Fibronectin fibril growth in the extracellular matrix of the Xenopus embryo

Rudolf Winklbauer* and Cornelia Stoltz
Max-Planck-Institut für Entwicklungsbiologie, Spemannstrasse 35, 72076 Tübingen, Germany
*Author for correspondence at present address: Universität zu Köln, Zoologisches Institut, Weyertal 119, 50931 Köln, Germany

SUMMARY

We show that the mechanism of fibronectin fibril formation on the blastocoel roof of the Xenopus embryo is comparable to that in other systems. Fibril assembly is inhibited by RGD peptide, by an amino-terminal fragment of fibronectin, and by cytochalasin B. When added exogenously, intact fibronectin, but not a 110 kDa cell binding fragment of fibronectin, is incorporated into fibrils. Thus, the blastocoel roof of Xenopus represents a valid model system for the study of fibronectin fibril formation in situ. Moreover, we show that fibril formation can be induced experimentally in this system. Examination of fibril elongation by double-labelling experiments reveals that individual, unbranched fibronectin fibrils grow only at one end, i.e. in a unipolar fashion. The rate of elongation is 4.7 μm/min. Most fibrils grow only for a short time, and the increase in total fibril length per cell is driven by the repeated initiation of new fibrils. Assembly of fibronectin into fibrils precedes cross-linking of fibronectin into multimers in this system.

Key words: fibronectin, extracellular matrix, Xenopus, embryo, matrix assembly

INTRODUCTION

The adhesive glycoprotein fibronectin (FN) mediates a number of important biological functions including cell adhesion and migration (Mosher, 1989; Hynes, 1990). FN is built from three types of repeating structural units, designated type I, II and III modules (Fig. 1). A series of nine type I modules at the amino-terminal region is interrupted by two type II units. Three further type I modules are located at the carboxy terminus. The middle portion of the molecule is occupied by a variable (15-17) number of type III repeats and a variably spliced region (Hynes, 1985). The main cell binding site, which is formed by an RGDS sequence of amino acids, is localized within the 10th type III module (Pierschbacher et al., 1981; Ruoslahti and Pierschbacher, 1987). In blood plasma, FN occurs as a soluble dimer. Two subunits which are identical except for splice variations are covalently linked together via disulfide bonds at their carboxy terminus (Hynes, 1985). However, the main functional form of FN is that of an insoluble multimer. Consequently, in the extracellular matrix of embryonic and adult tissues, FN is usually present as fibrils (McDonald, 1988; Mosher, 1989; Hynes, 1990). The assembly of these fibrils is beginning to be understood in terms of which sites on the FN molecule are involved in fibrillogenesis (Fig. 1).

An absolute requirement for the incorporation of FN into fibrils is the presence of the amino-terminal domain comprising the first five type I modules (McDonald et al., 1987; Schwarzbaumer, 1991; Sottile et al., 1991; Ichihara-Tanaka et al., 1992). Interaction of the RGD cell binding site of FN with a cellular integrin receptor is usually found to be essential (McDonald et al., 1987; Akiyama et al., 1989; Darribere et al., 1990; Winklbauer and Nagel, 1991; Yost, 1992; Darribere et al., 1992). But incorporation into fibrils of FN fragments lacking this region has been reported (Schwarzbaumer, 1991). An additional site involved in fibril formation is located within the first type III repeat (Chernousov et al., 1991; Morla and Ruoslahti, 1992; Darribere et al., 1992; Morla et al., 1994). Finally, only dimeric FN is integrated into fibrils, and hence the carboxy-terminal cysteine residues involved in subunit crosslinking are essential (Schwarzbaumer, 1991). Whereas the amino-terminal domain (McKeown-Longo and Mosher, 1985; Limper et al., 1991) and the RGD site are potentially able to mediate the interaction of FN with the cell surface during fibril formation, the first type III repeat is suited to promote direct FN-FN interaction (Morla and Ruoslahti, 1992; Morla et al., 1994).

Despite the identification of regions on the FN molecule relevant for fibril assembly, little is known yet about the spatial and temporal organization of this process. Basic data on the mode of growth of FN fibrils are lacking. It is not known whether FN is added to a growing fibril at one end only, at both ends, or by intercalation over the whole length of the fibril. Also, the rate of fibril elongation, which may well constrain models of FN fibril assembly, has not yet been determined. In the present article, we address such questions. We ask how individual FN fibrils grow in situ, on the blastocoel roof of the Xenopus embryo.

In the amphibian embryo, a network of FN fibrils develops on the blastocoel roof around the onset of gastrulation (Nakatsuji and Johnson, 1983b; Komazaki, 1988; Johnson et al., 1990; Darribere et al., 1990). This FN matrix supports mesoderm migration during gastrulation (Boucaut et al., 1984a, b; Darribere et al., 1988). Besides mediating cell adhesion to the blastocoel roof, it seems to be involved in reg-
ulation of mesoderm cell motility (Winklbauer, 1990; Winklbauer and Selchow, 1992), and in guiding mesoderm toward the animal pole of the embryo (Nakatsuji and Johnson, 1983a; Shi et al., 1989; Winklbauer and Nagel, 1991). Furthermore, left-right axial information is probably linked to the FN matrix (Yost, 1992). The defined onset of intense fibril formation, and the accessibility to experimental manipulation make the blastocoel roof a suitable model system for an analysis of FN fibril assembly under in situ conditions (Darribere et al., 1990, 1992). In the present article, we show that individual FN fibrils on the blastocoel roof of the Xenopus gastrula grow only at one end, at a rate of 4.7 \mu m/min, and for a short time only. The mechanism of fibril formation is shown to be similar to that in other systems, and therefore our results on the rate and mode of FN fibril growth may be of general significance.

**MATERIALS AND METHODS**

**Embryos**

Embryos of *Xenopus laevis* were obtained from induced spawnings and were staged according to Nieuwkoop and Faber (1967). Operations were carried out in Modified Barth’s Solution (MBS; Winklbauer, 1988).

**Incubation of embryos**

Middle or late blastulae (stage 8-9) were transferred to a drop of MBS containing FN fragments, antibodies, peptides etc. at the desired concentrations. The blastocoel was punctured and flushed with incubation medium. Embryos were allowed to heal and to develop further in this medium. In this way, the concentration of test substances could be accurately controlled.

**FN and FN fragments**

Bovine plasma FN, a 110 kDa chymotryptic cell binding fragment, and a 30 kDa heparin binding fragment from near the carboxy terminus of FN (Fig. 1) were prepared as described (Winklbauer, 1988). A 30 kDa amino-terminal fragment (Fig. 1) was prepared by limited digestion with trypsin (Sekiguchi and Hakomori, 1980).

**Histochemistry**

For FN fibril staining, blastocoel roofs were fixed in 4% formaldehyde and processed for immuno-histochemistry using a rabbit polyclonal antibody to *Xenopus* plasma FN (Winklbauer and Nagel, 1991; Winklbauer, unpublished) and FITC-conjugated secondary antibody. F-actin was stained with rhodamine-phalloidin after fixation and permeabilization of blastocoel roofs in cytoskeleton buffer (Symons and Mitchison, 1991) containing 0.5% Triton X-100 and 4% paraformaldehyde.

**Quantitation of FN fibril formation**

Double-labelling experiments were performed as described. Fibrils were allowed to elongate for 0.5 to 8 minutes. The lengths of newly formed FN fibrils or fibril segments were measured from projections of photographic negatives. Data from two experiments were pooled, and a total of 841 fibrils were evaluated. For each time point, the 3 longest fibrils of the respective sample were selected, and the average length of these fibrils was determined. In a second approach, stage 9 blastocoel roofs were used to condition substratum for varying times. Fibril formation was induced by pressing blastocoel roof against the bottom of a culture dish, with the aid of a strip of coverslip resting at both ends on silicone grease. Explants were removed after 0.75 to 18 minutes. Conditioned substrata were stained with FN antibody, and the lengths of fibrils were measured from projections of photographic negatives. Data from 3 experiments were pooled, and a total of approximately 2,000 fibrils were evaluated. For each time point, the 3 longest fibrils were selected, and treated as above. The lengths of all the fibrils from isolated cell-sized patches were added to give the total fibril length per cell. The number of fibrils per patch (excluding dots) was counted. For each time point, the three patches with the highest total fibril length, and the three patches with the highest fibril number, were selected and treated as above. A total of 184 cell-sized isolated patches was evaluated (many more patches were observed, but only the largest were examined).

**RESULTS**

**The mechanism of FN fibril formation in *Xenopus* is similar to that in other systems**

In *Xenopus*, it was demonstrated by SEM that assembly of a matrix fibril network on the blastocoel roof begins shortly before the onset of gastrulation at stage 10 (Nakatsuji and Johnson, 1983b). To show that it is in fact FN fibrils which develop at that time, we visualized FN by whole-mount immunofluorescence staining with antibodies to *Xenopus* plasma FN. The onset of FN fibril formation is detected shortly before or at stage 10 (Fig. 2a,b). Within the next hour of development, fibril density increases dramatically, and most of the FN network is formed (Fig. 2c). Further increase in FN fibril density up to stage 11 is relatively poor (Fig. 2d).

To characterize our system with respect to the mechanism

![Fig. 1. Diagram of a FN monomer outlining the locations of sites involved in matrix assembly, and of FN fragments relevant to this work. The three repeating units of FN are depicted as follows: type I repeats, hatched; type II repeats, lightly dotted; type III repeats, open boxes. Differentially spliced type III repeats E III A and E III B (heavily dotted) and the variably spliced region (III CS) are indicated. Heparin-, fibrin- and collagen-binding domains are outlined, and the RGD cell binding site in the 10th type III repeat is indicated.](image-url)
of FN fibril assembly, we first confirmed that RGD peptides inhibit FN fibril formation on the blastocoel roof of *Xenopus*. Embryos were incubated from the mid-blastula to the mid-gastrula stage in the presence of GRGDSP peptide or GRGESP control peptide. Strong inhibition of fibril formation was already observed at 1 mg/ml of GRGDSP peptide (not shown). At 4 mg/ml, GRGDSP peptide completely inhibits FN fibril formation (Fig. 3a), whereas GRGESP control peptide has no effect on fibril assembly (Fig. 3b). These results are in agreement with those of Winklbauer and Nagel (1991) and Yost (1992). They demonstrate the involvement of the RGD site in fibril formation. However, the presence of the RGD sequence alone is not sufficient for the incorporation of FN into fibrils. A fluorescein-labelled 110 kDa fragment of FN which includes the RGD site, but not the amino- and carboxy-terminal domains (Fig. 1), is not incorporated into the FN fibril network when applied at 0.5 mg/ml to early gastrulae (Fig. 3c). In contrast, labelled intact FN at the same concentration is readily integrated (Fig. 3d). This points to additional sites being required for FN fibrillogenesis.

In other systems, the amino-terminal domain of FN is essential for fibril formation, in addition to the RGD site, and corresponding fragments can inhibit fibrillogenesis (McKeown-Longo and Mosher, 1985; McDonald et al., 1987). This also holds true for *Xenopus*. Incubation in 0.16-0.5 mg/ml of an amino-terminal 30 kDa fragment (Fig. 1) inhibits fibril formation on the blastocoel roof (Fig. 3e), as compared to buffer-incubated controls (e.g. see Fig. 5b). As expected, a 30 kDa heparin binding fragment from near the carboxy terminus (Fig. 1) is not inhibitory at 0.5 mg/ml (not shown).

In fibroblasts, FN fibrils are often found colocalized with actin filament bundles, and cytochalasin treatment, which interferes with actin polymerization, leads to disintegration of FN fibril networks (Ali and Hynes, 1977; Mautner and Hynes, 1977). In *Xenopus*, development of FN fibrils is inhibited by cytochalasin B (Fig. 3f). Also, double staining of blastocoel roof whole-mounts for FN (Fig. 3g) and for F-actin (Fig. 3h) reveals a partial overlap of FN fibrils and F-actin condensations. However, FN fibril segments are also often not associated with F-actin concentrations, and much of the F-actin

Fig. 2. FN fibril formation on the blastocoel roof of the *Xenopus* embryo. Blastocoel roof from stage 9½ (a), stage 10 (b), stage 10+ (c) and stage 11 (d) embryos was stained with antibody to *Xenopus* plasma FN and FITC-conjugated secondary antibody, and mounted whole.
occurs in dots and short streaks outside of FN fibrils. Taken together, our results show that FN fibril formation in the *Xenopus* gastrula is very similar to FN fibril assembly in other systems, indicating that results obtained with the *Xenopus* embryo may be of general relevance.

**FN fibril elongation is unipolar**

To visualize the growth of FN fibrils, a double-labelling technique was developed (Fig. 4a). At the beginning of the experiment, all fibrils present on the blastocoel roof of stage 10+ to 10½ embryos are decorated with intact antibodies to *Xenopus* plasma FN by incubating opened embryos in 5 µg/ml of IgG for 2 minutes. In control experiments, continuous incubation in 25 µg/ml of the same antibody for 2 hours did not inhibit fibril formation (Fig. 5). After washing, embryos or isolated blastocoel roofs are further incubated in the absence
of antibody for various times ranging from 0.5 to 8 minutes (isolated blastocoel roofs) to 2 hours (healed embryos), to allow for fibril growth. After fixation in 4% formaldehyde, new fibrils that have formed during the experiment are labelled with Fab fragments of the same IgG antibody preparation. Whole IgG molecules and Fab fragments are visualized by staining with secondary rhodamine-conjugated antibodies specific for the Fc part, and fluorescein-conjugated antibodies directed against the Fab part, respectively. With this method, newly formed fibrils, which show fluorescein label only, can be distinguished from preexisting fibril tracts, which are also labelled by rhodamine (Fig. 4b,c). In controls, a faint bleed-through could be seen when fibrils stained heavily with fluorescein only were observed through the rhodamine filter. In the reciprocal control combination, break-through staining was stronger, and therefore secondary antibodies were chosen such that new fibrils were fluorescein-labelled. Exposure time was adjusted to minimize bleed-through from fluorescein to rhodamine in controls, and was then kept constant in all experiments.

In order to study fibril elongation, we looked for unbranched fibrils that were labelled showing that they were composed of both preexisting tracts and newly formed parts. Altogether, 296 such cases were found. In 274 of these cases newly added fibril material is exclusively present at only one end of a fibril (Fig. 4b,c). The remaining 22 cases are ambiguous; we could not decide whether pairs of separate fibrils were each growing at one end while being linked together at their non-growing ends, or whether single, sharply bent fibrils were elongating at both ends (not shown). However, no unequivocal case of true bipolar fibril growth was observed. This strongly suggests that FN fibrils typically elongate in a unipolar fashion.

To exclude any effect of antibody incubation on the mode of fibril elongation, we applied a second type of double-labelling procedure. Preexisting fibrils were labelled by incorporation of fluoresceinated bovine plasma FN (see Fig. 3d) instead of FN antibody. After incubation in the absence of exogenous labelled FN, newly formed fibrils were stained with FN antibody and rhodamine-conjugated secondary antibody. With this method, newly formed fibrils are labelled by rhodamine, and bleed-through can be more problematic, although it can always be distinguished from true staining by its color in the original preparations. Also, different fibrils seem to incorporate fluoresceinated FN with different efficiencies, leading sometimes to effects which are hard to interpret. Therefore, this technique was not routinely used. Nevertheless, the results with this method are generally consistent with those from our standard procedure (Fig. 4d).

To exclude the possibility that unipolar fibril elongation could be due to rearrangement of fibril tracts after fibril assembly proper, e.g. by end-to-end alignment of old and new fibril segments, double-labelling was combined with transfer of the forming fibrils to a plastic surface (Nakatsuji and Johnson, 1983a). FN fibrils attach to this substratum instantaneously, e.g. when the blastocoel roof is pressed against the bottom of a culture dish. Consequently, fibrils that form while the roof is being pressed down are deposited on the plastic surface as soon as they appear (unpublished results, and below). When we labelled preexisting fibrils with antibodies, and then pressed the blastocoel roof against plastic for some time to allow for further fibril growth, we found that under these conditions FN fibrils elongate also in a unipolar fashion (Fig. 4e). This makes it unlikely that the addition of new fibril length at one end is due to the end-to-end alignment of separately formed fibril tracts, and suggests instead true, continuous unipolar fibril growth.

New fibril segments are not only seen at the ends of preexisting fibrils, but they occur also as lateral additions, leading to fibril branching (Fig. 6a). The mechanisms of branching cannot be deduced from double-labelling experiments alone. However, arrays of fibrils are encountered, which suggest that secondarily connecting preformed fibrils may be involved; e.g. a short piece of preexisting fibril may appear to be added laterally to a longer, newly forming fibril (Fig. 6b). The notion that rearrangement of fibrils on the blastocoel roof plays a role in branching and network formation is consistent with the observation that on a conditioned substratum, branching is rare (Figs 4e, 8).

The rate of FN fibril growth

The maximum length of newly formed fibrils increases linearly in the double-labelling experiments as the time available for their formation rises from 0.5 to 4 minutes (Fig. 7a). From this, the maximum growth rate of FN fibrils can be estimated to be 4.7 µm/min. However, the majority of all newly formed fibrils is always very short. This could have two explanations: either that most fibrils grow at a much lower rate than the maximum, or that most fibrils do not grow all the time during an experiment. Fig. 7b shows the frequency distribution of the lengths of new fibril segments added to preexisting fibrils by unipolar growth. No significant change in the frequency distribution is observed as one goes from a 2 minute period of fibril addition to one of 8 minutes. This cannot be explained by lower growth rates producing shorter fibril segments, which should lead to a spreading of the distribution and a shift of its average toward higher fibril lengths with time. Instead, the result is consistent with the assumption that fibrils grow almost equally as fast, but only for short, variable times.

Fibril growth was further quantitated in a completely independent approach. The immediate transfer of forming FN fibrils to plastic during substratum conditioning allows us to follow fibril formation in the absence of secondary alignment of fibril tracts. Moreover, FN fibril deposition can be induced prematurely by the substratum conditioning procedure (Fig. 8). In the embryo, FN fibril formation begins between stages 9 and 10 (Fig. 2). However, when blastocoel roof from a stage 9 embryo is pressed against a plastic surface, fibril formation starts immediately. After a short time, isolated patches of fibrils are found which correspond in size to individual blastocoel roof cells. The number of patches increases with the time taken for conditioning, as does fibril density per patch (see below). Apparently, different cells start FN fibril assembly at different times, and only a few immediately after the process has been triggered by substratum contact. By concentrating on the most developed patches at each given time point, the course of FN fibril assembly of single cells can be reconstructed.

After 0.75 minute of substratum conditioning (Fig. 8a), only small groups of a few FN-containing dots and short fibrils can be found; 2.5 minutes later (Fig. 8b), larger fibril tracts are already present, and the number of short fibrils and dots has greatly increased. Such elaborate FN deposits are never observed after 0.75 minute of conditioning, and the simplest explanation is that patches are growing with time. At 8 minutes
Conditioning substratum with stage 9 blastocoel roof allows us to control exactly the start of FN fibril formation in experiments. When the substratum is conditioned for varying periods of time, the longest fibrils found for each time point can be taken to estimate the maximum growth rate, as above. This method yields a fibril elongation rate of 4.7 \( \mu \text{m/min} \) (Fig. 7a), which exactly coincides with that for fibrils growing on the blastocoel roof.

Substratum-induced fibril formation can also be used to measure in vitro the increase in total FN fibril length per cell (Fig. 9). Like the maximum length of single fibrils, total fibril length increases rapidly during the first 5 minutes, and then remains virtually constant. As can be seen from Fig. 8, both the elongation of individual fibrils and an increase in fibril number contribute to the growth of a cell’s fibril network. Quantitative evaluation shows that maximum fibril number per cell also increases sharply during the first 5 minutes to about 50 fibrils/cell (Fig. 9).

In contrast to the growth of single fibrils, the increases in total fibril length and in fibril number seem to be not linear, but slightly accelerated. However, to estimate roughly some parameters of the system, we can approximate the increase in total fibril length per cell during the first 5 minutes by an average of 18 \( \mu \text{m/min} \) (Fig. 9). To achieve this rate with a fibril elongation rate of 4.7 \( \mu \text{m/min} \), about 4 fibrils per cell must elongate actively at a time. After cessation of fibril growth, the total length of FN fibrils per cell is 90 \( \mu \text{m} \), and fibril number per cell is 50 (Fig. 9). Thus, average fibril length is 1.8 \( \mu \text{m} \), which is consistent with results from double-labelling experiments (Fig. 7b). With an elongation rate of 4.7 \( \mu \text{m/min} \), average fibrils should then grow for less than half a minute. To always have 4 fibrils actively growing, as deduced above, new fibrils must be initiated at a rate of about 10 fibrils/cell (Fig. 9). The longest fibrils formed in our experiments, both on conditioned substratum and on the blastocoel roof, are not much larger than 20 \( \mu \text{m} \). This would correspond to a fibril initiated near the start of the fibril formation process in a given cell, and growing throughout the whole 5 minute period of fibril forming activity. Apparently, this is a rare event. Overall, it appears that the growth of the fibril network of blastocoel roof cells in vitro and probably in situ is driven by the rapid initiation of new fibrils, which then grow at a rapid rate, but only for very short times.

Fig. 4. Visualization of FN fibril elongation by double-labelling. (a) Schematic representation of double-labelling technique. Existing fibrils are labelled with FN antibody (1). Then further fibril growth occurs in the absence of antibody (2). Newly added fibril segments are decorated with Fab fragments of the same IgG antibody preparation (3). Whole IgG molecules and Fab fragments are visualized by staining with rhodamine-conjugated antibodies specific for the Fe part (R) and fluorescein-conjugated antibodies directed against the Fab part (F), respectively (4). (b,b’) Double-labelling of growing FN fibrils. Fibrils were decorated with IgG at stage 10+, and with Fab fragment 2 hours later. (b) Rhodamine fluorescence, marking FN fibrils present at the beginning of the experiment. (b’) Same field as in (b), fluorescein label, visualizing both preexisting and newly formed fibrils. Fibrils that appear during the experiment are labelled with fluorescein only (arrow). To a preexisting fibril (ends marked with arrowheads in (b) and in (b’)) new fibril length has been added at one end. (c,c’) Further example, as in (b,b’). (d,d’) Modification of the double-labelling method. Existing fibrils (d) were labelled by the incorporation of fluorescein-FN. After further incubation (30 minutes) in the absence of labelled FN, blastocoel roofs were stained with FN antibody and rhodamine-conjugated secondary antibody (d’). Newly added fibrils are labelled with rhodamine only. Arrows and arrowheads as in (b,c). (e,e’) Combination of double-labelling with substrate conditioning. Preexisting fibrils were labelled with antibody and blastocoel roofs were then pressed against the bottom of the dish for 20 minutes. Antibody-decorated fibrils, and also fibril segments forming anew during conditioning, are transferred to the plastic substratum. The transferred fibril network was incubated with Fab fragments of the same antibody, and stained with secondary antibodies ((e), rhodamine; (e”), fluorescein) as described above. Arrowheads as in b-d; arrow, interrupted new fibril. Most fibrils, although short, did not elongate during the experiment. Bar, 10 \( \mu \text{m} \).

(Fig. 8c), a few large fibrils, which vary in thickness or may even appear interrupted, and very many short ones are typically present. This seems to represent a mature patch, since no significant change is observed as substratum conditioning is prolonged to 18 minutes (Fig. 8d). Longer, branched fibril tracts which cross cell borders are normally observed on the blastocoel roof, but they are probably formed by secondary alignment of fibrils. Thus, blastocoel roof cells can virtually complete fibril formation under experimental conditions about one hour before the process would start in the embryo. The cellular basis for this induction of fibril formation is under investigation.

Fig. 5. FN fibril formation in the presence of antibody. Continuous incubation from stage 9½ to stage 10+ in 25 \( \mu \text{g/ml} \) of IgG against Xenopus plasma FN does not inhibit fibril formation on the blastocoel roof (a), as compared to buffer-incubated controls (b). No fibrils were present at the beginning of the experiment in this batch of embryos (c). Bar, 10 \( \mu \text{m} \).
FN is not substantially multimerized during fibril elongation

Mature FN fibrils consist of covalently cross-linked, insoluble, multimeric FN. Multimerization is a relatively slow process (McKeown-Longo and Mosher, 1983). Given the high rate of fibril elongation determined above, it is to be expected that nascent FN fibrils are not extensively cross-linked and can be dissolved easily. This is in fact observed. When FN fibril formation is induced in stage 9 blastocoel roof and is allowed to proceed for 10 minutes, all FN fibrils assembled and deposited on plastic during this time (Fig. 10a) can be dissolved in 3 M urea, with only traces of fibrils being left (Fig. 10b). In contrast, when already formed FN fibrils from stage 10½ blastocoel roofs (i.e. 2 hours after the onset of fibril formation) are transferred to plastic, only part of the fibril network is dissolved, while other fibrils are resistant to 3 M urea (Fig. 10c), 8 M urea or 4 M urea at pH 11.5 (not shown), and can be stained with anti-FN antibody.

DISCUSSION

FN fibril formation has been mainly studied using various fibroblast cell lines cultured in vitro. In all cases, FN fibril assembly was found to be restricted to the surface of living cells and to require FN-cell and FN-FN interactions. Sites on the FN molecule involved in such interactions have been identified. Unfortunately, results obtained in different systems and with different approaches are not wholly compatible, perhaps in part due to possible differences in fibril structure and composition. However, a few sites are encountered again and again in the various systems, as being important for fibril formation. In particular, the amino-terminal five type I repeats (McDonald et al., 1987; Schwarzbauer, 1991; Sottile et al., 1991; Ichihara-Tanaka et al., 1992), a sequence within the first type III repeat (Chernousov et al., 1991; Morla and Ruoslahti, 1992; Darribere et al., 1992; Morla et al., 1994), the RGD cell binding site (McDonald et al., 1987; Akiyama et al., 1989; Darribere et al., 1990; Winklbauer and Nagel, 1991; Yost, 1992; Darribere et al., 1992), and the cysteine residues involved in cross-linking of FN into dimers (Schwarzbauer, 1991) appear to be essential.

FN fibril formation on the blastocoel roof of amphibian embryos shares the requirements defined above. In *Xenopus*, RGD peptide which competitively inhibits RGD-dependent integrin FN receptors blocks fibrillogenesis completely (Winklbauer and Nagel, 1991; Yost, 1992; this article). In *Pleurodeles*, where laminin is colocalized with FN in blastocoel roof matrix fibrils (Darribere et al., 1986), fibril formation is equally inhibited by RGD peptide, by antibodies to the RGD site of FN, or by antibodies to the integrin β1 subunit of the main FN receptor (Darribere et al., 1990, 1992). This demon-
strates the importance of the RGD cell binding site for fibril assembly in the amphibian embryo.

In *Pleurodeles*, a monoclonal antibody directed against the 9th type I/1st type III repeats inhibits FN fibril assembly (Darribere et al., 1992), suggesting involvement of this site in fibril formation in accordance with results from other systems (Chernousov et al., 1991; Morla and Ruoslahti, 1992; Morla et al., 1994). In the present paper, we show that a 30 kDa

---

**Fig. 8.** FN fibril growth on conditioned substratum. Plastic substratum was conditioned with stage 9 blastocoel roofs for 0.75 minute (a), 3.25 minutes (b), 8 minutes (c) and 18 minutes (d), and stained with FN antibody. Isolated patches of fibrils corresponding in size to single cells were photographed. Long fibrils sometimes appear to be interrupted (arrowheads). Bar, 10 µm.

**Fig. 9.** Quantitation of FN fibril growth on conditioned substratum. Substratum was conditioned with stage 9 blastocoel roofs for varying times. For isolated patches of fibrils corresponding to single cells, total fibril length, i.e. the sum of the lengths of all individual fibrils, and fibril number were determined. Dots were not counted as fibrils. For each time point, those three patches with the largest total fibril length, or the highest number of fibrils, were selected, and averages were calculated as for Fig. 7a. Vertical bars represent the range between the highest and the lowest value, as in Fig. 7a, not standard deviations. The average increase in total fibril length per cell (L) during the first 5 minutes is 18 µm/min, the average increase in fibril number (n) is 10 fibrils/min. The growth of single fibrils (Fig. 7a) is shown for comparison (l, broken line).
fragment from the amino terminus of FN inhibits fibril formation in Xenopus. This agrees with the notion that the first five type I repeats are essential for FN fibril assembly (McDonald et al., 1987; Schwarzbauer, 1991; Sottile et al., 1991; Ichihara-Tanaka et al., 1992). Surprisingly, an effect of this FN fragment on fibril formation could not be observed in Pleurodeles, although a 70 kDa fragment containing the amino terminus is inhibitory (Darribere et al., 1992). Taken together, FN fibril formation in amphibian embryos and in various fibroblasts cultured in vitro appears to be essentially alike.

FN fibril formation possesses two main aspects: the assembly of dimeric FN into fibrils, and multimerization of FN by covalent cross-linking of FN dimers. Fibril assembly proper is rapid, as shown in this article, while multimerization is a relatively slow process (McKeown-Longo and Mosher, 1983). It appears from this that we are dealing with two distinct processes which are to some extent separated in time. In fact, it has been shown in fibroblasts that FN newly incorporated into fibrils is still mostly dimeric after 15 minutes and can be easily extracted, whereas older FN fibrils are heavily cross-linked and resistant to extraction (McKeown-Longo and Mosher, 1983). In our experiments, newly formed FN fibrils, but not older fibrils, are completely soluble in urea and therefore appear not to be cross-linked substantially. Our results support the notion that FN cross-linking is not an integral part of the fibril assembly process, and is not required for fibril elongation to occur.

As regards the spatial and temporal organization of FN fibril assembly, it is usually assumed that FN fibrils do not appear in full length at once, but that they attain their final size by growth after an initial nucleation event (e.g. see McDonald, 1988; Fogerty and Mosher, 1990). Our results show that FN fibrils do in fact grow, and that elongation is unipolar. The addition of new fibril material always occurs at one end of a fibril. Intercalary growth was not observed, although we cannot exclude a contribution to fibril elongation by intercalation at a scale below the resolution of the fluorescence microscope.

We found, with two independent methods, that the rate of fibril elongation is 4.7 μm/min. This corresponds roughly to the lengthwise addition of one or two FN subunits per second. However, it must be noted that we did not follow the growth of individual fibrils directly and continually, but inferred elongation from static pictures. Therefore, we cannot exclude the possibility that on a small scale, growth is discontinuous, and the elongation rate determined above may properly be taken as an ‘apparent’ elongation rate.

Both on the blastocoel roof and on conditioned substratum, newly formed fibrils sometimes appear to be interrupted at one or more points. This suggests that the fibril assembly process is not self-propagating, but is guided by an underlying scaffold. Possible candidates would be actin filaments. For cells in vitro, some relationship between intracellular microfilament bundles and extracellular FN fibrils is evident from the partial colocalization of these structures (Hynes and Destree, 1978; Peters and Mosher, 1987). Disruption of actin filaments by cytochalasin leads to the loss of FN fibrils from the cell surface (Mautner and Hynes, 1977; Ali and Hynes, 1977). Moreover, cytochalasin inhibits the incorporation of exogenous FN into the desoxycholate-insoluble pool in fibroblasts (Barry and Mosher, 1988), suggesting a role for microfilaments in some aspect of FN matrix formation. In blastocoel roof cells of Xenopus, no extended stress fibers are present, but short streak-like condensations of F-actin, comparable in size to the average length of newly formed FN fibrils, are observed. These actin
bundes show a partial colocalization with FN fibrils. Moreover, cytchalasin B also inhibits FN fibril formation.

FN fibril elongation may be linked not only to the presence of microfilaments in general, but more specifically to actin polymerization. Microfilaments have a polar structure, and actin polymerization occurs preferentially at the barbed end of a filament. FN molecules are also added exclusively at one end of a fibril, and one possible explanation would be that unipolar fibril growth reflects the unipolar polymerization of an underlying actin filament scaffold. Moreover, the rate of FN fibril elongation, and measured in vivo rates of microfilament growth by actin polymerization, are similar. Displacement of the actin meshwork from the edge of fibroblast lamellipodia by actin polymerization occurs at a rate of 4.5 μm/min or more (Symons and Mitchison, 1991). Listeria bacteria move by actin polymerization through the cytoplasm of infected cells at about 6 μm/min on average (Theriot et al., 1992). Attachment of beads to lamellipodia of growth cones induces actin filament polymerization, which proceeds at an average rate of 5 μm/min (Forscher et al., 1992). The rate of 4.7 μm/min for FN fibril elongation is strikingly close to these values. This raises the possibility that FN fibril elongation is coupled to actin filament polymerization, e.g. by the fibril assembly machinery being physically linked to the end of a growing microfilament bundle. It must be admitted, however, that in general the evidence for actin filament involvement in FN fibril assembly is rather indirect at present. An alternative explanation for unipolar FN fibril elongation would be that FN fibrils themselves have a polar structure which restricts growth to one end, the rate of elongation equalling that of actin filaments by coincidence.

We thank Andrea Belkacemi for technical assistance and Martina Nagel for actin/fibronectin double staining. Part of this work, performed by R.W. at the Zoologisches Institut, Universität zu Köln, was supported by the Deutsche Forschungsgemeinschaft.

REFERENCES


(Received 31 August 1994 - Accepted 5 January 1995)