In Vivo Analyses of Integrin β_1 Subunit Function in Fibronectin Matrix Assembly

Thierry Darribère,* Kareen Guida,* Hannu Larjava,‡ Kurt E. Johnson,§ Kenneth M. Yamada,‡ Jean-Paul Thiery,‖ and Jean-Claude Boucaut*

*Laboratoire de Biologie Expérimentale, Université Pierre et Marie Curie, Unité Associée Centre National de la Recherche Scientifique, 1135, 75005 Paris, France; ‡Membrane Biochemistry Section, Laboratory of Molecular Biology, National Cancer Institute, Bethesda, Maryland 20892; §Department of Anatomy, The George Washington University Medical Center, Washington, DC 20037; and ‖Laboratoire de Physiopathologie du Développement, Centre National de la Recherche Scientifique-Ecole Normale Supérieure, 75230 Paris 05, France

Abstract. Early development of the urodele amphibian *Pleurodeles waltl* is accompanied by a process of progressive fibronectin (FN) fibrillogenesis. FN begins to assemble into fibrils on the inner surface of the blastocoele roof at the early blastula stage and progressively forms a complex extracellular matrix. We have analyzed the mechanisms of FN-fibril formation under normal and experimental conditions in vivo with the following probes: iodinated FN, fluorescein-labeled FN, synthetic peptides containing the Arg-Gly-Asp (RGD) cell surface recognition sequence of FN, and polyclonal antibodies against both β_1 subunit of the amphibian FN receptor and the cytoplasmic domain of β_1 subunit. We report that in living embryos, exogenous labeled mammalian FN injected into the amphibian blastocoele undergoes FN-fibril formation in spatiotemporal patterns similar to those of endogenous FN. This indicates regulation of fibrillogenesis by the cell surface rather than by changes in the type of FN. Fibrillogenesis is inhibited in a dose-dependent manner both by the GRGDS peptide and monospecific antibodies to amphibian integrin β_1 subunit. Furthermore, when injected intracellularly into uncleaved embryos or into selected blastomeres, antibodies to the cytoplasmic domain of integrin β_1 subunit produce a reversible inhibition of FN-fibril formation that follows early cell lineages and cause delays in development. Together, these data indicate that in vivo, the integrin β_1 subunit and the RGD recognition signal are essential for the proper assembly of FN fibrils in early amphibian development.

THE regulation of extracellular matrix assembly and cellular responses to these matrices are important for the control of morphogenesis and organogenesis. For instance, cell morphology, cell attachment and migration, tissue stability, cell polarity, and differentiation often require adhesion and specific interactions of a cell with its substratum. These interactions involve specific cell surface proteins that bind adhesive ligands of the extracellular matrix. A family of proteins that appears to provide such functions is known as the integrin superfamily (Hynes, 1987). Protein and cDNA sequencing of these receptors as well as immunological analysis have established the existence of two multigene families of glycoproteins detected throughout the animal kingdom corresponding to integrin α or β subunits (Tamkun et al., 1986; Argraves et al., 1987; Suzuki et al., 1987; DeSimone and Hynes, 1988; Marcantonio and Hynes, 1988; Cheresh et al., 1989). These families are divided into distinct subfamilies, in which members share a common β subunit that is noncovalently combined with a unique α subunit to form α/β heterodimers. The α subunit appears to confer ligand-binding specificity. To date, three major subfamilies have been described: the leukocyte adhesion proteins (Leu-

CAM) (Anderson and Springer, 1987), cytoadhesins (Ginsberg et al., 1988), and the very late antigens (VLAs)[1] (Horwitz et al., 1985). The integrin VLA family consists of heterodimers of six different α subunits each associated with a common β_1 chain. The VLA-5 molecule ($\alpha_5\beta_1$) is directly implicated via its extracellular domain in the recognition of fibronectin (FN) (Pytela et al., 1985; Wayner et al., 1988; Akiyama et al., 1989a). However, several other integrins also bind fibronectin, including $\alpha_3\beta_1$, $\alpha_{IIb}\beta_3$, and $\alpha_v\beta_x$. The relationship between these FN adhesion receptors and FN matrix assembly remains uncertain at present (reviewed in McDonald, 1988 and Akiyama et al., 1989b).

At the molecular level, FN matrix assembly could be modulated by at least three events (McDonald, 1988; Ruoslahti, 1988): the binding of FN to one or more cell surface receptors, FN-FN binding, and finally FN-FN cross-linking and fibril elongation through both disulfide bond formation and the action of a transglutaminase (McKeown-Longo and

---

Mosher, 1984; Homandberg and Erickson, 1986). For the first event, two cell surface receptors have been proposed: the FN cell adhesion receptor (an integrin) and a "matrix assembly receptor." Previous studies suggest that the RGD-containing cell adhesive domain and its integrin receptor may cooperate in FN-fibril assembly. For example, oncogenic transformation produces a loss of FN matrix and a diffuse distribution of FN receptors; on FN substrata, cells exhibit extracellular FN fibrils that colocalize with the FN-receptor complex (Chen et al., 1985; Damsky et al., 1985); RGD-containing peptides that induce detachment of fibroblasts from extracellular FN matrix lead to diffuse receptor localization (Chen et al., 1986); and particularly importantly, cell adhesive fragments, anti-FN and anti-$\alpha_5\beta_1$ antibodies that inhibit the binding of FN to its receptor, block FN-fibril formation by cultured fibroblasts (McDonald et al., 1987; Akiyama et al., 1989a; Roman et al., 1989).

On the other hand, evidence for the matrix assembly receptor identified by McKeown-Longo and Mosher (1983) appears equally compelling, although its molecular composition remains unclear. This postulated matrix assembly system appears to be primarily dependent on the amino-terminal domain of FN, a region that has little or no cell adhesive activity (McKeown-Longo and Mosher, 1983, 1985, 1988; Peters and Mosher, 1987; McDonald, 1988). Furthermore, heparan sulfate proteoglycan may also be implicated in matrix deposition and attachment of fibroblasts (Woods et al., 1986). A transmembrane heparan sulfate proteoglycan that binds to FN has been isolated (Rapraeger et al., 1986). Finally, a severely ganglioside-deficient cell line does not assemble FN into a matrix, and matrix assembly is restored by certain exogenous gangliosides, implicating gangliosides in this process as well (Spiegel et al., 1985; 1986).

In early amphibian development, an extracellular matrix containing FN fibrils undergoes assembly at the basal surface of cells that form the blastocoele roof (Boucaut and Darribère, 1983; Darribère et al., 1984, 1985; Johnson, 1981, 1985; Johnson et al., 1990; Lee et al., 1984; Nakatsuji et al., 1982, 1985). During gastrulation, these FN fibrils provide a substrate for mesodermal cell migration (Boucaut et al., 1984a,b; Nakatsuji and Johnson, 1984a,b). Furthermore, immunological inhibition studies performed in vitro and in vivo, demonstrate that a complex of glycoproteins of 90-140 kD is involved in the binding of mesodermal cells to FN fibrils (Darribère et al., 1988).

An important and still unanswered question concerns the actual mechanism of cell interactions with FN to form fibrils, as well as its regulation. We focused on the possibility that the β_1 subunit found in the integrin VLA-5 may mediate or modulate these interactions. We initiated experiments to analyze the matrix assembly activity of blastomeres in living amphibian embryos. We have used RGD-containing peptides and both antibodies to the whole molecule or the cytoplasmic domain of the integrin β_1 subunit to perturb its function. Our major findings are that exogenous FN incorporation into chimeric matrices is regulated in parallel with endogenous fibrillogenesis, and that RGD-containing peptides, polyclonal Fab' fragments of IgG to both the amphibian integrin β_1 subunit (extracellularly), and the cytoplasmic domain of β_1 (intracellularly) cause specific in vivo inhibition of the ability to incorporate labeled exogenous FN into extracellular matrices and to elaborate endogenous FN fibrils.

Materials and Methods

Embryos

Embryos of the urodele amphibian *Pleurodeles waltl* Michah were collected from natural matings. They were manually dejellied and maintained at 18°C in 10% modified Barth solution (MBS) (Barth and Barth, 1959) containing 50 µg/ml gentamycin (Gibco Laboratories, Grand Island, NY). Developmental stages of embryos were according to Gallien and Durocher (1957).

Purification of Fibronectin

FN was prepared from fresh-frozen bovine plasma by gelatin-Sepharose affinity chromatography (Engvall and Ruoslahti, 1977). Purity of FN was assessed by SDS-PAGE. If needed, FN was further purified using two cycles of gelatin-Sepharose chromatography.

Labeling of Fibronectin

Purified bovine plasma FN was labeled with ^{125}I or FITC. The iodination of FN was carried out with Bolton and Hunter (1973) reagent (74 TBq/mmol; Amersham Corp., Arlington Heights, IL). By SDS-PAGE, the iodinated material migrated as a doublet at an apparent molecular mass of 220 kD under reducing conditions. For FITC labeling, FN in a 0.1 M sodium carbonate/bicarbonate buffer (pH 9) was incubated with FITC (200 µg/mg of FN; Sigma Chemical Co.; St. Louis, MO) for 2 h at room temperature and with gentle stirring (Chernousov et al., 1985). Unreacted dye was removed by gel filtration on Sephadex G-50 eluted with MBS. For control experiments, BSA (fraction V; Sigma Chemical Co.) was labeled identically. Iodinated and fluorescein-conjugated FNs retained their biological activity in cell spreading assays described previously (Darribère et al., 1988).

Purification of Integrin β_1 Subunits

Batches of 1,000 larvae (stage 39) of *Pleurodeles waltl* were homogenized in a Polytron (12,000 rpm, 1 min, 4°C; Bioblock Scientific, France) in 40 mM octyl-β-glucopyranoside (OGP; Calbiochem-Behring Corp., San Diego, CA), 2 mM PMSF, 2 mM EGTA, 5 µg/ml aprotinin, 0.5 µg/ml leupeptin, 50 mM Tris-HCl pH 7.4 (buffer 2). Detergent extraction of proteins was carried out for 2 h at 4°C with stirring. Detergent-insoluble material was removed by centrifugation at 12,000 g for 15 min at 4°C. The resulting supernatant solution was incubated with Sepharose 4B beads (10 ml of packed beads; Pharmacia Fine Chemicals, Piscataway, NJ) for 1 h at 4°C in order to remove proteins that bind nonspecifically to Sepharose 4B. After sedimentation, the supernatant solution was mixed overnight at 4°C by end-over-end rotation with 10 ml packed volume of concanavalin A-Sepharose 4B (Sigma Chemical Co.). Bound proteins were eluted with 0.25 M α-methyl-D-mannoside (Sigma Chemical Co.) in buffer. Fractions that react by immunoblotting with anti-avian integrin IgG were pooled and dialyzed against buffer 2 at 4°C. They were then incubated with the IgG fractions of polyclonal antibodies against avian integrin VLA-5 coupled to CNBr-activated Sepharose 4B (Pharmacia Fine Chemicals) at a ratio of 5 mg antibodies per 1 ml of packed beads according to Hasegawa et al. (1985).

Antibodies and Peptides

Antibodies against the integrin β_1 subunit of *Pleurodeles waltl* (anti-β_1) were raised in New Zealand white rabbits. Subcutaneous injections using 0.2 mg of amphibian integrin β_1 emulsified 1:1 with Freund's complete or incomplete adjuvant were administered at 1-wk intervals. Rabbits were bled from an artery and IgG were purified from sera by chromatography on DEAE Trisacryl-M as recommended by the manufacturer (Industrie Biologie Française, France). Polyclonal antibodies against the cytoplasmic domain of integrin β_1 subunit (anti-β_1 COOH) were produced by immunization of a rabbit with a synthetic peptide with the sequence CKLLMIIHDR-REFAKFEKEKMNAKWDTGENPIYKSAVTTVVNPKYEGK (DeSimone and Hynes, 1988). The peptide was conjugated to keyhole limpet haemocyanin (Pierce Chemical Co., Rockford, IL) with the heterobifunctional reagent m-maleimidobenzoic acid-N-hydroxysuccinimide ester (MBS; Pierce Chemical Co.) essentially as described (Walker et al., 1985). This antibody immunoprecipitated the same human fibroblast integrins as an existing anti-β_1 monoclonal antibody (Akiyama et al., 1989a), and showed the same immunolocalization pattern; it also stained β_1 and preβ_1 subunits as expected in Western immunoblots of fibroblast extracts. Production of rabbit IgG fractions of polyclonal antibodies to *Ambystoma mexicanum* plasma FN

Mr   1   2   3   4

200 —

116 —

96 —

68 —

A   B
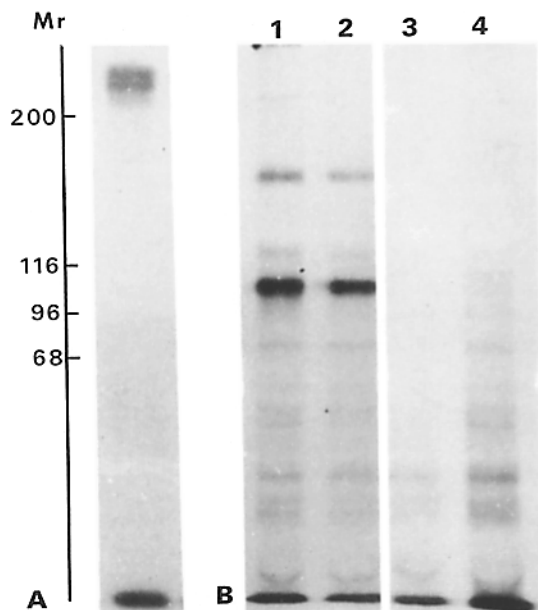
Figure 1. Specificity of anti-FN and anti-integrin β_1 subunit IgG. (*A*) Immunoprecipitation of [^{35}S]methionine-labeled proteins from *Pleurodeles waltl* midgastrula embryos with anti-FN IgG. Precipitation was performed with protein A–Sepharose (5 mg) and 30 μg of IgG. Sample was analyzed by 7.5% SDS-PAGE under reducing conditions. A doublet with an apparent molecular mass of 220 kD is clearly detectable. (*B*) Immunoprecipitation of embryonic [^{35}S]methionine-labeled proteins with 5 mg of protein A–Sepharose and 30 μg of anti-amphibian integrin β_1 subunit IgG (lane *1*) or IgG against the cytoplasmic domain of integrin β_1 subunit (lane 2). Samples were analyzed by 7.5% SDS-PAGE under nonreducing conditions. Controls were performed with protein A–Sepharose alone (lane *3*) or IgG preincubated with amphibian integrin β_1 subunit (lane *4*). The autoradiogram indicates that both antibodies recognize the amphibian integrin β_1 subunit of 100 kD. The faintly labeled polypeptides of 140 kD represent integrin α subunits. They are noncovalently associated with integrin β_1 subunits and are coprecipitated with β_1 subunits. M_r, molecular mass markers in kilodaltons.

(anti-FN) has been previously described (Boucaut and Darribère, 1983). The specificity of IgG fractions was evaluated by immunoprecipitation and SDS-PAGE using [^{35}S]methionine-labeled whole embryo homogenates (Fig. 1). Furthermore, anti-β_1 IgG inhibit mesodermal cell attachment and spreading on FN-coated substrata. Monovalent fragments of these antibodies (Fab') were prepared according to Brackenbury et al. (1977). Their purity was tested by SDS-PAGE. For control experiments we used nonimmune Fab', or we attempted to block the activity of Fab' fragments with antigen. In the later case, anti-β_1 Fab' or anti-β_1 COOH Fab' were mixed with purified amphibian integrin β_1 subunit at a weight ratio of 1:1 in sterile 10% MBS. Both control probes were used immediately after low speed centrifugation (1,000 g, 10 min) to sediment insoluble material. Control blastomere injections were also performed with IgG fractions from rabbit, mouse, and human sera.

The synthetic peptides used were Gly-Arg-Gly-Asp-Ser (GRGDS), and Cys-Gln-Asp-Ser-Glu-Thr-Arg-Thr-Phe-Tyr (P_2). They correspond to conserved and hydrophilic sequences from, the cell-binding and collagen-binding sites of FN, respectively (Yamada and Kennedy, 1984; Pierschbacher and Ruoslahti, 1984).

Metabolic Labeling, Immunoprecipitation, and Electrophoresis

Embryos at midgastrula stage (stage 10) were injected with 5 nl of [^{35}S]methionine (555 MBq/ml; Amersham Corp.) and allowed to develop in 10% MBS for 4–6 h. They were then homogenized in buffer 3:0.1 M NaCl, 1 mM MnCl$_2$, 40 mM OGP, 2 mM PMSF, 5 μg/ml aprotinin, 0.5 μg/ml leupeptin, 50 mM Tris-HCl, pH 7.4, and extracted for 15 min at 4°C. Yolk and insoluble materials were removed at 10,000 g for 30 min at 4°C.

Immunoprecipitations of FN, or of integrin β_1 subunit were performed with protein A–Sepharose CL-4B (Pharmacia Fine Chemicals). Nonspecific binding sites on beads were quenched by preincubating the beads with unlabeled extracts of embryonic proteins for 1 h at room temperature. Then, 5 mg of beads were incubated with 30 μg of the appropriate IgG in 1 ml of buffer 3 for 1 h at room temperature with stirring. After two washes with buffer 3, the beads were incubated with 1 ml of radioactive protein extract from 10 embryos overnight at 4°C with gentle stirring. After 10 washes with buffer 3, immunoprecipitates were analyzed by SDS-PAGE (Darribère et al., 1988). Gels were stained with Coomassie blue, processed for fluorography using Amplify (Amersham Corp.), dried, and exposed with an intensifying screen (Dupont Lightning Plus, France) using Kodak XAR film.

Injections

Embryos with diameter from 1.2 to 1.4 mm were collected when they reached the uncleaved, 2-cell, early, mid-, or late blastula stage (stages 0, 1, 5, 6, 7) and injected into blastomeres or the balstocoele. Iodinated FN, FITC-FN, or FITC-BSA were injected into early blastula blastocoeles (stage 5) in the absence or presence of peptides, anti-β_1 Fab', or preimmune antibodies. For injections, 200 nl of the blastocoele fluid was removed to prevent any overpressure during injection. Thereafter, 200 nl of a 0.05–1 mg/ml solution of reagents dissolved in 10% sterile MBS were injected (final amount per embryo: 10–200 ng). After injections embryos were incubated for 2, 4, 6, and 18 h in 10% MBS at 18°C. At each time, embryos were dissected in sterile MBS into animal (blastocoele roof, AP) and vegetal halves (VP). After careful washes, labeled FN binding was examined in both halves by immune precipitation, counting radioactivity, or direct fluorescence.

Anti-β_1 COOH Fab' were introduced into uncleaved embryos (stage 0) or one blastomere of two-cell embryos (stage 1). Injections into uncleaved embryos were performed in the equatorial region and at least 3 h after fertilization. Uncleaved embryos were injected with 10–50 nl (i.e., roughly 1/20–1/100 of the egg volume) of 0.05–10 mg/ml solutions of antibodies dissolved in 10% sterile MBS (final amount: 0.5–500 ng/embryo). Injections into blastomeres were performed with a volume of 5–30 nl (i.e., roughly 1/50–1/100 of the blastomere volume) of the same antibody solutions (final amount: 0.25–300 ng/blastomere) except that they also contained 50 mg/ml of lysinated rhodamine dextran (RLDx) (Gimlich and Cooke, 1983) to follow the fate of offspring of the injected blastomere. After injections, embryos were reared in 10% MBS at 18°C in the dark to minimize bleaching of the fluorescent dye. Embryos were dissected and fixed for whole-mount studies of FN-fibril formation.

Immunofluorescence and Scanning Electron Microscopy

For whole-mount observations, the roof of the blastocoele was dissected from 124 cells to late gastrulae (stages 4–13) after removal of the vitelline membrane. Then, the explants were treated for immunodetection of FN as described previously (Boucaut and Darribère, 1983). Scanning electron microscopy was carried out according to Nakatsuji et al. (1982).

Results

Fibronectin-fibril Formation

The time course and pattern of normal in vivo fibril formation was established by indirect immunofluorescence for FN. When the blastocoele begins to form in the eight-cell embryos (stage 3), there is no fluorescence for FN on the inner surface of blastomeres at the animal pole. In morulae (stage 4), no FN fibrils were yet observed. However, nonfibrillar fluorescent labeling was detected either as a ring around blastomeres near cell–cell contacts or as minute speckles on the surface of each blastomere (Fig. 2 *A*). The first FN fibrils were observed 2 h later in early blastulae (stage 5). They were located primarily at the edges of the smallest cell surfaces of the blastocoele roof (Fig. 2 *B*). In midblastulae (stage 6), fibrils appeared all around the cell periphery. They also
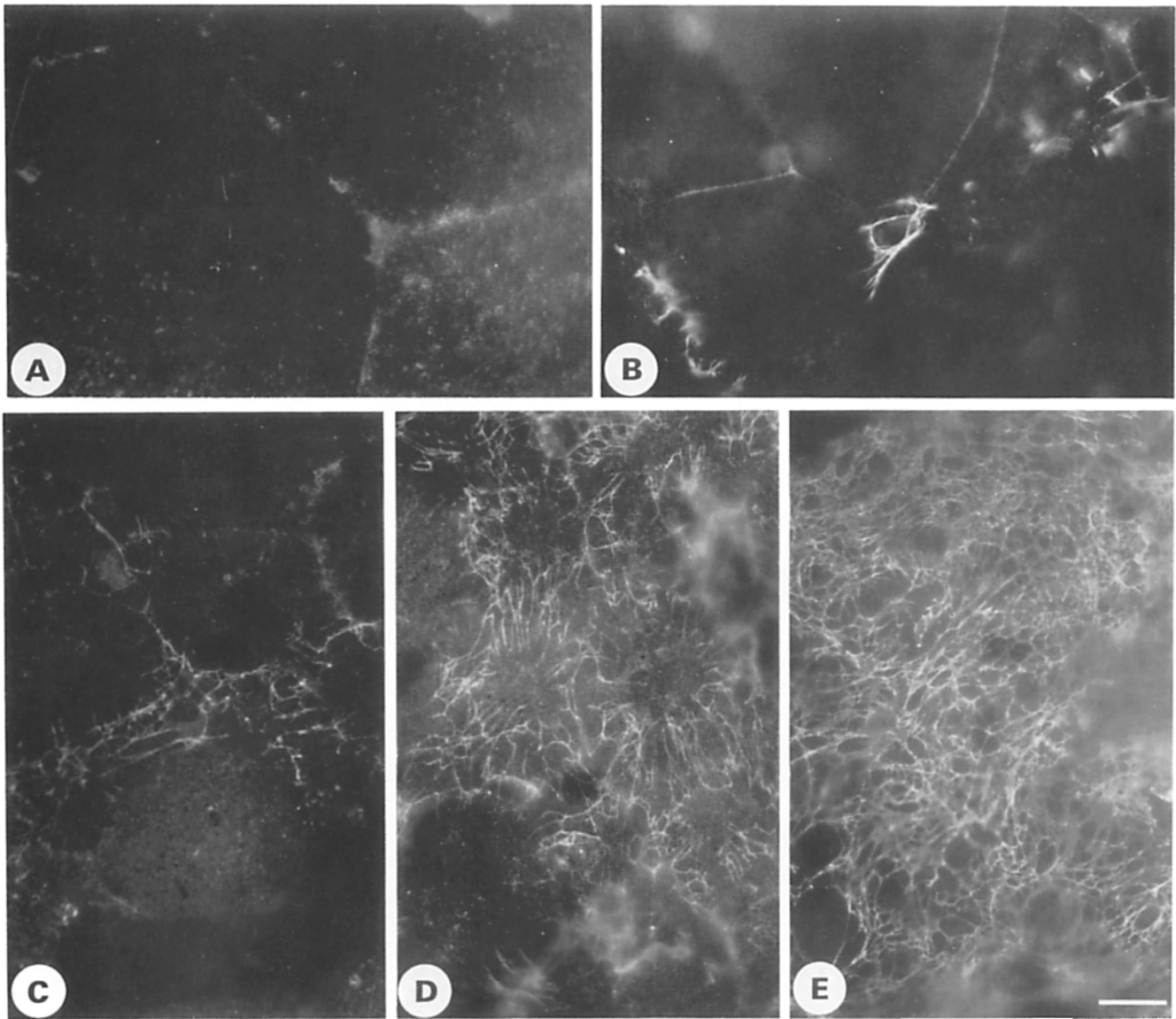
Figure 2. Time course analysis of FN-fibril formation. Whole mounts of the blastocoele roof from *Pleurodeles waltl* embryos were analyzed by indirect immunofluorescence with purified anti-FN IgG. (*A*) Inner face of blastocoele roof of morula stage (stage 4). Fluorescent staining is observed at areas of cell–cell contacts and more weakly on the cell surface. No FN fibrils are detected at this stage. (*B*) Early blastula stage (stage 5). Inner view of the blastocoele roof in the animal pole region. A faint fluorescent ring delineates blastomeres. The first FN fibrils appear at some blastomere edges. (*C*) Midblastula stage (stage 6). Fibrillar arrays of FN staining are concentrated at the periphery of blastomeres. (*D*) Late blastula stage (stage 7). FN fibrils extend from the cell periphery toward a central supranuclear zone. (*E*) Midgastrula stage (stage 10). A complex extensively anastomosed meshwork of FN covers the entire inner surface of the blastocoele roof. Bar, 5 µm.

crossed adjacent cell boundaries (Fig. 2 *C*). They were smaller than those occurring at first and arranged perpendicular to the plane of contact between blastomeres. Within a span of ∼6 h, fibrils elongated from these peripheral sites toward the central portion of cell surface over the nucleus. In late blastulae (stage 7), fluorescent staining for FN was now well developed over most of the cell (Fig. 2 *D*). FN fibrils were still more abundant at the periphery of each cell. At this stage, two kinds of fibrils can be distinguished: the first were short and thin, and the second were longer and thicker. 24 h after fertilization, in early gastrulae (stage 8a), FN fibrils formed a complex anastomosing fibrillar matrix, which covered the entire inner surface of the blastocoele roof. During gastrulation, the extracellular meshwork of FN fibrils showed further increases in density (Fig. 2 *E*).

Exogenous Fibronectin-Fibril Formation

We tested the hypothesis that the time course and pattern of FN assembly was independent of the FN molecules themselves using interspecies injections. Exogenous labeled FN was injected into the blastocoele of early blastula (stage 5), when few fibrils are present (Fig. 2 *B*). The binding of FN was studied with iodinated FN to quantify binding and with FITC-FN to localize injected FN.

The ^{125}I-FN binding was examined by immunoprecipitation and SDS-PAGE. Autoradiograms revealed that ^{125}I-FN was bound preferentially to the animal hemisphere. The polypeptides corresponding to FN were detected from 2 to 18 h after the injection in the animal half of embryos. The incorporation of FN increased in time-dependent fashion.

A

Time (hours)

B

Time (hours)
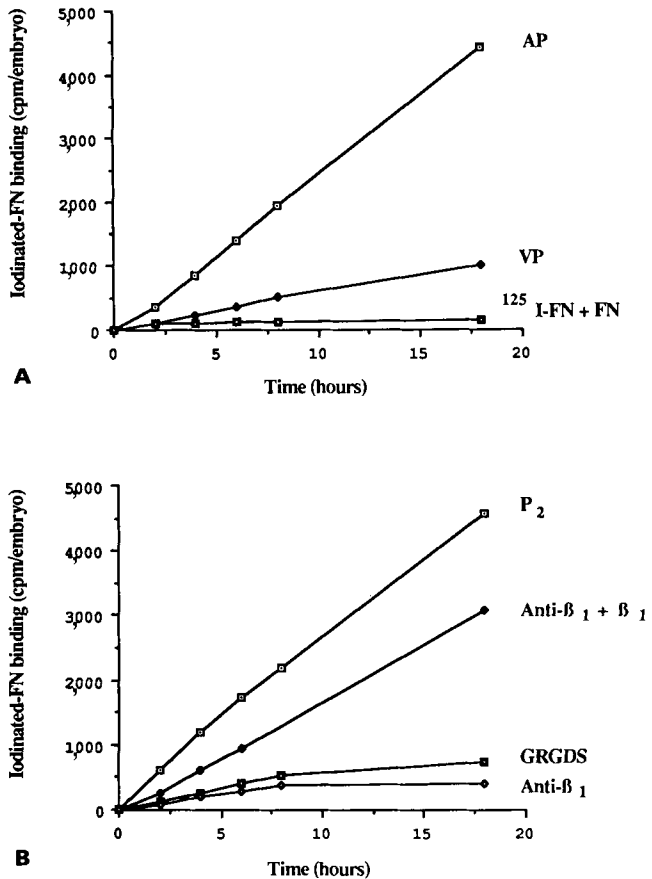
Figure 3. Time course of incorporation of ^{125}I-FN into *Pleurodeles waltl* embryos. (*A*) The same amount of ^{125}I-FN (50 μg/ml, 200 nl, 100,000 cpm) was injected into the blastocoele of early blastula embryos (stage 5). At each designated time point, 10 embryos were dissected into animal (*AP*) and vegetatal (*VP*) hemispheres. Radioactivity was determined for the two samples and presented as cpm/embryo. As a control for specific binding, labeled FN was injected in the presence of excess unlabeled FN (200 μg/ml), and the resultant binding to AP hemispheres is shown (^{125}I-FN + FN). In the presence of this excess unlabeled FN, binding is minimal. In contrast, the curves show a time-dependent incorporation of iodinated FN especially into AP compared with VP. Determinations represent averages from triplicate experiments. (*B*) The same quantities of ^{125}I-FN (50 μg/ml, 200 nl, 100,000 cpm) were injected into the blastocoele of early blastula embryos (stage 5) with control peptide P_2 (200 μg/ml), GRGDS (200 μg/ml), or anti-β_1 Fab′ (250 μg/ml). At the designated time points, 10 animal hemispheres were bissected and radioactivity was determined. The data represent the average of three experiments. They reveal a significant inhibition of incorporation of exogenous FN by either GRGDS or monovalent anti-β_1 antibodies. Controls were carried out with P_2 corresponding to the collagen binding domain of FN or anti-β_1 Fab′ absorbed with amphibian β_1 subunits (*Anti-β_1 + β_1*).

Conversely, virtually no incorporation of labeled-FN occurred in the vegetal half of embryos. As a control for specific binding of ^{125}I-FN, embryos (stage 5) were injected with a mixture of labeled (50 μg/ml, 100,000 cpm) and unlabeled FNs (200 μg/ml). No trace of FN could be detected on autoradiograms even after lengthy exposure of the gels (data not shown).

Bound FN was also measured by counting the radioactivity in the two embryonic halves. As shown in Fig. 3 *A*, iodi-

nated bovine plasma FN bound to the blastocoele roof. The binding was time dependent. In contrast, the binding of exogenous FN to the vegetal half occurred at substantially lower rates throughout the period of incubation. Addition of excess unlabeled FN (200 μg/ml), dramatically reduced the binding of ^{125}I-FN to the animal hemisphere (Fig. 3 *A*).

To determine where exogenous FN bound to the animal half, FN was also monitored by fluorescein labeling. Experiments were done with different concentrations of FN ranging from 10 to 100 μg/ml. Optimal incorporation was observed with 50 μg/ml. The results show that exogenous FN is transferred to the extracellular matrix with the same timing and pattern as endogenous FN. Fluorescent fibrils were absent in controls where FITC-FN were mixed with an excess of unlabeled FN (200 μg/ml) or where FITC-BSA was injected. When surfaces of endodermal cells facing the blastocoele were observed, no fibrils could be detected with FITC-FN even after 18 h of incubation. Furthermore, at late blastula stage (stage 7), when the extracellular matrix of the blastocoele roof was transferred onto slides (Shi et al., 1989) and incubated for 18 h with FITC-FN, no fluorescent fibrils were detected, suggesting that cellular processes mediate fibrillogenesis (data not shown).

Inhibition of Fibronectin-fibril Formation by GRGDS and Anti-integrin β_1 Subunit Antibodies Applied Extracellularly

Iodinated or fluoresceinated FN was injected into the blastocoele of early blastula (stage 5) in presence of either GRGDS or monovalent antibodies against integrin β_1 subunit. The binding of FN was assessed by immune precipitation, counting of radioactivity, and immunofluorescence.

Immunoprecipitation followed by SDS-PAGE and autoradiography demonstrated that GRGDS, as well as anti-β_1 Fab′, inhibited the binding of FN to the roof of the blastocoele. Indeed, no trace of radioactive FN could be detected in presence of GRGDS or anti-β_1 Fab′ even after an 18-h incubation. In contrast, polypeptides corresponding to FN were revealed after incubations with the collagen binding site of FN (peptide P_2).

Quantitative radioactivity binding data were obtained for blastocoele roof and vegetal hemisphere. The results confirm that both GRGDS and anti-β_1 Fab′ substantially inhibit the binding of ^{125}I-FN to blastomeres of the blastocoele roof. As shown in Fig. 3 *B*, the incorporation of ^{125}I-FN was minimal in the presence of GRGDS or anti-β_1 Fab′ when compared with control experiments performed with peptide P_2 or Fab′ absorbed with the integrin antigen.

Finally, in fluorescence localization studies with FITC-FN (50 μg/ml), FN did not become organized into fibrils when incubated with either GRGDS (200 μg/ml) or anti-β_1 Fab′ (250 μg/ml). Conversely, FN-fibril formation occurred normally in the presence of collagen-binding peptide P_2 (200 μg/ml) (data not shown).

Inhibition of Fibronectin-fibril Formation by Intracellular Injection of Antibodies to Cytoplasmic Domain of Integrin β_1 Subunit

Since FN adhesion receptors have been proposed to have transmembrane functions, we examined whether an intracellular perturbation of β_1 subunit of integrins might also affect extracellular matrix assembly.

Table I. Inhibition of Fibronectin-fibril Formation by Monovalent Antibodies against the Cytoplasmic Domain of the Integrin β_1 Subunit

| Time of development h	Morula 124 cell 10–12	stage 5 14	stage 6 22	stage 7 27	Gastrula stage 8a 31
MBS	−	+	+ +	+ + +	+ + + +
Fab' nonimmune					
(50 ng)	−	+	+ +	+ + +	+ + + +
Anti-β_1COOH					
(2.5 ng)	−	+	+ +	+ + +	+ + + +
(10 ng)	−	−	+	+ +	+ + +
(25 ng)	−	−	−	−	+
(50 ng)	−	−	−	−	−/+
(100 ng)	−	−	−	−	−

Various concentrations of anti-β_1 cytoplasmic domain monovalent antibodies were injected into uncleaved *Pleurodeles waltl* embryos. They were reared at 18°C in 10% MBS until they reached the indicated stage. The blastocoele roof was dissected, fixed, and then immunodetection of FN was performed. For each point, 10 embryos were observed. Quantities injected are expressed as nanograms of antibodies per embryo. MBS, sterile injection buffer; anti-β_1COOH, monovalent antibodies against the cytoplasmic domain of the integrin β_1 subunit; (−) no FN fibrils; (+) sparse FN fibrils at periphery of cells (as in Fig. 2 *B*); (+ +) FN fibrils all around blastomeres (as in Fig. 2 *C*); (+ + +) FN fibrils radially arranged on the cell surface (as in Fig. 2 *D*); (+ + + +) extensive, anastomosed FN fibrils (as in Fig. 2 *E*).

Monovalent antibodies were injected into fertilized eggs before the first cleavage. The results of intracytoplasmic injections performed with antibodies against the cytoplasmic domain of integrin β_1 subunit are summarized in Table I.

The main conclusion is that these monovalent antibodies cause dose-dependent and reversible defects in FN-fibril formation. There is a slight inhibition of FN-fibril formation at 10 ng of Fab'/embryo. This inhibition is characterized by a delay in the appearance of the first fibrils. Based on a sample of 10 embryos, this delay is estimated to be 7–8 h. With concentrations ranging from 25 to 50 ng of Fab'/embryo, the delay in FN-fibril formation increased to 17–24 h. In controls with injection buffer (10% MBS), or nonimmune Fab' at 50 ng/embryo, a normal sequence of FN-fibril formation occurred.

An example of the defective extracellular matrix of FN obtained after injection of 50 ng Fab' fragments is presented in Fig. 4. Observations at early, mid-, or late blastula (stages 5, 6, 7) showed the same results, i.e., no fluorescent fibrils were detected on the inner surface of the blastocoele roof (Fig. 4 *A*). It was not until the early gastrula stage (stage 8a) that the first fibrils appeared at the periphery of cells (Fig. 4 *B*). Their distribution was identical to that observed in control embryos at the much earlier midblastula stage (stage 6) (compare Figs. 4 *B* and 2 *C*). Later, FN-fibril formation was restored, but with a delay of ~24 h in forming patterns that matched those of control embryos (Fig. 4 *C*).

Antibodies were also injected at the two-cell stage. Fab' antibodies (25 ng) were introduced into one blastomere in the presence of RLDx. The noninjected blastomere provides an internal control for each injection, and the labeled dextran permits identification of the injected blastomere progeny. For example, Fig. 5 *A* shows an external view of a mid-
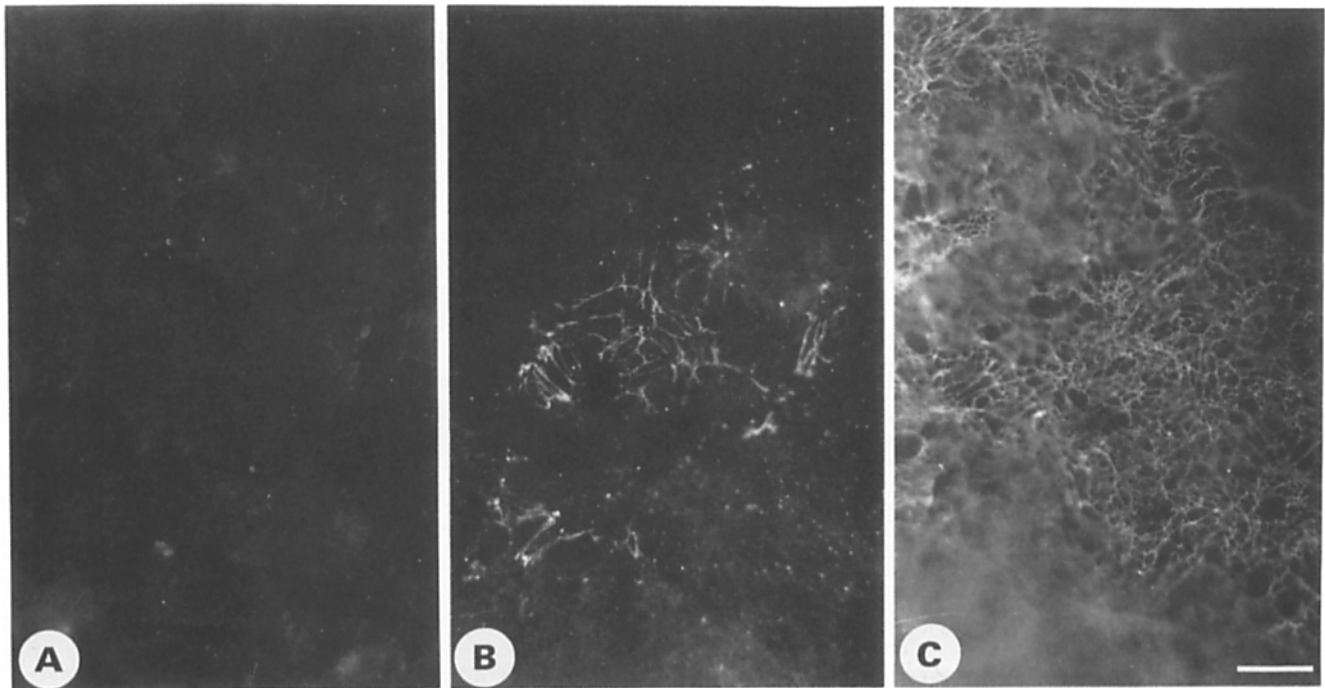
Figure 4. Inhibition of FN-fibril formation by Fab' to cytoplasmic domain of integrin β_1 subunit. Monovalent antibodies against cytoplasmic domain of integrin β_1 subunit (50 ng) were injected into uncleaved embryo. Embryos were maintained at 18°C, then dissected after the indicated times of incubation. Immunodetection of FN was then done on whole mounts of the bastocoele roof. (*A*) 24 h after injection, this embryo has reached the late blastula stage (stage 7), but no staining for FN can be detected. (*B*) The first FN fibrils appear at the early gastrula stage (stage 8a). They are generally distributed around the periphery of cells. Their pattern is comparable to normal FN-fibril formation observed at early blastula stages (17 h earlier). (*C*) Control involving injection of a mixture of anti-β_1 COOH antibodies and purified amphibian integrin β_1. Immunodetection of FN was performed in the same time as in *B*. A well-developed FN meshwork is revealed. Bar, 5 μm.
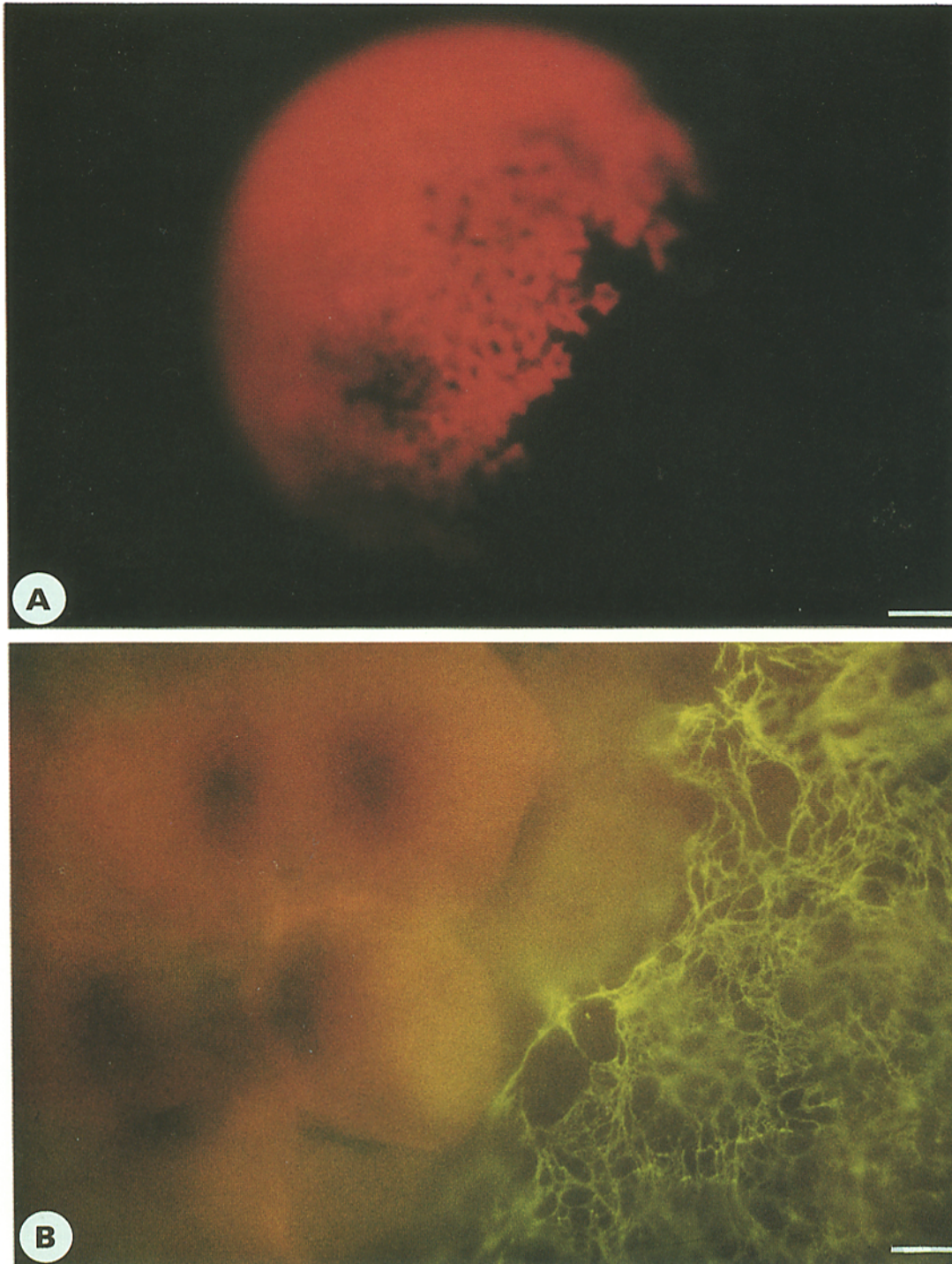
Figure 5. Inhibition of FN-fibril formation in selected blastomeres by Fab′ to cytoplasmic domain of integrin β_1 subunit. Monovalent antibodies against the cytoplasmic domain of integrin β_1 (25 ng/blastomere) were injected into one blastomere at the two-cell stage accompanied by lysinated rhodamine dextran (RLDx, 50 mg/ml). The embryos were cultured at 18°C. At the indicated stages, the blastocoele roof was dissected, fixed, and immunofluorescent detection of FN performed. (*A*) External view of living early blastula embryo (stage 6). Under rhodamine illumination, progeny of the injected blastomere could easily be determined. (*B*) Whole mounts of similar embryo as *A* when it reached the late blastula stage (stage 7). FN labeling visualized with fluorescein-specific optics shows absence of FN from the surfaces of rhodamine-labeled cells, while it was present in fibrillar arrays on the surface of unlabeled cells. Bars: (*A*) 0.13 mm; (*B*) 2.5 μm.

blastula stage (stage 6). It was injected at the two-cell stage with RLDx and anti-β_1 COOH Fab′ (25 ng). The cells divided normally and progeny of the injected blastomere was clearly identifiable. When such embryos reached the late

blastula stage (stage 7), the blastocoele roofs were dissected, fixed, and submitted to immunodetection of FN. Rhodamine-labeled cells lacked FN-fibrils on their inner cell surface. In the area of contact between labeled and unlabeled blasto-

meres, the surface of rhodamine-labeled cells was devoid of FN fibrils while neighboring unlabeled cells exhibited normal extracellular fibrils (Fig. 5 B). The same results are obtained at later stages of development. Treated cells lacking FN fibrils were surrounded by extracellular matrix-containing FN.

To assess the specificity of this inhibition of FN-fibril formation, we have performed five types of control experiments: (a) injection of nonimmune IgG of rabbit, mouse or human; (b) RLDx alone; (c) RLDx plus nonimmune Fab'; (d) an absorption experiment with RLDx and Fab' preincubated with amphibian integrin β_1 subunit; (e) RLDx plus Fab' mixed with the synthetic peptide used for immunizations. Injections of various nonimmune IgG as well as RLDx alone or Fab' preincubated with purified amphibian integrin β_1 subunit had no deleterious effects on fibrillogenesis. Finally, we attempted to block the activity of Fab' by mixing them with the synthetic peptide that was used as antigen; this probe itself produced a marked delay of the cleavage of labeled cells, whereas progeny of uninjected blastomere divided normally.

Experiments performed with exogenous iodinated or fluoresceinated FN confirmed the above findings. First, 100 ng of anti-β_1 COOH Fab' were injected intracellularly into fertilized embryos. When injected embryos reached the early blastula stage (stage 5), a solution of 50 μg/ml (final amount, 10 ng) of iodinated or fluoresceinated FN was injected into the blastocoele. 18 h later, when control embryos reached the early gastrula stage (stage 8a) radioactive binding data and immunofluorescence observations revealed that exogenous FN was not detectably bound at the inner ectodermal cell surface of treated embryos (not shown).

Development of Embryos Intracellularly Injected with Antibodies to Cytoplasmic Domain of Integrin β_1 Subunit

Because injected embryos showed striking defects in FN-fibril formation, and because these fibrils provide a substrate for mesodermal cell migration (Boucaut et al., 1984a,b; Darribère et al., 1988), we also studied the later development of injected living embryos.

Comparisons of the timing of gastrulation in uninjected embryos and in eggs injected with anti-β_1 Fab' at different concentrations showed that in all cases, the formation of the dorsal lip of the blastopore was unaffected. Nevertheless various degrees of subsequent defects in gastrulation were observed. The results obtained are summarized in Table II. They indicate that there is a variation not only in the percentage of embryos with defects but also in the severity of the gastrulation arrest. The three types of defects described are in agreement with those obtained when antibodies against avian integrins were injected into the blastocoele, i.e., extracellularly (Darribère et al., 1988). With low antibody concentrations (10 ng/embryo), no significant perturbation of gastrulation could be observed. With 25 ng of antibodies per embryo, which produces a delay of 7–8 h in FN-fibril formation (Fig. 4, A and B), effects ranging from a slight delay to complete inhibition of gastrulation were obtained (Table II). More severely affected embryos were obtained in response to injections of 100 ng of antibodies, 83% of the embryos showed complete inhibition of gastrulation (Table II).

A series of injections were performed to introduce anti-

Table II. Effects of Intracellular Injection of Antibodies to Cytoplasmic Domain of Integrin β_1 Subunit

Development	Amount of antibodies			
	10	25	50	100
		ng/embryo		
		%		
Normal	100	10	2	–
Type I	–	2	70	83
Type II	–	63	12	1
Type III	–	12	2	–
Necrosis	–	13	14	16

Various amounts of monovalent antibodies to the cytoplasmic domain of integrin β_1 were injected into fertilized eggs. They were allowed to develop for 60 h at 18°C and then observed. At this point (stage 14) normal embryos begin neurulation. Percent of defective embryos were pooled from results obtained in four independent experiments containing 25 embryos. *Type I*, no gastrulation, extensive convolution of the animal hemisphere. *Type II*, no gastrulation, slight convolution of the animal hemisphere. *Type III*, delay in gastrulation, small circular yolk plug.

bodies preferentially into lateral blastomeres of developing embryos. The results showed that intracytoplasmic injections of anti-β_1 COOH Fab' produced lateral defects of FN-fibril formation, which could be well correlated with a delay in mesodermal cell migration. The most obvious and consistent defects were found when neural folds failed to form properly. This was demonstrated by coinjecting 100 ng of antibodies and RLDx into one blastomere at the two-cell stage, in which case one-half of the neurula is derived from the injected cell. The labeled half of the embryo failed to form neural folds whereas the contralateral control was normal (Fig. 6).

Discussion

In this report, we provide new evidence that the integrin β_1 subunit and the Arg-Gly-Asp recognition signal are essential for FN-fibril formation in vivo. Our major results are: (a) interspecies injection of exogenous FN into the blastocoele of living embryos still permits assembly into extracellular fibrils with the same time-dependent pattern as endogenous FN; (b) fibril formation from exogenous FN is prevented by both RGD-containing peptides and polyclonal Fab' to the whole β_1 subunit of amphibian integrin applied extracellularly; (c) intracytoplasmic injections of antibodies to the carboxy-terminal domain of β_1 produce a reversible matrix assembly defect; and (d) defective mesodermal cell migration occurs in areas of embryos devoid of FN-fibrils as a result of antibody injection.

Before testing the ability of blastomeres to assemble exogenous FN into fibrils, we reexamined the kinetics of for-
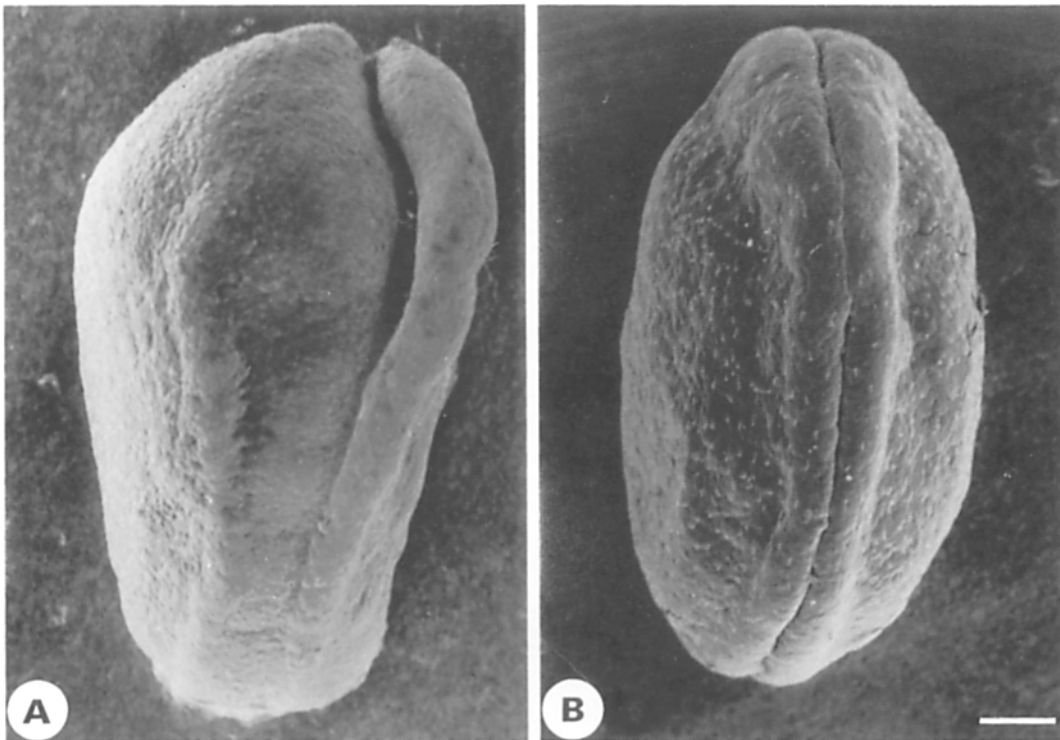
Figure 6. Embryos injected with antibodies to cytoplasmic domain of integrin β_1 subunit. (*A*) Embryo at the two-cell stage was injected into the left blastomere with 100 ng of anti-β_1 COOH Fab'. At the time of observation 72 h later, the left neural fold is defective. (*B*) Control experiment. Anti-β_1 COOH Fab' were preincubated with amphibian integrin β_1 and injected into the left blastomere at the two-cell stage. Neurulation occurs normally. Bar, 0.3 mm.

mation of the FN-containing extracellular matrix. Careful examination shows a reproducible, progressive, spatiotemporal organization of FN fibrils. In early blastulae (stage 5), they appear first at the periphery of blastomeres. Later on (by stage 7), they also appear over the surface of the central portion of cells. This pattern of formation is consistent with results described for cultured fibroblasts. For example, when fibroblasts were seeded onto FN-substrata, they apparently organized FN into fibrils from the periphery to the center of cells (Avnur and Geiger, 1981; Grinnell, 1986). This result is also in agreement with our recent observations of FN-fibril formation in *Rana pipiens* embryos (Johnson et al., 1990).

When exogenous labeled FNs were injected into the blastocoele at the initiation of FN-fibril formation, we were able to observe matrix assembly. Iodinated and fluoresceinated FNs were incorporated into fibrils in a slow time-dependent manner. The stage-dependent kinetics of FITC-FN assembly into fibrils is comparable with that for endogenous FN-fibril formation. It should be noted that fibril formation from exogenous FN is specific and saturable, because an excess of unlabeled FN prevents matrix assembly of labeled FN. Furthermore, on FN fibrils transferred to slides, no exogenous labeled FN is assembled into fibrils. This last observation suggests that in our experimental system, preexisting FN fibrils do not act as a primer for spontaneous exogenous FN fibrillogenesis and also that a direct cell surface interaction is needed. These results in vivo are generally consistent with those found using fibroblast model systems (McDonald, 1988), although this system displays a stage-specific regulation of FN assembly. Interestingly, we also find that in living

embryos, endodermal blastomeres are unable to organize FN into fibrils even in the presence of excess labeled FN. This result indicates cell-type specificity of matrix assembly.

The experimental system developed here thus provides a useful model for studies of FN-fibril formation in vivo. We have used this in vivo system to evaluate whether the integrin β_1 subunit is essential for FN-fibril formation.

We demonstrated that the assembly of exogenous FN by animal blastomeres is prevented both by RGD-containing peptides and by antibodies to the amphibian β_1 subunit. The simplest interpretation of these results is that FN-fibril formation in vivo requires the extracellular domain of integrin β_1 subunit. Moreover, the sensitivity to inhibition by an RGD-containing peptide suggests that the FN receptor (integrin VLA-5) is the molecule responsible, although the concurrent involvement of a FN-binding vitronectin receptor cannot be formally excluded. This is, to our knowledge, the first report that FN matrix assembly can be inhibited by RGD-containing peptides. Previous work with fibroblast model systems in vitro indicates that high levels of the large cell-adhesive domain of FN will partially inhibit assembly (McDonald et al., 1988), but the sensitivity to inhibition found in the present study was unexpectedly high. In fibroblasts in vitro, the non–cell-adhesive amino-terminal domain appears to play a particularly central role in FN binding to the postulated matrix assembly receptor. It is of interest that in similar experiments with human fibroblasts in vitro that have already initiated matrix assembly, RGD peptides do not cause any inhibition of FN binding to the matrix assembly receptor (Fogerty, F. J., and D. F. Mosher, personal communication),

suggesting that our in vivo system may use somewhat different mechanism. In this amphibian developmental system, the data suggest that the integrin (FN-receptor) system mediates not only the process of cell attachment and migration (Boucaut et al., 1984a,b; Nakatsuji and Johnson, 1984b; Darribère et al., 1988) but also mediates or modulates FN-fibril formation during extracellular matrix assembly. In fact, some of previously reported inhibition of gastrulation in this system after injection of synthetic peptides (Boucaut et al., 1984b) may involve inhibition of assembly of the fibrils necessary for mesodermal cell migration.

Intracellular injection of monovalent antibodies to the cytoplasmic domain of integrin β_1 subunit into fertilized eggs or into blastomeres results in direct inhibition of FN-fibril formation only in their progeny. Injections of IgG fractions from rabbit, mouse, and human sera or Fab' preadsorbed with amphibian integrin β_1 subunit have not produced such changes in FN-fibril formation. These controls indicate, respectively, that cytotoxic or nonspecific effects of antibodies are not responsible for the defects observed. These results represent the first direct injection evidence that the cytoplasmic domain of an integrin is essential for its function in vivo. One obvious mechanism by which these antibodies could inhibit FN matrix assembly is by disrupting integrin β_1 subunit interactions with the cytoskeleton. There is morphological and functional evidence that FN fib. ils may interact with the microfilament system via the fibronectin receptor. In cultured fibroblasts, microfilament bundles, α-actinin, and talin at least partially co-align with clusters of FN receptor (β_1 integrins; Chen et al., 1985; Damsky et al., 1985). Disruption of FN-receptor interactions using synthetic peptides or monoclonal antibodies against the α_5 or β_1 subunits of the FN receptor results in decreased microfilament bundle organization (Chen et al., 1986; Akiyama et al., 1989a). Biochemical observations suggest that there is direct interaction between detergent-solubilized chick integrin and talin, albeit with a low affinity (Horwitz et al., 1986). This binding is inhibited by a peptide corresponding to the cytoplasmic domain of integrin β subunit (Buck and Horwitz, 1987). Another possibility is that antibodies prevent the noncovalent association of β_1 with the α_5 subunit, which is needed for ligand specificity.

References

Akiyama, S. K., S. S. Yamada, W.-T. Chen, and K. M. Yamada. 1989a. Analysis of fibronectin receptor function with monoclonal antibodies: roles in cell adhesion, migration, matrix assembly, and cytoskeletal organization. J. Cell Biol. 109:863–875.

Akiyama, S. K., K. Nagata, and K. M. Yamada. 1989b. Cell surface receptors for extracellular matrix proteins. Biochim. Biophys. Acta. In press.

Anderson, D. C., and T. A. Springer. 1987. Leucocyte adhesion deficiency: an inherited defect in the Mac-1, LFA-1, and p150,95 glycoproteins. Annu. Rev. Med. 38:175–194.

Argraves, W. S., S. Suzuki, M. Arai, K. Thompson, M. D. Pierschbacher, and E. Ruoslahti. 1987. Amino acid sequence of the human fibronectin receptor. J. Cell Biol. 105:1183–1190.

Avnur, Z., and B. Geiger. 1981. The removal of extracellular fibronectin from areas of cell-substrate contact. Cell. 25:121–132.

Barth, L. G., and L. J. Barth. 1959. Differentiation of cells of the Rana pipiens gastrula in unconditioned medium. J. Embryol. Exp. Morph. 7:210–222.

Boucaut, J. C., and T. Darribère. 1983. Fibronectin in early amphibian embryo: migrating mesodermal cells contact fibronectin established prior to gastrulation. Cell Tissue Res. 234:135–145.

Boucaut, J. C., T. Darribère, H. Boulekbache and J. P. Thiery. 1984a. Prevention of gastrulation but not neurulation by antibodies to fibronectin in amphibian embryos. Nature (Lond.). 307:364–367.

Boucaut, J. C., T. Darribère, T. J. Poole, H. Aoyama, K. M. Yamada, and J. P. Thiery. 1984b. Biologically active synthetic peptides as probe of embryonic development: a competitive peptide inhibitor of fibronectin function inhibits gastrulation in amphibian and neural crest cell migration in avian embryos. J. Cell Biol. 99:1822–1830.

Brackenbury, R., J. P. Thiery, U. Ruthishauser, and G. M. Edelman. 1977. Adhesion among neural crest cells of the chick embryo. I. An immunological assay for molecules involved in cell-cell binding. J. Biol. Chem. 252:6835–6840.

Bolton, A. E., and W. M. Hunter. 1973. The labeling of proteins to high specific radioactivities by conjugation to a ^{125}I containing acylating agent. Biochem. J. 133:529–539.

Buck, C. A., and A. F. Horwitz. 1987. Integrin, a transmembrane glycoprotein complex mediating cell-substratum adhesion. J. Cell Sci. Suppl. 8:231–250.

Chen, W.-T., E. Hasegawa, T. Hasegawa, C. Weinstock, and K. M. Yamada. 1985. Development of cell surface linkage complexes in cultured fibroblasts. J. Cell Biol. 100:1103–1114.

Chen, W.-T., J. Wang, T. Hasegawa, S. S. Yamada, and K. M. Yamada. 1986. Regulation of fibronectin receptor distribution by transformation, exogenous fibronectin, and synthetic peptides. J. Cell Biol. 103:1649–1661.

Cheresh, D. A., J. W. Smith, H. M. Cooper, and V. Quaranta. 1989. A novel vitronectin receptor integrin ($\alpha_v\beta_x$) is responsible for distinct adhesive properties of carcinoma cells. Cell. 57:59–69.

Chernousov, M. A., M. L. Metsis, and V. E. Koteliansky. 1985. Studies of extracellular fibronectin matrix formation with fluoresceinated fibronectin and fibronectin fragments. FEBS (Fed. Eur. Biochem. Soc.) Lett. 183:365–369.

Damsky, C. H., K. A. Knudsen, C. A. Bradley, and A. F. Horwitz. 1985. Distribution of the cell substratum attachment (CSAT) antigen on myogenic and fibroblastic cells in culture. J. Cell Biol. 100:1528–1539.

Darribère, T., D. Boucher, J. C. Lacroix, and J. C. Boucaut. 1984. Fibronectin synthesis during oogenesis and early development in amphibian Pleurodeles waltlii. Cell Differ. 14:7–14.

Darribère, T., H. Boulekbache, D. L. Shi, and J. C. Boucaut. 1985. Immunoelectron microscopic study of fibronectin in gastrulating amphibian embryos. Cell Tissue Res. 239:75–80.

Darribère, T., K. M. Yamada, K. E. Johnson, and J. C. Boucaut. 1988. The 140-kD fibronectin receptor complex is required for mesodermal cell adhesion during gastrulation in the amphibian Pleurodeles waltlii. Dev. Biol. 126:182–194.

DeSimone, D., and R. O. Hynes. 1988. Xenopus laevis integrins: structural conservation and evolutionary divergence of integrin β subunits. J. Biol. Chem. 263:5333–5340.

Engvall, E., and E. Ruoslahti. 1977. Binding of soluble form of fibroblast surface protein, fibronectin, to collagen. Int. J. Cancer. 20:1–15.

Gallien, L., and M. Durocher. 1957. Table chronologique du développement chez Pleurodeles waltlii Michah. Bull. Biol. Fr. Belg. 91:97–114.

Gimlich, R. L., and J. Cooke. 1983. Cell lineage and the induction of second nervous system in amphibian development. Nature (Lond.). 306:471–473.

Ginsberg, M. H., J. C. Loftus, J. Ryckwaert, M. D. Pierschbacher, R. Pytela, E. Rouslahti, and E. F. Plow. 1988. Immunological and amino-terminal sequence comparison of two cytoadhesins indicates they contain similar or identical beta subunits and distinct alpha subunits. J. Biol. Chem. 262:5437–5440.

Grinnel, F. 1986. Focal adhesion sites and the removal of substratum-bound fibronectin. J. Cell Biol. 103:2697–2706.

Hasegawa, T., E. Hasegawa, W.-T. Chen, and K. M. Yamada. 1985. Characterization of a membrane-associated glycoprotein complex implicated in cell adhesion to fibronectin. J. Cell. Biochem. 287:307–318.

Homandberg, G. A., and J. W. Erickson. 1986. Model of fibronectin tertiary structure based on studies of interaction between fragments. Biochemistry. 25:6917–6925.

Horwitz, A. F., K. Duggan, R. Gregges, C. Decker, and C. Buck. 1985. The cell substrate attachment CSAT antigen has properties of a receptor for laminin and fibronectin. J. Cell Biol. 101:2134–2144.

Horwitz, A. F., K. Duggan, C. Buck, M. C. Beckerle, and K. Burridge. 1986. Interaction of plasma membrane fibronectin receptor with talin. A transmembrane linkage. Nature (Lond.). 320:531–532.

Hynes, R. O. 1978. Integrins: a family of cell surface receptors. *Cell.* 48:549-554.

Johnson, K. E. 1981. Normal frog gastrula extracellular materials serve as a substratum for normal and hybrid cell adhesion when covalently coupled with CNBr-activated sepharose beads. *Cell Differ.* 10:47-55.

Johnson, K. E. 1985. Frog gastrula cells adhere to fibronectin-Sepharose beads. *In* Molecular Determinants of Animal Form. G. M. Edelman, editor. Alan R. Liss, Inc., New York. 291-306.

Johnson, K. E., T. Darribère, and J. C. Boucaut. 1990. Fibronectin and integrin in normal *Rana pipiens* gastrulae and in arrested hybrid gastrulae *Rana pipiens* ♀ × *Rana esculenta* ♂. *Dev. Biol.* 137:86-99.

Lee, G., R. O. Hynes, and M. Kirschner. 1984. Temporal and spatial regulation of fibronectin in early *Xenopus* development. *Cell.* 36:729-740.

Marcantonio, E. E., and R. O. Hynes. 1988. Antibodies to the conserved cytoplasmic domain of the integrin β_1 subunit react with proteins in vertebrates, invertebrates, and fungi. *J. Cell Biol.* 106:1765-1772.

McDonald, J. A. 1988. Extracellular matrix assembly. *Annu. Rev. Biochem.* 57:375-413.

McDonald, J. A., B. J. Quade, T. J. Broekelmann, R. LaChance, K. Forsman, E. Hasegawa, and S. K. Akiyama. 1987. Fibronectin's cell-adhesive domain and an amino-terminal matrix assembly domain participate in its assembly into fibroblasts pericellular matrix. *J. Biol. Chem.* 262:2957-2967.

McKeown-Longo, P. J., and D. F. Mosher. 1983. Binding of plasma fibronectin to cell layers of human skin fibroblasts. *J. Cell Biol.* 97:466-472.

McKeown-Longo, P. J., and D. F. Mosher. 1984. Mechanism of formation of disulfide-bonded multimers of plasma fibronectin in cell layers of cultured human fibroblasts. *J. Biol. Chem.* 259:12210-12215.

McKeown-Longo, P. J., and D. F. Mosher. 1985. Interaction of the 70,000-mol-wt amino-terminal fragment of fibronectin with the matrix-assembly receptor of fibroblasts. *J. Cell Biol.* 100:364-374.

McKeown-Longo, P. J., and D. F. Mosher. 1988. The assembly of the fibronectin matrix in cultured human fibroblast cells. *In* Fibronectin. D. F. Mosher, editor. Academic Press Inc., New York. 163-179.

Nakatsuji, N., and K. E. Johnson. 1984a. Experimental manipulation of a contact guidance system in amphibian gastrulation by mechanical tension. *Nature (Lond.).* 307:453-455.

Nakatsuji, N., and K. E. Johnson. 1984b. Ectodermal fragments from normal frog gastrulae condition substrata to support normal and hybrid mesodermal cell migration *in vitro*. *J. Cell Sci.* 68:49-67.

Nakatsuji, N., A. C. Gould, and K. E. Johnson. 1982. Movements and guidance of migrating mesodermal cells in *Ambystoma maculatum* gastrulae. *J. Cell Sci.* 56:207-222.

Nakatsuji, N., M. A. Smolira, and C. C. Wylie. 1985. Fibronectin visualized by scanning electron microscopy immunocytochemistry on the substratum for cell migration in *Xenopus laevis* gastrula. *Dev. Biol.* 107:264-268.

Pierschbacher, M. D., and E. Ruoslahti. 1984. Variants of the cell recognition site of fibronectin that retain attachment-promoting activity. *Proc. Natl. Acad. Sci. USA.* 81:5985-5988.

Peters, D. M. P., and D. F. Mosher. 1987. Localization of cell surface sites involved in fibronectin fibrillogenesis. *J. Cell Biol.* 104:121-130.

Pytela, R., M. D. Pierschbacher, and E. Ruoslahti. 1985. Identification and isolation of a 140 kD cell surface glycoprotein with properties expected of a fibronectin receptor. *Cell.* 40:191-198.

Rapraeger, A. M., M. Jalkenen, and M. Bernfield. 1986. Cell surface proteoglycan associates with the cytoskeleton at the basolateral cell surface of mouse mammary epithelial cells. *J. Cell Biol.* 103:2683-2696.

Roman, J., R. M. LaChance, T. J. Broekelmann, C. J, Kennedy, E. A. Wayner, W. G. Carter, and J. A. McDonald. 1989. The fibronectin receptor is organized by extracellular matrix fibronectin: implications for oncogenic transformation and for cell recognition of fibronectin matrices. *J. Cell Biol.* 108:2529-2543.

Ruoslahti, E. 1988. Fibronectin and its receptors. *Annu. Rev. Biochem.* 57:375-413.

Shi, D. L., T. Darribère, K. E. Johnson, and J. C. Boucaut. 1989. Initiation of mesodermal cell migration and spreading relative to gastrulation in the urodele amphibian *Pleurodeles waltl.* *Development (Camb.).* 105:351-363.

Spiegel, S., K. M. Yamada, B. E. Hom, J. Moss, and P. M. Fishman. 1985. Fluorescent gangliosides as probes for the retention and organization of fibronectin by ganglioside deficient mouse cells. *J. Cell Biol.* 100:721-726.

Spiegel, S., K. M. Yamada, B. E. Hom, J. Moss, and P. M. Fishman. 1986. Fibrillar organization of fibronectin is expressed coordinately with cell surface gangliosides in a variant murine fibroblast. *J. Cell Biol.* 102:1898-1906.

Suzuki, S., W. S. Argraves, H. Azai, L. R. Languino, M. D. Pierschbacher, and E. Ruoslahti. 1987. Amino-acid sequence of the vitronectin receptor α subunit and comparative expression of adhesion receptor mRNA. *J. Biol. Chem.* 262:14080-14085.

Tamkun, J. W., D. W. De Simone, D. Fonda, R. S. Patel, C. A. Buck, A. F. Horwitz, and R. O. Hynes. 1986. Structure of integrin, a glycoprotein involved in the transmembrane linkage between fibronectin and actin. *Cell.* 42:271-282.

Wayner, E. A., W. G. Carter, R. S. Piotrowicz, and T. J. Kuniki. 1988. The function of multiple extracellular matrix receptors in mediating cell adhesion to extracellular matrix: preparation of monoclonal antibodies to the fibronectin receptor that specifically inhibit cell adhesion to fibronectin and react with platelet glycoprotein Ic-IIa. *J. Cell Biol.* 107:1881-1891.

Walker, L. E., T. A. Ketler, R. A. Houghten, G. Schulz, A. Chersi, and R. A. Reisfeld. 1985. Human major histocompatibility complex class I antigens: residues 61-83 of the HLA-B7 heavy chain specificity on alloreactive site. *Proc. Natl. Acad. Sci. USA.* 82:539-542.

Woods, A., J. R. Couchman, S. Johansson, and M. Hook. 1986. Adhesion and cytoskeletal organization of fibroblasts in response to fibronectin fragments. *EMBO (Eur. Mol. Biol. Organ.) J.* 5:665-670.

Yamada, K. M., and D. W. Kennedy. 1984. Dualistic nature of adhesive protein function: fibronectin and its biologically active peptide fragments can auto-inhibit fibronectin function. *J. Cell Biol.* 99:29-36.