**Abstract.** During *Xenopus laevis* gastrulation, the basic body plan of the embryo is generated by movement of the marginal zone cells of the blastula into the blastocoel cavity. This morphogenetic process involves cell adhesion to the extracellular matrix protein fibronectin (FN). Regions of FN required for the attachment and migration of involuting marginal zone (IMZ) cells were analyzed in vitro using FN fusion protein substrates. IMZ cell attachment to FN is mediated by the Arg-Gly-Asp (RGD) sequence located in the type III-10 repeat and by the Pro-Pro-Arg-Arg-Ala-Arg (PPRRAR) sequence in the type III-13 repeat of the Hep II domain. IMZ cells spread and migrate persistently on fusion proteins containing both the RGD and synergy site sequence Pro-Pro-Ser-Arg-Asn (PPSRN) located in the type III-9 repeat. Cell recognition of the synergy site is positionally regulated in the early embryo. During gastrulation, IMZ cells will spread and migrate on FN whereas presumptive pre-involuting mesoderm, vegetal pole endoderm, and animal cap ectoderm will not. However, animal cap ectoderm cells acquire the ability to spread and migrate on the RGD/synergy region when treated with the mesoderm inducing factor activin-A. These data suggest that mesoderm induction activates the position-specific recognition of the synergy site of FN in vivo. Moreover, we demonstrate the functional importance of this site using a monoclonal antibody that blocks synergy region–dependent cell spreading and migration on FN. Normal IMZ movement is perturbed when this antibody is injected into the blastocoel cavity indicating that IMZ cell interaction with the synergy region is required for normal gastrulation.

**Fibronectin (FN)** is a well characterized adhesive glycoprotein of the extracellular matrix (ECM) that is implicated in several developmental events including gastrulation, neural crest cell migration, somitogenesis, and heart formation (for reviews see Hynes, 1990; DeSimone, 1994). FNs are dimers composed of identical or near identical subunits joined at their COOH-terminal ends by a pair of disulfide bonds. Each subunit consists of a series of distinct functional domains that participate in matrix formation, cell binding, and binding to heparin, collagen, and fibrin (Sekiguchi and Hakomori, 1980; Yamada, 1990; see also Fig. 1). Multiple forms of FN are generated by alternative splicing of a common transcript (Schwarzbauer, 1991; Yamada, 1991).

Cell adhesion to FN is complex and involves multiple cell interactive sites on the molecule. The tripeptide sequence Arg-Gly-Asp (RGD), located in the type III-10 repeat of FN, is essential for the binding of many cell types (Pierschbacher and Ruoslahti, 1984). An additional site that acts cooperatively with the RGD site to support cell adhesion is located in the type III-9 repeat and corresponds to the sequence Pro-His-Ser-Arg-Asn (PHSRN) (Bowlitch et al., 1994; Aota et al., 1994). This site is termed the “synergy” site. Other cell-binding sites are located in the alternatively spliced V-region of the molecule (Humphries et al., 1987; Guan and Hynes, 1990). V-region cell-binding sites include the Glu-Ile-Leu-Asp-Val (EILDV) (Garcia-Pardo et al., 1990; Komoriya et al., 1991; Wayner and Kovach, 1992) and Arg-Glu-Asp-Val (REDV) (Humphries et al., 1987; Mould et al., 1991) sequences. Some cell types are also reported to bind to the COOH-terminal he-
Cells bind to FNs and other ECM proteins using integrin receptors (for review see Hynes, 1992). The integrins are a family of heterodimeric transmembrane glycoproteins, composed of distinct α and β subunits that interact with both the ECM and the cytoskeleton. Different integrins recognize distinct regions of FN. For example, α5β1 binds to the EILDV site (Garcia-Pardo et al., 1990; Komoriya et al., 1991; Wayner and Kovach, 1992) while α2β1 binds to the RGD sequence (Pytel et al., 1985) in cooperation with the synergy site (Aota et al., 1994; Danen et al., 1995). Several other integrins are also reported to bind FN (Hynes, 1992). Nonintegrin receptors for FN include cell surface proteoglycans, some of which bind the HepII site (Saunders and Bernfield, 1988; Ruoslahti, 1989; Wight et al., 1992). These receptors appear to be important in the formation of focal contacts and in cell motility (Wood et al., 1986). Thus, a cell may modulate its adhesion to FN by regulating the types of integrins and cell-surface proteoglycans it expresses.

In Xenopus, cDNAs encoding FN and several integrin subunits have been cloned and characterized (DeSimone and Hynes, 1988; DeSimone et al., 1992; Whitaker and DeSimone, 1993; Ransom et al., 1993; Joos et al., 1995; Lalier et al., 1996). FN is secreted by cells from all regions of the early embryo and becomes localized to the ECM that forms along the blastocoel roof (Lee et al., 1984). During gastrulation involuted marginal zone (IMZ) cells, which include both mesoderm and endoderm, come in contact with this FN matrix. In Xenopus and urodèle embryos, mesoderm migration is inhibited following the injection of anti-FN antibodies into the blastocoel (Boucaut et al., 1984a; Howard et al., 1992; Ramos et al., 1996). Additionally, dispersed Xenopus IMZ cells will spread and migrate on the inner surface of excised blastocoel roofs and on FN-coated petri dishes (Nakatsuji and Johnson, 1983; Winklebauer, 1988, 1990). Although all cells of the early embryo are able to attach to the RGD-containing central-cell-binding domain of FN, only IMZ cells spread and migrate on this region in vitro (Ramos et al., 1996).

Gastrulation is the morphogenetic process that establishes the three primary germ layers in their relative positions in the embryo. Mesoderm induction plays an essential role by initiating the cellular movements associated with this event (for review see Dawid and Taira, 1994). Smith et al. (1988) have developed an in vitro mesoderm induction assay that mimics normal inductive signals that arise from the vegetal pole of the embryo. In this system, animal pole tissue normally fated to become ectoderm is induced to form mesoderm and undergo gastrulation-like movements when exposed to activin-A, a member of the TGF-β family of growth factors. Animal cap cells, which normally only attach to FN, gain the ability to spread and migrate on this ECM protein following exposure to activin (Smith et al., 1990; Howard and Smith, 1993).

In this study we address the contributions of individual FN cell-binding sites to the adhesion, spreading, and migration of IMZ and activin-induced animal cap cells. We show that three cell-interactive regions of FN are used by these cells: the RGD site, the synergy region, and the HepII domain. The RGD site and the HepII region both support the attachment of all cells of the early embryo, however, IMZ cell spreading and migration require a synergy site in combination with the RGD site. Moreover, an antibody that inhibits synergy region–dependent cell migration on FN, but not interaction with the RGD site, perturbs gastrulation. These data demonstrate that IMZ cell recognition of the synergy region is positionally specified in vivo in response to mesoderm inducing signals.

Materials and Methods

Xenopus Embryos

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

PCR Amplification of 9.11, EIII A, EIIIB, and V-regions

Forward and reverse oligodeoxynucleotide primers containing engineered restriction sites were produced using a PCR-Mate oligodeoxynucleotide synthesizer (Applied Biosystems, Foster City, CA). Primer sequences are listed in Table I. EIIIA, EIIIB, and V-region amplifications were carried out in separate 100-μl reactions containing 1 μg of forward and reverse primers and 20 ng of template cDNA (Xenopus FN clone pNaL; DeSimone et al., 1992). Target regions were amplified in 30 cycles (94°C, 1 min; 50°C, 2 min; 72°C, 3 min) using Taq DNA polymerase (Perkin-Elmer, Foster City, CA). Similarly, the type III-9 to type III-11 segment of FN was amplified using 1 μg of each primer and 100 ng of template cDNA clone pF7 (DeSimone et al., 1992) in 20 cycles (94°C, 1 min; 48°C, 1 min; 72°C, 3 min) using Vent polymerase (New England Biolabs, Beverly, MA).

Each amplified DNA was gel purified and sequenced using the dyeoxy chain-termination method (Sanger et al., 1977) after subcloning into suitable pGEX expression vectors (Smith and Johnson, 1988, Guan and Dixon, 1991) as described below.

Construction of Fibronectin Fusion Proteins

To express specific regions of FN as fusion proteins, PCR amplified cDNAs were digested with appropriate restriction enzymes and ligated into pGEX-2T (GST-A and GST-B) or pGEX-KG (GST-V). Additional FN fusion proteins were constructed by subcloning DNA fragments from previously isolated Xenopus FN clones (DeSimone et al., 1992) into the pGEX-2T (GST-4.13A0, GST-10, GST-9.10 and GST-1.9) or pGEX-KG (GST-12.12 and GST-12.15) plasmids. The recombinant plasmids were constructed and pGEX-9.11 was used as a control. Site-directed mutagenesis of pGEX-9.11 was performed by inverse PCR (Hemsley et al., 1989). Similarly, the V-region was deleted from pGEX-12.15 using inverse PCR deletion mutagenesis. In summary, forward and reverse primers (Table I) were designed to abut one another (pGEX-12.15VO) so that the amplified product would contain both vector and altered insert sequences. The forward primer for the GST-9.11e construct contains a single base change resulting in a glutamic acid to aspartic acid substitution at position 1617 of XFN while the GST-9.11a primer contains a single base change resulting in an arginine to alanine substitution at position 1533. In 100-μl reactions, 1 μg of each primer (JR3 and JR4, JR5 and JR6, or JR1 and JR2) was mixed with 200 ng of template DNA (pGEX-9.11 or pGEX-
12.15) and the target region amplified in 25 cycles (94°C, 1 min; 48°C, 2 min; 72°C, 12 min) using Pfu polymerase (Stratagene, La Jolla, CA). The amplification products were blunted with Klenow, treated with T4 poly- nuclotide kinase, gel purified, and ligated. Each construct was sequenced to verify the success of the mutagenesis.

**Construction of GST-6.15, GST-6.15e, GST-6.15V0, and GST-6.15eV0**

The constructs described above were subsequently used along with clones pN4 and p4L (DeSimone et al., 1992) to build a series of fusion protein constructs extending from type III-6 to type III-15, and differing in their content of the RGD and V-regions (see Fig. 1). Inverse PCR site-directed mutagenesis was performed on pN4 as described above for pGEX-9.11e constructs extending from type III-6 to type III-15, and differing in their content from pN4, p4L, and pGEX-12.15 using convenient restriction sites. Similarly, pGEX-6.15e was constructed from p4Ne, p4L, and pGEX-12.15; pGEX-6.15V0 from pN4, p4L, and pGEX-12.15V0; and pGEX-6.15eV0 from pN4, p4L, and pGEX-12.15V0. The final constructs were sequenced to confirm orientation, identity, and continuous reading frame.

**Antibodies**

The GST-9.11 fusion protein was used to immunize BALB/c mice (Hilltop) by subcutaneous and interperitoneal injection (100 μg). Hybridoma fusion and cloning were performed using standard methods (Chapman et al., 1984) and culture supernatants screened by ELISA and Western blot for immunoreactivity to Xenopus plasma FN (pFN), GST-9.11, or GST. Two hybridomas were selected on the basis of immunoreactivity with pFN and GST-9.11 (1F7) or GST alone (1C9). A third hybridoma (4H2), which also recognizes pFN and GST-9.11, was obtained from a previous fusion where intact Xenopus pFN was used as immunogen (Ramos et al., 1996). Monoclonal antibodies from all three hybridomas were isotype as IgG1. Ascites fluids were generated by injecting 106 hybridoma cells into IRCF1 mice, primed 10 days earlier with pristane (Hoogenraad and Waight, 1986). Purified IgG1 was obtained from ascites by recombinant protein G affinity chromatography (Pharmacia LKB Biotechnology, Piscataway, NJ). FN type III-repeat epitope mapping for the 4H2 and 1F7 antibodies was done by Western blot using a series of Xenopus FN fusion proteins.

**Preparation of Substrates and Cell Adhesion and Migration Assays**

Protocols for substrate preparation, attachment, and spreading assays are described in detail in Ramos et al. (1996). Briefly, marked areas of plastic petri dishes (Falcon: Becton-Dickinson Co., Lincoln Park, NJ) were coated overnight at 4°C with a 50-μl drop of phosphate buffer (pH 7.5) containing 0.5 μM human plasma FN (HgFN), GST fusion proteins or 5 μg/ml RGD peptide (Peptite-2000; Telios, La Jolla, CA). Dishes were washed with 0.1 × MBS followed by a 30-min incubation in Modified Stern’s Solution (MSS; DeSimone and Johnson, 1991) containing 0.5 μg/ml BSA. In parallel experiments, the anti-GST mAb 1C9 was used by ELISA to confirm that each fusion protein was adsorbed to the plastic surface at approximately equimolar amounts.

A plug of involuted embryonic cells containing presumptive mesoderm and endoderm was dissected out of stage 11–15 embryos and dispersed in Ca"++ and Mg"++-free 1× MBS (CMF-MBS) for 30 min (see Fig. 2). Identical methods were used to isolate additional embryonic regions, such as those indicated in the diagram in Fig. 8. Approximately 600 dispersed cells per experiment were added to coated substrates by mouth pipette and incubated for 1 h in MSS (pH 8.3) containing 0.5% BSA, 1 mM CaCl₂ and 1 mM MgCl₂. Active induction assays were performed by dissecting animal caps from stage 8 embryos and dispersing the cells in CMF-MBS until control embryos reached stage 9.5 (Fig. 2). Dispersed cells were added to coated substrates and incubated for 2 h in MSS as above or in MSS containing 20 μg/ml of activin A, kindly provided by Dr. Jim Smith (MRC, Mill Hill, London, UK). In each experiment three to five marked regions were photographed and the plates inverted in buffer for 5 min. The same regions were then photographed again. Cells counted as spread were attached to the substrate, flattened, and had at least two lamellipodia (Fig. 3, A and B). Cells counted as attached were spherical and remained bound to the plate during the inversion in buffer (Fig. 3, C and D). Cells counted as nonattached were spherical and fell away during inversion. Cells were photographed on a Zeiss Axiovert microscope equipped with Hoffman optics. Most experiments were repeated at least eight times.

For migration assays, embryonic cells were obtained as described above and applied to Xenopus pFN or fusion protein coated substrata. Cell cultures were monitored on a Zeiss Axiovert using time-lapse video microscopy (4-s interval). Tracings of individual cells were made from the monitor following video playback. At least 10 cells per substrate per experiment were followed at 5-min intervals and the rates of migration calculated.

**Antibody and GAG Blocking Assays and Microinjection**

Blocking antibodies were tested in vitro using fusion protein and pFN substrates prepared as described above. Substrates were blocked with 0.5% BSA in MSS, rinsed, and then incubated with various FN antibodies (1 mg/ml) at 25°C for 1 h. The coated petri dishes were then washed three times with MSS before the addition of dispersed IMZ cells. Similar methods were used to pre-treat substrates with 1 μg/ml each of heparan sulfate (low mol wt form), dextran sulfate, or chondroitin sulfate (Sigma Chemical Co.). In all cases, cell attachment and spreading were quantified as described above.

The 4H2 control and 1F7 migration blocking antibodies were used for microinjection at a concentration of 10 ng/ml in Danilchik’s solution (Shih and Keller, 1992). Antibodies were centrifuged at 16,000 g for 5 min at 4°C just before injection. Siliconized glass needles (PL-100; Medical Systems Corp., Greenvale, NY) were pulled and the tips bevelled using a Narishige PB-7 puller and EG-1 grinder (Narishige USA, Greenvile, NY), respectively. A pressure driven injector (PL-100: Medical Systems Corp.) was used to deliver a 30-nl pulse of antibody solution into each of the two separate sites of the blastocoel at stage 9.5. All embryos were injected and cultured in 0.1 × MBS and scored for gastrulation defects. Embryos were imaged using a Zeiss STEMI-2000 dissecting microscope equipped with a Kodak DCS420c digital camera.

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**Table I. Sequence of PCR Primers Used in Fusion Protein Construction**

| Region amplified | Primer sequence |
|------------------|-----------------|
| V-region forward | 5'-AGTTCTAGACCATCCGCTGGTTACA-3' |
| V-region reverse | 5'-CCGAAATTCATAGATATCCAAAATG-3' |
| EIIIB forward    | 5'-CCGAAATTGGTACCTGATT-3' |
| EIIIB reverse    | 5'-CCGAAATTCATAACATTGCAATCCCA-3' |
| EIIIA reverse    | 5'-CCGAAATTCGTTGCTCGATCCCA-3' |
| III-14 (JR1)     | 5'-TGTTCCTCATTCGCC-3' |
| III-15 (JR2)     | 5'-GGGCCCCAGTGTTAAT-3' |
| III-10 (JR3; RGD>RGE) | 5'-AGGAGGAAGAGTCCAGC-3' |
| III-10 (JR4)     | 5'-CCAGCTACGTGATAAAGC-3' |
| III-15 (JR2)     | 5'-CCTACCGGAACTCTATC-3' |
| III-9 (JR5; SRN>SAN) | 5'-AGGTACCGCTCTCTCTT-3' |
| III-9 (JR6)      | 5'-CGGTGCACTGCTTTGTGAAATGTG-3' |
| III-10 (JR8)     | 5'-CGGTGCACTGCTTTGTGAAATGTG-3' |
Whole Mount In Situ Hybridization

Whole mount in situ hybridizations were performed on albino embryos as described by Harland (1991) using digoxigenin-rUTP-labeled (Boehringer Mannheim) transcripts synthesized in vitro from a *Xenopus* brachyury cDNA (Xbra) obtained from Dr. Jim Smith (Smith et al., 1991). Embryos were cleared in 2:1 (vol/vol) benzyl-alcohol/benzyl-benzoate before recording with the DCS420c camera on a Zeiss Axiophot using bright-field optics.

Results

Marginal Zone Cell Adhesion Requires Both the RGD and Synergy Sites

We reported previously the cloning of cDNAs encoding *Xenopus* fibronectin (XFN; DeSimone et al., 1992). In the current study we used these cDNAs to construct three general groups of FN-GST fusion proteins (Fig. 1) in order to identify regions of the molecule involved in embryonic cell adhesion and migration. The first group was designed to evaluate the importance of the RGD-containing central cell-binding domain of FN (Fig. 1, e.g., GST-9.11 series). The second group was used to test the contributions of the HepII and V-regions (Fig. 1, e.g., GST-12.15 series). The GST-6.15 series and GST-1.9 were used to confirm results obtained with the smaller fusion proteins and to test for possible cis-acting functional complementarity. Other fusion proteins include nonadhesive controls (Fig. 1, e.g., GST-B, GST-A, and GST alone). Fusion proteins were used as substrates in in vitro adhesion assays with isolated *Xenopus* involuted marginal zone (IMZ) or animal cap cells (Fig. 2). Representative examples of cell adhesion to these fusion proteins are presented in Fig. 3 and discussed below.

Our initial experiments addressed whether the RGD site of FN is sufficient to support adhesive and migratory behaviors of IMZ cells, as has been suggested in previous studies (Boucaut et al., 1984b; Winklbauer, 1990; Smith et al., 1990). Several RGD-containing fusion proteins (GST-4.13A0, GST-9.11, and GST-9.10) support the attachment and spreading of IMZ cells to the same extent as HpFN (Figs. 3 and 4 A). The smallest of these fusion proteins (GST-9.10) corresponds to the type III-9 to type III-10 repeat regions of FN. The RGD containing type III-10 repeat alone (GST-10) supports attachment but not spreading (Fig. 4 A). Therefore, the type III-9 repeat is also required for the spreading of these cells. Not surprisingly,
Figure 3. Morphology of embryonic cells on representative cell interactive sites of FN. Cells of the involuted mesoderm extend several lamelliform protrusions becoming polygonal in shape on (A) HpFN and (B) GST-9.11. We refer to this morphology as "spread." Involuted mesoderm cells attach but remain round on (C) RGD (Peptite) and (D) GST-9.11a, but can extend filopodia (arrow in C). We refer to this morphology as "attached." Bar: (D) 100 μm.

RGD peptide (Peptite) supports cell attachment but not cell spreading. The amino acid sequence PHSRN (PPSRN in *Xenopus*) located in the type III-9 repeat of FN is reported to be required for the spreading of many cell types (Aota et al., 1994; Bowditch et al., 1994). IMZ cell adhesion to a fusion protein in which this sequence is mutated to PPSAN (GST-9.11a) is indistinguishable from that on type III-10 alone (Fig. 4 A; GST-10). Thus, RGD is the minimum sequence required for IMZ attachment, but the PPSRN-containing synergy site located in the type III-9 repeat is necessary to support cell spreading.

The importance of the RGD site is confirmed by the fusion protein GST-9.11e, which is identical to fusion protein GST-9.11 with the exception of an aspartic acid (D) to glutamic acid (E) mutation in the RGD site. GST-9.11e does not support attachment or spreading (Fig. 4 A). In experiments where GST-9.11e and peptite were coated together, no spreading was noted (data not shown). Therefore, the RGD sequence is the primary site of IMZ cell attachment to the GST-9.11 fusion protein acting in cis with a synergy region to support cell spreading. The synergy region alone is not sufficient to support attachment or spreading (GST-9.11e; Fig. 4 A).

**IMZ Cells Attach to the Heparin-binding Region but Not the V-Region**

Adhesion to the COOH-terminal half of FN was investigated by comparing the relative contributions of the HepII and V-regions. Fusion proteins GST-12.12 and GST-12.15 (Fig. 1) contain both the V- and HepII regions, the latter of which corresponds to the type III-13 and type III-14 repeats (McCarthy et al., 1988). To distinguish the relative adhesive activities of the HepII and V-regions the V-region alone (GST-V) and a V-region minus version of GST-12.15 (GST-12.15V0) were also tested. Cells do not attach to GST-V indicating that this region is not recognized by IMZ cells (Fig. 4 B). Fusion proteins containing the HepII region (GST-12.12, GST-12.15, and GST-12.15V0) support comparable levels of IMZ attachment but not spreading;
the presence or absence of the V-region having no detectable influence on adhesive activity in these assays (Fig. 4 B). Cells derived from embryos injected with integrin α4 transcripts will adhere to GST-V indicating that the recombinant fusion protein has functional activity (Ramos et al., 1996).

To investigate further the specificity of embryonic cell attachment to the HepII region fusion protein substrates we preincubated with chondroitin sulfate, dextran sulfate, or heparan sulfate before the application of IMZ cells. Both heparan sulfate and dextran sulfate interfere with IMZ attachment while chondroitin sulfate has no affect (Fig. 5). None of the glycosaminoglycans tested had any affect on IMZ adhesion to GST-9.11. These data suggest that IMZ cells may bind to the HepII region using cell surface proteoglycans. We examined the cation dependency of cell attachment to the HepII region by performing adhesion assays in the presence (Fig. 6, A and C) or absence (Fig. 6, B and D) of free calcium. Cells were plated on substrates containing HepFN, GST-9.11, GST-12.15, and GST-12.15V0 alone (Fig. 6, A and B) or preincubated with free heparan sulfate (Fig. 6, C and D). Removal of calcium from the medium has no affect on IMZ attachment to HepII containing fusion proteins (Fig. 6 B; GST-12.15 and GST-12.15V0), however, attachment to the RGD/synergy fusion protein (GST-9.11) is inhibited (Fig. 6 B). As predicted, IMZ cells attach to intact HepFN under these conditions but do not spread (Fig. 6 B). Preincubation of the substrates with free heparan sulfate has no affect on IMZ attachment to GST-9.11 but inhibits attachment to HepII containing fusion proteins (Fig. 6 C). Interestingly, both preincubation of the substrate with free heparan sulfate and removal of cations from the solution are required to inhibit completely IMZ attachment to the entire FN molecule (Fig. 6 D; HepFN). These studies indicate that IMZ cell attachment to HepII containing fusion proteins is cation independent and suggests a nonintegrin-dependent interaction involving recognition by cell-surface proteoglycans.

The sequence PPRRAR (PPRRPR in Xenopus) has been shown to support the adhesion of some cell lines to the HepII region (Barkalow and Schwarzbauer, 1991). We used a rat FN fusion protein containing a Thr and Met substitution for two of the arginines in this sequence (PPRRAR > PPTMAR) in order to determine if this site is required for IMZ cell attachment to the HepII region. Our data indicate that IMZ cells will not attach to a fusion protein containing this substitution (MBP-HVOTM). The control fusion protein (MBP-HVO) supports IMZ adhesion equivalent to that described for the Xenopus FN fusion proteins that correspond to this region (Figs. 4 B, 5, and 6 A). Cells from the endoderm and ectoderm of gastrulae also attach (Fig. 8 E) but do not spread on the

![Figure 5](image-url)

*Figure 5.* Free glycosaminoglycans (GAGs) were used to investigate the specificity of cell adhesion to the HepII domain of FN. Heparan sulfate and dextran sulfate but not chondroitin sulfate (substrates pre-incubated with 1 μg/ml of each GAG) block the attachment of IMZ cells to the HepII containing fusion protein GST-12.15, but have no affect on the adhesion of cells to the RGD/synergy containing GST-9.11. At least 500 cells were counted per substrate per assay. Each assay was repeated two times (n = 2). Error bars indicate standard deviations.

![Figure 6](image-url)

*Figure 6.* Adhesion of involuted marginal zone cells to the HepII region of FN is Ca++ independent. IMZ cells attached (gray bars) and spread (black bars) are indicated. (A) IMZ cells spread on HepFN and GST-9.11, both of which contain the RGD site and synergy region, but only attach to GST-12.15 and GST-12.15V0 which contain the HepII site. (B) In Ca++-free MBS containing 10 μM EDTA, the cells do not attach to GST-9.11 but remain attached to HepFN, GST-12.15, and GST-12.15V0, all of which contain the HepII site. (C) When the substrates are pre-incubated with heparan sulfate (1 μg/ml) and washed before performing the adhesion assay, the cells no longer attach to the HepII containing fusion proteins, but adhesion to RGD/synergy-containing proteins, GST-9.11 and HepFN, is unaffected. (D) When substrates are preincubated in the presence of free heparan sulfate and the adhesion assay is performed in the presence of 10 μM EDTA, no cells attach. (E) Attachment to the HepII-containing region of rat FN is lost when the cells are plated on a fusion protein containing a PPRRAR to PPTMAR mutation (Barkalow and Schwarzbauer, 1991). At least 500 cells were counted per substrate per assay in E. Each assay was repeated three times (n = 3). Error bars indicate standard deviations.
The presence of the HepII region indicates that this attachment, like that to the RGD site alone, is not specific for IMZ cells.

**IMZ Adhesion to RGD/Synergy Is Unaffected by the Presence of the HepII Region**

It is possible that RGD/synergy, HepII, and V-region adhesive activities differ when presented in cis. To address this question, fusion proteins spanning all three of these regions and differing only in their content of the RGD site and V-region were constructed (Fig. 1). When both the HepII and RGD/synergy sites are present in the same fusion protein (GST-6.15, GST-6.15V0, and GST-4.13A0), attached cells behave as they would on RGD/synergy alone (e.g., GST-9.11; Fig. 4, A and C). Additionally, when GST-9.11 is used in combination with equimolar amounts of fusion proteins containing the HepII region, the cells behave as they do on the RGD/synergy proteins alone (data not shown). Thus, with respect to IMZ attachment and spreading, there is no evidence for cis or trans acting complementation among these regions in our assays. When HepII and the synergy site are present without the RGD site (GST-6.15e and GST-6.15eV0), however, some cell spreading can be detected (Fig. 4 C). When IMZ cells are cultured on mixed fusion protein substrates containing equimolar concentrations of HepII (GST-12.15V0) and the synergy site (GST-9.11e) they do not spread (data not shown). Therefore, these data suggest that some cis complementarity of the synergy region-containing central cell-binding domain with the Hep II region is possible in the absence of an intact RGD site. In all cases, we have been unable to detect any adhesive role for the V-region in these assays consistent with a lack of integrin α4 expression by these cells (Ramos et al., 1996).

We also investigated whether additional regions of FN support IMZ attachment in vitro. GST-1.9 corresponds to the NH2-terminal half of FN and contains the low affinity heparin-binding site (HepI) and a portion of the synergy region. GST-A and GST-B contain only the alternatively spliced EIIIA and EIIIB domains, respectively. Neither attachment nor spreading of cells was supported by any of these fusion proteins even at plating concentrations up to 10 mM (Fig. 4 B and data not shown).

**Activin Induced Animal Cap Cells Recognize the RGD/Synergy Site**

Treatment of *Xenopus* blastula stage animal cap cells with 20 U/ml activin-A results in the attachment, spreading, and migration of these cells on FN (Smith et al., 1990). Untreated cells attach but remain rounded. We were interested in determining whether the mechanism by which spreading and migratory behaviors of activin induced animal cap cells is regulated involves the recognition of multiple cell-interactive sites on FN. Consequently, animal caps were dissected from stage 8-9 embryos and the cells dispersed in CMF-MBS (Fig. 2). Dispersed cells were applied to fusion protein coated substrata in the presence or absence of 20 U/ml activin-A (Fig. 7). Greater than 90% of noninduced cells attach to the RGD sequence and the HepII region, alone or in combination (Fig. 7 A), similar to that observed for IMZ cells (Fig. 4). Unlike IMZ, however, these cells do not spread on RGD/synergy-containing fusion proteins. Animal cap cells do not attach to the RGE-containing fusion protein (GST-9.11e) or to the V-region alone (GST-V, Fig. 7 A).

Animal cap cells treated with activin-A also attach to both the RGD and the HepII region, however, they spread only on RGD/synergy site containing fusion proteins (Fig. 7 B) in a manner indistinguishable from that of endogenously induced IMZ. The cells do not spread on fusion proteins in which the synergy site has been mutated (GST-9.11a) or is absent (GST-10). Therefore, activin induction leads to animal cap cell “recognition” of the synergy site. Moreover, this change in animal cap cell adhesive behavior begins within 30–60 min after application of activin and

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**Figure 7.** Only activin-A-induced animal cap cells recognize the synergy region of FN. Solid bars represent a percentage of cells attached; gray bars represent a percentage of cells spread. (A) Noninduced dissociated animal cap cells attach but do not spread on the RGD/synergy region (GST-4.13A0, GST-9.11, and GST-9.10) and on Hep II (GST-12.12, GST-12.15, and GST-12.15V0). (B) Activin-induced animal cap cells attach and spread on FN fusion proteins containing an intact RGD and synergy site (GST-4.13, GST-9.10, and GST-9.11), but do not spread on either site alone (GST-10, GST-9.11e, GST-9.11a, and PepTite). Activin-induced animal cap cells (B), like IMZ cells, do not spread on Hep II (GST-12.15 and GST-12.15V0). Noninduced cells (A) do not spread on any of the substrata. Neither induced or noninduced cells are able to attach to the synergy (GST-9.11e) or V regions (GST-V) alone. At least 500 cells were counted per substrate per assay. Each assay was repeated eight times (n = 8). Error bars indicate standard deviations.
does not depend on new integrin expression (Ramos et al., 1996).

**Synergy Region-dependent Adhesive Behavior Is Temporally and Spatially Regulated in the Embryo**

The fusion protein studies indicate that the adhesive behaviors of activin-induced animal cap and explanted IMZ cells on specific FN cell-interactive sites are indistinguishable in vitro. This suggests that normal mesoderm induction leads to the position-specific activation of RGD/synergy region recognition in vivo. To test this hypothesis, regions of stage 8 and stage 11 embryos were dissected before and after involution as shown in Fig. 8, and applied to fusion protein-coated substrates.

Cells obtained from each of several different regions of blastula stage embryos attach but are unable to spread on intact human pFN or on any of the *Xenopus* FN fusion protein substrates (Fig. 8, B–E). These cells appear the same on each fusion protein with the synergy region mutant (GST-9.11a) supporting slightly less attachment than the others (Fig. 8 D). As gastrulation begins, cells of the dorsal lip and IMZ move into the embryo. Involuting cells gain the ability to attach and spread on the RGD/synergy fusion protein (Fig. 8 C; GST-9.10), but are unable to spread on a fusion protein containing the synergy region mutation (Fig. 8 D; GST-9.11a). Cell attachment to the HepII site remains unchanged before and after involution (Fig. 8 E; GST-12.15V0). These data suggest that IMZ cells come in contact with endogenous signals which, like activin-A, induce them to recognize the synergy site.

**The RGD/Synergy Site Is Required for Embryonic Cell Migration on FN**

Fusion proteins containing the RGD/synergy region (GST-4.13A0 and GST-9.11) support migration of both explanted IMZ and activin-induced animal cap cells at a rate similar to that on HpFN (~180 μm per hour; Fig. 9). In vivo, the mesoderm advances along the blastocoel roof at a comparable rate of 120 μm per hour (Keller and Winklbauer, 1992). Time lapse video microscopy reveals that motile IMZ cells migrate in random directions on these fusion proteins as described previously for intact bovine pFN (Winklbauer and Selchow, 1991). The RGD-containing peptide (Peptite) does not support cell migration (Fig. 9). Interestingly, the GST-9.11a fusion protein, which contains the mutated synergy site, supports a distinct mode of migration from that observed on GST-9.11. The cells remain round (Fig. 3 D), occasionally extend filopodia and translocate across the substrate in an apparent “rolling” fashion (data not shown). The cells will often stop abruptly and sometimes spread briefly. Antibody 1F7, which binds to the type III-9 repeat of FN, blocks completely this “migration” on GST-9.11a (Fig. 9), however, the cells remain adherent (Fig. 10 B). These data suggest that region(s) other than the PPSRN site in type III-9 are able to contribute to this alternate form of IMZ cell migratory activity on FN.

The HepII region (GST-12.15 and GST-12.15V0) is unable to support the migration of IMZ cells and when both the HepII and RGD/synergy regions are present (GST-4.13A0) the cells migrate at approximately the same rate as on RGD/synergy region alone (Fig. 9). This indicates that the HepII region, which supports IMZ attachment (Figs. 5 and 6), does not affect the rate of migration.

Previous studies suggest that mesodermal cell–cell adhesive interactions are important for directional migration in vitro (Winklbauer et al., 1992) and may be required to organize cell movements during gastrulation in vivo (Niehrs et al., 1994). Therefore, we also performed adhesion and migration assays using explanted head mesoderm (data not shown) in order to compare results obtained with dispersed IMZ cells. Fragments of head mesoderm adhere very tightly to the RGD/synergy region (GST-9.11) and cells spread and migrate away from the center of the ex-
Figure 9. Rates of embryonic cell migration on FN fusion proteins. Explanted involuted marginal zone (Mesoderm), explanted animal cap cells (A.C.), and explanted animal cap cells incubated in activin-A (A.C. + Activin) were applied to HpFN, PepTite, or various fusion proteins. IMZ cells were also applied to GST-9.11a and XpFN that had been pre-incubated with mAb 1F7. The cells were filmed for 1 h by time-lapse videomicroscopy. Cell paths were traced using the center of the cell as the origin. The path length was measured and used to generate an average rate of migration for each substrate. Activin-induced and uninduced animal cap cells are not included in the 1F7 blocking experiments. n > 10 cells for each value. Error bars indicate standard deviation.

Figure 10. Monoclonal antibody 1F7 blocks spreading of IMZ cells. Adhesion assays were done using fusion protein coated substrata previously incubated in either mAb 4H2 or mAb 1F7. The percentage of cells attached (gray bars) and spread (black bars) was determined as described in the Materials and Methods. (A) Preincubation with control mAb 4H2 has no affect on cell attachment and spreading on XpFN, GST-9.11, or GST-12.15V0 (compare to Fig. 4 A). (B) Preincubation of substrates with mAb 1F7 prevents cells from spreading on the RGD/synergy-site containing proteins (XpFN and GST-9.11) but has no affect on attachment to the HepII-containing fusion protein GST-12.15V0. At least 100 cells were counted per assay. Each assay was repeated eight times (n = 8). Error bars indicate standard deviations.
Figure 11. mAb 1F7 perturbs gastrulation when injected into the blastocoel. Representative 4H2- and 1F7-injected embryos are shown at stages corresponding to late gastrula (stage 12; A and D), late neurula (stage 21; B and E), and tailbud (stage 35; C and F). Blastopore closure is delayed in (D) mAb1F7-injected embryos compared to (A) control mAb 4H2-injected embryos. (E) Approximately 75% of mAb 1F7-injected embryos do not neurulate normally and (F) form severely truncated tailbud stage embryos lacking head and tail structures (compare to C).

Discussion

We present evidence that the FN-dependent adhesive and migratory behaviors of cells that comprise the early Xenopus embryo are regionally regulated during the process of gastrulation. Only IMZ cells spread and migrate on the RGD/synergy region of FN while other cells of the gastrula do not. Animal cap ectoderm artificially induced to become mesoderm by activin-A display adhesive behaviors similar to endogenously induced IMZ. In each case, spreading is dependent on synergy region recognition and does not involve HepII. We show that although these cells are less adherent to FN fusion proteins containing a mutation in the synergy site, they are still able to migrate as single cells. This motility is distinct from normal RGD/synergy-containing proteins, however, and head mesoderm explants do not spread or migrate when applied as intact tissue. Finally, we present evidence that IMZ cell interaction with the synergy region of FN is required for normal gastrulation movements in vivo.

The most significant regionally specified change in embryonic cell adhesion that we report in this study is the ability of IMZ to recognize the synergy region of FN as gastrulation movements commence. Previous reports noted that all the cells of the early gastrula are able to attach to FN via the RGD site in vitro, but the adhesive characteristics of regional cell populations differ (Winklbauer, 1988, 1990; Smith et al., 1990). We now show that differences in the major types of adhesive behaviors observed (e.g., attached vs spread and migratory) arise from recognition of the synergy site in response to mesoderm inducing signals. The molecular mechanism by which activin induces animal cap cells to switch rapidly from nonmotile attachment via RGD binding to motile attachment and spreading via the RGD/synergy region is not understood. We report else-

along the anterior-posterior axis and lack head and tail structures (Fig. 11 F). These data confirm that type III-9-mediated migration on FN is required for normal gastrulation movements and mesoderm patterning in Xenopus.

Table II. Antibody Injection Experiments

| Experiment | Antibody | Number injected | Number normal | Number abnormal | Number dead |
|------------|----------|-----------------|---------------|-----------------|-------------|
| 1          | 4H2      | 53              | 50            | 0               | 3           |
|            | 1F7      | 64              | 2             | 60              | 2           |
| 2          | 4H2      | 71              | 66            | 0               | 5           |
|            | 1F7      | 115             | 30            | 79              | 4           |
| 3          | 4H2      | 50              | 50            | 0               | 0           |
|            | 1F7      | 50              | 10            | 39              | 1           |

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 three representative experiments are shown.
where, however, that this switch in adhesive activity is independent of new integrin receptor expression at the cell surface after exposure to activin (Ramos et al., 1996). These data suggest that an integrin already present on the embryonic cell surface is "activated" following activin-induction in such a way as to alter receptor affinity and promote recognition of the synergy site. Similar inside-out signaling mechanisms have been proposed for integrins in other systems (for reviews see Hynes, 1992; Williams et al., 1994). For example, αβ1 is activated in T cells after antigen binding. This activation leads to increased adhesion of the cells to FN without changing the surface level expression of αβ1 (Shimizu et al., 1990; Chan et al., 1991). Our results could, therefore, be explained by a change in the activation state of αβ1 on induced animal cap cells in vitro and on presumptive mesoderm cells in vivo. The αβ1 receptor is a likely candidate for mediating changes in RGD/synergy site-dependent adhesive response in *Xenopus* embryos, in part because it is expressed at the surface of all cells from cleavage stages through gastrulation (Joos et al., 1995).

A putative integrin-binding site in the synergy region of human FN (PHSRN) has been identified and shown to be recognized by the αβ1 (Bowditch et al., 1994) and αβ1 (Aota et al., 1994) receptors. The arginine residue in this sequence is important for adhesive function (Aota et al., 1994) and is conserved in FNs from all species including *Xenopus* (PPSRN; DeSimone et al., 1992). We show that spreading of *Xenopus* IMZ and activin-induced animal cap cells is inhibited when the cells are cultured on fusion proteins in which this arginine has been mutated to alanine. This suggests that cell recognition of this region in response to inductive signals leads to a conformational change that alters the ligand-binding "pocket" of αβ1 so that it can accommodate the synergy site. Recent studies by Danen et al. (1995) demonstrate that activated forms of αβ1 can bind the RGD site of FN with increased affinity independent of PHSRN whereas "low affinity" forms of the receptor are able to recognize the synergy site. One possibility is that local inductive interactions maintain αβ1 in a low affinity state on the IMZ, thereby enabling these cells to bind the synergy region and spread and migrate on FN.

This hypothesis predicts that in the absence of mesoderm inducing signals the receptor could exist in a default high affinity state in other regions of the embryo, for example, on the surfaces of blastocoel roof ectodermal cells, which assemble the FN matrix in vivo. Interestingly, it has been reported recently that FN matrix assembly is dependent on integrin activation state with only high affinity forms of the receptor capable of supporting fibrillogenesis (Wu et al., 1995). Thus, early embryonic inductive interactions may play a role in specifying the spatial and temporal regulation of diverse integrin-dependent adhesive functions.

Recognition of the PPSRN site has important consequences for the spreading and migration of IMZ cells. We confirm that the presence of this site is required for IMZ cell spreading as described for other cell types (Aota et al., 1994; Bowditch et al., 1994). Of particular interest in the present study, however, is the demonstration that recognition of PPSRN can be regulated by inside-out signals. Although cell spreading is inhibited on PPSRN mutated fusion proteins such as GST 9.11a, significant motility of dispersed cells can still be observed (Fig. 9). The 1F7 mAb completely abolishes this migration, suggesting that additional sequences within type III-9 are likely to be contributing to synergy region adhesive activity. Interestingly, GST 9.11a, unlike GST 9.11 or intact pFN, does not support the motility of intact explants. This also suggests that cell–cell interactions may influence adhesion to the synergy region of FN. This latter point is important in view of the fact that most of the IMZ translocates directionally across the blastocoel roof as a cohesive population (Keller and Winklbauer, 1992).
We also report that all cells of the gastrula can attach to the HepII region of FN and that this interaction requires the presence of the PPRRAR sequence located in the type III-13 repeat, as described previously for other cell types (Barkalow and Schwarzbauer, 1991; Busby et al., 1995). We confirm that embryonic cell attachment to HepII is integrin independent and unlike adhesion to RGD/synergy, is not positionally regulated in the early embryo. Winklbauer (1990) reports that adhesion to pFN substrates is inhibited in the presence of RGD peptides suggesting that RGD-independent adhesion does not play a role in mesoderm attachment to FN. Although we cannot account conclusively for these differences in results, our earlier observations that IMZ cells will attach to FN in the presence of RGD peptide (Smith et al., 1990) are consistent with the present demonstration that the HepII site is responsible for this additional adhesive activity. In any event, the presence or absence of the HepII region has no obvious effect on RGD/synergy site-dependent cell migration in our studies whether present in cis or trans (Fig. 4), however, HepII is able to substitute partially for RGD in supporting cell spreading when present in cis with the synergy site (Fig. 4 C; GST-6.15e). Although we cannot rule out a role for HepII in gastrulation, mAb perturbation experiments (Fig. 11 and Ramos et al., 1996) indicate that HepII is not sufficient to "compensate" for a loss of IMZ cell adhesion to the RGD site and/or synergy region. Several reports suggest that cell surface–associated heparan sulfate proteoglycans are important in the binding of the growth factors FGF and activin (Brickman and Gerhardt, 1994; Sokol, 1994). Removal of HSPGs from the cell surface inhibits induction of mesoderm by these factors and thus perturbs gastrulation. It is possible, therefore, that the attachment of IMZ and activin-induced cap cells to the HepII region in vitro does not represent an adhesive function for this region in vivo.

The hypothesis that FN plays a role in supporting mesodermal cell movements during amphibian gastrulation was addressed in several previous studies. Polyclonal antibodies directed against FN are reported to perturb gastrulation in Pleurodeles (Boucaut et al., 1984a) and Xenopus (Howard et al., 1992) embryos after microinjection into the blastocoel. RGD peptides inhibit Xenopus mesoderm adhesion and migration on FN in vitro (Winklbauer, 1990) and a monoclonal antibody that blocks attachment to the RGD site in vitro, perturbs gastrulation in vivo (Ramos et al., 1996). Hence, mesoderm interaction with the RGD site of FN is required for this process. These studies do not address whether FN is required simply as an adhesive ligand or if active cell migration on this ECM protein is essential for normal gastrulation to proceed. This distinction has important consequences for understanding the mechanisms involved in gastrulation movements. It has been proposed that the primary force driving gastrulation movements in Xenopus is the process of convergence and extension, in which cells of the IMZ intercalate to form a narrower and longer tissue array (Keller and Winklbauer, 1992). The present studies demonstrate that IMZ recognition of the synergy region in type III-9 is required for cell spreading and migration on FN in vitro (Fig. 4) and gastrulation in vivo (Fig. 11). The 1F7 mAb, which binds to the type III-9 repeat, blocks the spreading and migration of IMZ and induced animal cap cells on intact Xenopus pFN (Figs. 9 and 10). When injected into the blastocoel, this mAb causes a disruption in the anterior progression of the IMZ (Fig. 12), but has relatively little effect on blastopore closure, and, presumably, convergence extension movements. Although qualitatively less severe, the phenotypes of 1F7-injected embryos are very similar to those obtained after injection of a mAb that blocks adhesion to the RGD site in type III-10 (Ramos et al., 1996). Taken together, these data suggest that FN-dependent cell migration is required for normal mesodermal movements along the blastocoel roof. In the absence of cell interactions with the RGD/synergy region of FN, presumptive mesoderm invaginates but fails to reach its normal position in the embryo. The resulting range of phenotypes observed (e.g., truncation along anterior-posterior axis, defects in head and axial structures) is consistent with this model. Interestingly, FN homozgous null mouse embryos share many of these features (George et al., 1993).

In conclusion, these studies demonstrate that adhesion to the RGD site and synergy region of FN is required for gastrulation in Xenopus. We also show that the initiation of cell migration on FN can be regulated by inductive signals and that the switch to a motile state involves integrin-dependent activation of synergy site recognition. Additional efforts will be required to confirm the identities of the integrins involved in this process and to elucidate the molecular mechanisms responsible for regulating positional and temporal changes in RGD/synergy site-dependent adhesive behaviors by inside-out signals.

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