
# Heparin-fibronectin interactions in the development of extracellular matrix insolubility

**Irene Raitman [a], Mia L. Huang [b], Selwyn A. Williams [a], Benjamin Friedman [a], Kamil Godula [b] and Jean E. Schwarzbauer [a]**

***a* - Department of Molecular Biology,** *Princeton University, Princeton, NJ 08544-1014, USA*
***b* - Department of Chemistry and Biochemistry,** *University of California San Diego, La Jolla, CA 92093-0358, USA*

***Correspondence to Jean E. Schwarzbauer:*** *jschwarz@princeton.edu*
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## Abstract

During extracellular matrix (ECM) assembly, fibronectin (FN) fibrils are irreversibly converted into a detergent-insoluble form which, through FN's multi-domain structure, can interact with collagens, matricellular proteins, and growth factors to build a definitive matrix. FN also has heparin/heparan sulfate (HS) binding sites. Using HS-deficient CHO cells, we show that the addition of soluble heparin significantly increased the amount of FN matrix that these cells assemble. Sulfated HS glycosaminoglycan (GAG) mimetics similarly increased FN assembly and demonstrated a dependence on GAG sulfation. The length of the heparin chains also plays a role in assembly. Chains of sufficient length to bind to two FN molecules gave maximal stimulation of assembly whereas shorter heparin had less of an effect. Using a decellularized fibroblast matrix for proteolysis, detergent fractionation, and mass spectrometry, we found that the predominant domain within insoluble fibril fragments is FN's major heparin-binding domain HepII (modules $III_{12–14}$). Multiple HepII domains bind simultaneously to a single heparin chain in size exclusion chromatography analyses. We propose a model in which heparin/HS binding to the HepII domain connects multiple FNs together to facilitate the formation of protein interactions for insoluble fibril assembly.



## Introduction

The biophysical properties of the extracellular matrix (ECM) are essential for generating and maintaining the shape, rigidity, stability, and structure of all tissues. The proportions of fibrous glycoproteins, collagens, and polysaccharides that are cell-assembled into an ECM framework determine tissue properties and support tissue-specific cell activities [1,2]. Fibronectin (FN) is a ubiquitous ECM protein and a main component of the ECM in most tissues. It is assembled into a fibrillar matrix in a cell-mediated process that requires cell contractility and FN-FN interactions. Initially fibrils are soluble in deoxycholate (DOC) detergent but over time nascent fibrils are irreversibly converted into a form that is insoluble in DOC [3–5]. This insoluble FN matrix serves an important role as a foundational matrix for other ECM proteins to build upon [6–8]. As such, FN's multi-domain structure provides binding sites for deposition of other ECM proteins including collagens, matricellular proteins, proteoglycans/glycosaminoglycans (GAGs), and FN itself [3]. Its N-terminal FN-binding domain is essential for fibril assembly [9], and its primary interaction site is in the first type III modules $III_{1–2}$ [10,11]. The N-terminal domain also interacts with other FN-binding sites located throughout the molecule [10–14]. Two of these sites are in heparin binding domains, HepII (spanning modules $III_{12–14}$) and HepIII ($III_{4–6}$) which differ in their heparin binding properties [15]. Heparan sulfate (HS) on proteoglycans is abundant in the ECM and on the cell surface. In fact, the HepII domain has been implicated in modulating cell responses to FN through interactions with cell surface syndecan proteoglycans [16–19].

There is evidence that GAGs are involved in matrix assembly. Mutant Chinese hamster ovary (CHO) cells that lack HS GAGs do not assemble a FN matrix [20]




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and show a deficiency in cell contractility [21]. In contrast, FN assembly by BHK or HT1080 cells that produce HS can be reduced by adding excess heparin or GAGs to the cell culture [17,20] suggesting that the level of HS determines whether it promotes or inhibits matrix assembly. Interactions involving the cytoplasmic domain of transmembrane syndecan-2 inside and its GAG chains outside have roles in matrix assembly [17,18,20]. Given the prevalence of HS binding sites on ECM proteins and evidence for syndecan-ECM interactions in assembly, it is surprising that we do not have a better understanding of the role of GAGs in fibrillogenesis.

To test the requirement for HS in FN assembly, we examined the matrix formed by CHO-677 cells, which do not synthesize HS [22]. CHO cells have been used extensively in matrix assembly studies to identify requirements for ECM proteins and domains, ECM receptors, and intracellular signaling [18,20,23–26]. Here we show that HS-deficient CHO cells require exogenous heparin or HS to stimulate the initiation of assembly of DOC-soluble fibrils and to promote the conversion to the DOC-insoluble form. GAG sulfation levels and GAG length are key parameters in promoting fibril assembly. We propose that heparin/HS acts early in the assembly process to bring together FN molecules to promote interactions required for fibrillogenesis.

## Results

### Heparin addition is required for FN fibril assembly by CHO-677 cells

Mutant CHO-677 cells do not synthesize HS [22] due to significantly reduced expression of EXT1, a glycosyltransferase required for HS elongation [27]. Hamster FN was not detected in whole cell lysates or conditioned medium of CHO-677 or wild type CHO-K1 cells (data not shown). Matrix assembly was analyzed for confluent cell monolayers (Fig. S1) and neither cell line produced a significant amount of FN matrix in the absence of an exogenous supply of FN (Fig. 1A). Therefore, all subsequent experiments were performed in the presence of exogenous FN. While CHO-K1 cells assembled a dense FN matrix when the culture medium was supplemented with rat plasma FN, assembly by CHO-677 cells was significantly lower with a 0.7 ± 0.04 mean fluorescence intensity of the FN matrix compared to the wild type cells ($p = 0.001$) (Fig. 1A). To investigate the effect of heparin on matrix assembly, increasing concentrations of heparin were added along with FN. Heparin significantly increased the amount of FN matrix fibrils assembled by CHO-677 cells, with a 1.3 ± 0.1-fold increase at 50 μg/ml heparin and 1.5 ± 0.1-fold increase with 100 μg/ml heparin compared to the no heparin condition for these cells ($p < 0.05$) (Fig. 1B). Higher magnification shows that the FN matrix assembled in response to heparin stimulation has the typical fibrillar organization of a fibroblast matrix (Fig. 1C). The matrix assembled by CHO-K1 cells was more robust than that of CHO-677 cells and therefore, heparin did not have a significant effect on fibril formation or organization (Fig. 1B, C).

The amounts of DOC-insoluble FN matrix assembled by wild type and mutant cells without and with heparin were quantified. When increasing concentrations of heparin were added to the mutant cells, the amounts of DOC-insoluble FN matrix were not statistically different after 24 h with 50, 100, or 200 μg/ml heparin, but insoluble FN was decreased when 25 μg/ml heparin or less was added (Fig. 2A). The 50 μg/ml heparin concentration was thus chosen for subsequent experiments. Heparin addition did not have an effect on insoluble FN levels in wild type CHO-K1 cells at either 12 or 24 h. Levels of DOC-insoluble material compared to 12 h-no heparin samples differed by 0.9 ± 0.3 and 1.4 ± 0.4 for 12 h and 24 h heparin treatments, respectively (Fig. 2B left). Addition of heparin to CHO-677 cells had a significant effect on insoluble FN levels. Compared to 12 h-no heparin samples, heparin induced an average fold increase in DOC-insoluble FN of 2.7 ± 0.4 at 12 h and 3.8 ± 0.95 at 24 h (Fig. 2B right). Heparin treatment for 24 h is able to rescue the assembly defect of CHO-677 cells since the addition of heparin yielded DOC-insoluble matrix levels equivalent to CHO-K1 cells at the same time. With time, there was a modest increase in insoluble FN without heparin (2.1 ± 0.6-fold at 24 h compared to 12 h) suggesting that the HS-deficiency slows rather than completely stops assembly. The effects of heparin addition were not due to changes in cell numbers or morphology

**Fig. 1.** Heparin increases FN matrix in HS-deficient CHO cells. Wild-type CHO-K1 and HS-deficient CHO-677 cells were grown for 3 days and, except for the "No FN" samples, exogenous FN was added at 25 μg/ml for the last 24 h. Samples were fixed and immunostained for FN, matrix was visualized by fluorescence microscopy, and fluorescence intensities were quantified. (A) Cells were grown with (+FN) or without FN (No FN). The fold change in mean fluorescence intensities of +FN samples for CHO-677 compared to CHO-K1 cells is shown in yellow (mean ± SE) ($n = 3$, $^*p = 0.001$). Scale bar = 50 μm. (B) CHO-K1 and CHO-677 cells were supplemented with FN alone (left), or together with 50 μg/ml (middle) or 100 μg/ml (right) heparin for 24 h. The fold change from the no heparin condition (No Hep) is noted for the CHO-677 cells (mean ± SE, n = 3, $^*p < 0.05$). Scale bar = 50 μm. (C) Higher magnification images of CHO-K1 and CHO-677 cells in (B) with FN and with or without heparin addition. Scale bar = 20 μm. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

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