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The N-terminal 70-kDa fragment of fibronectin binds to cell surface fibronectin assembly sites in the absence of intact fibronectin

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Abstract

Binding of the N-terminal 70-kDa (70K) fragment of fibronectin to fibroblasts blocks assembly of intact fibronectin and is an accurate indicator of the ability of various agents to enhance or inhibit fibronectin assembly. Such binding is widely thought to be to already assembled fibronectin. We evaluated this hypothesis with fibronectin-null mouse fibroblasts plated on laminin-1 in the absence of intact fibronectin. As a proteolytic fragment or recombinant protein, 70K bound fibronectin-null cells specifically in linear arrays that extended outwards from the periphery of spread cells. At early time points, these arrays were similar to those formed by intact fibronectin. 70K arrays formed within 5 min following ligand addition at concentrations as low as 5 nM, indicating rapid and high affinity binding. Bound 70K was extractable with Triton X-100 or deoxycholate but became insoluble when cross-linked with a membrane-impermeable agent into large SDS-stable complexes. Intact fibronectin, in contrast, became progressively non-extractable in the absence of cross-linking. The detergent-resistant arrays of cross-linked 70K localized to tips of cellular extensions and partially overlapped with α6 and β1 integrin subunits at the base of the extensions. α5 did not localize with 70K arrays, but became progressively co-localized with assemblies of intact fibronectin over time. These results support a model in which the 70-kDa region of fibronectin binds to linearly arrayed cell surface molecules of adherent cells to initiate assembly, display of the arrays is controlled by the integrin that mediates adhesion, and fibronectin-binding integrins promote fibronectin–fibronectin interactions during progression of assembly.

Keywords: Fibronectin; N-terminal 70-kDa fragment; Assembly; Fibrillogenesis; Initiation; Integrin

1. Introduction

The fibrillar form of fibronectin (FN) provides information important for cell adhesion, migration, survival and proliferation, and for connective tissue remodeling (Pankov and Yamada, 2002; Sottile and Hocking, 2002). Much is known about the transformation of soluble FN into its fibrillar form (Magnusson and Mosher, 1998; Mao and Schwarzbauer, 2005; Wierzbicka-Patynowski and Schwarzbauer, 2003), but the precise mechanism is yet to be elucidated. On adherent cells, FN assembly is efficiently initiated by interaction of soluble FN with cell surface molecules. Following this initial binding, progression of assembly involves FN–FN interactions, insolubilization of FN and formation of elongating fibrils (Mao and Schwarzbauer, 2005; McKeown-Longo and Mosher, 1983; Peters and Mosher, 1987).

FN is a dimer, with each subunit being composed of three types of modules with some variability due to alternative splicing (Mao and Schwarzbauer, 2005; Pankov and Yamada, 2002). The five N-terminal type I modules are essential for fibrillogenesis (McDonald et al., 1987; McKeown-Longo and Mosher, 1985; Schwarzbauer, 1991; Sottile et al., 1991). The N-terminal 70-kDa catheptic fragment (70K), containing these modules and the adjacent gelatin-binding region, binds to cell monolayers with the same affinity as intact fibronectin and blocks assembly of intact fibronectin, but does not become incorporated into detergent-insoluble extracellular matrix

Abbreviations: FN, fibronectin; 70K, N-terminal 70-kDa region of fibronectin; r70K, recombinant 70K; LPA, lysophosphatidic acid; BSA, bovine serum albumin; FITC, fluorescein isothyocyanate; PBS, phosphate-buffered saline; TBS, tris-buffered saline; FUD, functional upstream domain; PFA, paraformaldehyde.

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(McKeown-Longo and Mosher, 1985). Bound 70K co-localizes with pre-existing FN fibrils (Chernousov et al., 1985), albeit incompletely (Zhang et al., 1994). 70K also binds to FN that is adsorbed to surfaces and subjected to tension (Zhong et al., 1998). Thus, the N-terminal region of FN has been proposed to mediate binding to stretched or otherwise conformationally altered FN molecules (Hocking et al., 1994, 1996; Wierzbicka-Patynowski and Schwarzbauer, 2003; Zhong et al., 1998). This proposed interaction is incorporated into a model in which FN fibrillogenesis begins by binding of an integrin recognition sequence in FN, e.g., RGD in repeat III_{10}, to an integrin, e.g., $\alpha5\beta1$(Mao and Schwarzbauer, 2005; Wierzbicka-Patynowski and Schwarzbauer, 2003). Interaction with integrins is hypothesized to promote extension of FN and thereby facilitate interactions of the N-terminal 70K region with other parts of FN (Aguirre et al., 1994; Bultmann et al., 1998; Hocking et al., 1994, 1996).

An alternative hypothesis is that tethering of the 70K region to cell surface molecules causes a conformational change in FN and exposes the FN regions involved in FN–integrin and FN–FN interactions. This hypothesis is consistent with findings that the N-terminal modules of FN are available in solution to bind to various molecules (Ingham et al., 1988; Isaacs et al., 1989; Khan et al., 1990), whereas the integrin-binding midpiece is cryptic in solution but is exposed in complexes of FN and substances that bind the N-terminal modules (Ensenberger et al., 2004; Ugarova et al., 1995). A model in which interactions with the N-terminal modules cause a conformational change that opens up the integrin-binding site, rather than vice versa, has a precedent in studies of bacteria–FN interactions (Ozeri et al., 1998). In the case of *Streptococcus pyogenes*, binding of protein F1 to the N-terminal region of FN enables cellular uptake of bacteria–FN complexes by a process that is inhibitable by RGD peptides, involves integrins and likely contributes to bacterial virulence (Cue et al., 2000; Nyberg et al., 2004; Ozeri et al., 1998). Thus, cellular uptake of *S. pyogenes* may represent a subversion of the mechanism whereby binding via the N-terminal region of FN exposes the cryptic integrin-binding type III_{10} repeat. Furthermore, neither monomeric nor dimeric $III_{7–10}$ fragment binds integrins stably; a trimer is required (Coussen et al., 2002). This finding suggests that cell-associated FN must be assembled into a multimeric form to engage integrins.

FN−/− fibroblasts provide the opportunity to test the two alternative models. If 70K only binds to conformationally altered FN, there should be no binding of 70K to FN−/− cells. If the 70K region binds to molecules at assembly sites that are not FN, 70K should bind to FN−/− cells with the same distribution as intact FN. We report here that 70K does bind to FN−/− cells in a system lacking intact FN and provide evidence that the binding sites have the characteristics of assembly sites. We then probed 70K and integrin localization to discern the role of integrins in assembly. Our results are compatible with a mechanism, whereby assembly is initiated by binding of the 70K portion of FN to linearly arrayed binding sites that are not FN, display of these sites is controlled by integrin-mediated adhesion, and progression of assembly, with concomitant FN–FN interactions, involves translocation of assembling FN tethered to FN-binding integrins.

2. Results

2.1. 70K binds to cells in linear arrays in the absence of intact FN

FN−/− cells assemble exogenous FN in short-term assays when cultured on substrata coated with FN or laminin but not when cultured on vitronectin (Bae et al., 2004). To learn whether the correlation between 70K binding and FN assembly extends to the different effects of the substrates on FN−/− cells, we compared the binding of FN and 70K to cells cultured on FN, vitronectin or laminin. As shown in Fig. 1, all substrates promoted adhesion and spreading of FN−/− cells to a similar extent, FITC–70K only bound to cells adherent to FN or

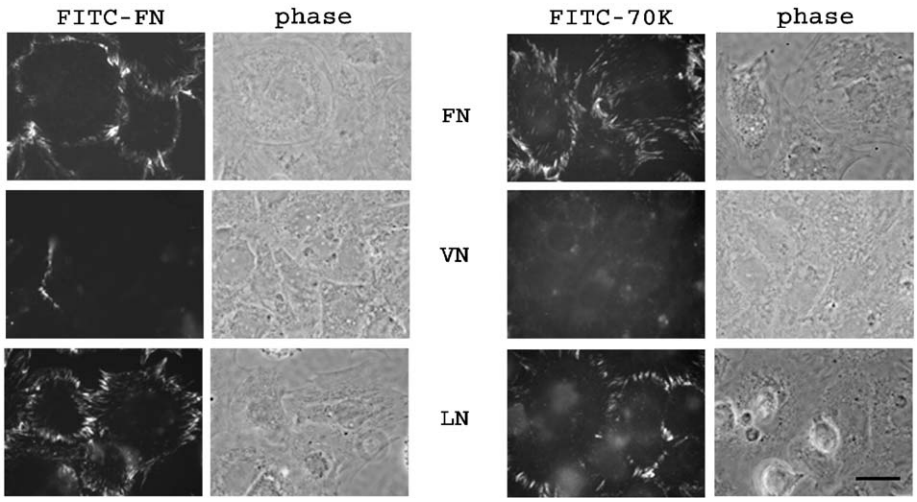

Fig. 1. FITC–70K and FITC–FN form linear arrays on FN−/− cells adhered to FN or laminin but not to vitronectin. FN−/− cells were plated for 2 h on coverslips coated with FN, laminin or vitronectin. FITC–FN (20 nM) or FITC–70K (40 nM) were incubated with the cells for 1 h in DMEM containing 400 nM LPA and 0.2% BSA. Coverslips were washed, fixed and observed under fluorescence and phase contrast microscopy. Bar=10 μm.