Proteolysis of Aggregated Fibronectin

A Model for In Vivo *Matrix Degradation*

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INTRODUCTION

The extracellular matrix (ECM) is a network of several proteins representing both a barrier delimiting the tissues and a substratum for cell adhesion, migration, and differentiation.[1] Its degradation by mammalian Zn^{2+}-proteinases referred to as MMPs (for matrix metalloproteinases) is implied in many normal or pathological processes (development, inflammation, metastasis, dissemination, etc.).[2]

Fibronectin (FN) is a major structural and functional protein of the ECM. It is a dimeric protein mainly composed of three different types of homologous modules that are grouped in compact domains interconnected by flexible strands.[3] Each domain presents numerous binding sites (cell binding domain, collagen, heparin, DNA binding domains, etc.) (see FIGURE 1, *upper*). FN modules are highly resistant to proteolysis, but observable cleaved sites are located on the connecting strands. Furthermore, many FN proteolytic fragments have been shown to present functions that are not observed in the intact protein.[4] Precise knowledge of the *in vivo* mechanisms of FN fragment production is thus of high interest.

This study presents an original approach to the kinetics of multiple cleavage site proteolysis of large proteins such as FN (M_r, 500×10^3). We have determined the proteolytic cascade leading to apparition of different FN fragments and the velocity at which cleavages occur, using thermolysin as a model for MMPs. Proteolysis has been studied using FN under two forms: as a soluble form (as found in plasma) and as a reticulated insoluble form that mimics the aggregated form that is incorporated into ECM. Hence, contrary to the usual studies dealing with enzymology under heterogeneous conditions, the substrate is here immobilized and the soluble enzyme diffuses into it.

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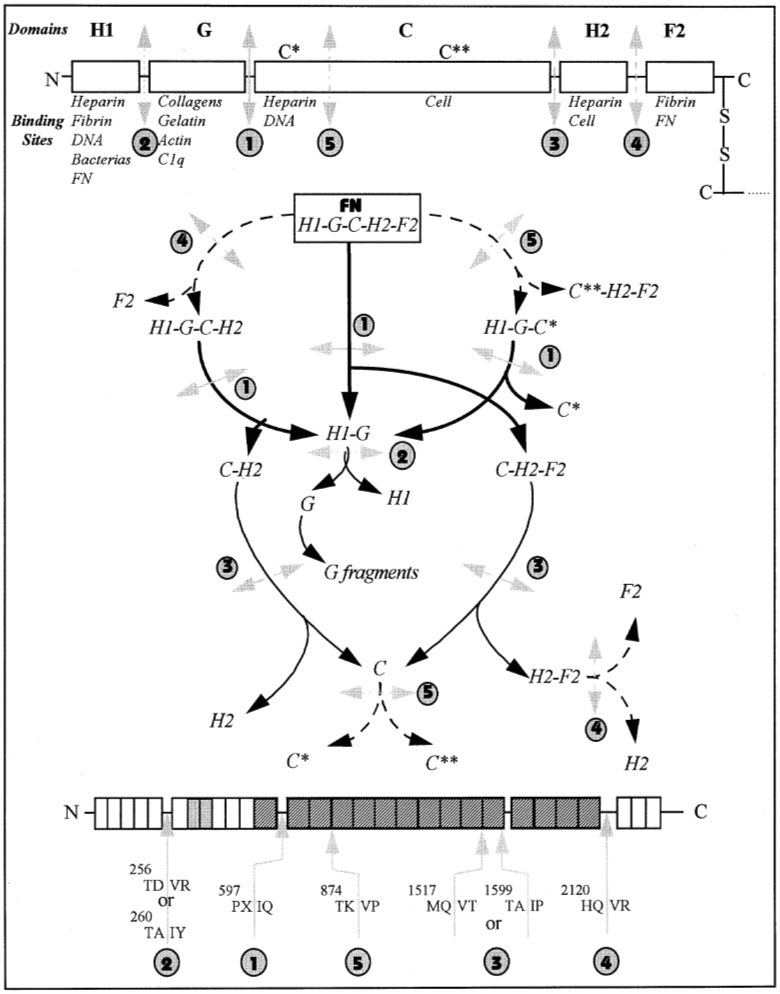

FIGURE 1. Proteolytic cascade for soluble FN thermolysin-catalyzed cleavage. *Upper*: Disposition of the different domains in FN (H1, G, C, H2, F2), respective binding sites, and location of the five cleavages. *Middle*: Proteolytic cascade. Segments are designated according to their domain sequence. *Lower*: Location of the cleavage sites in the intact FN sequence: type I module □; type II module ▭; type III module ▨. Amino acids are designated according to the one-letter code.

MATERIALS AND METHODS

FN Purification

Human plasma FN was purified according to a three-step protocol using successive gelatin-, heparin-, and gelatin-affinity chromatographies.

Enzyme Kinetics

Proteolysis was performed at 37 °C with 1 nM thermolysin (Sigma). The reaction was stopped with EDTA. FN and fragments were subjected to SDS-PAGE followed by silver nitrate staining. Stained gels were scanned and analyzed by a densitometric software (IPGel®).

FN Immobilization

Lyophilized soluble purified FN was dissolved in 1% (v/v in H_2O) glyceraldehyde, frozen overnight at –20 °C, softly thawed at 4 °C, and subsequently washed with 6 × 20 mL H_2O. Reticulated FN is stable for more than 5 days in Tris buffer, pH 7.4, at 37 °C.

RESULTS AND DISCUSSION

Soluble FN Hydrolysis

The kinetics of the entire FN consumption have been studied and the efficiency parameter has been estimated to be $k_{cat}/K_M = 2.5 \times 10^5\ M^{-1}\ s^{-1}$. As the FN maximal solubility ($\approx$ 4 μM) seems largely lower than K_M, individual values of k_{cat} and K_M could not be estimated separately. This value of k_{cat}/K_M nevertheless indicates that FN is a better substrate for thermolysin than the model oligopeptides usually used for thermolysin kinetic studies.[5]

The order of fragment appearance allows us to estimate the relative velocity of each cleavage. The respective quantity of each fragment can be used to approach the steady state levels resulting from its production and consumption. Finally, estimation of the fragment sizes, as observed through electrophoretic mobility, correlated with the affinity of each fragment for gelatin and heparin, allows us to place each fragment in the sequence of intact FN.

Through correlation of all these data, we were able to build the proteolytic cascade leading to fragment appearance (FIGURE 1, *middle*). FN presents five cleavage sites for thermolysin. The velocity of cleavage 1 (N_t to the central cell binding domain) is the highest and concerns principally the entire FN molecule. Cleavages 2 and 3 (N_t to the gelatin binding domain and to the H2 heparin binding domain, respectively) are less frequent and occur at approximately the same velocity. These two cleavages are less implied in intact FN proteolysis. Cleavage 4 (N_t to the F2 fibrin

binding domain) is less frequent than cleavages 2 and 3 and thus mainly concerns FN fragments. Finally, cleavage 5 is atypical—it occurs with low velocity, but seems relatively specific for thermolysin-catalyzed proteolysis; thus, resulting fragments are present in the early stage of the reaction, but in very low quantity. Furthermore, this cleavage is not located in a connective strand, but in the central cell binding domain.

Putative Cleavage Site Identification

Possible sites corresponding to the observed cleavages have been sought in the intact FN sequence. A cleaved sequence must both correspond to the known thermolysin specificity and be located in a proteinase-accessible part of the protein. Seven possible sequences have been identified (FIGURE 1, *lower*) that correspond to thermolysin-specific sequences and they are located in the closest vicinity (4 or 5 amino acids max.) of sequences for which proteolysis by other proteinases (chymotrypsin, trypsin, plasmin) has been demonstrated.[6]

Aggregated FN Hydrolysis

Proteolysis of FN by thermolysin is modified when FN is reticulated, that is, in an insoluble form that mimics the way that FN is incorporated in ECM. Only low molecular weight fragments (<100 kDa) are liberated in solution as a consequence of proteolysis. The order of fragment appearance is altered as compared to that observed with soluble FN, as well as fragment respective quantities (TABLE 1).

Identity of most of the produced fragments seems conserved regardless of the FN form used as a substrate. Immobilization thus would not greatly alter the sequences recognized by thermolysin, but rather would cause modifications in the relative velocities of the different cleavages.

Nevertheless, a 43-kDa major, but late-appearing fragment is observed only with

TABLE 1. Comparison of Thermolysin Product Specificity with Soluble or Reticulated FN as Substrate[a]

Substrate	Order of Fragment Appearance	Fragment Quantity
Soluble FN	1. H1-G; C-H2; H1-G-C*; G 2. C; C-H2-F2 3. G fragments; H2 4. H1-G-C-H2; C**	1. H1-G 2. C-H2; G; C 3. H2; H1-G-C*; H1-G-C-H2
Reticulated FN	1. G fragments; C** 2. H1-G 3. H1-G-C*; G	1. G fragments; 53 kDa 2. H2; G; H1-G 3. H1-G-C*

[a]Fragments are designated according to their domain sequence as in FIGURE 1. Each lane lists fragments appearing almost simultaneously (order of appearance) or in equal quantity (fragment quantity).

reticulated FN (TABLE 1). Hence, *in vivo* FN proteolysis could produce fragments that are not observed under *in vitro* soluble conditions, such as the observed 43-kDa fragment. This is of importance as this means that the functions and binding affinities demonstrated for fragments produced *in vitro* from soluble FN could be physiologically not relevant. *In vivo* produced fragments would be different and thus present different and possibly new functions.

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