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Revisiting the mystery of fibronectin multimers: the fibronectin matrix is composed of fibronectin dimers cross-linked by non-covalent bonds

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Abstract

Fibronectin (FN) matrix fibrils have long been thought to be formed by disulfide-bonded FN multimers, although there is no direct evidence that they are covalently linked with each other. To understand the biochemical properties of these fibrils, we extracted a crude FN matrix from FN-YPet transfected 3T3 cell culture using 0.2% deoxycholate and DNase. The insoluble extracted matrix preserved fibrillar structures and a major portion of the extracted proteins migrated as FN monomers on an SDS gel under reducing conditions. Under non-reducing conditions, some FN molecules appeared to be trapped at the top of the stacking gel. We tested this by mixing fluorescently labeled FN dimers with the extracted matrix just before loading on an SDS gel, and found that most of them were trapped with the extracted proteins at the top of the stacking gel. These results suggested that some components of the extracted matrix plugged the stacking gel and FN dimers were trapped with them. Rotary shadowing electron microscopy showed that the extracted matrix had some fibers that resembled fibrillin microfibrils. Peptide mass fingerprinting confirmed the presence of fibrillin in the extracted matrix. Fibrillin is known to form disulfide-bonded multimers and it is likely to be one of the components that plug the stacking gel and trap FN molecules in this system. The phenomenon by which FN molecules appear to migrate as multimers on SDS gels is thus an artifact arising from the presence of other large components in the extract. We conclude that FN matrix fibrils are made of FN dimers that are further cross-linked by non-covalent protein-protein bonds.

Keywords

Fibronectin; disulfide bond; matrix; SDS gel; fibrillin

1. Introduction

Native fibronectin (FN) molecules are secreted from cells as disulfide-bonded dimers, which are present in plasma and tissue fluids (Hynes 1990). Soluble dimeric FN assembles into an insoluble fibrillar matrix on the cell surface. The assembly of FN matrix fibrils requires integrins (Wu et al. 1995; Wennerberg et al. 1996; Sechler et al. 2000; Takahashi et al. 2007) and their translocation by the actin cytoskeleton (Pankov et al. 2000; Ohashi et al. 2002). In cell culture, FN forms a fibrillar network that can be visualized by immunofluorescence staining. Electron microscopy (EM) shows that these matrix fibrils are composed of bundles

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of fine FN fibers, which are 5 to 10 nm in diameter (Chen et al. 1978; Singer 1979). In addition, live cell imaging demonstrated that FN matrix fibrils are not a stationary structure but are dynamic and elastic (Ohashi et al. 1999; Sivakumar et al. 2006; Davidson et al. 2008).

Although the gross structure of the extracellular FN matrix has been well characterized, the bonds connecting molecules are more obscure. It had been believed that FN matrix fibrils are composed of disulfide-bonded multimers because they migrated as large multimers in an SDS gel under non-reducing conditions (Hynes and Destree 1977; Keski-Oja et al. 1977; Yamada et al. 1977; McConnell et al. 1978). But in 1996, Chen and Mosher (1996) comprehensively studied the FN matrix by cleaving FN molecules with cyanogen bromide and formic acid, and concluded that there is no evidence that FN forms disulfide-bonded multimers. It has therefore been a mystery why FN molecules in the matrix behave like large disulfide-bonded multimers on an SDS gel.

In this study, we isolated a crude FN matrix from cell culture and discovered that a majority of FN dimers in the matrix were physically trapped at the top of the stacking gel during SDS-PAGE. We propose that fibrillin is the major candidate responsible for plugging the stacking gel and trapping FN in 3T3 cells.

2. Results and Discussion

We originally tried to isolate FN matrix fibrils with 0.5-2% deoxycholate (DOC) as reported previously (Hynes and Destree 1977; Chen et al. 1978; Hedman et al. 1979; Sechler et al. 1996). However, it was difficult to isolate them because DNA made the extracts very viscous and DNase did not work at these DOC concentrations. We found that using shear force to break the DNA and reduce viscosity caused variable, sometimes almost complete, solubilization of the “DOC-insoluble” FN matrix. In order to perform the extraction while still preserving the FN matrix, we tested DNase treatment under several conditions using DOC, SDS, Triton X-100, Tween 20, high pH buffer (CAPS) and urea, and found that extraction with 0.2% DOC and DNase gave the best results. We used a culture of 3T3 cells expressing FN-YPet. As shown in Fig. 1, the extracted matrix preserved fibrillar structures that contained FN-YPet (Fig. 1A and C), although most of the extract was a large aggregate with no clear substructure. Chen et al. (1978) also reported that isolated matrix fibrils tend to aggregate. DIC (differential interference contrast) images also showed fibrillar structures at the edge of the aggregates (Fig. 1B and D). A similar result was also obtained by extraction with 0.1% SDS and DNase.

The insoluble pellet from the initial 0.2% DOC extract was treated with a second extraction (TBS alone, 0.5% DOC or 6 M urea) to test whether FN molecules could be solubilized under other conditions. The soluble and insoluble fractions from this second extraction were run on SDS-PAGE (Fig. 2). Under reducing conditions, the major component was FN monomer as seen in the pellet from the TBS extraction. Insoluble FN molecules in the initial extract stayed insoluble in 0.5% DOC, but a majority of the FN molecules were solubilized with 6 M urea, which is consistent with previous reports (Hedman et al. 1979; Sasaki et al. 1996). A small amount of protein was also seen on the top of the stacking gel. Some of this may be products formed by transglutaminase which has been shown to covalently cross-link FN and collagen (Mosher et al. 1980). Under non-reducing conditions the major component was FN dimer, however the intensity of the dimer band was much less than that of the monomer band under reducing conditions. This suggests that in the absence of reducing agents the missing FN molecules are trapped at the top of the stacking gel (there is a prominent band there, but it cannot be quantitated).

When increasing amounts of the extracted matrix were run on an SDS gel, the amount of FN monomer increased proportionally under reducing conditions (Fig. 3). On the other hand, the

amount of FN dimer was relatively constant for all loading volumes under non-reducing conditions. These results suggest that FN dimers are easily trapped at the top of the stacking gel, especially as the amount of the total extract is increased. To verify this, we mixed fluorescently labeled FN dimers with the extracts before loading on the gel. As expected, a majority of fluorescently labeled FN dimers were trapped at the top of the stacking gel under non-reducing conditions (Fig. 4). This confirmed that the extracted matrix plugged the stacking gel under non-reducing conditions and trapped even FN that was added to the matrix as soluble dimers.

Thirty years ago, McConnell et al.(1978) suggested that a majority of FN dimers formed disulfide-bonded multimers, which were responsible for plugging the stacking gel, and that some non-bonded FN dimers were trapped with them. However, the study by Chen and Mosher (1996) clearly demonstrated that FN dimers do not form disulfide-bonded multimers, so FN dimers must be trapped by something else. To study this further, we examined the crude extracted matrix by rotary shadowing EM. Like light microscopy, most of the extract was a large aggregate, so it was hard to see any structural details. However, in some areas we observed structures at the edge of the aggregate as shown in Fig. 5. The most prominent structures were strings with periodically arranged beads which were approximately 20 nm in diameter. The distance between beads varied from 20 to 100 nm and the beads appeared to be connected with fine filamentous structures. These features are characteristic of fibrillin microfibrils (Fleischmajer et al. 1991; Keene et al. 1991; Baldock et al. 2001). A recent study showed that the beads are composed of the C-terminal portions of multiple fibrillin molecules (Hubmacher et al. 2008). In addition to fibrillin microfibrils, there were various macrostructures (arrows in Fig. 5) but it was impossible to discern any fine structure in them. Individual FN molecules were also seen as short strings (arrowheads in Fig.5), as reported previously (Erickson et al. 1981).

The close relation between fibrillin and FN during the initial stage of fibrillin microfibril assembly has recently been reported (Kinsey et al. 2008; Sabatier et al. 2008). In addition, fibrillin appears to directly interact with FN (Sabatier et al. 2008). Soluble fibrillin was first isolated from the conditioned medium of a human fibroblast culture as a ~350 kDa glycoprotein and a major component of microfibrils (Sakai et al. 1986). Fibrillin is a cysteine-rich molecule and assembles into microfibrils via interchain disulfide bonds (Reinhardt et al. 2000; Hubmacher et al. 2008). SDS gel images of fibrillin from these studies showed that disulfide-bonded fibrillin was trapped at the top of the stacking gel under non-reducing conditions. Under reducing conditions, fibrillin migrated as a monomer above the FN band. We have seen a similar band on our SDS gels (arrows in Figs. 2, 3, 4 and 6). Like fibrillin, this band is only seen under reducing conditions. We performed peptide mass fingerprinting and identified this protein as mouse fibrillin2. Mouse fibrillin1 and fibrillin2 share 76% amino acid sequence identity with each other and have highly homologous structures (Zhang et al. 1994). However, they show differential gene expression patterns during development. It has been reported that fibrillin2 can self-assemble into microfibrils, although it can also co-assemble with fibrillin1 (Charbonneau et al. 2003).

The formation of fibrillin microfibrils in cell culture has been reported to be slower than FN matrix assembly (Kinsey et al. 2008; Sabatier et al. 2008). To reduce fibrillin contamination, we isolated a crude matrix from a one day culture. The initial cell density was increased four-fold and the final suspension volume was reduced by half to compensate for the quantity of the extract. Unlike the extract from a four day culture, a negligible amount of fibrillin was seen on an SDS gel under reducing conditions. Under non-reducing conditions a large amount of FN dimers was detected, relative to the four day culture (Fig. 6). These results support our speculation that fibrillin is the major component that plugged the stacking gel and trapped FN in our system.

Peptide mass fingerprinting also identified another prominent protein band as mouse myosin 9, which runs below FN under reducing conditions (arrowheads in Figs. 2, 3, 4 and 6). Some of the cytoskeleton proteins such as myosin and actin are insoluble with detergent extraction (Chen et al. 1978). Like FN, myosin appears to be trapped at the top of the stacking gel under non-reducing conditions (Fig. 2, 3, 4 and 6). Unfortunately, we were unable to identify other proteins in the extract, probably because the purity and/or quantity of those proteins were insufficient for the assay. Therefore we also tested a recombinant FN fragment (~120 kDa) and a tenascin-C (TN-C) fragment (~150 kDa) by mixing them with the extracts and found that these molecules were not trapped as FN and myosin were (data not shown). This suggests that only very large molecules (>200 kDa) tend to be trapped at the top of the stacking gel.

Overall, our results show that the detergent extracted matrix contains some proteins that plug the top of the stacking gel and cause most of the FN dimers to be trapped with them. fibrillin microfibrils, which are known to form large disulfide-bonded multimers and are present in the extract, seem the most likely candidate. In cell lines that do not express fibrillin, other molecules may form covalently linked multimers that are responsible for plugging the stacking gel. To experimentally test this hypothesis, we mixed purified FN with purified TN-C, which forms a 1,300 kDa disulfide-bonded hexamer (Erickson and Iglesias 1984), and ran them on an SDS gel. TN-C did not enter the running gel under non-reducing conditions and appeared to plug the stacking gel and trap some FN (Fig. 7). The presence of these covalently linked multimers can account for FN dimers failing to enter the gel under non-reducing conditions; it is not necessary to postulate disulfide-linked multimers of FN. We conclude that FN matrix fibrils are composed of FN dimers that are further cross-linked by non-covalent protein-protein bonds of still unknown nature.

3. Materials and Methods

FN-YPet stably transfected cells

To generate stably transfected cells, the FN-YPet expression vector was constructed by modifying the pAIPFN vector (Akamatsu et al. 1996; Ohashi et al. 1999). In this expression vector, we inserted monomeric YPet (Ohashi et al. 2007) between FN type III (FNIII) domains 6 and 7 (FNIII6...PLSPggrMVS...YPet...KTSggrPLSP...FNIII7, the YPet sequence is underlined). Note that we added four extra amino acids derived from FNIII7 at the C-terminus of FNIII6 to stabilize this domain, because a C-terminal extension seems to be important for stabilizing some FNIII domains (Hamill et al. 1998; Niimi et al. 2001). We also added the PCR amplified neomycin cassette from the EYFP-C1 vector into the Kpn I site of the expression vector. We call this construct FN-YPet/Neo. The FN-YPet/Neo vector was transfected into NIH3T3 fibroblasts with lipofectamine (Invitrogen). The stably transfected clones were selected with G418 (0.7 mg/ml, Invitrogen) and screened for visible FN matrix fibrils by fluorescence microscopy.

Matrix extraction

To isolate FN matrix fibrils, the FN-YPet transfected cells ($\sim 5 \times 10^6$) were plated in a T-75 tissue culture flask and cultured with 40 ml DMEM (Invitrogen) containing 10% calf serum (HyClone) for 4 days. For extraction, the culture medium was removed from the flask and 10 ml extraction buffer (0.2% DOC, 20 mM Tris (pH 8.0), 2mM PMSF and 2 mM iodoacetamide) containing DNase (10 μ g/ml) was added. We avoided adding magnesium for DNase activation, because excess magnesium enhanced FN degradation, probably by activating a protease during extraction. Fortunately, endogenous magnesium from the culture cells seemed sufficient to activate DNase and FN degradation appeared to be minimal, although DNA digestion took approximately 2 hours at room temperature under these conditions. A small amount of the insoluble matrix was still attached to the bottom of the culture flask after extraction. It is likely

that many of the molecules were denatured by attachment to the plastic or would be mechanically denatured by attempts to scrape them off, so they were left behind. After digestion, extracts were transferred to 15 ml tubes and were centrifuged at 2,000 ×g for 5 min. The insoluble pellets were rinsed with 5 ml extraction buffer, then transferred with 1 ml extraction buffer into 1.5 ml centrifuge tubes and centrifuged at 2,000 ×g for 5 min. The pellets were rinsed twice with 1 ml TBS (20 mM Tris containing 0.15 M NaCl, pH 8.0) and stored with 50 µl TBS at 4°C until use. To test the solubility of the extracted matrix, some pellets were treated with a second extraction with 50 µl 0.5% DOC in 20 mM Tris (pH 8.0) or 6 M urea in 20 mM Tris (pH 8.0) overnight then centrifuged at 20,000 ×g for 5 min. For SDS-PAGE, an equal volume of SDS loading buffer containing either 50 mM DTT under reducing conditions or 50 mM iodoacetamide under non-reducing conditions was added to the final pellets. SDS-PAGE was carried out using standard methods.

Fluorescence-labeled FN

Fluorescein maleamide (Invitrogen) was used to label purified FN as reported previously (Baneyx et al. 2001). To expose the two buried free thiols in FNIII domains 7 and 15, 6M urea was added to 0.25 mg/ml purified FN in PBS. Maleamide dye labeling was performed following manufacturer's instructions. After labeling, free dye and urea were removed with a PD-10 desalting column (Amersham). The labeling efficiency was approximately 70%. The fluorescein labeled domains are unlikely to refold properly, but refolding is unnecessary since they will be denatured by SDS during the assay. Fluorescein labeled FN was added to the insoluble matrix, which was resuspended with loading buffer. After heat denaturation, samples were run on an SDS gel and fluorescence images were captured before Coomassie blue staining.

Light microscopy and rotary shadowing electron microscopy

For light microscopy, the DOC extracted insoluble pellet was pipetted out and mounted on a glass slide with TBS. A cover glass was pressed down to spread the insoluble extract. The extracted matrix was observed with a light microscope (Zeiss Axiophot) and the images were acquired with a cooled CCD camera (CoolSNAPHQ, Roper Scientific).

For rotary shadowing EM, the insoluble extracts were resuspended with 20 mM ammonium bicarbonate containing 30% glycerol and directly sprayed onto freshly split mica, dried in vacuum and rotary shadowed with platinum (Fowler and Erickson 1979).

Peptide mass fingerprinting

The bands of interest were excised from the Coomassie blue stained SDS gel of samples that were run under reducing conditions and cut into small pieces (~1 mm³). Gel pieces were washed with 25 mM ammonium bicarbonate/50% acetonitrile and dried. In-gel digestion was performed with 50 µg/ml trypsin (Sigma, proteomics grade) in 25 mM ammonium bicarbonate. The trypsin-digested sample was cleaned up with a Ziptip (Millipore), mixed with α -cyano-4-hydroxycinnamic acid (1:10), mounted on a plate and dried for MALDI analysis. MALDI-TOF mass spectrometry was performed with a Voyager DE (Applied Biosystems). Spectra were obtained from the accumulation of 160-200 laser shots. The data were analyzed by Aldente (a peptide mass fingerprinting tool; <http://www.expasy.org/tools/aldente/>).

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Fig. 1. Isolation of a crude FN matrix from FN-YPet transfected cell culture

A crude FN matrix was extracted with 0.2% DOC plus DNase from cultured cells producing FN-YPet. Most of the isolated matrix formed aggregates, but in some areas fibrillar structures were seen at the edge of the aggregates. Fluorescence images showed that the matrix extracts contained FN-YPet (A and C). DIC images also showed fibrillar structures (B and D).

Fig. 2. The solubility of FN in the crude FN matrix extracts

The 0.2% DOC extracted crude matrix was treated with a second extraction with TBS, 0.5% DOC or 6 M urea, and centrifuged to separate the soluble and insoluble portions. Samples of the final supernatant (S) and pellet (P) were run on an SDS gel and stained with Coomassie blue. A majority of the matrix proteins was solubilized with 6 M urea. Note that FN from the extracts migrates as a diffuse band because it derives from three different sources: endogenous mouse FN from fibroblasts, calf serum FN and transfected FN-YPet, all of which have slightly different molecular weights. Purified FN (~250 kDa) was used as a reference. An arrow and arrowhead indicate fibrillin and myosin, respectively, on the reducing gel.

Fig. 3. FN dimers trapped at the top of the stacking gel under non-reducing conditions
 Increasing amounts of the extracted matrix were run on an SDS gel and stained with Coomassie blue. Under reducing conditions the amounts of FN monomers increased along with the total extracts. Under non-reducing conditions, the amounts of FN dimers did not increase substantially when the amounts of the extracts were increased, and FN dimers appear to be trapped at the top of the stacking gel. An arrow and arrowhead indicate fibrillin and myosin, respectively.

Fig. 4. Fluorescently labeled FN dimers trapped by the extracted matrix at the top of the stacking gel under non-reducing conditions

Fluorescein-maleamide labeled FN was run on an SDS gel with or without the matrix extract and stained with Coomassie blue (left panel) or observed by fluorescence (right panel). Under reducing conditions FN-fluorescein was detected as monomers both in the presence and absence of the extracted matrix; a small fraction appeared trapped at the top when mixed with matrix. On a non-reducing gel, on the other hand, a majority of fluorescein-labeled FN dimers were trapped at the top of the stacking gel in the presence of the extract. An arrow and arrowhead indicate fibrillin and myosin, respectively.

Fig. 5. Rotary shadowing EM images of the isolated matrix

Distinctive fibrillin microfibrils were seen as strings of beads at the edge of aggregates. Some macromolecular structures were also observed, although the arrangements of molecules were not clear at this resolution (arrows). Single FN molecules (arrowheads) and some globular molecules were also seen throughout the field.

Fig. 6. A comparison of the extracts from one and four day culture

The extracts from one and four day culture were run on an SDS gel and stained with Coomassie blue. A majority of the matrix proteins in both extracts migrated as FN monomers under reducing conditions. A significant amount of fibrilllin was only observed in the extract from four day culture. Under non-reducing conditions, a large amount of FN dimers was detected in the extract from one day culture. An arrow and arrowhead indicate fibrilllin and myosin, respectively.

Fig. 7. Disulfide bonded TN-C plugs the stacking gel and traps FN dimers

Fluorescein-maleamide labeled FN was run on an SDS gel with or without TN-C and stained with Coomassie blue (left panel) or detected by fluorescence (right panel). FN-fluorescein was observed as monomers both in the presence and absence of TN-C under reducing conditions. On a non-reducing gel, on the other hand, some fluorescein-labeled FN dimers were trapped at the top of the stacking gel in the presence of TN-C. An arrow indicates TN-C (small splicing variant, ~220 kDa).