Control of Cell Cycle Progression by Fibronectin Matrix Architecture

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Developmental patterning and differentiation, maintenance of parenchymal cell function, and the size, shape, and invasiveness of tumors are all orchestrated by cell interactions with the extracellular matrix. Here we show that the fibrillar structure of fibronectin (FN) matrix encodes essential regulatory cues and controls cell proliferation and signaling through changes in matrix architecture. A matrix assembled from native FN stimulated cell growth. In contrast, a mutant FN (FNΔIII1–7) that contains all known cell binding motifs but forms a structurally distinct matrix inhibited progression from G0/G1 into S phase. Furthermore, FNΔIII1–7, that contains all known cell binding motifs but forms a structurally distinct matrix inhibited progression from G0/G1 into S phase. Furthermore, FNΔIII1–7, that contains all known cell binding motifs but forms a structurally distinct matrix inhibited progression from G0/G1 into S phase. Therefore, FNΔIII1–7 suppressed the stimulatory capacity of native FN and induced different levels of tyrosine phosphorylation of pp125FAK. The differential effects on cell growth were ablated by blocking formation of matrix fibrils. Thus, modification of matrix architecture provides a novel approach to control cell proliferation.

In tissues and tumors, cells live within a multidimensional fibrillar extracellular matrix. The surrounding matrix fibrils thus are uniquely poised to supply environmental signals to cells, suggesting that the structural organization of the matrix itself contributes to the control of cell behavior. Extracellular matrix regulation of cell adhesion, migration, and gene expression occurs through binding to integrins and other cell surface receptors (1–3). As an integral component of extracellular matrices, FN1 matrix fibrils interact with integrin receptors through well characterized binding sites (4, 5). Multiple FN domains are required to maintain the integrity of the matrix and deletion or mutation of specific sites in FN can affect matrix structure and assembly (6–11). In particular, a recombinant FN, FNΔIII1–7, that contains all known cell binding sites but lacks the first seven type III repeats (Fig. 1A) exhibits an altered rate of matrix assembly with unique intermediates as compared with native FN (11). FNΔIII1–7 and FN matrices also differ in their capacities to re-organize the actin cytoskeleton (12), suggesting that the structurally distinct architecture of native and altered FN matrices may have significantly different intracellular consequences.

Matrix engagement of specific receptors represents an important control point for determining cell shape, cytoskeletal geometry, and the organization of intracellular components (13, 14) as well as initiating the signaling events that lead to cell cycle progression (15–17). We now show that the architecture of the FN matrix can also regulate this process. FN matrices with distinct morphologies had opposite effects on cell growth by specifically altering the rate of G0/G1 to S phase progression. These effects required FN fibril formation demonstrating that the structure of the matrix plays an active role in modulating cell signaling.

EXPERIMENTAL PROCEDURES

Proteins and Cells—SVT2 (SV40-transformed 3T3) cells were grown in Dulbecco’s modified Eagle’s medium plus 10% calf serum. Growth conditions for CHOo5 cells and expression and purification of pFN and recombinant FNs were as described previously (11). FNΔIII1–7 is a baculovirus-expressed full-length recombinant FN lacking the first two type III repeats.

Cell Synchronization—SVT2 and CHOo5 cells were seeded at a density of 2 × 10⁵ cells in either four-well (Nunc) plastic chamber slides (for SVT2 cells) or 24-well dishes with glass coverslips (CHOo5), allowed to attach and spread for 16–24 h, then incubated in serum-free medium for 16–22 h to obtain a population of cells in G0. As is the case for most transformed cell lines, serum starvation was not sufficient to totally synchronize SVT2 or CHOo5 cells but did enrich a G0 population as determined by propidium iodide staining and FACS analysis. For synchronization at G0/S, CHOo5 and SVT2 cells were seeded in 24-well dishes at a concentration of 2.5 × 10⁵ cells/well. After a 16-h incubation in complete medium, SVT2 cells were cultured with 0.5 mM hydroxyurea for 14 h and CHOo5 cells in 1 mM hydroxyurea for 18 h to synchronize cells at late G0/S phase. Cells were released from hydroxyurea, washed, and reseeded with complete medium containing FN-depleted serum and 50 μg/ml pFN or FNΔIII1–7. Cells were transfected with propidium iodide (Cycle Test Plus DNA Reagent Kit, Becton Dickinson), and 3 × 10⁶ cells were analyzed by FACS 10 h after release from hydroxyurea to monitor progression through S phase and G0/M. A set of nonsynchronized cells and cells after hydroxyurea incubation were also stained and analyzed by FACS using Cell Quest software (Becton Dickinson).

Matrix Assembly and Cell Adhesion—After synchronization, cells were transferred into complete medium containing FN-depleted serum plus 50 μg/ml pFN, 50 μg/ml FNΔIII1–7, or other recombinant FNs or without added FN. For the mixture of FNΔIII1–7 with pFN, 50 μg/ml of each protein were added. For 70-kDa inhibition of fibril formation, 50 μg/ml pFN or FNΔIII1–7 was mixed with 250 μg/ml 70-kDa fragment. Cells were allowed to assemble FN matrix for the indicated time periods. For immobilized proteins, 96-well microtiter plates were coated overnight at 4 °C with 10 μg/ml pFN or FNΔIII1–7, for BrdUrd incorporation or 5, 10, and 15 μg/ml for adhesion. Serum-starved CHOo5 cells were trypsinized and plated on coated wells at a concentration of 2 × 10⁴ cells/well and then cultured in medium containing FN-depleted serum for a 10–20 h time course.

BrdUrd Labeling—BrdUrd was added to a final concentration of 10 μM and incubated for 30 min. BrdUrd-positive cells were detected with an anti-BrdUrd antibody followed by an alkaline phosphatase-conjugated secondary antibody and developed with nitro blue tetrazolium and X-phosphate substrate solution (Boehringer Mannheim). The numbers of total and BrdUrd-positive cells were counted for several fields (750–1000 total cells) and the percentage of positive cells calculated. Alternatively, cells were labeled with 10 μM BrdUrd and processed for BrdU Detection Kit III (Boehringer Mannheim). Plates were read on a microtiter plate reader at 405 nm with a reference wavelength of 490 nm.

Immunoblots—For adhesion, serum-starved CHOo5 cells were trypsinized and plated at a concentration of 2 × 10⁵ cells onto a 48-well
Results and Discussion

Native and Mutant FN Matrices Differentially Regulate Cell Proliferation—Native FN and FN(III1–7) matrices have markedly different effects on cell proliferation as shown in Fig. 1B. CHOα5 cells, which fail to synthesize a FN matrix (11), were serum-starved to obtain an enriched population of quiescent cells in G0. Cells were then released from serum-free conditions and incubated with medium containing native pFN, baculovirus-expressed mutant FN(III1–7), or no exogenous FN. During the incubation, CHOα5 cells bind and assemble exogenous FN into a fibrillar matrix at the cell surface (11). Labeling of newly synthesized DNA by incorporation of BrdUrd was then used to monitor cell cycle progression. Compared with cells without FN matrix, pFN stimulated and FN(III1–7) inhibited entry into S phase (Fig. 1B). Twice as many cells with a native FN matrix were BrdUrd-positive 16 h after release from serum-free conditions as compared with cells with FN(III1–7) matrix (Fig. 1C). With native FN matrix, cells entered S phase at least 8 h earlier than cells with FN(III1–7) matrix. Cells with no FN matrix progressed into S phase at a rate intermediate to native FN and FN(III1–7). BrdUrd incorporation by cells with a full-length recombinant FN matrix was the same as cells with pFN, indicating that differences in growth were not due to the source of the baculovirus-expressed recombinant protein.

Not only does FN(III1–7) matrix inhibit cell growth, it also suppresses the stimulatory effects of native FN. A matrix composed of equal proportions of pFN and FN(III1–7) reduced BrdUrd incorporation by 30% relative to cells in the presence of native FN matrix (Fig. 1D). This result shows that the FN(III1–7) matrix has a dominant-negative effect on growth stimulation by native FN.

FN(III1–7) matrix also slowed the proliferation of SVT2 cells that assemble a matrix using endogenously produced FN (Fig. 2A). Incubation of cells with exogenous pFN or FN(III1–7) results in co-assembly with endogenous SVT2 FN as well as a significant increase in the overall level of matrix-associated FN (9). The G0 to S phase interval of SVT2 cells with FN(III1–7)-containing matrix was 4 h longer than that of cells assembling a pFN matrix, suggesting a dominant inhibitory effect of the FN(III1–7) matrix on cell growth. Morphologically, SVT2 cell matrices containing pFN and FN(III1–7) are distinct. Immunofluorescence staining of cells with exogenous pFN shows an ordered fibrillar matrix (Fig. 2B). In contrast, FN(III1–7) matrix appears less uniform and is characterized by fibrils of varying length and thickness (Fig. 2, C and D). Together, these results demonstrate that co-assembly of FN(III1–7) with SVT2 FN has a dominant-negative effect on cell growth and, as with CHOα5 cells, this effect correlates with differences in matrix fibril organization.

The effects of matrix assembly on cell growth are restricted to events controlling G0/G1 progression. CHOα5 and SVT2 cells synchronized at G0/S with hydroxyurea were released into complete medium containing pFN, FN(III1–7), or no FN and stained with propidium iodide to monitor DNA synthesis and cell cycle progression by FACS analysis. Neither native FN or FN(III1–7) matrix altered the progression of cells through S or G2/M as the percentages of cells traveling through these phases and back to G1 were identical (not shown).

Growth Responses Are Abolished by Inhibition of FN Fibril Formation—pFN and FN(III1–7) both contain all known cell binding sites and both use α5β1 integrin to initiate matrix assembly (11, 18, 19), yet the two types of matrices have opposite effects on cell growth. Cell proliferation would be affected if there are different levels of integrin-mediated binding and adhesion to these two FNs (16, 17, 20). To address this possibility, attachment and growth of CHOα5 cells on immobilized pFN or FN(III1–7) protein were measured. Equal numbers of cells attached to pFN and FN(III1–7) substrates in 30 min (not shown). Attached cells showed identical levels of BrdUrd incorporation on the two proteins (Fig. 3A). Therefore, cell adhesive
interactions with native and mutant FNs are indistinguishable. An alternative explanation for the opposite growth responses is that the distinct architectures of the two matrices may influence cell proliferation. If fibrillar matrix structure controls the rate of growth, then inhibition of fibril formation should eliminate the differences between native FN and FN\(_{DIII1–7}\) matrices. Inclusion of excess 70-kDa amino-terminal fragment of FN during matrix assembly blocks FN-FN interactions via the assembly domain (see Fig. 1A) thus preventing fibril formation by pFN (6, 7) and FN\(_{DIII1–7}\) (11). 70-kDa fragment does not interfere with FN-integrin interactions. Inhibition of fibril formation by addition of 70-kDa fragment reversed the growth stimulatory effects of native FN matrix and the inhibitory effects of FN\(_{DIII1–7}\) matrix (Fig. 3B). In both cases, a block in fibril assembly resulted in BrdUrd incorporation comparable with that of cells with no matrix. Further evidence for the role of fibrillar matrix structure in regulating cell growth was provided by experiments with two other mutant recombinant FNs, FN\(_{DIII1–2}\) and FN(syn-). FN\(_{DIII1–2}\) is defective in FN binding and polymerization, while FN(syn-) does not bind well to \(\alpha_5\beta_1\) integrin (21). As a result of these defects, each can only form short fibrils but cannot assemble into an extensive matrix of the type shown in Fig. 2. Cells grown in the presence of either of these two mutant FNs progressed into S phase at a rate similar to cells with no FN matrix (Fig. 3B). These results demonstrate that the differential effects of native FN and FN\(_{DIII1–7}\) on cell growth occur only when each is interacting with cells from within a fibrillar matrix. More importantly, the opposite effects of these FNs on cell proliferation

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\(^2\) J. L. Sechler and J. E. Schwarzbauer, unpublished observations.
are directly attributable to the structural differences between native FN and FNIII1–7 matrix fibrils.

Induction of Intracellular Signaling Responses—Cell interactions with FN activate a number of intracellular signal transduction cascades (2, 22, 23). In particular, phosphorylation of focal adhesion kinase (pp125FAK) is an early biochemical response to integrin-mediated adhesion to FN substrates (24–26). If FNIII1–7 matrix structure can perturb cell cycle progression, then intracellular signaling in response to matrix should be affected. Phosphorylation levels of pp125FAK were compared in cells assembling different fibrillar matrices. Cells assembling native FN matrix exhibited a 1.5–2-fold increase in pp125FAK phosphorylation over cells with either no FN or FNIII1–7 matrix (Fig. 4A). The differential effects of these matrices were maintained even at the earliest time point where a transient increase in pp125FAK phosphorylation was observed under all three conditions, probably due to the addition of serum (2). The high levels of phosphorylation in response to native FN matrix were sustained throughout the entire time course. However, pp125FAK phosphorylation in cells with FNIII1–7 matrix remained low until the 24-h time point when it gradually increased to within 1.5-fold of levels with pFN (Fig. 4A). In contrast to the effects of fibrillar matrix assembly, cell adhesion on immobilized pFN or FNIII1–7 protein resulted in a rapid phosphorylation of pp125FAK to equivalent levels (Fig. 4B). Moreover, on both substrates, pp125FAK phosphorylation was transient with obvious decreases after 3 h. A second focal adhesion protein, p130CAS (27, 28), did not exhibit differential phosphorylation (Fig. 4C). p130CAS phosphorylation was transient, and comparable levels were observed with both pFN and FNIII1–7 during matrix assembly and cell adherence. Therefore, cell interactions with fibrillar matrix elicit specific responses that are not observed with cell binding to immobilized protein substrates. Similar to the stimulatory effect on cell proliferation, native FN matrix induced pp125FAK phosphorylation. FNIII1–7 matrix, on the other hand, inhibited both pp125FAK phosphorylation and cell cycle progression. Clearly, the structure of the FN matrix can regulate receptor signaling and downstream pathways.

Matrix architecture could regulate the induction of cell proliferative signals by modulating the spatial distribution of FN’s cell binding sites. As shown here, a network of matrix fibrils can vary the degree of proliferation allowing different matrix structures to stimulate, inhibit, or maintain cell growth rates. Inhibition of smooth muscle cell growth by treatments that can block FN fibril formation has also been reported (29). By placing unique three-dimensional structural constraints on the disposition of integrins, FN matrix fibrils could act from all sides of the cell to specify the organization of the actin cytoskeleton. Cytoskeletal geometry plays a central role in activating intracellular signaling pathways that lead to cell cycle progression (14, 30), and inhibition of pp125FAK localization to focal adhesions correlates with decreased cell proliferation (31). Thus reorganization of the cytoskeleton by specific alterations in matrix structure would ultimately influence cell growth. This concept is reinforced by the correspondence between differences in actin stress fiber arrangements and distinct native FN and FNIII1–7 matrix structures (12). Extracellular matrix rigidity can modify the strength of connections between integrins and the cytoskeleton (32) and may constitute another link between matrix architecture and cell growth control. Interestingly, our results also show that altering the structure of the FN matrix can be more detrimental than having no matrix at all. pFN polymers have been shown to have antimetastatic activity (33), and they may be acting through effects on matrix organization. Thus the design of reagents that can modify existing matrix architecture through dominant-negative effects could be a novel strategy for controlling cell and tumor growth and spread.

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