Cross-linking of the NH$_2$-terminal Region of Fibronectin to Molecules of Large Apparent Molecular Mass

CHARACTERIZATION OF FIBRONECTIN ASSEMBLY SITES INDUCED BY THE TREATMENT OF FIBROBLASTS WITH LYSOPHOSPHATIDIC ACID*

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Cell surface molecules on adherent cells that bind 125I-labeled fibronectin or its 70-kDa N-terminal fragment were identified by cross-linking with factor XIIIa and by photoaffinity labeling. Such cross-linking caused the 70-kDa fragment to become associated irreversibly to cell layers and was greater in cells treated with lysophosphatidic acid, an enhancer of fibronectin assembly and strong modulator of cell shape. Cross-linking of the 70-kDa fragment with factor XIIIa was to molecules that migrated in discontinuous sodium dodecyl sulfate-polyacrylamide gels at the top of the 3.3% stacking gel and near the top of the separating gel. Estimated sizes of these large apparent molecular mass molecules (LAMMs) were >3 MDa and ~3 MDa. The label in 70-kDa fragment conjugated with 125I-sulfosuccinimidyl 2-(p-azidosalicyl)aminodiole (3'-dithiopropionate) was associated with >3-MDa LAMMs without reduction and with ~3-MDa LAMMs after reduction and transfer of the cleavable label. The LAMMs were expressed on monolayer cells shortly after adherence, required both 1% Triton X-100 and 2 M urea for efficient extraction, and were susceptible to digestion with trypsin but not to cathepsin D digestion. Complexes of 125I-70-kDa fragment and LAMMs were also susceptible to limited acid digestion and Glu-C protease digestion but were not cleaved by chondroitin lyase or heparitinase. Neither the uncleaved complexes nor the cleavage products were immunoprecipitated with anti-fibronectin antibodies directed toward epitopes outside the 70-kDa region. Thus, cell surface molecules that are either very large or not dissociated in sodium dodecyl sulfate comprise the labile matrix assembly sites for fibronectin.

Fibronectin is a major extracellular protein that is necessary for normal embryogenesis (1). Fibronectin exists as a soluble protein at near micromolar concentrations in blood plasma and other body fluids and in an insoluble multimeric form in extracellular matrix (2, 3). Insoluble tissue fibronectin has several likely biological functions, including promotion of cellular migration during embryogenesis and wound healing (4–6). During gastrulation of Xenopus laevis, polarized fibronectin fibrils are thought to guide migrating mesoderm to its target region (7). Local perturbation of the fibronectin-rich extracellular matrix of Xenopus gastrulae correlates with localized randomization of left-right asymmetries later in development (8).

Since fibronectin is primarily functional in its insolubilized state, the polymerization of fibronectin has importance similar to that of the fibrinogen-to-fibrin conversion. There is no evidence that a modifying proteolytic event, as with the cleavage of fibrinogen to fibrin monomer, triggers polymerization of fibronectin. Unlike collagens and laminins (9), plasma fibronectin does not self-polymerize in physiologically relevant solutions. Strategies that have worked to induce self-polymerization of plasma fibronectin reproducibly include incubation with denaturants (10), reduction of disulfides (11, 12), incubation with peptides based on a specific sequence of fibronectin (13), and exposure to shear forces at air-liquid or liquid-solid interfaces (14, 15). There is little passive accumulation of fibronectin in pre-existing extracellular matrix (16, 17). Rather, assembly of fibronectin requires cells and takes place at specialized sites on cell surfaces (18). Binding to these sites is mediated by the N-terminal modules of fibronectin, especially modules I-1 through I-5 (19–21). A number of cells synthesize and secrete forms of fibronectin that are spliced differently from the plasma form, but all have the N-terminal modules. These cellular isoforms have a greater tendency for self-polymerization than plasma fibronectin (2). Cultured fibroblasts, however, directly assemble both plasma fibronectin (present in serum-containing culture medium) and fibroblast-derived, alternatively spliced fibronectin (17, 22, 23). Thus, the rules for assembly seem the same for both plasma and cellular fibronectin. Reagents that block assembly of fibronectin in cell culture also block assembly of fibronectin in the blastula (24, 25). Cultured cells and granulation tissue of healing wounds have co-linear transmembranous associations of fibronectin-containing extracellular matrix fibers and bundles of actin microfilaments localized at dense submembranous plaques (26). Somehow, therefore, the intracellular cytoskeleton determines the pattern of assembly of extracellular matrix and/or the extracellular matrix determines the pattern of assembly of intracellular cytoskeleton.

We had studied previously the effects of activated factor XIII (factor XIIIa, plasma transglutaminase) on the incorporation of fibronectin and its N-terminal fragments into extracellular matrix by cultured fibroblasts (16, 27). When factor XIIIa was included in the binding medium, accumulation of 125I-fibronectin in the deoxycholate-insoluble matrix was increased in the form of cross-linked nonreducible high molecular weight aggregates. Factor XIIIa also cross-linked 125I-labeled 27- and 70-kDa N-terminal fragments into high molecular weight aggregates that could not be extracted from cell layers with deoxycholate. The characteristics of the cross-linked partners were similar to those reported when bound 27-kDa fragment was cross-linked with the membrane-impermeable reagent.

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bis(sulfosuccinimidyl)suberate (28) or cellular fibronectin was derivatized, bound to cells, and cross-linked with photoactivatable agents (29).

These previous cross-linking studies are clouded by several unknowns: presence of pre-existing matrix, long incubation times, and, when photoactivatable cross-linkers were not used, possibility of cross-linking of receptors to other molecules or to multiple labeled ligand during the exposure to cross-linkers. Another shortcoming inherent to this analytical approach is the absence of an unequivocal way to distinguish between cross-linking events with cell surface molecules critical for fibrillogenesis ("productive") and events that are specific yet irrelevant to matrix assembly ("nonproductive"). We reasoned that we could deal with these problems using cells stimulated with lysophosphatidic acid (LPA), a specific up-regulator of fibronectin binding and assembly (30). Because fibronectin binding is as responsive to stimulation by LPA when cells spread shortly after dense seeding as when cells become confluent days after sparse seeding, we could compare cross-linking in monolayers of cells with little or no matrix shortly after seeding and abundant matrix upon reaching confluence. Furthermore, the addition and withdrawal of LPA cycles cells so rapidly between assembly-competent and assembly-deficient phenotypes (30) that one would expect minimal changes in the composition of molecules on the surface of cells and in the extracellular matrix. Thus, binding to molecules not at assembly sites, i.e. to molecules at sites irrelevant to and nonproductive of assembly, should be the same in control and LPA-treated cultures, and a comparison of the cross-linked complexes following the binding in the absence and presence of LPA should identify the labile receptor molecules.

**EXPERIMENTAL PROCEDURES**

**Materials**—LPA, fatty acid-free bovine albumin, danacladaverine, formic acid, cathepsin D, Glu-C protease, and guanidine isothyiocyanate were from Sigma. Fluorescein isothiocyanate (FITC) was from Molecular Probes (Eugene, OR). Kinase inhibitor H7 was from Seikagaku America Inc. (St. Petersburg, FL). Chondroitin ABC lyase and heparitinase were from Miles Laboratories (Elkhart, IN) and ICN (Costa Mesa, CA), respectively. Rabbit antisera to the N-terminal 70-kDa fragment of fibronectin generated by cathepsin D were isolated, and to the type III module region of fibronectin (11) were prepared in our lab (31). Rabbit antibodies to the N-terminal 70-kDa fragment following manufacturer’s instructions. 

**Enzyme Digestion**—Cells were incubated with 125I-70-kDa fragment and cross-linked with factor XIIIa. Cell layers were then digested with 2% SDS, 5 mM EDTA. Formic acid was added to a final concentration of 70%, digestion was carried out at 37 °C for 24 h (36). The digests were then dialyzed against 0.1% SDS containing 1 mM EDTA and 1 mM N-ethylmaleimide, lyophilized, resuspended in water, and analyzed by electrophoresis or immunoprecipitation.

**Immunoprecipitation of Radioactive Proteins**—Labeled proteins were bound and cross-linked to LPA-treated confluent human foreskin fibroblasts. Cell layers were extracted with 2% SDS, 5 mM EDTA. Extracts, with or without acid or enzyme digestion, were diluted 5-fold in 1.25% Triton X-100 and incubated with preimmune rabbit serum followed by goat anti-rabbit IgG coupled to Sepharose. Nonspecific binding in the presence of 500 µg/ml unlabeled 70-kDa fragment (approximately 10−30% of total binding for both intact fibronectin and the 70-kDa fragment) was subtracted from total binding to calculate specific binding. Total cell protein was also determined (34).

**Acid Digestion**—Cells were incubated with 125I-70-kDa fragment and cross-linked with factor XIIIa. Cell layers were extracted with 2% SDS, 5 mM EDTA. Formic acid was added to a final concentration of 70%, and digestion was carried out at 37 °C for 24 h (36). The digests were then dialyzed against 0.1% SDS containing 1 mM EDTA and 1 mM N-ethylmaleimide, lyophilized, resuspended in water, and analyzed by electrophoresis or immunoprecipitation.

**Cross-linking of Bound Labeled Protein**—with thrombin-activated factor XIII (factor XIIIa) was done using the techniques and reagents previously described (27) except that recombinant factor XIII catalytic subunit kindly provided by Dr. Paul Bishop of Zymogenetics (Seattle, WA) was used rather than tetrameric enzyme purified from plasma. Experiments were done in Tyrode’s buffer supplemented with 2 mM calcium chloride or, for control purpose, 2 mM EDTA. Photolinking was also carried out with sulfosuccinimidyl 2-[(p-azidosalicylamido)-1,3-dithiopropionate; (SASD), a heterobifunctional, iodo-, cleavable, photocross-linking agent purchased from Pierce. SASD was iodinated and coupled to the 70-kDa N-terminal fragment following manufacturer’s instructions. 125I-SASD-70-kDa was purified from iodination mixture by G-25 spin column and bound to cells, and finally the photoreactive group was activated by UV light. The manipulations were carried out as described for studies that led to identification of the receptor for interleukin-3 (35). In control experiments, 125I-labeled fibronectin or 70-kDa fragment was subjected to photolysis in solution or after binding to cell surfaces.

**Acid Digestion**—Cells were incubated with 125I-70-kDa fragment and cross-linked with factor XIIIa. Cell layers were extracted with 2% SDS, 5 mM EDTA. Formic acid was added to a final concentration of 70%, digestion was carried out at 37 °C for 24 h (36). The digests were then dialyzed against 0.1% SDS containing 1 mM EDTA and 1 mM N-ethylmaleimide, lyophilized, resuspended in water, and analyzed by electrophoresis or immunoprecipitation.

**Enzyme Digestion**—Cells were incubated with 125I-labeled fibronectin or 70-kDa fragment and cross-linked with factor XIIIa. Cell layers were treated with 10 µg/ml cathepsin D for 3 h at 37 °C in 50 mM sodium acetate, pH 3.5, containing 1 mM phenylmethylsulfonyl fluoride (37). The digestion was carried out with 50 µg/ml pepstatin. Cells remained intact by phase contrast microscopy. Extract of 125I-labeled fibronectin or 70-kDa fragment cross-linked to cells was also digested with 10 µg/ml Glu-C protease in 50 mM ammonium bicarbonate, 0.5% SDS, pH 7.8, for 2 h at 37 °C (38). The treatment was stopped by boiling for 2 min in 2% SDS-2 mM phenylmethylsulfonyl fluoride. The digests were then analyzed by electrophoresis or immunoprecipitation.

**Immunoprecipitation of Radioactive Proteins**—Labeled proteins were bound and cross-linked to LPA-treated confluent human foreskin fibroblasts. Cell layers were extracted with 2% SDS, 5 mM EDTA. Extracts, with or without acid or enzyme digestion, were diluted 5-fold in 1.25% Triton X-100 and incubated with preimmune rabbit serum followed by goat anti-rabbit IgG coupled to Sepharose. Nonspecifically precipitated radioactivity, which was about 10−20% of input, was removed by centrifugation, and the supernatants were divided and immunoprecipitated with rabbit preimmune serum or antisera to the 70-kDa fragment or type III module region (11) of human plasma fibronectin or to αβ integrin followed by anti-rabbit IgG-Sepharose. Precipitates were collected by centrifugation and analyzed by electrophoresis. 

**SDS-PAGE and Autoradiography**—The radioactive samples were analyzed with or without reduction by discontinuous SDS-PAGE (3.3% stacking gel, 6–10% separating gel) containing 5–20% of labeled ligand and cross-linked complexes were quantified by phosphorimaging (Molecular Dynamics, Inc., Sunnyvale, CA). High molecular weight markers from rabbit pesoa (skeletal muscle) myofibrils (40) were kindly provided by Dr. Marion Greaser (University of Wisconsin-Madison). Dithiothreitol-treated fibronectin cross-linked in solution by factor XIIIa (41) served as a second mix of high molecular weight stand-
The presence of 200 nM 1-oleoyl LPA for 60 min at 37 °C. For localization of thrombin-activated factor XIII or Ca^2+ kinase inhibitor. In control studies, no cross-linking was seen if enhanced 3–4-fold in cell layers treated with LPA and diminished and in the stacking gel of lanes containing the cross-linked 70-kDa fragment and rhodamine-labeled secondary antibodies. Coverslips were mounted with glycerol gel, and cells were viewed on a Nikon epifluorescence microscope.

RESULTS

The purpose of the present studies was to characterize the cell surface molecules responsible for binding and assembly of fibronectin. Fibronectin matrix assembly is highly regulated, such that addition or withdrawal of LPA rapidly causes a 2–4-fold stimulation or less, respectively, of the binding of fibronectin or the 70-kDa fragment to cell layers of MG63 osteosarcoma cells or human foreskin fibroblasts (30, 33). Binding of the 70-kDa fragment is to a single class of binding sites on unstimulated and LPA-stimulated cells, with increases of both avidity and number of binding sites upon stimulation (30). Protein kinase C inhibitors, in contrast, cause a 5–10-fold decrease in binding (42). Cross-linking studies of cells treated to be assembly-competent or assembly-deficient, therefore, will yield information about the molecules that comprise the labile binding sites.

The N-terminal 70-kDa fragment of fibronectin binds reversibly to cells in monolayer culture with the same avidity as the initial reversible binding of intact fibronectin (20). The fragment consists of an N-terminal domain of 27 kDa and a 40-kDa gelatin-binding domain (2, 3). Near the N terminus is a glutamine residue that is attacked by factor XIIIa (43). The five type I modules of the 27-kDa domain are most important for binding (19–21), whereas the adjacent gelatin-binding domain does not bind specifically to cell layers (44). Nevertheless, the gelatin-binding domain must contribute to the binding, because the whole 70-kDa fragment has approximately 10-fold higher avidity for the binding site ($K_a$ is 3 nM on LPA-treated cells (30)) than the isolated 27-kDa domain (20, 44). Our cross-linking studies, therefore, were done with the 70-kDa fragment used at concentrations in which binding sites would be 5–20% saturated with ligand.

Fig. 1 shows PhosphorImager scans of discontinuous SDS gels of 125I-70-kDa fragment bound to MG63 osteosarcoma cells for 60 min, cross-linked with factor XIIIa for 5 min, and reduced prior to electrophoresis. The fragment was cross-linked into complexes that migrated at the top of separating gel or rhodamine-labeled secondary antibodies. Coverslips were mounted with glycerol gel, and cells were viewed on a Nikon epifluorescence microscope.

When increasing amounts of unlabeled 70-kDa fragment were incubated with LPA-treated cell layers, the dose responses for inhibition of binding of 125I-70-kDa fragment to cell layers and for inhibition of formation of cross-linked complexes at the tops of the stacking and separating gels were identical (Fig. 3). At the highest concentration of unlabeled 70-kDa fragment, binding and cross-linking were 18–20% of control. Cross-linking was also inhibited by monodansylcadaverine, which competes for protein-protein cross-linking and incorporates into factor XIIIa-reactive glutamines (Fig. 4). Quantification with a PhosphorImager indicated that the radioactivity missing from the top could be accounted for by radioactivity at the position of the monomer. Cross-linking to form the labeled band at the top of the separating gel, however, was not inhibited at the same rate as cross-linking to form the labeled band at the top of the stacking gel.

In order to study the influence of preformed fibronectin-containing extracellular matrix, osteosarcoma cells and foreskin fibroblasts were studied shortly after plating on vitronectin-coated plates or at confluence without or with LPA stimulation. Immunofluorescence studies demonstrated scant organized fibronectin in freshly seeded cells (see Fig. 5B) and copious fibronectin matrices in confluent cultures (data not shown). Under all conditions, >60% of 125I-labeled 70-kDa fragment or fibronectin remained insoluble in 1% Triton X-100 when cell layers were extracted after cross-linking; the Triton-insoluble fractions are shown in Fig. 5. Treatment with LPA resulted in increased cross-linking of the 70-kDa fragment to both cell types under both culture conditions (Fig. 5A). Similarly, 125I-fibronectin was cross-linked to bands at the top of the stacker and at the top of the separating gel in all four types of samples (Fig. 5B). The major difference in cross-linking patterns was a band somewhat larger than monomeric fibronectin (300 kDa) that was seen when the 70-kDa fragment was cross-linked. This band was most prominent in extracts of foreskin fibroblasts after 3 days in culture. Occasionally, several bands with sizes less than 200 kDa were observed, but this was not a reproducible finding.

The fact that the 70-kDa fragment did not form a dimer >
trimer > tetramer . . . ladder upon cross-linking indicates that the formation of large complexes is not due to the 70-kDa fragment cross-linking to itself. Cellular targets of the cross-linking, however, potentially could be cross-linked to one another or to other molecules as well as to the labeled ligand during the exposure to factor XIIIa. Such cross-linking could account for the extraordinarily large apparent size of the complexes. Therefore, we compared results obtained with factor XIIIa with those obtained with SASD, a heterobifunctional, iodonitrate, cleavable, photoreactive cross-linking agent that has been proven useful in a variety of situations, such as identification of the receptor for interleukin-3 (35). The advantages of SASD are that the only cross-linking events should be between the ligand and the molecules that bind the ligand and that the label is in part transferred (trans-labeling) to the binding molecules after reduction.

FIG. 2. Association of 125I-70-kDa fragment of fibronectin with osteosarcoma cells after cross-linking. Cells were incubated for 60 min with 125I-70-kDa fragment and 200 nM LPA, washed, and cross-linked with factor XIIIa, 10 μg/ml, for 5 min. A, cell layers with (XL) or without (No XL) factor XIIIa cross-linking were incubated for 6 h with Tyrode’s buffer containing 0.2% fatty acid-free bovine albumin. At the designated time points, small portions of medium were sampled to quantify dissociation of specifically bound 70-kDa fragment. Each point represents the average of duplicate values that varied <5%. B, the factor XIIIa cross-linked cell layers were extracted with 1% Triton X-100 or 2 M urea or 1% Triton X-100 plus 2 M urea for 10 min. The remaining pellets were extracted with 2% SDS. The fractions were analyzed by SDS-PAGE and autoradiography. Int, interface of the 8% running and 3% stacking gel; Top, top of the stacking gel.

FIG. 3. Dose responses of inhibition of binding and factor XIIIa-mediated cross-linking of 125I-70-kDa fragment by unlabeled fragment. Fore skin fibroblasts were incubated 60 min with 125I-70-kDa fragment and LPA followed by a wash and 5-min incubation with factor XIIIa. Cell layers were extracted with 2% SDS sample buffer and analyzed by SDS-PAGE and autoradiography. Variable amounts of unlabeled 70-kDa fragment were present in the 60-min incubation. Binding of labeled fragment (70-kDa) and amounts of cross-linked fragment at the top of the stacker (Top) and near the top of the separating gel (Int) were quantified by phosphorimaging and expressed as % of values determined when no unlabeled fragment was present. Points represent the mean ratios ± S.D. (n = 3).

FIG. 4. Competition for cross-linking of 125I-70-kDa fragment by monovalent amine. Osteosarcoma cells were incubated with 125I-70-kDa fragment and LPA for 60 min followed by a wash and 5-min incubation with factor XIIIa except that variable amounts of dansylcadaverine were present during the 5-min incubation with factor XIIIa. Actual counts from PhosphorImager scans are plotted versus dansylcadaverine concentration. Radioactivity lost from the top of the stacking gel (Top) and from near the top of the separating gel (Int) was found at the position of the monomer (70-kDa). The reaction schemes show how incorporation of dansylcadaverine (CadD) into a reactive glutamine (Q) of protein P1 competes for εγ-glutamyllysine (EK) cross-links between proteins P1 and P2.

treated by photolysis in solution or bound to cell surface (data not shown). Cross-linked 125I-SASD-ligand was not solubilized with 1% Triton (data not shown). Upon reduction, part of the radioactive signal was transferred to the molecules that migrated near the top of the separating gel, with an apparent size similar to that obtained with factor XIIIa-mediated cross-linking of 70-kDa fragment. The major difference between factor...
FIG. 5. Cross-linking of 125I-labeled 70-kDa fragment (A) or fibronectin (B) to osteosarcoma cells (MG63) or human foreskin fibroblasts (HSF). Cell layers were studied 4 h after dense seeding in vitronectin-coated wells in the absence of serum or 3 days after sparse seeding and culture in fetal bovine serum until confluent. Layers were incubated for 60 min with labeled protein without (−) or with (+) 200 nM LPA, washed, and cross-linked with factor XIIIa, 10 μg/ml, for 5 min. Cell layers were first extracted by 1% Triton X-100, and the Triton-insoluble materials were collected with 2% SDS sample buffer and analyzed. Shown are phosphorimages of dried mini-gels, with a 3% stacker, and 10% running gel.

FIG. 6. Cross-linking with a bifunctional photoactivable reagent, 125I-SASD-70-kDa. Confluent human foreskin fibroblasts were incubated for 1 h with 125I-SASD-70-kDa fragment and 200 nM LPA in Tyrode’s buffer containing 0.2% albumin. After washing, cells were photolyzed for increasing amounts of time (0–10 min) and then extracted with SDS sample buffer. Cell lysates were analyzed on a 3.3/8% SDS-PAGE with or without reduction followed by scanning of radioactive bands. The 70-kDa fragment in some lanes ran off the gel. Phosphorimaging quantitation indicated that upon reduction the signal from the top band decreased from 26,000 to 8,000, without rFXIIIa precross-linking and from 39,000 to 9,000 with rFXIIIa pretreatment, whereas the signal from the interface band increased from 5,000 to 11,000 for the former and from 6,000 to 13,000 for the latter.

XIIa- and SASD-mediated cross-linking was the persistence of label at the top of the stacking gel in the factor XIIa cross-linked samples after reduction. In order to learn whether this was an artifact of factor XIIa cross-linking of the cellular targets to each other or other large molecules, the cell surface molecules were first exposed to factor XIIa and then probed with 125I-SASD-70-kDa fragment (Fig. 7). Upon reduction, the radioactive signal present at the top of the stacker became diminished, and increased label was detected at the top of the separating gel and at the position of the 70-kDa fragment, just as in the sample generated without the pretreatment with factor XIIa. This observation suggests that the redistribution of the label upon reduction of the samples probably represents the dissociation of multiple 70-kDa fragments from the targets.

FIG. 7. Photoaffinity labeling of factor XIIa cross-linked cell surface molecules with 125I-SASD-70-kDa. Freshly seeded fibroblasts were (+) or were not (−) cross-linked by 10 μg/ml factor XIIa for 5 min. 125I-SASD-70-kDa fragment was then bound to the cells for 1 h and photolyzed for 10 min. The cell lysates were analyzed by reducing and nonreducing SDS-PAGE and autoradiography. Phosphorimaging quantitation indicated that upon reduction the signal from the top band decreased from 26,000 to 8,000, without rFXIIIa precross-linking and from 39,000 to 9,000 with rFXIIIa pretreatment, whereas the signal from the interface band increased from 5,000 to 11,000 for the former and from 6,000 to 13,000 for the latter.

In previous studies, when cellular fibronectin was coupled to photoactivatable chemical cross-linkers and activated after binding to cells, the high molecular weight complex was sensitive to chondroitin ABC and AC lyases and to trypsin (29). The complexes formed with 125I-70-kDa fragment were not degraded by chondroitin ABC lyase after cross-linking by factor XIIa (protocol III of Table I). Furthermore, pretreatment of cells with chondroitin ABC lyase (protocol I) or treatment with chondroitin ABC lyase after binding and before cross-linking (protocol II) did not result in decreased formation of complexes. Treatment with heparitinase also did not result in decreased amounts of complexes (data not shown). After factor XIIa-catalyzed cross-linking, 70-kDa fragment complexed to LAMMs was degraded rapidly with low concentrations of trypsin (data not shown). This result is difficult to interpret, because the 70-kDa fragment itself is susceptible to trypsin cleavage. However, the labeled LAMMs near the top of the separating gel obtained with 125I-SASD-70-kDa fragment under reducing condition (trans-labeled LAMMs) were also sensitive to trypsin digestion of intact labeled cells (10 μg/ml trypsin for 10 min) (data not shown). After factor XIIa-catalyzed cross-linking, 125I-70-kDa fragment complexed to LAMMs were not degraded by the digestion with cathepsin D (data not shown). Cathepsin D, it should be noted, was the enzyme used to generate the 70-kDa fragment in the first place. The results indicate that LAMMs are proteins that are susceptible to cleavage with trypsin but resist degradation with cathepsin D.

Candidates for the cell surface binding molecules include pre-existing assembled and polymerized fibronectin and αβ₃ integrins as described in detail under “Discussion.” To explore whether the 70-kDa fragment might be binding to fibronectin, we co-localized FITC-labeled 70-kDa fragment and the IST-2 monoclonal antibody to the type III module region of fibronectin.
**Table I**  
*Chondroitin ABC lyase treatment*

Cross-linking of 125I-70-kDa fragment to MG63 cells without (−) or with (+) treatment with chondroitin lyase ABC (cABC). Three protocols (29) were used. I, cells were treated with 0.5 units/ml chondroitin ABC lyase for 1 h at 37 °C prior to a 1-h binding period and cross-linking by 10 μg/ml factor XIIIa for 5 min at 37 °C. II, cells were incubated with 125I-70-kDa fragment, then treated with chondroitin ABC lyase, and cross-linked. III, cells were incubated with 125I-70-kDa fragment, cross-linked, and treated with chondroitin ABC lyase. Cell lysates were run on a 10% polyacrylamide gel with 3% stacking. Radioactive signals at the top of the stacking gel (Top), near the top of the separating gel (Int), and position of the 70-kDa fragment (70 kDa) were quantified by phosphorimaging.

| cABC | Confluent cells | Freshly seeded cells |
|------|----------------|---------------------|
|      | Top | Int | 70 kDa | Top | Int | 70 kDa |
| I −  | 3.1 | 3.3 | 6.1    | 3.8 | 2.1 | 3.4    |
| +   | 3.4 | 3.5 | 6.1    | 3.3 | 1.6 | 2.9    |
| II − | 2.6 | 2.1 | 3.7    | 2.0 | 1.0 | 1.9    |
| +   | 2.3 | 1.8 | 3.3    | 2.8 | 1.1 | 1.8    |
| III −| 3.5 | 2.6 | 5.0    | 2.7 | 1.3 | 1.8    |
| +   | 2.9 | 2.3 | 4.2    | 3.7 | 1.3 | 1.7    |

**Fig. 8.** Localization of bound 70-kDa fragment and pre-existing fibronectin on cell surface. MG63 cells were cultured for 4 h on a vitronectin-coated coverslip and then incubated for 1 h with FITC-70-kDa fragment, 15 μg/ml, and 200 nM LPA in Tyrode’s solution containing 0.2% albumin. The coverslip was washed, and cells were fixed with 3% paraformaldehyde. Fibronectin was stained with IST-2 followed by rhodamine-labeled goat anti-mouse IgG, A, FITC-70-kDa fragment; B, IST-2; C, phase. The open arrow points to a linear array of 70-kDa fragment on the lateral cell surface where there is little IST-2 epitope. The arrowhead points to a linear array of 70-kDa fragment where four cells come together. In controls in which the 40-kDa gelatin-binding domain of fibronectin was substituted for the 70-kDa fragment, no specific fluorescence was seen.

The antibody was detected using rhodamine-labeled anti-mouse IgG. Localization was after a 1-h incubation of FITC-labeled 70-kDa fragment with osteosarcoma cells 4 h after seeding (Fig. 8A). IST-2 co-localized with the fragment in some areas (Fig. 8B), but linear arrays of 70-kDa fragment were also noted where no IST-2 epitopes were visualized (Fig. 8, open arrow). Similar results were obtained with C6F10, a monoclonal antibody to III8–11, and polyclonal antibodies to the type III module region produced by limited trypsination of reduced and alkylated fibronectin to yield a core of type III modules minus the surrounding type I modules (data not shown). The anti-type III region antibodies were also used in immunoprecipitation assay. While anti-type III region antibodies immunoprecipitated 125I-fibronectin and its cross-linked complexes, the anti-type III module antibodies did not immunoprecipitate 125I-70-kDa fragment and the cross-linked complexes of 70-kDa fragment when compared with preimmune serum (Fig. 9 and data not shown for the preimmune serum). As positive controls, anti-70-kDa fragment antibodies immunoprecipitated complexes of both cross-linked intact 125I-fibronectin and cross-linked 125I-70-kDa fragment (Fig. 9). Anti-α5β1 integrin antibodies did not precipitate the complexes (data not shown).

The LAMMs could represent a conglomerate of proteins that resist dissociation in discontinuous SDS-PAGE. Therefore, complexes were analyzed using electrophoretic conditions that might better dissociate and separate large molecules. Cross-linked 70-kDa fragment or fibronectin migrated just below the interface even on a 3.5% separating gel (Fig. 10A). Diffuse radioactivity was present in the stacking gel and extending below the top of the running gel. In a polyacrylamide gel system that separate the large proteins of myofibrils (40), cross-linked 70-kDa fragment just below the interface migrated at approximately the same position as muscle titin, which is 3 MDa (45) (data not shown). Exclusion or inclusion of 6 M urea along with SDS during sample preparation had no effect (data not shown). Cross-linked samples were also analyzed on a 4% continuous SDS gel cast with 6 M urea, so that the samples were exposed to 6 M urea throughout the electrophoresis and not concentrated in the stacking gel. The complexes migrated at the top of the gel (Fig. 10B). Finally, complexes were treated with 6 M guanidine isothiocyanate for 1 h at 37 °C followed by dialysis against 2% SDS at 60 °C and electrophoresis. Even after this harsh treatment, the complexes migrated with the same characteristic mobilities (data not shown).

In order to explore whether the 70-kDa fragment is cross-linked specifically to a discrete molecule in the LAMMs, samples were subjected to digestion in 70% formic acid which cleaves proteins selectively at Asp-Pro bonds (36). Bands of 130 and 150 kDa containing label from 125I-70-kDa fragment were observed in the acid-digested material (Fig. 11A). The yield of the 130- and 150-kDa bands was less in samples processed after cross-linking of 125I-70-kDa fragment to cells not treated with LPA or when excess unlabeled 70-kDa fragment was present prior to cross-linking (data not shown). The 130- and 150-kDa bands could not be immunoprecipitated by anti-type III region antibodies but were immunoprecipitated by anti-70-kDa fragment antibodies (Fig. 11A). Samples were also digested by Glu-C protease, which cleaves selectively at certain Glu-C bands in the presence of SDS (38). A band of about 140 kDa containing label from 125I-70-kDa fragment was observed in the Glu-C protease-digested material (Fig. 11B). The band was immunoprecipitated by anti-70-kDa fragment antibodies but could not be immunoprecipitated by anti-type III region antibodies, whereas all the bands generated by the Glu-C protease digestion about cross-linking of bound 125I-fibronectin to cells were immunoprecipitated by both antibodies (Fig. 11B). These observations indicate that the immediate...
cross-linking partner on cell surface for the 70-kDa fragment is, in part at least, a protein that yields discrete fragments.

DISCUSSION

We have used cross-linking strategies to identify the cell surface molecules that mediate labile binding of fibronectin or its 70-kDa N-terminal fragment in cultures of fibroblasts and osteosarcoma cells. To test specificity, binding sites were up-regulated with LPA and down-regulated with the H7 kinase inhibitor. As described in the Introduction, LPA is a powerful and specific modulator of cell shape and early matrix assembly. LPA rapidly increases the isometric tension of chick embryo fibroblasts in collagen gels (46). Cells begin to retract as early as 2 min after addition of LPA, correlating with dramatic reorganization of cytoskeleton, appearance of filopodia, and increase of fibronectin binding (30). Fibronectin matrix assembly is 3-fold in 1.25% Triton X-100 and incubated with preimmune rabbit serum followed by goat anti-rabbit IgG coupled to Sepharose. Nonspecifically precipitated radioactivity was removed by centrifugation. The nonspecifically precipitated radioactivity accounts for 15% of input and was present in the bands at the tops of the stacking gel and separating gel. The supernatants were divided and immunoprecipitated with rabbit antiserum to the 70-kDa fragment (anti-70-kDa) or type III module region (anti-III) of human plasma fibronectin followed by anti-rabbit IgG-Sepharose. 80% of 125I-fibronectin associated with cells was precipitated by either the anti-70-kDa fragment or anti-III antibodies, whereas 15% was precipitated with preimmune serum. Of 125I-70-kDa associated with cells, 20% was precipitated by anti-III antibodies or preimmune serum, whereas more than 80% was precipitated by anti-70-kDa fragment antibodies. Precipitates were analyzed after reduction by electrophoresis on a 3/10% polyacrylamide slab gel. Starting extracts are shown in the left panel of the phosphorimage and immunoprecipitated proteins on the right. The 300-kDa band is indicated.

FIG. 10. Separation of the cross-linked complexes under different conditions. Labeled fibronectin (Fn) or 70-kDa fragment (70-kDa) was bound to LPA-treated human foreskin fibroblasts, without (−) or with (+) cross-linking with factor XIIIa (rFXIIIa). Cell layers were extracted with 2% SDS sample buffer. Samples were analyzed after reduction by electrophoresis on a 3/3.5% discontinuous polyacrylamide slab gel (A) or a 4% continuous SDS gel cast with 6 M urea (B).

FIG. 9. Radioimmunoprecipitation of cross-linked 125I-labeled fibronectin (Fn) or 70-kDa fragment (70-kDa) complexes with anti-type III module region of fibronectin or anti-70-kDa fragment antibodies. Labeled proteins were bound and cross-linked to LPA-treated confluent human foreskin fibroblasts by factor XIIIa. Cell layers were extracted with 2% SDS, 5 mM EDTA. Extracts were diluted 5-fold in 1.25% Triton X-100 and incubated with preimmune rabbit serum followed by goat anti-rabbit IgG coupled to Sepharose. Nonspecifically precipitated radioactivity was removed by centrifugation. The nonspecifically precipitated radioactivity accounts for 15% of input and was present in the bands at the tops of the stacking gel and separating gel. The supernatants were divided and immunoprecipitated with rabbit antiserum to the 70-kDa fragment (anti-70-kDa) or type III module region (anti-III) of human plasma fibronectin followed by anti-rabbit IgG-Sepharose. 80% of 125I-fibronectin associated with cells was precipitated by either the anti-70-kDa fragment or anti-III antibodies, whereas 15% was precipitated with preimmune serum. Of 125I-70-kDa associated with cells, 20% was precipitated by anti-III antibodies or preimmune serum, whereas more than 80% was precipitated by anti-70-kDa fragment antibodies. Precipitates were analyzed after reduction by electrophoresis on a 3/10% polyacrylamide slab gel. Starting extracts are shown in the left panel of the phosphorimage and immunoprecipitated proteins on the right. The 300-kDa band is indicated.

FIG. 11. Formic acid and Glu-C protease digestions of the cross-linked complexes. Human foreskin fibroblasts were incubated for 60 min with 125I-70-kDa fragment or fibronectin and 200 nM LPA, washed, and cross-linked with factor XIIIa, 10 µg/ml, for 5 min and extracted with 2% SDS, 5 mM EDTA. A, the cross-linked 125I-labeled 70-kDa fragment after formic acid digestion was immunoprecipitated with anti-type III module region (Anti-III) or anti-70-kDa fragment (Anti-70-kDa) antibodies. B, the cross-linked 125I-labeled fibronectin (FN-XL-HFSF) or 70-kDa fragment (70-kDa-XL-HFSF) to human foreskin fibroblasts (HFSF) after Glu-C protease digestion was immuno-precipitated with anti-type III module region (Anti-III), anti-70-kDa fragment (Anti-70-kDa) antibodies, or preimmune serum (Pre-immune). s, the starting material; p, the pre-clear material. Arrow indicates the 140-kDa band generated by Glu-C cleavage of the cross-linked complexes.

bly on substrate-attached fibroblasts requires intact cytoskeletal structure, as cytochalasin B treatment abolishes the cell’s assembly ability (16, 47). Similarly, fibronectin assembly occurs around fibroblasts cultured in collagen gels under tension but not in relaxed collagen gels (48). Correlative microscopic and binding studies (30) suggest that LPA-induced tension on the adherent cells stretches labile assembly site molecules and opens multiple cryptic binding sites for the N-terminal modules of fibronectin. H7, in contrast, causes loss of actin cytoskeleton and filopodia. The cross-linking studies with factor XIIIa together with photoaffinity labeling experiments indicate that molecules with LAMMs by SDS-PAGE bind the 70-kDa region of fibronectin and have the properties of regulated fibronectin assembly sites. Cross-linking correlated with binding of labeled 70-kDa fragment, since cross-linking and binding were blocked with the same dose response by unlabeled 70-kDa fragment. There were no intermediate complexes of <3 MDa observed when increasing amounts of a monovalent cross-linking competitor.
dansylcadaverine, were present, suggesting that factor XIIIa-mediated cross-linking occurs in a single step. Both factor XIIIa-mediated cross-linking and photoaffinity trans-labeling were subject to stimulation by LPA. Neither ligand nor factor XIIIa penetrate the membrane under the experimental conditions (18, 49), and the molecules were sensitive to light trypsinization of intact cells. Thus, the labeled molecules are proteins on the outside of cells. The cross-linked complexes described in the previous experiment and the ability of cellular fibronectin, as opposed to plasma fibronectin, to bind to chondroitin sulfate (55).

Assembly Sites for Fibronectin

Chondroitin ABC lyase or heparitinase digestion did not affect the binding or cross-linking of the 70-kDa fragment. Sulfate-depleted and chlorate-treated cells deficient in glycosaminoglycan bound the same amount of fibronectin as untreated cells and responded identically to LPA. The heparitinase and sulfate depletion results are consistent with previous studies showing no effects on matrix assembly by treatment with heparitinase (54). The chondroitin lyase results are different from those obtained by photoactivable cross-linking of cellular fibronectin after an 18-h incubation with cell layers (29). This difference may be due to the long incubation time in the previous experiment and the ability of cellular fibronectin, as opposed to plasma fibronectin, to bind to chondroitin sulfate.

Studies with purified proteins have demonstrated an interaction between the N-terminal modules of fibronectin and the III-1/III-2 module region of fibronectin (56–59). Furthermore, the 70-kDa fragment codistributes with III-1 and III-10 modules when added to fibroblasts under serum-free conditions (58). In our studies, the 70-kDa fragment bound in a highly restrictive manner to areas of the cell surface where there was often pre-existing fibronectin. However, there was no complete co-localization of the 70-kDa fragment with fibronectin. Occasionally, long linear arrays of 70-kDa fragment were localized where there was no fibronectin staining detectable by the sensitive technique of immunofluorescent microscopy. This result was found with several different antibodies to the type III module region of fibronectin. These observations suggest that the 70-kDa fragment may bind to pre-assembled fibronectin and to molecules other than fibronectin. However, we found no evidence of 70-kDa fragment-fibronectin cross-linking. Antibodies to fibronectin regions other than the N-terminal 70-kDa region did not immunoprecipitate cross-linked complexes of 125I-70-kDa fragment or the digested products of cross-linked complexes of the 70-kDa fragment. Furthermore, cross-linked 70-kDa fragment complexes could not be digested with cathepsin D, even though this enzyme effectively generates the 70-kDa fragment from plasma fibronectin and is known to digest fibronectin deposited in extracellular matrix (37). These results indicate that if pre-polymerized fibronectin does represent some or all of the LAMMs, multiple antigenic epitopes and sites of proteolytic digestion must be lost during the pre-polymerization process. Further, extraordinarily rapid and specific condensation of cellular fibronectin must be hypothesized to account for the rapid appearance of LAMMs on cells freshly seeded on vitronectin-coated dishes.

The notion that fibronectin binds and cross-links to a moiety other than fibronectin at cell surfaces has a precedent in the cross-linking of fibronectin to fibrin (41). Purified plasma fibronectin is susceptible to factor XIIa-catalyzed cross-linking to itself but only if aggregate formation is induced by reduction of disulfides or denaturation (10, 11, 41). In contrast, native plasma fibronectin is cross-linked efficiently to fibrin (41). The cross-linking involves lysyl residues in fibrin and factor XIIa-reactive glutaminyl residues in fibronectin (60). We do not know the order of cross-linking at the cell surface. Molecules with the electrophoretic mobilities of the LAMMs incorporate dansylcadaverine when cultured cells are exposed to the monofunctional amine and factor XIIa (61). Thus, the order of cross-linking could be lysine in fibronectin or 70-kDa fragment to glutamine in LAMMs, or vice versa, or both. When we failed in multiple strategies to dissociate the cross-linked 70-kDa fragment-LAMMs complex prior to and during SDS-PAGE. Cellular titin, a 2.5-MDa fibroblast protein (62), and shape change, development of filopodia, and display of the assembly site.
muscle titin, an elastic component of myofibril (45), have a similar size to the ~3-MDa LAMMs by SDS-PAGE. The titins, however, are intracellular proteins. The fact that the complexes had apparent size of about 3 MDa does not mean that the band represents a single 3-MDa protein. There are a number of well-studied examples of SDS-stable protein complexes, e.g. stabilization of the streptavidin tetramer by biotin (63) and of the α and β subunits of class II major histocompatibility complex proteins by peptides in the binding groove (64). Also, integral membrane proteins such as glycophorin (65) and phospholamban (66) have been shown to form SDS-stable complexes by virtue of interaction among residues in the membrane-spanning α-helices. One can well imagine the LAMMs being a conglomerate of one or several proteins. The appearance of the radioactive 150–150-kDa bands in formic acid or Glu-C protease digests of cross-linked125I-70-kDa fragment indicates that the 70-kDa fragment of fibronectin is cross-linked to a specific stretch of polypeptide of a very large protein or of a smaller protein within the conglomerate. We are trying to purify the 70-kDa fragment bound to cross-linked partner from the digests of the LAMM complexes in order to characterize the cellular binding moiety.

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