In Vivo Analyses of Integrin $\beta_1$ Subunit Function in Fibronectin Matrix Assembly

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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 $\beta_1$ subunit of the amphibian FN receptor and the cytoplasmic domain of $\beta_1$ 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 $\beta_1$ subunit. Furthermore, when injected intracellularly into uncleaved embryos or into selected blastomeres, antibodies to the cytoplasmic domain of integrin $\beta_1$ 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 $\beta_1$ 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 substra- tum. 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 immuno-
logological analysis have established the existence of two multi-
gene families of glycoproteins detected throughout the animal kingdom corresponding to integrin $\alpha$ or $\beta$ subunits (Tamkun et al., 1986; Argraves et al., 1987; Suzuki et al., 1987; DeSi-
mone and Hynes, 1988; Marcantonio and Hynes, 1988; Cheresch et al., 1989). These families are divided into distin-
gt subfamilies, in which members share a common $\beta$ sub-
unit that is noncovalently combined with a unique $\alpha$ subunit to form $\alpha/\beta$ heterodimers. The $\alpha$ 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 (Gins-
berg et al., 1988), and the very late antigens (VLAs) (Hor-
witz et al., 1985). The integrin VLA family consists of heterodimers of six different $\alpha$ subunits each associated with a common $\beta$ 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 $\alpha_5\beta_1$, $\alpha_6\beta_1$, and $\alpha_v\beta_3$. 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; Ruos-
lahvi, 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

1. Abbreviations used in this paper: FN, fibronectin; MBS, 10% modified Barth solution; RLDx, lysinated rhodamine dextran; VLA, very late anti-
gen.
binds to FN has been isolated (Rapraeger et al., 1986). Fibrillar and both antibodies to the whole molecule or the cytoplasmic domain of integrin VLA-5 may mediate these interactions. We initiated experiments that the ~ subunit found in the integrin VLA-5 may mediate FN-fibril formation by cultured fibroblasts (McDonald 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, the/~ subunit (extracellularly), and the cytoplasmic do-

trix deposition and attachment of fibroblasts (Woods et al., 1984a, b, 1985). During gastrulation, these FN fibrils provide a substrate for mesodermal cell migration (Boucaut et al., 1984a,b; Nakatsuji and Johnson, 1984a,b). 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 blastocoelic roof (Boucaut and Darribere, 1983; Darribere 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 (Darribere 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 ~ 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 ~, 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 ~, subunit (extracellularly), and the cytoplasmic domain of ~ (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 Michiels 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 ~g/ml gentamycin (Gibco Laboratories, Grand Island, NY). Developmental stages of embryos were according to Gallien and Durocher (1975).

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 125I 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 acetate/bicarbonate buffer (pH 9) was incubated with FITC (200 ~g/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 FN's retained their biological activity in cell spreading assays described previously (Darribere et al., 1988).

Purification of Integrin ~, 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-~-glucopyranoside (OGP; Calbiochem-Behring Corp., San Diego, CA); 2 mM PMSE; 2 mM EGTA; 5 ~g/ml aprotinin, 0.5 ~g/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 mixed overnight at 4°C by end-over-end rotation with 10 ml packed volume of concanavalin A-Sepharose 4B conjugated to NF (Sigma Chemical Co.). Bound proteins were eluted with 0.25 M a-methyl-D-mannoside (Sigma Chemical Co.) in buffer. Fractions that react by immunoblotting with anti-avian integrin ~g were pooled and dialyzed against buffer 2 at 4°C. These were then incubated overnight with 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 ~, subunit of Pleurodeles waltl (anti-3,1) were raised in New Zealand white rabbits. Subcutaneous injection using 0.2 mg of amphibian integrin ~g 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 Biologique Francaise, France). Polyclonal antibodies against the cytoplasmic domain of integrin ~g, subunit (anti-3,1 COOH) were produced by immunization of a rabbit with a synthetic peptide with the sequence REFAKFEKEKMNAKWDGPIENLYKSSATTVNPKYEGK (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., 1983). This antibody immunoprecipitated the same human fibroblast integrins as an existing anti-~g monoclonal antibody (Akiyama et al., 1989a), and showed the same expected in Western immunoblots of fibroblast extracts. Production of rabbit IgG fractions of polyclonal antibodies to Ambystoma mexicanum plasma FN
The specificity of IgG fractions was evaluated by immunoprecipitation and 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 blastocoel. Iodinated FN, FITC-FN, or FITC-BSA were injected into early blastula blastocoel (stage 5) in the absence or presence of peptides, anti-β1 Fab', or preimmune antibodies. For injections, 200 nl of the blastocoel fluid was removed to prevent any overpressure during injection. Thereafter, 200 nl of a 0.005-1 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 into sterile MBS into animal (blastocoele roof, 2N) and vegetal halves (VP). After careful washes, labeled FN binding was examined in both halves by immune precipitation, counting radioactivity, or direct fluorescence.

Anti-β1 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 30 mg/ml of lysinated rhodamine dextran (RLDx) (Gilmich 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 blastocoel 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 blastocoel 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 blastocoel roof (Fig. 2 B). In midblastulae (stage 6), fibrils appeared all around the cell periphery. They also

**Metabolic Labeling, Immunoprecipitation, and Electrophoresis**

Embryos at midgastula stage (stage 10) were injected with 5 nl of [35S]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 MnCl2, 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 β1 subunits were performed with protein A-Sepharose 6-4B (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.

**Figure 1.** Specificity of anti-FN and anti-integrin β1 subunit IgG. (A) Immunoprecipitation of [35S]methionine-labeled proteins from *Pleurodeles waltl* midgastula 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 [35S]methionine-labeled proteins with 5 mg of protein A–Sepharose and 30 μg of anti-α-amphibian integrin β1 subunit IgG (lane 1) or IgG against the cytoplasmic domain of integrin β1 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 β1 subunit (lane 4). The autoradiogram indicates that both antibodies recognize the amphibian integrin β1 subunit of 100 kD. The faintly labeled polypeptides of 140 kD represent integrin α subunits. They are noncovalently associated with integrin β1 subunits and are coprecipitated with β1 subunits. Mr, molecular mass markers in kilodaltons.
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 **125I-FN binding was examined by immunoprecipitation and SDS-PAGE. Autoradiograms revealed that **125I-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.
Iodinated or fluoresceinated FN was injected into the blastocoel of early blastula (stage 5) in presence of either GRGDS or monovalent antibodies against integrin β1 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-β1 Fab', inhibited the binding of FN to the roof of the blastocoel. Indeed, no trace of radioactive FN could be detected in presence of GRGDS or anti-β1 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 P2).

Quantitative radioactivity binding data were obtained for blastocoel roof and vegetal hemisphere. The results confirm that both GRGDS and anti-β1 Fab' substantially inhibit the binding of ¹²⁵I-FN to blastomeres of the blastocoel roof. As shown in Fig. 3 B, the incorporation of ¹²⁵I-FN was minimal in the presence of GRGDS or anti-β1 Fab' when compared with control experiments performed with peptide P2 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-β1 Fab' (250 μg/ml). Conversely, FN-fibril formation occurred normally in the presence of collagen-binding peptide P2 (200 μg/ml) (data not shown).

Inhibition of Fibronectin-fibril Formation by Intracellular Injection of Antibodies to Cytoplasmic Domain of Integrin β1 Subunit

Since FN adhesion receptors have been proposed to have transmembrane functions, we examined whether an intracellular perturbation of β1 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 β1 Subunit

| Time of development | Morula 124 cell stage | Stages of development | Gastrula stage 8a |
|---------------------|-----------------------|-----------------------|------------------|
| h                   | 10-12                 | 14                    | 22               |
|                     |                       | 27                    | 31               |
| MBS                 | –                     | +                     | ++                |
| Fab' nonimmune      | (30 ng)               | –                     | +                 |
| Anti-β1 COOH        | (2.5 ng)              | –                     | +                 |
|                     | (10 ng)               | –                     | +                 |
|                     | (25 ng)               | –                     | –                 |
|                     | (50 ng)               | +                     | –                 |
|                     | (100 ng)              | –                     | –                 |

Various concentrations of anti-β1 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-β1 COOH, monovalent antibodies against the cytoplasmic domain of the integrin β1 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).

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 5. Inhibition of FN-fibril formation in selected blastomeres by Fab' to cytoplasmic domain of integrin β1 subunit. Monovalent antibodies against the cytoplasmic domain of integrin β1 (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.
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 β1 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 β1 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-β1 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).

Table II. Effects of Intracellular Injection of Antibodies to Cytoplasmic Domain of Integrin β1 Subunit

| Development | Amount of antibodies |
|-------------|----------------------|
|             | 10 ng/embryo         |
|             | 25 ng/embryo         |
|             | 50 ng/embryo         |
|             | 100 ng/embryo        |

|                  | %                  |
|------------------|--------------------|
| Normal           | 10                 |
|                  | 2                  |
|                  | 70                 |
|                  | 83                 |
| Type I           | 63                 |
|                  | 12                 |
|                  | 1                  |
| Type II          | 12                 |
|                  | 2                  |
|                  | 16                 |

Various amounts of monovalent antibodies to the cytoplasmic domain of integrin β1 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.

Discussion

In this report, we provide new evidence that the integrin β1 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 blastocoel 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 β1 subunit of amphibian integrin applied extracellularly; (c) intracytoplasmic injections of antibodies to the carboxy-terminal domain of β1 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-
formation 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 blastocoel 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 \( \beta_5 \) 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 \( \beta_5 \) subunit. The simplest interpretation of these results is that FN-fibril formation in vivo requires the extracellular domain of integrin \( \beta_5 \) 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).

Figure 6. Embryos injected with antibodies to cytoplasmic domain of integrin \( \beta_5 \) subunit. (A) Embryo at the two-cell stage was injected into the left blastomere with 100 ng of anti-\( \beta_5 \) COOH Fab'. At the time of observation 72 h later, the left neural fold is defective. (B) Control experiment. Anti-\( \beta_5 \) COOH Fab' were preincubated with amphibian integrin \( \beta_5 \) and injected into the left blastomere at the two-cell stage. Neurulation occurs normally. Bar, 0.3 mm.
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 (Bouc, et al., 1984; Nakatsuji and Johnson, 1984; Barri-bere 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 (Bouc, 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, subunit into fertilized eggs or into blastomers 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, 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, subunit interactions with the cytoskeleton. There is morphological and functional evidence that FN fibrils may interact with the microfilament system via the fibronectin receptor. In cultured fibroblasts, microfilament bundles, actin-talin and talin at least partially co-align with clusters of FN receptor (β, integrins; Chen et al., 1985; Damsky et al., 1985). Disruption of FN-receptor interactions using synthetic peptides or monoclonal antibodies against the α or β 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, with the α subunit, which is needed for ligand specificity.

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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. Biochem. 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.

Argawal, 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. 105:1183-1192.

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. 1989. Differentiation of cells of the Rana pipiens gastrula in unconditioned medium. J. Embryol. Exp. Morph. 7:210-222.

Barth, L. G., and L. J. Barth. 1989. Differentiation of cells of the Rana pipiens gastrula in unconditioned medium. J. Embryol. Exp. Morph. 7:210-222.

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

Bouc, J. C., T. Darribère, T. J. Poole, H. Aoyama, K. M. Yamada, and J. P. Thiery. 1984a. 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. P. Thiery, U. Rathibusher, and G. M. Eidelberg. 1977. Adhesion among neural crest cells of the chick embryo. I. An immunological assay for molecules involved in cell-cell binding. J. Biol. Chem. 252:6833-6840.

Bolton, A. E., and W. M. Hunter. 1973. The labeling of proteins to high specific radioactivities by conjugation to a[14C] containing acylating agent. Biochem. J. 133:529-539.

Buck, A. C., and A. F. Horwitz. 1987. Integrin, a transmembrane glycoprotein complex mediating cell-substrate 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. 101:1103-1114.

Chen, W.-T., T. 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. Quimanta. 1989. A novel fibronectin receptor integrin (α5β1) is responsible for distinct adhesive properties of carcinoma cells. Cell. 57:59-69.

Chernousov, M. A., M. L. Messis, and V. E. Koteliansky. 1985. Studies of extracellular fibronectin matrix formation with fluoresceinated fibronectin and fibronectin fragments. FEBS (Fed. Eur. Biochem. Soc.) Lett. 185:365-369.

Damsky, C. H., K. A. Knudsen, C. A. Bradley, and A. F. Horwitz. 1985. Identification of the cell substrate 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 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. Hyne. 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. Durroux. 1957. Table chronologique du dé veloppement 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. Lotus, J. Ryckwaert, M. D. Pierschbacher, R. Pytel, E. Ruoslahti, and E. F. Pow. 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.

Grinell, F. 1986. Focal adhesion sites and the removal of substrate-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.
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.

Maccarone, E. E., and R. O. Hynes. 1988. Antibodies to the conserved cytoplasmic domain of the integrin β1 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. Brockelmann, R. LaChance, K. Forsman, E. Hasegawa, and S. K. Aktyama. 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 conserved transmembrane linkage between fibronectin and actin. 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.

Pytel, 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. Jalken, 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. Brockelmann, 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.