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Human plasma and cerebrospinal fibronectins differ in the accessibility of the epitopes on the N-terminal domains

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Three monoclonal antibodies specific to the central cell-binding and the C- and N-terminal domains of fibronectin (FN) were used to test antigenic epitope accessibility on human plasma and cerebrospinal fibronectins. In the plasma group, the mean N-terminal FN domain immunoreactivity was about one fourth that of the cell-binding and C-terminal domains, whereas in cerebrospinal fluid they were nearly equal. In the presence of 0.5–6 M urea N-terminal domain immunoreactivity in the plasma increased 3–6-fold, but it decreased 0.7–3-fold in the cerebrospinal fluid. Analysis of fibronectin domain immunoreactivities of the cell-binding and N-terminal domains by a panel of specific monoclonal antibodies may reveal N-terminal fibronectin domain accessibility for reaction with biological partner ligand(s) and/or processes in which FN could be implicated. Such determinations may have important clinical implications.

Keywords: fibronectin, N-terminal fibronectin domain, C-terminal fibronectin domain, cell-binding fibronectin domain, cerebrospinal fluid.

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INTRODUCTION

Fibronectin (FN) is a large ubiquitous multidomain glycoprotein that is an abundant soluble and insoluble component of the blood plasma and extracellular matrix (ECM), respectively. Plasma FN originates mainly from hepatic synthesis, whereas tissue FN is produced locally by many cell types (e.g., fibroblasts, lymphocytes, and endothelial cells), and can be expressed on a cell and/or included in the ECM (Mao & Schwarzbauer, 2005). In the cerebrospinal fluid FN appears to be product of local synthesis, mainly by astrocytes and other cell types of nervous and immunological system origin (Liesi *et al*., 1986; Torre *et al*., 1993).

Plasma FN is believed to be biologically inactive. However, in the presence of endothelial injury, the plasma FN can enter the extravascular space and become incorporated into the ECM (Yi *et al*., 2003; Moretti *et al*., 2007). In tissues FN is essential for the assembly and maintenance of the ECM preserving its proper structural integrity, organization, and regulation of normal tissue metabolism (Vogel *et al*., 2001; Cukierman *et al*., 2002). FN is involved in various biological processes, such as the maintenance of normal cell functions including morphology, adhesion, differentiation, migration, and hemostasis. FN also plays a role in thrombosis, wound healing, and oncogenic transformation (reviewed by Pankov & Yamada, 2002).

FN is a mosaic protein comprising polypeptides composed of series of modular short segments I, II, and III (with 12, 2 and 15–17 copies, respectively). The modules are arranged into independently folded, functional domains (Fig. 1A) with binding sites for ECM proteins (e.g., collagen), cell surface receptors (integrins, bacterial FN receptors), blood protein derivatives (fibrin) and glucosaminoglycans (heparin) (reviewed by Magnusson & Mosher, 1998; Pankov & Yamada, 2002; Plata *et al*., 2009). The FN domains are mainly folded regularly, having their own globular shape, whereas the linking sequences between the domains are flexible random coils and are extremely sensitive to degradation (Spitzfaden *et al*., 1997; Hynes, 1999; Mao & Schwarzbauer, 2005).

In humans, FN exists in many structural forms; however, the basic monomeric subunit is composed of two nearly identical 200–250-kDa chains having an antiparallel configuration. The polypeptides are exclusively linked by disulphide bonds near their C-termini, whereas the N-terminal parts remain unbound (Pankov & Yamada, 2002; Mao & Schwarzbauer, 2005). Such elastic arrangements of dipolypeptides allow conformational alterations from compact globular (Fig. 1B) to extended fibrillar (Fig. 1C) forms (Sakai *et al*., 1996; Hynes, 1999; Mao & Schwarzbauer, 2005). It has been shown that FN has a globular shape in the blood, but it tends to form a multimeric network of fibers when assembled by cells. Some laboratory experiments have also shown that FN reveals large-scale flexibility and local order and can exist in unfolded and refolded forms (Johnson *et al*., 1999; Olszowski *et al*., 2003; Patel *et al*., 2006). It is claimed that the FN unfolding and extension within the cell matrix is controlled by cytoskeletal tension (Baneyx *et al*., 2002). This event is known to reveal some of the FN biological functions by exposing cryptic sites that were buried in the globular state, by deforming the conformation of a binding site, or by changing the distance between two or more binding sites that bind to the same receptor molecule (Vogel *et al*., 2001).

In the present study we compared the accessibility of the epitopes to antibodies on the central cell-binding (^{CBD}FN), C-terminal (^{Ct}FN), and N-terminal (^{Nt}FN) domains of fibronectins in normal plasma and normal cerebrospinal fluid. It was expected that, in contrast to the

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Abbreviations: FN, fibronectin; ^{CBD}FN, cell-binding domain of fibronectin; ^{Nt}FN, N-terminal domain of fibronectin; ^{Ct}FN, C-terminal sequences of fibronectin containing disulfide bridges; ECM, extracellular matrix

globular plasma FN, the cerebrospinal FN would have an extended shape with epitopes exposed on the Nt-domain. We also analyzed alterations in the presentation of the CBD, Ct and Nt domains of FN after treatment with a denaturing agent, 0.5–6 M urea. We assumed that the concentration ratios of the central ^{CBD}FN to the terminally located ^{Nt}FN and ^{Ct}FN domains, determined using monoclonal antibodies with well-defined specificity, would reflect the differences in epitope accessibility depending on the source of FN.

MATERIALS AND METHODS

Samples. Samples of cerebrospinal fluid (30) and blood plasma (76) were taken from patients (3–17 years old, mean age: 9 ± 4.5 years) receiving care at the Department and Clinic of Pediatrics and Infectious Diseases, Medical University of Wroclaw (Wroclaw, Poland) in the years 2005–2007. The children visited the Clinic due to various meningeal symptoms, or they were summoned for medical control after acute meningitis. All patients were evaluated from the results of clinical examination and routine laboratory blood plasma and cerebrospinal fluid parameters and only samples from patients whose diagnoses were normal were included into the studies.

The cerebrospinal fluid and plasma samples used here were surplus remaining after routine diagnostic procedures had been performed. All samples were collected with the informed consent of the individual patient's parents and the study was approved by the local ethics committee (no. KB-955/05). The samples were centrifuged at $1000 \times g$ for 20 min to separate cells from the supernatant, aliquoted and stored at –76 °C until use. Cerebrospinal fluid samples contaminated with blood were discarded. Frozen samples were thawed at 20 °C before use.

Quantification of FN domain expression. The expression of the cellular (^{CBD}FN) and the first fibrin-heparin N-terminal (^{Nt}FN) domains and the C-terminal region containing disulfide bridges (^{Ct}FN) in the FN molecule (Fig. 1) were determined based on the immunoreactivity of conformationally accessible epitopes on FN with three specific and well-defined monoclonal antibodies, anti-^{CBD}FN (FN 30-8; M010), anti-^{Ct}FN (FN 1-1; M013), and anti-^{Nt}FN (FN 9-1; M001) (TaKaRa, Shuzo Co., Shiga, Japan), by three independent sandwich-type solid-phase enzyme-linked immunoassays (ELISA) according to a procedure described earlier (Hirnle & Kątnik-Prastowska, 2007). Shortly: the respective monoclonal antibodies, anti-^{CBD}FN (diluted 1:10000), anti-^{Ct}FN (diluted 1:5000), and anti-^{Nt}FN (diluted 1:2000) were used as coating agents in the wells of a microtiter plate (Nalge Nunc International, Naperville, IL, USA) to bind FN in the sample. Before the analysis the plasma samples were diluted with Tris-buffered saline (TBS) containing 0.05% Tween-20 (TBS-T), 4000- and 8000-fold for ^{CBD}FN and ^{Ct}FN determinations, but only 250- and 500-fold for ^{Nt}FN. The samples of cerebrospinal fluid were diluted 25- and 50-fold with TBS-T for ^{CBD}FN, ^{Ct}FN, and ^{Nt}FN measurements. The amount of FN bound by the monoclonal antibody was quantified by rabbit anti-FN polyclonal antibodies (Sigma Chemical Co, St. Louis, MO, USA) diluted 1:5000. Peroxidase-conjugated goat anti-rabbit immunoglobulins (Sigma Chemical Co, St. Louis, MO, USA) diluted 1:20000, 1:10000, and 1:5000 for ^{CBD}FN, ^{Ct}FN, and ^{Nt}FN, respectively, were the secondary antibodies. The amount of FN was assayed by a colorimetric reaction using *o*-phenylenediamine dihydrochloride/H_2O_2 as the enzyme substrate and measured in a Stat Fax 2100 Microplate Reader (Awareness Technology Inc., Palm City, FL, USA) at 492 nm with 630 nm as the reference filter. All ELISA immunobinding and washing steps were carried out in TBS containing 0.1% Tween

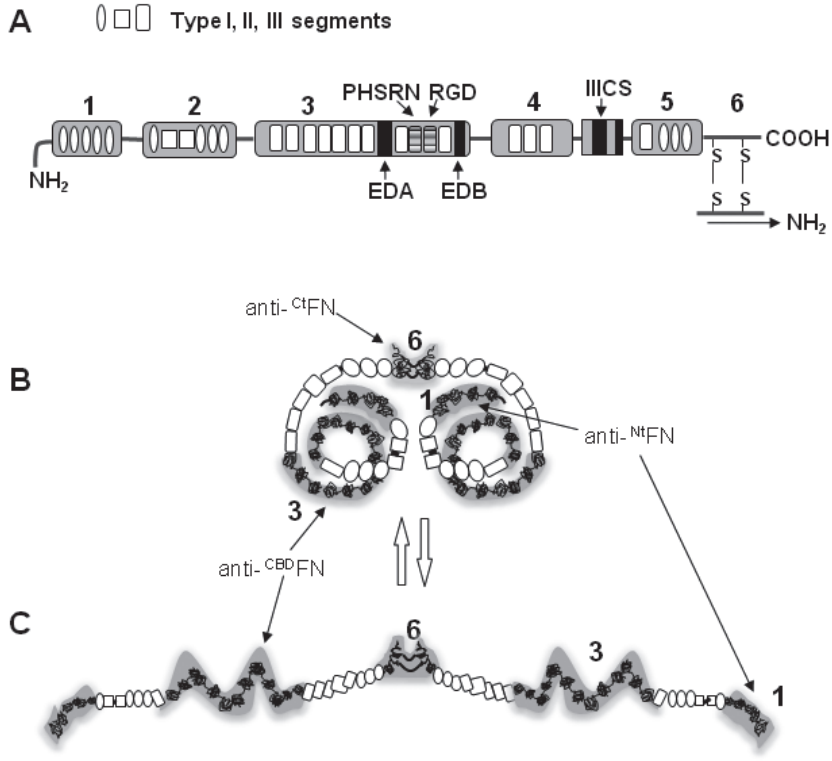

Figure 1. Scheme of FN domain arrangement and differences in N-terminal domain exposure in globular and fibrillar forms
Series of segments I, II, and III (ovals and rectangles) are arranged into functional domains **(A)**: N-terminal (Nt) binding fibrin and glucosaminoglycans (1), collagen (2), cell surface receptors (3), glucosaminoglycans (4), second fibrin (5) and C-terminal (Ct) sequences containing two disulphide bridges (6). RGD and PHSRN are sequences recognized by cell surface integrins. Segments alternatively spliced (EDA, EDB, and the variable IIICS) are marked by black filled rectangles. The globular modules can be independently folded into a soluble compact globular molecule **(B)** with hidden epitopes, particularly at the N-termini, or can form an extended fibrillar molecule **(C)** with exposed epitopes at the N-termini (Hynes, 1999; Mao & Schwarzbauer, 2005). Long arrows indicate the (1) ^{Nt}FN, (3)^{CBD}FN, and (6)^{Ct}FN domains analyzed here with domain-specific monoclonal antibodies.

Table 1. Fibronectin domain expression in plasma and cerebrospinal fluid

Group and number of samples	FN concentration mg/l			Ratio ^{CBD}FN to:	
	^{CBD}FN	^{Ct}FN	^{Nt}FN	^{Ct}FN	^{Nt}FN
Cerebrospinal fluid, n = 30	1.83 ± 0.8	1.73 ± 0.9	2.28 ± 0.8	1.16 ± 0.5	0.83 ± 0.3
Plasma, n = 76	267.4 ± 98	347.5 ± 163	74.6 ± 37	0.84 ± 0.2	4.14 ± 1.8

Expression of FN domains was estimated by ELISA (Hirnle & Kątnik-Prastowska, 2007) using domain-specific monoclonal antibodies (TaKaRa) to the central cellular (^{CBD}FN), carboxyl- (^{Ct}FN) and amino- (^{Nt}FN) terminal FN domains. The ratios of ^{CBD}FN to ^{Ct}FN and ^{CBD}FN to ^{Nt}FN were calculated for each sample and then averaged. Data are presented as mean values from four determinations ± S.D.

20, pH 7.4. A human FN preparation (Sigma Chemical Co., St. Louis, MO, USA) was used as the standard in the ^{CBD}FN-, ^{Ct}FN-, and ^{Nt}FN-ELISAs. The FN standard was prepared according to the manufacturer's instructions to a concentration of 1 mg in 1 ml TBS. Then the solution was diluted twice with glycerol and divided into aliquots which were stored at –30 °C until use. Under such conditions the FN solution is unfrozen. Before ELISA, the working aliquot was kept for 1 h at room temperature and then diluted with TBS-T to the required FN concentrations (from 3 to 50 ng/well). The background absorbance (with buffer instead of standard or sample, but with all other reagents) ranged from 0.02 to 0.06 A_{492} depending on the microtitre plate and the day of the experiment. The differences in absorbance units (AU) at 492 nm for the FN standard concentrations using the anti-^{CBD}FN, anti-^{Ct}FN, and anti-^{Nt}FN antibodies had ranges of approx. 0.3–1.5 AU, 0.15–1.2 AU, and 0.1–0.8 AU, respectively.

The samples were analyzed at the same time in two dilutions, each in duplicate. The ^{CBD}FN, ^{Ct}FN, and ^{Nt}FN concentrations given in mg/l are presented as the mean ± standard deviation (S.D.).

Unfolding the protein by urea. Samples of plasma (5) and cerebrospinal fluid (3) were incubated at 37°C for 20 h with urea powder added to final concentration of 0.5, 1.0, 2.0, 4.0 or 6.0 M. Then the samples were cooled to room temp., diluted, and immediately subjected to ELISA to determine the FN concentration and to immunoblotting in order to check the FN pattern.

SDS/PAGE and Western immunoblot analysis. Samples of cerebrospinal fluid and blood plasma containing 200 ng of FN were subjected to SDS/7.5%-polyacrylamide gel electrophoresis under reducing conditions (Laemmli, 1970). The separated proteins were subsequently blotted (Towbin *et al.*, 1979) and FN bands were developed with monoclonal antibody (FN30-8, TaKaRa, Shuzo Co., Shiga, Japan) diluted 1 : 10 000 and then with rabbit anti-mouse immunoglobulins conjugated with horseradish peroxidase diluted 1 : 10 000 (Sigma Chemical Co., St Louis, MO, USA). The color reaction was developed with 3,3'-diaminobenzidine. The molecular masses of the FN subunits were estimated from the R_F values of bands as described previously (Hirnle & Kątnik-Prastowska, 2007).

RESULTS

FN domain expression

In the cerebrospinal fluid group the mean values of the apparent ^{CBD}FN, ^{Ct}FN, and ^{Nt}FN concentrations (Table 1) were nearly the same (1.83 ± 0.8 mg/l, 1.73 ± 0.9 mg/l, 2.28 ± 0.8 mg/l, respectively), whereas in the plasma group the ^{Nt}FN concentration (74.6 ± 37 mg/l) was about one fourth that of ^{CBD}FN (267.4 ± 98 mg/l) and ^{Ct}FN (347.5 ± 163 mg/l). Thus the apparent ^{CBD}FN to ^{Ct}FN ratios in the plasma (0.84 ± 0.2) and cerebrospinal fluid (1.16 ± 0.5) were similar, whereas the ^{CBD}FN to ^{Nt}FN ratios were significantly higher in the plasma (4.14 ± 1.8) than in the cerebrospinal fluid (0.83 ± 0.3).

Fibronectin domain exposure after urea treatment

The immunoreactivty of plasma and cerebrospinal ^{CBD}FN, ^{Ct}FN, and ^{Nt}FN modified by urea-induced FN molecular expansion are shown in Table 2. They decreased with increasing urea concentrations for the cerebrospinal fluid reaching the lowest values with 4–6 M of urea; the ^{CBD}FN and ^{Nt}FN levels decreased by a factor of 3 and of ^{Ct}FN by almost 10.

The immunoreactivty of ^{CBD}FN in the plasma increased slightly after treatment with 0.5 and 1 M urea

Table 2. Epitope exposure to antibody on plasma and cerebrospinal fibronectins after urea treatment

Samples after urea treatment*	Urea mol/l	Relative immunoreactivity of FN domain to antibody (%)		
		^{CBD}FN	^{Ct}FN	^{Nt}FN
Blood plasma n = 7	0	100	100	100
	0.5	110 ± 17	79 ± 23	297 ± 68
	1	103 ± 15	72 ± 23	338 ± 124
	2	90 ± 18	50 ± 13	461 ± 255
	4	77 ± 21	29 ± 5	439 ± 202
	6	56 ± 9	23 ± 5	318 ± 188
Cerebrospinal fluid n = 3	0	100	100	100
	0.5	57 ± 9	48 ± 8	70 ± 12
	1	49 ± 9	34 ± 8	58 ± 18
	2	50.7 ± 19	22 ± 22	36 ± 31
	4	34.2 ± 6	13 ± 12	32 ± 23
	6	29 ± 3	12 ± 6	27 ± 16

*Samples of plasma and cerebrospinal fluid were incubated with urea for 20 h. FN domain expression without urea treatment was assumed to be 100 % and the data are expressed as the percentage of the FN level without urea treatment. Data are presented as mean values ± S.D.

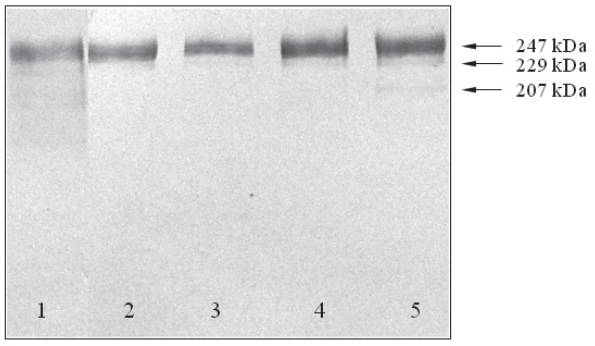

Figure 2. Representative immunoblotting patterns of intact and modified plasma and cerebrospinal fluid fibronectins
Samples were subjected to 7.5% SDS/PAGE under reducing condition and then Western blotting was performed using a monoclonal antibody (FN30-8) specific to the largest, central cell-binding domain (CBD) of FN. The lanes represent: native cerebrospinal fluid **(1)**, native plasma FN **(2)**, plasma FN after 0.5, 1, and 2 M urea treatments (**3–5**, respectively).

and decreased by 56% in 6 M urea, whereas the ^{Ct}FN level successively decreased, showing only 23% of control level in 6 M urea. In contrast, the apparent ^{Nt}FN concentration increased by factors of 3, 4, and 6 with 0.5 M, 1 M and 2 M urea, then decreased slightly with 4 M and 6 M urea, although it was still more than 4-times higher than that estimated for untreated plasma.

Immunoblotting (Fig. 2) of urea-treated FN samples revealed the presence of bands having molecular masses (247 kDa, 229 kDa, and 207 kDa) corresponding to the polypeptides of FN isoforms and a lack of fragmentation products.

DISCUSSION

Our results indicate that the epitopes located at the N-terminal part (^{Nt}FN) of plasma fibronectin, in contrast to its exposed central cell-binding (^{CBD}FN) and carboxy-terminal (^{Ct}FN) domains and to the exposed ^{CBD}FN, ^{Ct}FN, and ^{Nt}FN of cerebrospinal fibronectin, were hardly accessible to a specific monoclonal antibody (Table 1).

The results of the antibody binding to FN, although expressed in concentration units, reflect the relative FN domain immunoreactivity rather than the actual FN concentration in the samples. The accessibility of epitopes in the multidomain large FN molecule depends on the overall protein architecture, size and topography of the individual segments, and the arrangement of modules. The ^{CBD}FN-to-^{Nt}FN ratio calculated by us reflects the differences between the epitope expressions on two domains, ^{CBD}FN located in the central part of the polypeptide, whose the measurements correspond almost exactly to the actual FN concentration, and ^{Nt}FN which forms heparin-fibrin domain located at the N-terminus of polypeptide, hidden in globular plasma FN. Our data indicate that the ^{CBD}FN-to-^{Nt}FN ratio (Table 1) of the plasma FN (4.14 ± 1.8) and of the cerebrospinal fibronectin (0.83 ± 0.3) reflects the significant differences in the epitope accessibility for a specific monoclonal antibody on the N-terminal domains. Such a contrasting N-terminal FN domain exposure is not limited to the plasma and cerebrospinal fibronectins but was earlier reported by us (Pupek *et al.*, 2009) for plasma and pleural effusion fibronectins. The pleural effussion FN shows nearly equal levels of ^{Nt}FN and ^{CBD}FN, as was also found here for the cerebrospinal fluid. Moreover, the commercial FN preparation from human plasma (used by us in ELISA experiments as FN standard) and FN from plasma samples differed in their the anti-^{Nt}FN antibody binding (not shown). The purified plasma FN preparation (Sigma) showed nearly four times higher reaction with the anti-^{Nt}FN monoclonal antibody than the native FN of plasma samples. At least two factors could be considered to explain this difference. First, the preparation procedure of the FN from human plasma could produce a partly denatured molecule with unfolded segments, revealing the N-terminal epitopes (Fig. 1C), normally hidden in the three-dimensional structure of globular native plasma FN (Fig. 1B). A similar event was observed by us after denaturation (Table 2) of plasma FN with 0.5–6 M urea, which led to the gradual revealing of the N-terminal epitopes. Second, the plasma FN preparation is free of other plasma components, while FN present in the body fluid can be linked with other molecules, such as domain ligands, which might interfere with domain-specific antibody binding. Thus, the differences in the antibody binding may stem not only from FN unfolding but also might result from FN purification process removing other proteins or other molecules present in biological fluids, which could interact with FN.

An intriguing question is why cerebrospinal fluid fibronectin has more accessible antibody-binding sites on the N-terminal domains than does plasma fibronectin. To understand the data it is necessary to consider the different FN origins and environments in the plasma and cerebrospinal fluid. The two fibronectins are produced by different cells: plasma fibronectin by hepatic and cerebrospinal fibronectin locally by nerve and immunological cells (Liesi *et al.*, 1986; Torre *et al.*, 1993). The different FN molecular arrangements might result from alternative splicing (Ffrench-Constant, 1995) through inclusions or not of the EDA, EDB, or variable IIICS regions between the FNIII segments (Fig. 1A). We observed that cerebrospinal fibronectin contains an EDA domain which is absent in normal plasma FN (not shown). Finally, plasma-derived fibronectin could be blocked at the N-terminus by a possible ligand(s) (Ito *et al.*, 2005; Makogonenko *et al.*, 2007) that is absent in the cerebrospinal fluid.

Another explanation could be connected with the differences in distribution of electrostatic forces and hydrogen bonds maintaining the conformation of FN. It is well documented that in solution under physiological conditions, the $^{1\text{-}2}$FNIII segments of the central cell-binding domain are in a closed conformation and associate weakly with the N-terminal domain built from FNI segments (Fig. 1A), thus maintaining closed conformation (Fig. 1B) of native plasma fibronectin (Vakonakis & Campbell, 2007; Vakonakis *et al.* 2007). The interaction among the FNIII domains includes a large electrostatic component which is substantially reduced at higher ionic strength (Vakonakis *et al.*, 2009). Although the compositions of plasmatic and cerebrospinal electrolytes are very similar, the total protein concentration of cerebrospinal fluid is about 0.5% that of plasma. Moreover, the specific protein concentrations in cerebrospinal fluid are not proportional to their plasma levels because of the specificity of the ultrafiltration process (Sedor, 2000; Brown *et al.*, 2004). Thus the distribution of electrostatic forces

stabilizing the protein structures is probably different in the plasma and cerebrospinal fluid.

Some *in vitro* experiments show that native fibronectin can undergo dramatic structural changes from a globular to an extended conformation after treatment with low doses of hypochlorite (Olszowski *et al*., 2003), or after urea-induced denaturation (Lairez *et al*., 2003; Patel *et al*., 2006; Nelea *et al*., 2008). In such cases the accessibility of fibronectin epitopes for an antibody increases. Olszowski *et al*. (2003) have shown that the number of accessible fibronectin epitopes increases when fibronectin adopts an extended conformation in complex with the antibody. In our experiment under the mildly denaturing conditions of 0.5–2 M urea, the hidden epitopes of the N-terminal and some of the CBD domains were probably exposed and made more accessible for reaction with the respective antibodies. Thus the apparent concentration of ^{Nt}FN increased about fourfold but that of ^{CBD}FN only slightly (Table 2, Fig. 1C). At higher concentrations of urea (4–6 M), the epitopes seemed to lose their ability to bind antibody, most probably because the conformational determinants lost their native conformation.

In conclusion, the results of an analysis of fibronectin domain expression by a panel of specific monoclonal antibodies may reflect the conformation-dependent functional states of fibronectin and/or processes in which fibronectin could be implicated. Such determinations may have important clinical implications. However, additional studies should be performed to reveal the nature of the observed differences in domain accessibility and exposure depending on the protein origin.

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