of the early embryo retain the ability to attach to this region, only involuting cells arising from the dorsal and ventral lips of the blastopore are able to spread and migrate on fibronectin in vitro. This change in adhesive behavior is mimicked by treating animal cap cells with activin-A. Activin-induced changes in adhesion are independent of new transcription, translation, or changes in receptor expression at the cell surface. We demonstrate that ectopic expression of integrin $\alpha_4\beta_1$ in

animal cap cells results in attachment to the non RGD-containing V-region of fibronectin. Further, these cells acquire the ability to spread on the V-region following activin induction. Thus, $\alpha_4\beta_1$ adhesion to the V-region, like endogenous integrin binding to the central cell-binding domain, is responsive to activin signalling. These data indicate that cell adhesion to the central cell-binding domain is regulated in both space and time, and is under the control of inductive signals that initiate gastrulation movements. We suggest that position-specific inductive interactions are likely to represent a novel and general mechanism by which integrin adhesion is modulated throughout development.

Key words: integrin, cell adhesion, gastrulation, *Xenopus*

INTRODUCTION

The regulation of cell adhesion to fibronectins (FNs) and other extracellular matrix (ECM) proteins is of fundamental importance to a variety of biological processes that include embryogenesis, differentiation and the maintenance of cell and tissue boundaries (Hynes and Lander, 1992; Adams and Watt, 1993). Cell adhesion to the ECM is mediated in large part by the integrins, which are a functionally diverse family of heterodimeric transmembrane receptors (Hynes, 1992). Integrins function as mechanical links between the ECM and the cytoskeleton (Sastry and Horwitz, 1993) and also participate in a variety of adhesion-dependent cell signalling events (Clark and Brugge, 1995). Integrin ligand-binding specificity is determined by subunit composition and more than 20 different receptors are known to assemble from the 8 β and 16 α subunits that have been identified. Cells and tissues often express distinct repertoires of integrins on their surfaces and come in contact with matrices composed of varied ligands. Not surprisingly, therefore, most of the available information concerning integrin/ECM involvement in development is limited to descriptions of where and when these molecules are expressed in embryos (Lallier et al., 1994). Gene targeting experiments in mice demonstrate conclusively that FN (George et al., 1993) and at least some integrins (Yang et al., 1993, 1995; Fassler and Meyer, 1995; Stephens et al., 1995) are essential for normal development, however, the specific

involvement of these molecules in the regulation of morphogenetic change at the cellular level remain unclear.

In amphibian embryos, FN is localized to the roof of the blastocoel where it comes in contact with involuting marginal zone (IMZ) cells during gastrulation (Boucaut and Darribere, 1983; Lee et al., 1984). Microinjection of anti-FN antibodies or Arg-Gly-Asp (RGD) peptides is reported to inhibit gastrulation, presumably by interfering with IMZ adhesion and migration (Boucaut et al., 1984a,b; Howard et al., 1992). In contrast, extensive mesodermal cell migration through the primitive streak is evident in FN-deficient mice (George et al., 1993) indicating that adhesion to FN is unlikely to represent an essential feature of normal gastrulation movements common to all vertebrates. Moreover, experiments with *Xenopus* suggest that cell adhesion to FN is not required to initiate mesoderm involution, convergence and extension movements, or blastopore closure (Keller et al., 1985; Yost, 1992; Keller and Jansa, 1992). The precise role of FN in *Xenopus* gastrulation, therefore, is uncertain.

In recent years, considerable effort has been devoted to elucidating molecular mechanisms involved in mesoderm induction and the control of gastrulation movements in *Xenopus* (reviewed by Kessler and Melton, 1994; Slack, 1994). Progressive inductive interactions between vegetal pole endoderm and the marginal zone of the embryo are responsible for mesoderm formation and subsequent dorsal-ventral patterning of this tissue. *Xenopus* animal cap cells isolated

from blastula stage embryos alter their adhesive properties following treatment with the mesoderm inducing factor activin, a member of the TGF β family (Smith et al., 1990; Howard and Smith, 1993; Briehner and Gumbiner, 1994). These experiments suggest that inductive interactions may control local cell-adhesive functions in vivo. Although embryonic region-specific variations in cell adhesion to FN at the gastrula stage have been reported (Winklbauer, 1988; 1990), the involvement of integrins in the establishment of these spatial differences has not been addressed.

In this study, we demonstrate that FN is required for gastrulation in *Xenopus* and that integrin-dependent cell adhesion to this ECM protein is spatially and temporally regulated. These data establish that changes in integrin adhesive activity are linked to a cell-autonomous gastrulation 'timer' (Cooke and Smith, 1990) that is independent of cell division, transcription and protein synthesis. We propose that the position-specific activation of integrin adhesive function in the embryo is controlled by inside-out signalling mechanisms triggered by mesoderm induction.

MATERIALS AND METHODS

Xenopus embryos

Adult wild-type and albino *Xenopus laevis* were purchased from Xenopus 1 (Ann Arbor, MI). Eggs and embryos were obtained as described by Newport and Kirschner (1982). Embryos were cultured in 0.1 \times Modified Barth's Saline (MBS; Gurdon, 1977) and staged according to Nieuwkoop and Faber (1994).

Antibodies

A glutathione S-transferase (GST) fusion protein containing the CCB δ of FN (GST-9.11) and *Xenopus* plasma FN (XpFN) were used to immunize mice in order to generate monoclonal antibodies (mAbs) using standard methods (Chapman et al., 1984). Several hybridomas were selected on the basis of immunoreactivity to XpFN and GST-9.11 (mAbs 4H2 and 4B12) and GST (mAb 1C9). Ascites fluids were generated and purified IgG1 obtained by recombinant protein G affinity chromatography (Pharmacia LKB). Epitope mapping for the 4H2 and 4B12 antibodies was done by western blot using a series of *Xenopus* FN fusion proteins (Ramos and DeSimone, 1996). Hybridoma fusions and ascites production were performed by the Lymphocyte Culture Center at the University of Virginia. The polyclonal antibody 363 is directed against the C-terminal 39 amino acids of the integrin β_1 subunit (Marcantonio and Hynes, 1988). D3FAP and D2AP are affinity purified polyclonal antibodies directed against the C-terminal 20 amino acids of the *Xenopus* integrin α_3 (Meng and DeSimone, unpublished) and α_4 (Whittaker and DeSimone, unpublished) subunits, respectively. The anti-*Xenopus* α_5 antibody 881 (Joos et al., 1995) was kindly provided by Dr. Peter Hausen (Tuebingen). The anti- α_v antibody was obtained from Chemicon (AB1930).

Antibody injections

Purified IgGs were prepared in Danilchik's solution at a concentration of 10 mg/ml (Shih and Keller, 1992) and centrifuged at 16,000 g for 5 minutes at 4°C just prior to injection. Antibodies were delivered into 2 separate sites (30 nl each, yielding a total of 600 ng mAb) of the blastocoel roof at stage 9.5 by pressure injection (PLI-100, Medical Systems Corp.). All embryos were injected and cultured in 0.1 \times MBS.

Whole-mount in situ hybridizations were performed on injected embryos as described by Harland (1991) using digoxigenin-rUTP-

labelled (Boehringer Mannheim) transcripts synthesized in vitro from a *Xenopus brachyury* cDNA obtained from Dr Jim Smith (Smith et al., 1991). The 12-101 antibody (Kintner and Brockes, 1984) was used for whole-mount immunostains following the procedure described by Klymkowsky and Hanken (1991). All embryos were imaged using a Zeiss Axiophot or STEMI-2000 dissecting microscope equipped with a Kodak DCS420c digital camera.

FN fusion proteins and adhesion assays

All FN fusion proteins were prepared using the pGEX-KG vector (Guan and Dixon, 1991) and expressed in bacteria as described by Smith and Johnson (1988). Details regarding the construction and purification of the GST-9.11, GST-12.15V0 and GST-V fusion proteins are described by Ramos and DeSimone (1996).

Marked areas of 35 mm plastic Petri dishes (Falcon) were coated overnight at 4°C with human pFN or fusion proteins at a concentration of 0.23 μ M in phosphate buffer (pH 7.5). The Petri dish was then washed with 0.1 \times MBS and incubated in Modified Stern's Solution (MSS; DeSimone and Johnson, 1991) containing 0.5% bovine serum albumin (BSA) for 45 minutes. ELISAs were performed on coated Petri dishes using the anti-GST mAb 1C9 in order to confirm that the fusion proteins were adsorbed onto the plastic surface at approximately equimolar amounts. IMZ cells consisting of presumptive mesoderm and endoderm were dissected from stage 11-11.5 embryos and dispersed in Ca²⁺ and Mg²⁺ free 1 \times MBS (CMF-MBS) on agarose-coated dishes for 30 minutes. Identical methods were used to isolate cells from additional embryonic stages and regions, as indicated in Fig. 3. Dispersed cells were added to substrates and incubated in MSS containing 0.5% BSA, 1 mM CaCl₂ and 1 mM MgCl₂. Activin induction assays were similarly performed using dispersed animal cap cells incubated for 2 hours in MSS alone or in MSS containing 10-20 U/ml of activin A (generously provided by Dr Jim Smith, London).

For each experiment, three to five fields at 10 \times magnification were photographed and the plates inverted in buffer for 5 minutes. The same fields were then photographed again. Cells counted as spread were attached to the substrate, flattened in appearance, and had at least two lamellipodia. Cells counted as attached were spherical and remained bound to the plate during the inversion in buffer. Cells counted as non-attached were spherical and fell away during inversion. Each experiment was repeated at least 8 times. For blocking experiments, antibodies were tested in vitro using fusion protein and XpFN substrates incubated with mAbs (1 mg/ml) at 25°C for 1 hour. After washing, cells were added and attachment and spreading quantified as described above.

Inhibition of transcription and translation

Dispersed animal cap cells were incubated in MSS alone or MSS containing α -amanitin (100 μ g/ml; Sigma) or cycloheximide (10 μ g/ml; Sigma). α -amanitin-treated cells were immediately pipetted onto the GST-9.11 substrate and cultured continuously in the presence of the inhibitor. Activin-A (20 U/ml) was added to the culture 30 minutes after the cells were plated. Cycloheximide-treated cells were plated initially onto agarose-coated dishes and 20 U/ml activin-A added after 30 minutes. Following an additional 30 minutes incubation in the presence of both cycloheximide and activin the cells were washed and pipetted onto GST-9.11-coated dishes and cultured in MSS containing 20 U/ml activin-A until the equivalent of control stage 11. TCA precipitations of [³⁵S]methionine-labelled cells were used to confirm translation inhibition.

α_4 transcript injections

Full length cDNAs encoding *Xenopus* integrin α_4 were isolated from a stage 17 cDNA library using a partial *Xenopus* α_4 cDNA (Whittaker and DeSimone, 1993). Details regarding the cloning and sequencing of the α_4 cDNAs will be described elsewhere (Whittaker, Ramos and DeSimone, unpublished). Synthetic transcripts were prepared as described by Cunliff and Smith (1992), dissolved in H₂O and injected

into the animal poles of fertilized eggs using standard methods (Moon and Christian, 1989).

Immunoprecipitations

Animal caps were dissected from 500 stage 8 embryos and dispersed to single cells as described above. In other experiments, total dispersed cells from 50 stage 8 or 11 embryos were prepared. Cells were then surface biotinylated (Müller et al., 1993), lysed in 500 µl of RIPA buffer (100 mM Tris-HCl (pH 7.5), 1% deoxycholate, 1% Triton X-100, 0.5% SDS, 100 mM KCl, 5 mM MgCl₂, 2 M PMSF, 1 µg/ml aprotinin, 1 µg/ml pepstatin, and 1 µg/ml leupeptin) and immunoprecipitated as described by Hens and DeSimone (1995). Immunoprecipitates were transferred to nitrocellulose and biotinylated proteins visualized by enhanced chemiluminescence (Amersham).

RESULTS

Mesoderm adhesion to fibronectin is required for gastrulation

Previous studies with salamander embryos suggest an important role for FN in mesodermal cell adhesion and migration at gastrulation (Boucaut et al., 1984a,b). The significance of FN adhesion in the gastrulation of anurans such as *Xenopus*, however, has been controversial (reviewed by Keller and Winklbauer, 1992; DeSimone, 1994). In order to investigate the role of FN in this process, we developed a monoclonal antibody (mAb) that interferes specifically with the adhesion of mesodermal cells to the central cell-binding domain (CCBD) of FN. mAb 4B12 is an IgG1 directed against the RGD-containing Type III-10 repeat of *Xenopus* FN (Fig. 1). A series of glutathione S-transferase/FN (GST-FN) bacterial fusion proteins (Fig. 1) were used as adhesive substrates in order to test the specificity of antibody blocking in vitro. mAb 4B12 blocks completely the attachment and spreading of explanted mesodermal cells (involved marginal zone, IMZ) on the GST-9.11 fusion protein (Fig. 2A). Attachment to the HepII containing GST-12.15VO fusion protein is unaffected by the 4B12 antibody. The 4H2 control mAb is an IgG1 that binds the Type III-9 repeat of FN (Fig. 1) and has no effect on the adhesion of IMZ cells to fusion protein substrates or to intact *Xenopus* plasma fibronectin (XpFN; Fig. 2A). Thus, mAb 4B12 blocks mesoderm adhesion to the RGD-containing CCBD of FN but not the integrin-independent attachment of these cells to a fusion protein containing the HepII region. As predicted from these data, 4B12 blocks the spreading of IMZ cells on XpFN but not cell attachment to the HepII region of the intact protein (Fig. 2A).

We next used these antibodies to investigate the importance of mesodermal cell adhesion to FN in vivo. Purified mAbs were injected at a concentration of 10 mg/ml (60 nl containing 600 ng of mAb) into the blastocoels of stage 9-9.5 embryos prior to the onset of gastrulation. Embryos were analyzed at various time points from gastrulation through tailbud stages (Fig. 2B; stages 13, 22 and 31). Mesoderm involution

Table 1. Antibody injection experiments

Experiment	Antibody	Number injected	Number normal	Number abnormal	Number dead
1	4H2	100	97	0	3
	4B12	105	1	99	5
2	4H2	50	49	0	1
	4B12	51	0	46	5
3	4H2	52	50	0	2
	4B12	50	0	48	2
4	4H2	102	94	2	6
	4B12	101	0	96	5

Stage 9-9.5 embryos were injected with 600 ng of purified antibody as described in Materials and Methods. Number of abnormal refers to embryos arrested as late gastrulae. Data from 4 representative experiments are shown.

proceeds in 4B12 injected embryos but blastopore closure is delayed relative to 4H2 and uninjected controls (Fig. 2B, stage 13), however, partial to complete closure of the blastopore will occur in approximately 70% of cases by control stage 14. The most obvious defect in these embryos is failure of the mesoderm to maintain contact with the inner surface of the blastocoel roof at the animal pole, the latter of which is evident as a thin and translucent layer of ectodermal cells by the end of gastrulation. Table 1 summarizes data obtained from four representative experiments.

The early mesodermal marker *brachyury* (*Xbra*; Smith et al., 1991) was used to confirm the location of the mesoderm in mAb injected gastrulae. *Xbra* staining is confined to a circum-blastoporal ring of mesoderm in 4B12 injected embryos at stage 12 (Fig. 2C, *Xbra*). In contrast, uninjected (not shown) and 4H2 control embryos reveal the presence of a notochord, indicating normal axial mesodermal patterning. Differences between 4B12 injected and control embryos become more striking as development proceeds (Fig. 2B, stages 22 and 31). Affected embryos fail to neurulate properly and in all cases lack anterior neural folds. Elongation of the anterior-posterior

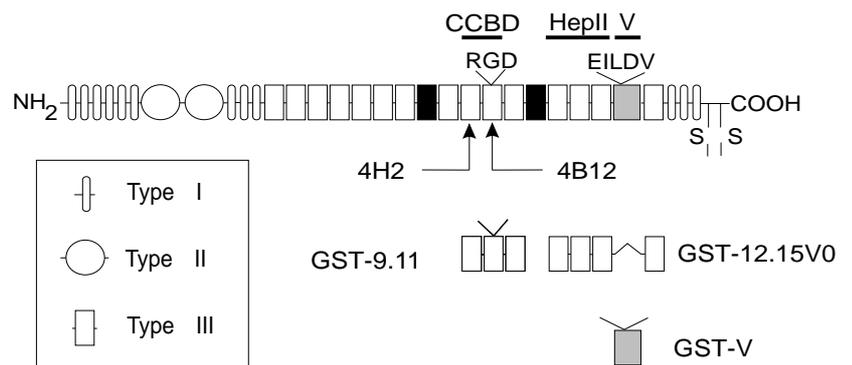
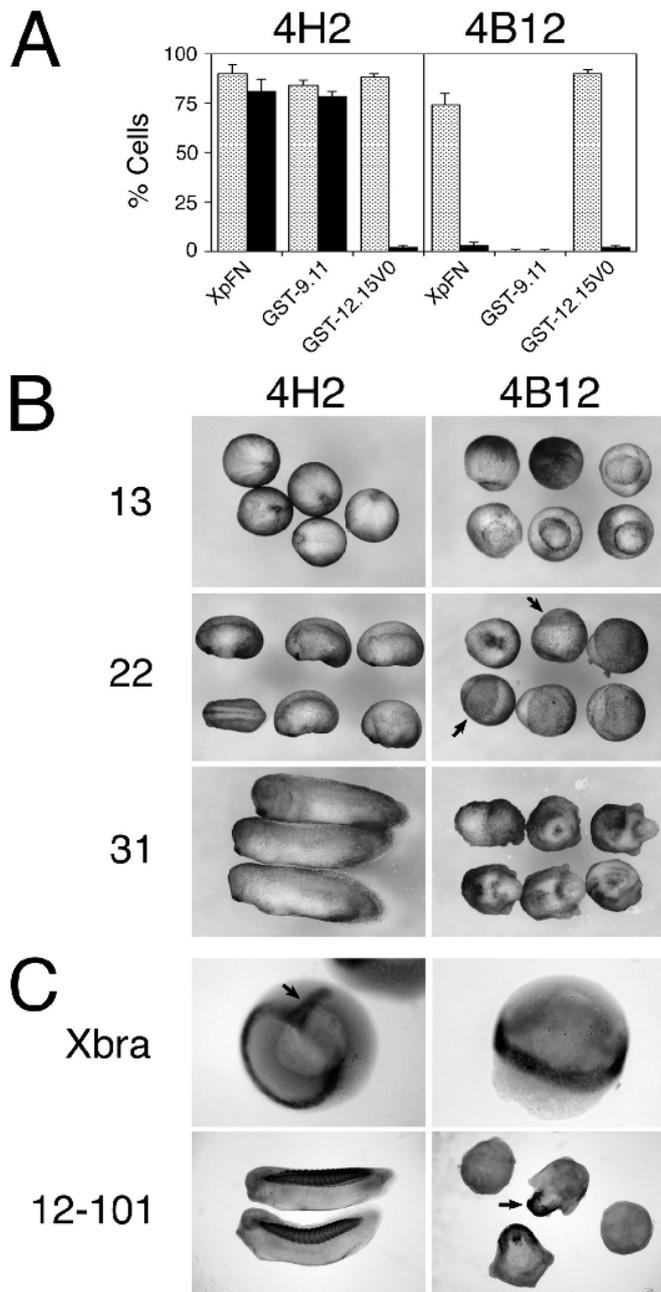


Fig. 1. The structure of *Xenopus* FN (XFN) and related fusion proteins. One subunit of the XFN dimer is shown with the known splice variants included (black and shaded boxes). All FNs are made up of a series of repeats termed Types I, II and III. Regions of FN with known cell-binding activity are underlined and include the RGD-containing central cell-binding domain (CCBD), the non-integrin dependent heparin binding domain (HepII), and the LDV-containing variable region (V). The approximate locations of the epitopes recognized by the 4H2 (Type III-9) and 4B12 (Type III-10) mAb antibodies are also indicated. All fusion proteins were produced in the pGEX bacterial expression system and contain glutathione S-transferase at their N termini.



axis is inhibited resulting in truncated embryos that lack head and tail structures. The 12-101 antibody (Kintner and Brockes, 1984) was used to visualize somitic tissue in stage 31 embryos (Fig. 2C). Normal somitic fields are evident in 4H2 injected controls. In contrast, 12-101 staining is absent in approximately 50% of the 4B12 injected embryos at this stage. The remainder of these embryos show variable levels of 12-101 staining and in some cases, blocks of somites are evident. These data confirm that cell adhesion to the CCBD of FN is required for normal gastrulation movements and the patterning of mesodermal tissues in *Xenopus*.

Mesoderm adhesion to the CCBD of FN is under temporal and spatial control

The injection studies demonstrate the functional importance of

Fig. 2. Antibody blocking experiments. (A) In vitro adhesion assay demonstrates that mAb 4B12 blocks attachment of dorsal IMZ cells to the CCBD (GST-9.11) but not to the HepII-containing GST-12.15V0. Mab 4B12 also blocks IMZ spreading on *Xenopus* plasma FN (XpFN) but not cell attachment. The 4H2 mAb does not block normal attachment or spreading behaviors on any of these substrates. Bars represent mean percentages of attached (grey) and spread (black) cells per field of view. At least 1000 cells per substrate were counted. Each assay was repeated 8 times ($n=8$). Error bars indicate standard deviations. (B) 4H2- and 4B12-injected embryos are shown at stages corresponding to early neurula (13), late neurula (22) and tailbud (31). Arrows (22, 4B12) indicate translucent area of typical blastocoel roofs devoid of attached mesoderm. (C) Whole-mount in situ hybridization of mAb-injected embryos at stage 12 (top panels) showing localization of *Brachyury* (*Xbra*) mRNA. Arrow (*Xbra*) indicates normal notochord in 4H2 control embryo. (Bottom panels) Whole mounts of mAb-injected embryos at stage 31 stained with the somite-specific 12-101 antibody. Arrow (12-101) indicates somitic staining representative of approximately 50% of 4B12 injected embryos.

the CCBD of FN in gastrulation but how adhesion to this ECM protein is regulated is not known. One possibility is that FN-dependent adhesive behavior develops as a consequence of mesoderm induction and differentiation (Smith et al., 1990; DeSimone et al., 1994). If this hypothesis is correct, then the ability to bind the CCBD should parallel both the timing and position of mesoderm formation in vivo.

Explants of cells from defined regions of blastulae, early and mid-gastrulae were dissociated and placed on the GST-9.11 fusion protein and attachment and spreading behavior quantified (Fig. 3). At the blastula stage, cells from all regions of the embryo attach to GST-9.11 but no spreading is observed (Fig. 3A). The ability to attach to GST-9.11 is retained by all cells through the mid gastrula stage, however, regional differences in adhesive behavior become apparent just prior to stage 10 when mesoderm involution begins. Leading-edge head mesoderm and endoderm (dorsal IMZ) are first to develop the ability to spread on GST-9.11 (Fig. 3B) as they come in contact with the FN matrix, which is localized along the blastocoel roof by this stage. Acquisition of spreading behavior accompanies the involution of additional cells at the dorsal lip and later the lateral and ventral margins of the blastopore as it closes (Fig. 3C). These data indicate that, while all cells of the blastula and gastrula are able to recognize and attach to the RGD-containing CCBD, FN-dependent spreading and migratory activity are spatiotemporally controlled.

Cell spreading on the CCBD is a consequence of mesoderm induction

In earlier studies, we and others reported that stage 8 animal cap cells attach to pFN but do not spread or migrate on this substrate unless treated with mesoderm inducing factors such as activin (Smith et al., 1990; Howard and Smith, 1993; Symes et al., 1994). In the current study we use this assay as a model system for analyzing the regulation of integrin adhesive activity. Dispersed stage 8 animal cap cells attach to pFN or GST-9.11 but remain round and immotile unless treated with 20 U/ml of the mesoderm inducing factor activin-A, whereupon the cells will spread (e.g., Fig. 6C) and migrate (Ramos and DeSimone, 1996). This is in contrast to dorsal IMZ cells, which spread on these substrates in the absence of

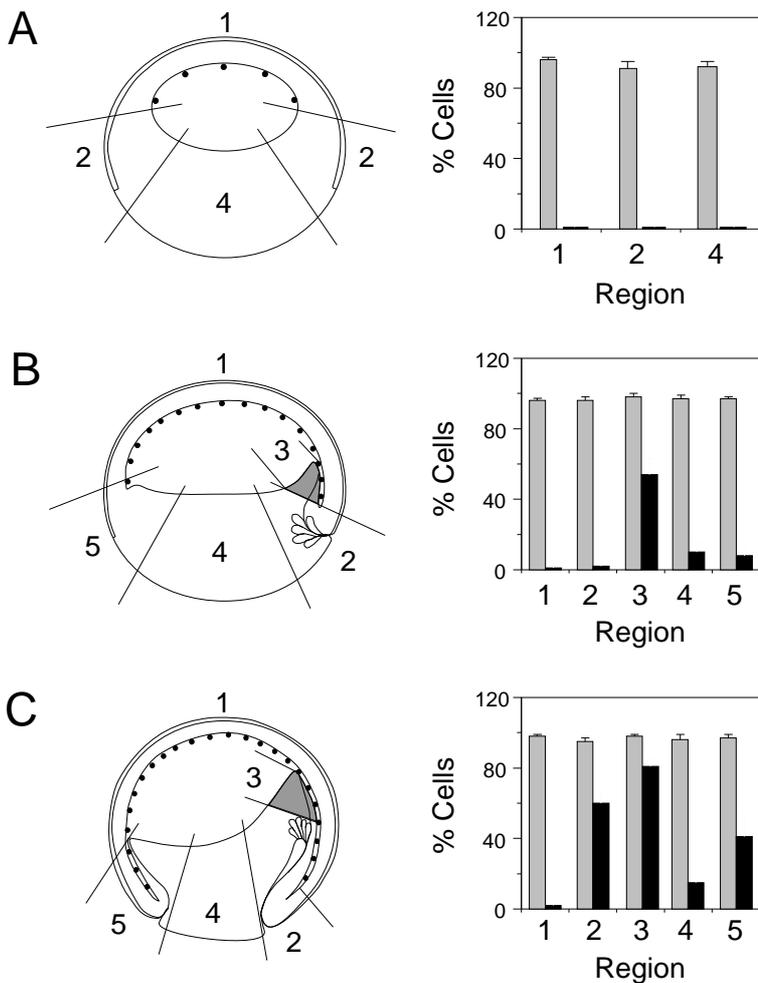


Fig. 3. FN adhesive activity is temporally and spatially regulated in the early embryo. Diagrams on the left depict location of regions (numbered 1-5) explanted from embryos at various stages. The leading edge of the dorsal IMZ (3) is shaded. Black dots on blastocoel roof indicate location of FN matrix. Graphs on the right represent mean percentages of cells attached (grey) and spread (black) on the GST-9.11 fusion protein. Numbers along the x axis correspond to regions indicated in the diagrams. At least 300 cells per region were counted for each substrate. Each assay was repeated 8 times ($n=8$). Error bars indicate standard deviations. (A) Stage 8 mid-blastula. Cells from all regions of the embryo attach but none spread. (B) Stage 10 gastrula. More than 50% of attached dorsal IMZ cells (3) spread. (C) Stage 11 gastrula. Approximately 80% of region 3 cells spread. Additional cells gain the ability to spread on the CCBF coincident with their involution at the dorsal (2) and ventral (5) lips of the blastopore. Animal cap (1) and yolk plug (4) cells attach but display little or no spreading behavior during gastrulation.

exogenously applied inducers (Fig. 2A). Attachment of cap cells to GST-12.15V0 is unaffected by activin (data not shown) suggesting that the initiation of spreading is not a general consequence of induction but requires a specific substrate. Collectively, these experiments indicate that the RGD-containing CCBF is both sufficient and necessary to support the observed changes in cell adhesive behavior that occur during mesoderm induction.

As a first step toward understanding the mechanisms involved in controlling the initiation of mesodermal cell migration on FN *in vivo*, we examined more closely the timing of animal cap cell spreading in response to activin. It was shown previously that stage 8 animal cap cells cultured in activin will not spread on FN until intact sibling control embryos reach stage 10 (Smith et al., 1990), which marks the onset of gastrulation movements. It has also been reported that the initiation of animal cap spreading behavior occurs over a range of activin concentrations and is cell autonomous (Smith et al., 1990; Howard and Smith, 1993; Symes et al., 1994).

In order to determine whether the timing of cap cell exposure to activin affects when the cells spread, we explanted animal cap cells from stage 8 embryos and cultured them on human plasma FN. Activin was then added to these cultures at several time points from 0.5 to 6.5 hours after plating. The number of spread cells was counted at times corresponding to various sibling control stages. Regardless of when the cells are

exposed to activin, cell spreading is only observed between sibling stages 9.5-10 (Fig. 4A). Cells that receive activin 6.5 hours or more after plating (sibling stage 11 or older) do not spread. Thus it appears that the cell autonomous, activin-dependent spreading behavior is tightly regulated and timed to begin only at the equivalent of stage 9.5-10, regardless of when the inducing signal is received. However, animal cap cell competence to adhere to FN in response to inductive signals is lost shortly after stage 10 when gastrulation movements are underway.

We next performed an experiment to determine the minimum response time from application of activin to initiation of spreading behavior. Dispersed animal cap cells were cultured on human plasma FN until sibling stage 10 and then exposed to 20 U/ml activin. Cells were photographed every 30 minutes following the addition of activin and the number of spread cells counted (Fig. 4B). These data indicate that cell spreading begins in as little as 30-60 minutes following exposure to activin (Fig. 4B).

Recognition of the CCBF does not require new integrin synthesis or transport to the cell surface

Our data demonstrate that the spreading behavior of IMZ cells on pFN and GST-9.11 is mimicked by animal cap cells treated with activin. This suggests that the activin-dependent switch in animal cap adhesive behavior involves changes in integrin

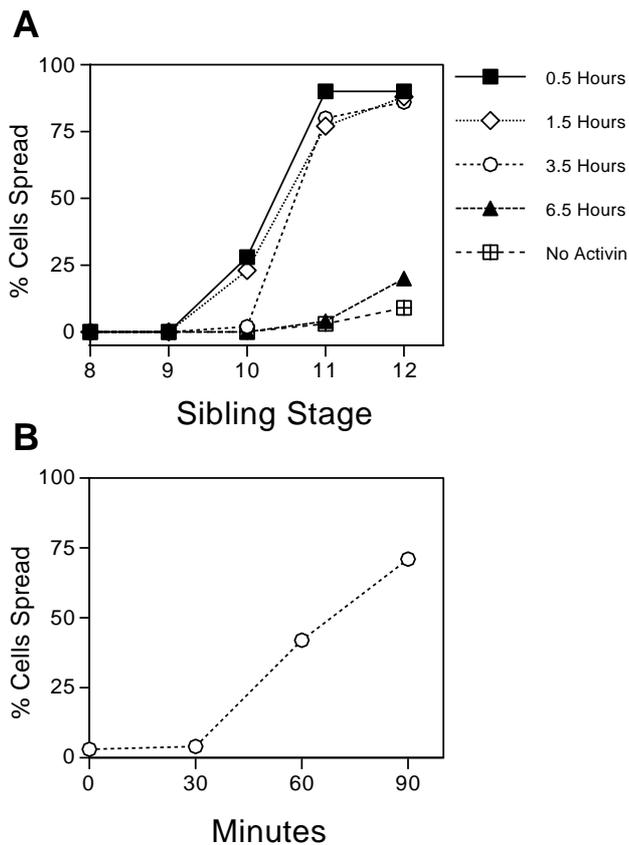


Fig. 4. Timing of activin-induced animal cap cell spreading activity. (A) Stage 8 animal cap cells were plated on human plasma FN (HpFN) and 20 U/ml activin-A added to the cultures at the times indicated. Percentage of attached cells that spread were recorded at times corresponding to the developmental stage of intact sibling control embryos. (B) Stage 10 animal cap cells were plated on HpFN in the presence of 20 U/ml of activin-A. The percentage of attached cells spread was recorded at 30 minute intervals. Data from 1 representative experiment are shown for both A and B.

interactions with the CCBd of FN that are likely to parallel those occurring during mesoderm involution *in vivo*. We report also that activin-A induced spreading of dispersed animal cap cells on FN can occur in as little as 30-60 minutes following exposure to the growth factor. It is unlikely, therefore, that new integrin synthesis could account for the rapid change in behavior of these cells on the CCBd. In order to exclude the possibility that activin induction leads to a change in integrin synthesis we repeated the animal cap adhesion assays in the presence of inhibitors of transcription and translation.

Stage 8 animal cap cells were incubated in the presence of α -amanitin (10 μ g/ml) or cycloheximide (10 μ g/ml), both of which inhibit further cell division following application. Cycloheximide treatment results in a 80-90% reduction in overall protein synthesis (not shown) as reported previously by Cascio and Gurdon (1987). Neither of these treatments had a substantial affect on the ability of the cap cells to spread on the GST-9.11 fusion protein following incubation in 20 U/ml of activin (Fig. 5A). Furthermore, the timing of the activin-induced response occurs within 60 minutes (not shown), similar to that of cells cultured in the absence of inhibitors (Fig. 4B).

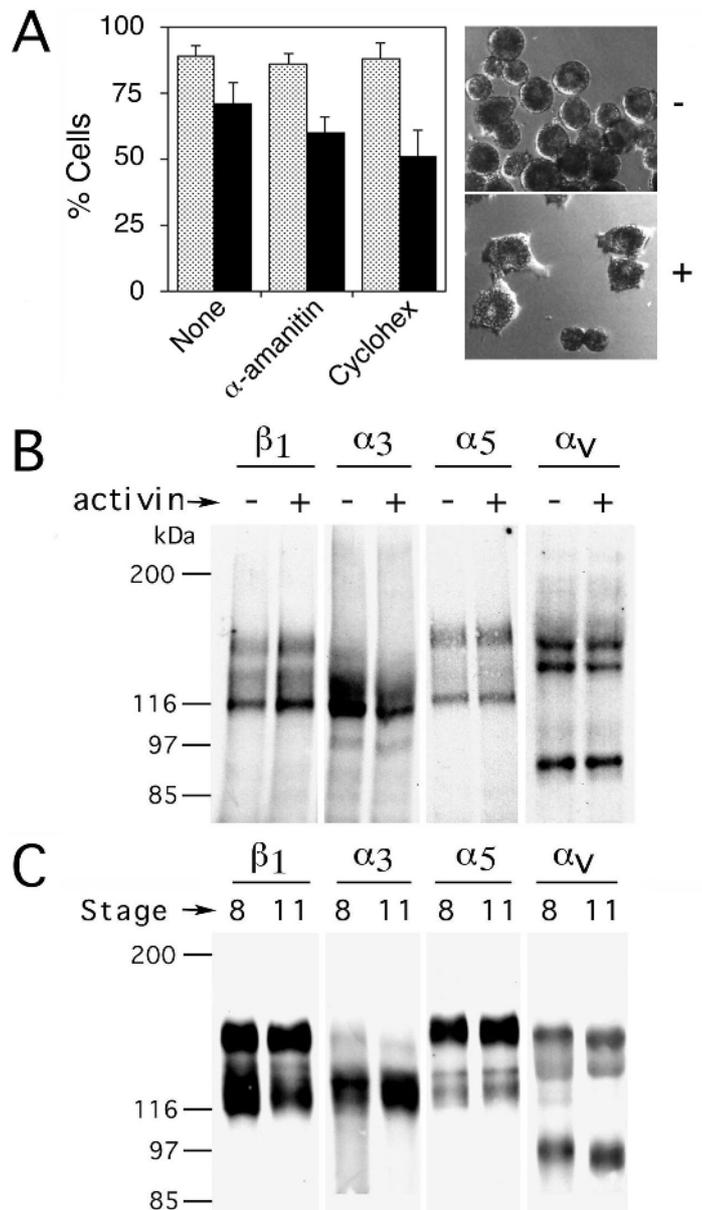


Fig. 5. Activin-induced cell spreading on FN is independent of transcription, translation or changes in integrin expression at the cell surface. (A) Stage 8 animal cap cells were plated on GST-9.11 in the presence of 20 U/ml activin-A and treated with 10 μ g/ml α -amanitin or 10 μ g/ml cycloheximide as described in the Materials and Methods. Bars represent mean percentages of attached (grey) and spread (black) cells per field of view. At least 500 cells were counted per treatment and each assay was repeated 8 times ($n=8$). Error bars indicate standard deviations. Micrographs of uninduced (-) and activin-A induced (+) animal cap cells cultured in cycloheximide are shown on the right. (B) Cell-surface biotinylated integrins from uninduced (-) and activin-A induced (+) animal cap cells immunoprecipitated with anti- β_1 , α_3 , α_5 , and α_V antibodies (50 animal cap equivalents per sample). 8% SDS-PAGE, non-reducing conditions. (C) Immunoprecipitation of cell-surface biotinylated integrins from ten stage 8 and 11 embryos, antibodies and electrophoresis conditions same as in B.

These data indicate that it is unlikely that the change in adhesive behavior on FN that occurs during mesoderm

induction is dependent on new transcription or translation. It is probable, therefore, that post-translational changes in integrin surface expression or functional activation state are responsible. To investigate differences in the distribution of integrins at the cell surface following mesoderm induction, animal cap cells and dissociated embryos were surface-labelled with biotin, solubilized and subjected to immunoprecipitation analyses using anti-integrin α_3 , α_5 , α_V , and β_1 subunit-specific antibodies. Of the 10 integrins reported to bind the CCBD of FN we have evidence that $\alpha_3\beta_1$ (Gawantka et al., 1994; Meng and DeSimone, unpublished), $\alpha_5\beta_1$ (Joos et al., 1995), and $\alpha_V\beta_7$ (Alfandari et al., 1995) are each expressed during amphibian gastrulation (Whittaker and DeSimone, 1993).

Cell-surface labelling experiments confirm that the overall pattern of expression for β_1 integrins on animal cap cells remains unchanged following activin treatment (Fig. 5B). Similar results are observed for the $\alpha_3\beta_1$, and $\alpha_5\beta_1$ receptors. The pattern of α_V containing receptors also stays the same following induction, however, lack of suitable cross reacting antibodies prevents us from identifying the *Xenopus* β subunit that pairs with α_V at this stage. The $95 \times 10^3 M_r$ α_V -associated band is possibly β_3 based on relative mobility and the presence of β_3 mRNA at gastrulation (Ransom et al., 1993). Two variant forms of the α_V subunit are also expressed at the cell surface in a pattern similar to that reported for the salamander, *Pleurodeles* (Alfandari et al., 1995).

We next tested whether any change in the surface expression of these integrins could be detected on blastomeres obtained from dissociated embryos at stages 8 (mid-blastula) and 11 (gastrula). No significant differences in the levels or pattern of CCBD-binding integrins could be discerned between these two stages (Fig. 5C), further suggesting that the induction of mesoderm in vivo is not accompanied by a change in integrin expression at the cell surface. Therefore, the inhibitor and immunoprecipitation data are consistent with a model, which predicts that changes in adhesion to FN during mesoderm induction are mediated by posttranslational changes in the functional activities of pre-existing integrins expressed at the cell surface.

Ectopically expressed integrin α_4 supports activin induced changes in adhesive activity

Our data indicate that there are several possible candidate integrins (e.g., $\alpha_3\beta_1$, $\alpha_5\beta_1$ and $\alpha_V\beta_7$) that either individually or in combination, are likely to mediate the developmentally regulated changes in adhesion to the CCBD that we report. It is also possible that a novel integrin or a non integrin-dependent adhesive interaction plays an

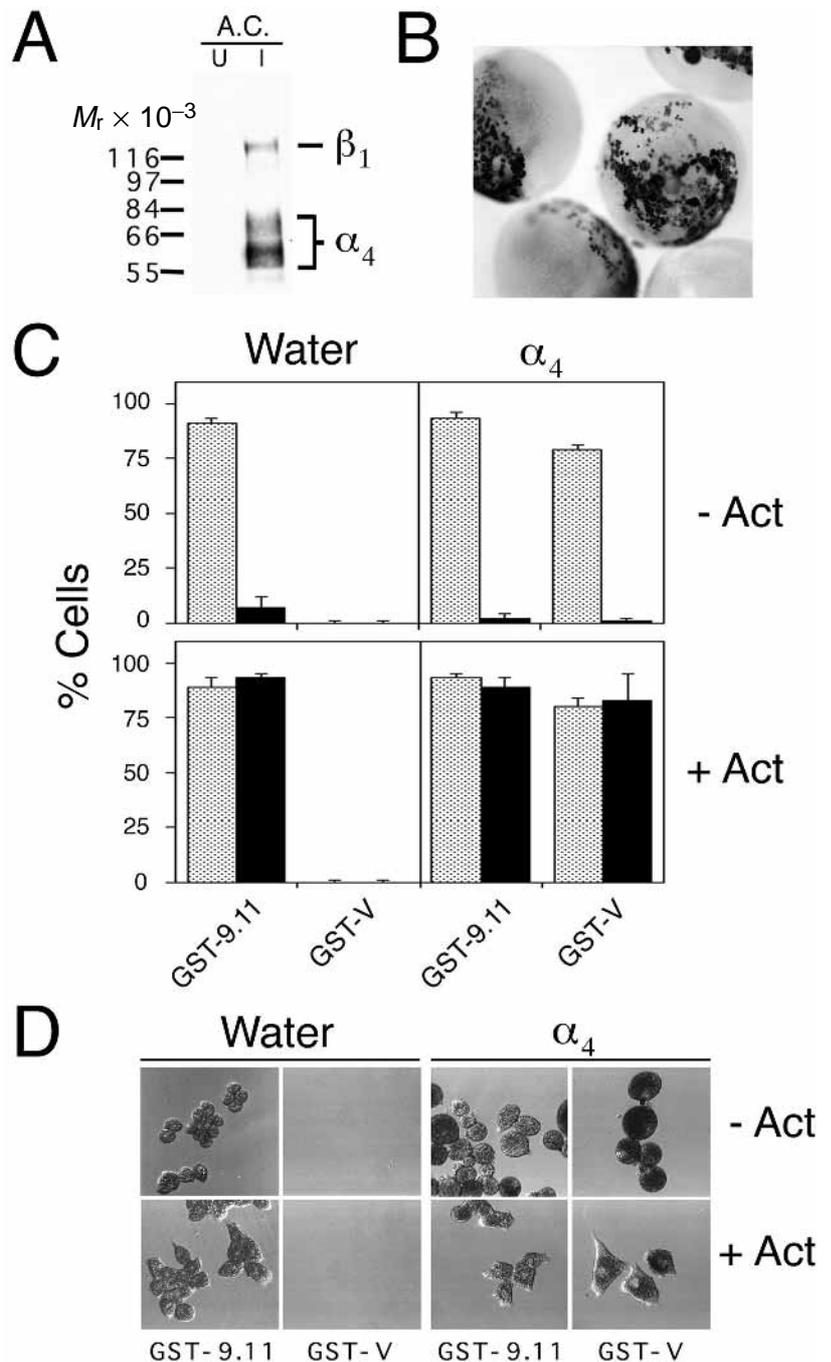


Fig. 6. Ectopic expression of the α_4 integrin subunit in animal cap cells. (A) α_4 immunoprecipitation of cell-surface biotinylated animal cap cells from 50 uninjected (U) or α_4 transcript-injected (I) embryos. The cleaved $60\text{--}80 \times 10^3 M_r$ α_4 bands run below the $125 \times 10^3 M_r$ β_1 subunit (8% SDS-PAGE, non-reducing conditions). (B) Whole-mount, anti- α_4 immunostaining of stage 8 albino embryos injected with α_4 transcript. (C) Animal cap cells from water- or α_4 transcript-injected embryos were plated on GST-9.11 or GST-V in the presence (+Act) or absence (-Act) of 20 U/ml activin-A. Bars represent mean percentages of cells spread (black) or attached (grey) per field of view. At least 300 cells were counted for each substrate. Each assay was repeated 8 times ($n=8$). Error bars indicate standard deviations. Note that only those cells expressing α_4 attach to the V-region substrate. Blastomeres that do not express the α_4 subunit were washed away prior to counting the percentage of attached versus spread cells on GST-V. Nearly all cells (80-90%) attach to GST-9.11. (D) Micrographs of representative cells corresponding to the experimental conditions in C.

important role. Our working hypothesis is that mesoderm induction results in a change in the activation state of an integrin receptor present at the cell surface via an 'inside-out' signalling mechanism. If this is correct then it should be possible to express ectopically an integrin with distinct ligand-binding characteristics that would mediate attachment and, in response to induction, cell spreading and migration.

Dorsal IMZ and stage 8 animal cap cells are unable to attach or spread on the LDV-containing V-region of FN (Ramos and DeSimone, 1996). Consistent with this observation is the lack in these cells of $\alpha_4\beta_1$, which is the receptor responsible for binding to the V-region. Fertilized eggs were injected into the animal pole with synthetic transcripts encoding the full-length *Xenopus* α_4 subunit. Cell-surface labelling experiments confirm that embryos injected with α_4 transcript express $\alpha_4\beta_1$ whereas uninjected embryos do not (Fig. 6A). Furthermore, whole-mount immunostains of transcript-injected embryos demonstrate that the α_4 subunit is widely expressed throughout the animal pole by stage 9 (Fig. 6B).

Injected embryos were cultured until stage 8 when the animal caps were removed, and the cells dissociated and plated on the GST-9.11 or GST-V fusion proteins. As predicted, cells from both α_4 and water-injected control embryos attach to GST-9.11 and spread on this fusion protein in the presence of activin (Fig. 6C,D). However, attachment to GST-V is observed only with cap cells obtained from α_4 -injected embryos. These cells are able to spread on the V-region following the addition of activin (Fig. 6C,D). Thus, activin-induced change in adhesive behavior can be replicated in animal cap cells expressing an exogenous integrin that binds to a domain of FN not normally recognized by these cells.

DISCUSSION

We report two major findings. The first is that integrin-dependent IMZ cell adhesion to the RGD-containing CCBD of FN is required for gastrulation in *Xenopus*. The second is that integrin adhesive activity to this region is controlled by distinct temporal and spatial mechanisms in early embryos. These data provide support for the hypothesis that inductive interactions direct the position-specific activation of adhesive molecule function by an inside-out signalling mechanism and that this is an important step in the initiation of cell migration at gastrulation.

A role for fibronectin in *Xenopus* gastrulation

A major goal of the current study has been to reexamine the role of FN during gastrulation using approaches designed to identify the contributions of specific cell-interactive sites on the protein. Based on our results, we propose that FN is required to maintain the close apposition of involuted mesoderm along the blastocoel roof and that this adhesive interaction is crucial for subsequent mesodermal patterning and inductive events. In support of this model we confirm that the ability of IMZ cells to spread on FN is acquired during involution and depends upon specific interactions with the RGD site located in the CCBD. We show that cell spreading on FN is inhibited by a mAb directed against the RGD-containing Type III-10 repeat of the protein and that a control mAb that recognizes the adjacent Type III-9 repeat does not block

cell spreading. Finally, we report that the blocking mAb specifically disrupts gastrulation following microinjection into the blastocoel. We observe mesoderm involution in 100% of viable mAb injected embryos with blastopore closure occurring in approximately 70% of these cases. In each instance, involuted cells fail to cover the inner surface of the blastocoel roof at the animal pole. Thus, it appears that convergence extension movements and blastopore closure are largely autonomous of FN-dependent adhesive interactions as suggested by earlier experiments (Keller and Winklbauer, 1990; Keller and Jansa, 1992). However, our data indicate that ECM-dependent interactions of involuted mesoderm with the blastocoel roof represent an essential step in the overall process of gastrulation.

The disruption of normal mesodermal positioning that occurs in mAb-injected *Xenopus* embryos at the gastrula stage is consistent with the range of phenotypes that later emerge as development proceeds. These include truncation and disruption of the anterior-posterior axis, deficiencies in the patterning of axial and paraxial mesoderm, and the failure to form normal heads. Interestingly, many of these phenotypes are shared by mouse embryos lacking FN (George et al., 1993). Both FN null mice and mAb-injected *Xenopus* embryos appear to initiate gastrulation normally with considerable mesodermal cell movement evident through the primitive streak and blastopore, respectively. This suggests, therefore, that the processes of murine and amphibian gastrulation may share more similarities than previously thought and that the deficit in mesodermal structures reported for FN null mice may be due, at least in part, to subtle disruptions in mesoderm migration and positioning following ingression.

Regional specification of adhesive activity

There is considerable precedent for the localization of adhesion molecules to specific regions of the embryo during development, which suggests that the control of gene expression is key to regulating adhesive function in vivo (Lallier et al., 1994). In this study, we provide evidence that regional differences in embryonic cell adhesion may also be regulated by local changes in the functional activities of pre-existing integrins expressed at the cell surface. Although all cells of the late blastula and early gastrula attach specifically to the RGD-containing CCBD of FN, only IMZ cells are able to spread and migrate on this region. Additional cells gain the ability to spread on the CCBD as their position in the embryo changes during involution. No changes in the patterns of FN receptor expression at the cell surface are detected as gastrulation begins or in response to mesoderm induction.

Thus, we can superimpose on the gastrula a dynamic 'map' of integrin-dependent adhesive activities that are posttranslationally regulated in both space and time by inductive interactions. This model does not require the predetermined localization or segregation of specific integrins to the site of mesoderm involution and migration. Instead, we propose that ubiquitous expression followed by regional changes in activation state are sufficient to initiate adhesion-dependent morphogenetic events in this instance. We suggest that this is likely to represent a general mechanism used throughout development to regulate integrin function.

It has also been reported that C-cadherin dependent cell-cell adhesive activity decreases during mesoderm induction

(Brieher and Gumbiner, 1994). This decrease in adhesion does not involve changes in levels of C-cadherin expressed at the cell surface. Interestingly, the reduction in cadherin adhesion coincides with the observed change in integrin adhesion that we have detected. Therefore, these alterations in adhesive activity may be thought of as complementary consequences of inductive signals that are required to support morphogenetic movements at gastrulation. Decreased cell-cell adhesion may facilitate the cellular rearrangements involved in mesoderm involution. At the same time, increases in adhesion to FN become necessary as involuting cells make contact with the blastocoel roof. It will be of interest to determine the extent to which these distinct adhesion-dependent signalling pathways converge during mesoderm induction.

Timing of integrin adhesive activation

The initiation of gastrulation movements in *Xenopus* is governed by a cell autonomous 'timer' that is independent of cell cycle, zygotic gene expression, protein synthesis, and nuclear cytoplasmic ratio (reviewed by Cooke and Smith, 1990). The loss of competence to respond to mesoderm induction is also likely to be a feature of this timing mechanism (Grainger and Gurdon, 1989). Similarly, the animal cap experiments reported in this study indicate that activin-dependent changes in integrin adhesion occur in the absence of protein and nucleic acid synthesis and are not dependent on cell division. Animal cap cells spread 'on time' regardless of when the inducing signal is received within the period of mesodermal competence that ends shortly after stage 10. These data are consistent with earlier observations showing that the initiation of convergence and extension movements in animal caps is independent of the timing of activin exposure (Symes and Smith, 1987). Howe et al. (1995) recently reported that cyclin A degradation is also linked to a gastrulation timer. In the current study, we report that activation of integrin-dependent adhesion serves as an additional molecular indicator of this putative clock. The α_4 expression studies (Fig. 6) demonstrate that a single integrin can respond to the internal timer by mediating a cell autonomous change in adhesive behavior on the V-region of FN at a time when gastrulation movements begin in vivo. Further analyses of integrin activation, therefore, may provide insight into the molecular pathways involved in regulating the timing of gastrulation.

Activation of integrin function

Inside-out signalling mechanisms have been shown to be involved in the regulation of integrin ligand binding affinity in a number of cells including platelets and lymphocytes (reviewed by Hynes, 1992; Clark and Brugge, 1995) and we propose that a similar mechanism is responsible for changes in *Xenopus* IMZ cell adhesion to FN during gastrulation. An alternative explanation is that initiation of spreading on the CCBD does not require an inside-out change in receptor binding affinity but involves post ligand-binding, 'inside-in' changes in cytoskeletal organization (Danilov and Juliano, 1989). Our recent studies suggest that global changes in cytoskeletal organization alone cannot account for the integrin-dependent switch in adhesive behavior that we observe. The spreading and migration of animal cap or IMZ cells on the CCBD of FN requires the presence of the RGD sequence in addition to a synergy site (Ramos and DeSimone, 1996)

located in the type III-9 repeat of *Xenopus*, avian and mammalian FNs (Aota et al., 1994). Moreover, recognition of the synergy site by animal cap cells occurs only in response to mesoderm inducing signals (Ramos and DeSimone, 1996). Because recognition of this site does not depend on new integrin expression (Fig. 5) it is likely that an inside-out signalling mechanism is involved in controlling receptor activation state (i.e., changes in the extracellular conformation of the integrin).

The integrin $\alpha_5\beta_1$ receptor is a likely candidate for regulating embryonic cell attachment, spreading and migration on the CCBD because (1) it recognizes both the RGD and synergy sites (Aota et al., 1991) depending on the activation state of the receptor (Danen et al., 1995), (2) it can be activated by inside-out signals (Faull et al., 1993) and, (3) it is expressed at high levels on all cells of the early *Xenopus* embryo through gastrulation (Joos et al., 1995). It is also possible that an additional integrin(s) such as $\alpha_V\beta_3$, which recognizes RGD but not the synergy site of FN (Bowditch et al., 1994), is responsible for the attachment of non-induced cells. Although we must await final confirmation of the specific integrins involved in binding the CCBD, our data demonstrate clearly that a single integrin receptor is sufficient to support initial cell attachment and subsequent spreading behavior following activin treatment. Non-induced animal cap cells that express exogenous α_4 attach to the V-region of FN. These cells then spread on the V-region when treated with mesoderm inducing factor. Consistent with this observation, $\alpha_4\beta_1$ is reported to exhibit multiple ligand-binding activation states (Wayner and Kovach, 1992; Matsumoto and Hemler, 1993). Future studies of the endogenous integrins involved in binding to the CCBD will help establish the relative contributions of inside-out signalling mechanisms to the position-specific activation of adhesive function in the embryo.

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