The Inclusion of the Type III Repeat ED-B in the Fibronectin Molecule Generates Conformational Modifications That Unmask a Cryptic Sequence*

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We have previously reported an anti-fibronectin monoclonal antibody (mAb) (BC-1) which reacts with an ED-B-containing β-galactosidase-fibronectin fusion protein but not with an identical β-galactosidase-fibronectin fusion protein in which the ED-B sequence is omitted. In further experiments aimed at localizing more precisely the epitope recognized by this mAb, we demonstrate that 1) the mAb BC-1 is indeed specific for ED-B-containing fibronectin (FN) molecules even though the epitope recognized by this mAb is localized on the type III homology repeat 7 (the one which precedes the ED-B sequence) and 2) in fibronectin molecules lacking the ED-B sequence, this epitope is masked. We further demonstrate that, to mask the epitope recognized by the mAb BC-1, the presence of at least half of the FN type III homology repeat 9 is necessary. We also report the production of the mAb IST-6 which recognizes only FN molecules in which the ED-B sequence is lacking. These data clearly demonstrate that the presence of the ED-B sequence within FN molecules generates conformational modification in the central part of the molecules that unmask previously cryptic sequences and masks others.

Fibronectins (FNs) are high molecular mass adhesive glycoproteins present in the extracellular matrix and in body fluids. These molecules are involved in different biological phenomena such as the establishment and maintenance of normal cell morphology, cell migration, hemostasis and thrombosis, wound healing, and oncogenic transformation (for reviews, see Altalal and Vaheri (1982), Yamada (1983), Hynes (1985), Ruoslahti (1988), Hynes (1990), Owens et al. (1986)).

FN polymorphism is due to alternative splicing patterns in three regions (HICS, ED-A, and ED-B) of the single FN primary transcript (see Fig. 1) as well as to post-translational modifications. The alternative splicing of the FN pre-mRNA is regulated in a cell-, tissue-, and developmentally specific manner (see above-mentioned reviews and references therein). Furthermore, it has been demonstrated that the splicing pattern of FN pre-mRNA is deregulated in transformed cells and in malignancies (Castellani et al., 1986; Bors et al., 1987; Vartio et al., 1987; Zardi et al., 1987; Barone et al., 1987; Carnemolla et al., 1989; Oyama et al., 1989, 1990; Borsi et al., 1992). In fact, the FN isoforms containing the IICS, ED-A, and ED-B sequences are expressed to a greater degree in transformed human cells and in tumor tissues than in their normal counterparts. In particular, the FN isoform containing the ED-B sequence, which, with some very rare exceptions, is undetectable in normal adult tissues, has a much greater expression in fetal and tumor tissues as well as during wound healing (Norton and Hynes, 1987; Schwarzbackground et al., 1987; Gutman and Kornbluth, 1987; Carnemolla et al., 1989; ffrench-Constant et al., 1989; ffrench-Constant and Hynes, 1989, Laitinen et al., 1991). The ED-B oncofetal domain, a complete type III homology repeat composed of 91 amino acids and coded for by a single exon, is the most conserved FN region with 100% and 96% homology with rat and chicken FN, respectively (Norton and Hynes, 1987; Zardi et al., 1987). This could indicate either a more recent evolution of the ED-B sequence, with less time to diverge, or a more stringent requirement due to some unknown function(s) performed by this sequence. While the alternative spliced sequence IICS contains a cell type-specific cell binding site, the biological functions of the ED-A and ED-B are still a matter of speculation (Humphries et al., 1986).

We describe two monoclonal antibodies, BC-1 and IST-6, which are specific for FN isoforms containing and not containing the ED-B sequence, respectively. Given this specificity, we assumed that the epitope recognized by the mAb BC-1 was localized within the ED-B sequence (Carnemolla et al., 1989). However, we now demonstrate that this epitope is localized within the type III repeat 7 (which precedes the ED-B) and that it is cryptic in FN molecules lacking the ED-B, while it is unmasked in molecules containing this sequence.

MATERIALS AND METHODS

Cell Lines, Monoclonal Antibodies (mAbs), Radioimmunoassay, and Purification of FN and Its Proteolytic Fragments—The cultured normal human fibroblast cell line GM-5659 (from non-fetal skin) and the SV40-transformed WI-38-VA cell line were purchased from NIGMS, Human Genetic Mutant Cell Repository (Camer, NJ) and from the American Tissue Type Culture Collection, respectively. The mAb IST-6 was prepared as described using splenocytes from mice immunized with FN purified from human plasma (Zardi et al., 1989). The mAb BC-1 has been described previously (Carnemolla et al., 1989). FNAs were purified from human plasma and from the conditioned medium of the various cell lines using a modification (Zardi et al., 1980) of the procedure of Engvall and Ruoslahti (1977). The radioimmunoassay experiments, using purified FNAs or FN fragments, were carried out as reported by Zardi et al. (1980). The 120-kDa (containing the ED-B) and 110-kDa (lacking the ED-B) FN domain 4 isoforms (see Fig. 1) were purified from thermolysin digests as previously described (Zardi et al., 1987; Borsi et al., 1991).

cDNA Clones and Fusion Proteins—cDNA clones AF2 and XFe, obtained as previously reported (Carnemolla et al., 1989), cover the
type III homology repeats 7, 8, and half of 9 (from residues 1138 to 1380) (Kornblihtt et al., 1985). However, the ED-B sequence was included in the λF2 clone while it was omitted in the λF6c clone. In order to facilitate the reading of the manuscript, we have here named these clones according to the type III repeats or fractions of type III repeats they contain: λF2 = λF7.8.B.8.9/2; λF6c = λF7.8.9/2 (see Fig. 1A). The cDNA clones λF7.8/18 and λF7 were obtained from the λF7.8.9/2 FN insert by digestion with BspM1 and PV2, respectively. The λF7.8/18 cDNA clone includes the type III homology repeat 7 and few amino acids of the 8 (from residues 1138 to 1239), while the inserts of the clones XF7.8 and XF713.8 were obtained from polymerase chain reaction amplification of the XF7.8.912 using Xgtll and appropriate oligonucleotides. PCR reactions were performed for 35 cycles (1 min at 94 °C, 1 min at 48 °C, and 1 min at 68 °C) in a final volume of 100 μl containing 50 mM KCl, 10 mM Tris-Cl, pH 8.3, 1.5 mM MgCl2, 0.01% (w/v) gelatin, 200 mM concentration of each dNTP, 100 pmol of each oligonucleotide, 5 units of Taq DNA polymerase (Amplitaq, Perkin-Elmer Cetus), and 10 ng of purified λF7.8/3.8 DNA as template. All cloning and subcloning procedures were carried out, using the expression vector Xgtll phage, according to previously described (Carnemolla et al., 1989; Carnemolla et al., 1989), and their immunoenzymatic reaction with mAbs IST-6 and BC-1 was carried out using a Protoblot immunoscreening system kit purchased from Promega Biotec. SDS-PAGE and immunoblotting were carried out as previously reported (Carnemolla et al., 1989).

RESULTS AND DISCUSSION

Fig. 1A depicts the domain structure of a human FN subunit. We have tested the mAbs BC-1 and IST-6 with fibronectins containing different percentages of ED-B-containing molecules. We have previously reported that the ED-B-containing FN is absent in plasma and present in a very low percentage in FN from cultured normal skin fibroblasts, while it is present in a high percentage in FN from SV40-transformed WI-38-VA cells (Borsi et al., 1992; Carnemolla et al., 1989). Fig. 1 shows the results obtained by radioimmunoassay experiments using the mAbs BC-1 and IST-6 on FNs from these three different sources. The mAb BC-1 does not react with plasma FN and barely reacts with FN from normal skin fibroblasts, while it reacts strongly with FN from the SV40-transformed WI-38-VA cell line. On the contrary, the mAb IST-6 gives a strong reaction with FNs from plasma and normal skin fibroblasts while it shows a weaker reaction with FN from WI-38-VA cells. Fig. 2 shows the results obtained by immunoblotting using the monoclonals BC-1 and IST-6 on lysis plaques generated by the clones λF7.B.8.9/2 and λF7.8.9/2 (Fig. 2). The mAb BC-1 reacts with the fusion protein λF7.B.8.9/2 but not with λF7.8.9/2. On the contrary, the mAb IST-6 does not react with the fusion protein λF7.B.8.9/2 but does with the fusion proteins λF7.8.9/2. Identical results were obtained in immunoblotting experiments after SDS-PAGE using the λF7.B.8.9/2 and λF7.8.9/2 fusion proteins (Fig. 3) and the 120 (ED-B+) and 110 (ED-B–) kD

![Fig. 1. A, model of the domain structure of a human FN subunit. The IIICS, ED-A, and ED-B regions of variability, due to alternative splicing of the FN pre-mRNA, are indicated. The figure also indicates the internal homologies, macromolecules interacting with the various FN domains, and the β-galactosidase-FN fusion proteins expressed in Escherichia coli containing (λF7.B.8.9/2) and not containing (λF7.8.9/2) the ED-B sequence. B, radioimmunoassay analysis of plasma FN (PL), FN purified from the conditioned media of normal human skin fibroblast cell line, GM-5659, and of WI-38-VA, SV40-transformed embryonic lung fibroblast, using the mAbs BC-1 (black bars) and IST-6 (white bars).](#)

![Fig. 2. λF7.8.9/2 and λF7.B.8.9/2 clones were plated on E. coli cells, overlaid with a nitrocelullose filter paper previously saturated with isopropyl β-D-thiogalactopyranoside, and probed with mAbs BC-1 and IST-6. BC-1 gives positive signals with λF7.B.8.9/2 lysis plaques only. On the contrary, IST-6 gives positive signals with λF7.8.9/2 lysis plaques only.](#)

![Fig. 3. On the left, a 4–18% SDS-PAGE gradient of proteins from E. coli-injected by the expression vector λgt11 and by the clones λF7.B.8.9/2 and λF7.8.9/2, respectively. On the right, immunoblots using the mAb BC-1 and IST-6, respectively. The values on the left indicate the molecular masses, in kDa, of the standards.](#)
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![Image](image-url)

**Fig. 4.** On the left, a 4–18% SDS-PAGE gradient of the purified ED-B+ (lane 1) 110- and ED-B− 120-kDa (lane 2) domain 4. On the right, immunoblots of a similar gel using the mAbs BC-1 and IST-6. The values (in kDa) on the left indicate the molecular masses of the two FN fragments.

**Fig. 5.** On the left (top), the FN sequences contained in the fusion proteins expressed in E. coli infected by the clones AF7.8.9/2, AF7.8, AF7/3.8, AF7/8.18, AF7, and the reactivity of these fusion proteins with the mAbs BC-1 and IST-6. On the right (top), a 4–18% SDS-PAGE of proteins from E. coli infected by the clones described above. The values on the right indicate the molecular masses, in kDa, of the standards. At the bottom, immunoblots of similar gel using the mAb BC-1 and IST-6. The values in the middle of the figure indicate the molecular masses, in kDa, of the standards.

domains 4 (Zardi et al., 1987) with the mAbs BC-1 and IST-6 (Fig. 4). These results demonstrate that BC-1 and IST-6 are specific for FN molecules containing and not containing the ED-B sequence, respectively.

In immunoblotting experiments using different FN-β-galactosidase fusion proteins, we have more precisely localized the epitope recognized by the mAb BC-1. Even though the experiments shown in Figs. 1–4 could suggest that this epitope is localized within the ED-B sequence, since it is specific only for ED-B-containing FN molecules or fragments, the results shown in Fig. 5 demonstrate that it is localized within the type III homology repeat 7 and that the presence of the type III homology repeat 9 makes this epitope cryptic, while the expression of the ED-B sequence unmasks it. In fact, a FN-β-galactosidase fusion protein identical with the AF7.8.9/2 but lacking the repeat 9 reacts strongly with the mAb BC-1. In addition, the β-galactosidase-FN fusion protein AF7 which contains only the repeat 7 also shows the same positive reaction with the mAb BC-1. The mAb BC-1 also reacted with a β-galactosidase-FN fusion protein which contains the repeats 7 and ED-B (data not shown) but did not show any reaction with β-galactosidase-FN fusion proteins containing, respectively, only the repeat ED-B or the repeat 9 (data not shown).

The epitope recognized by the mAb IST-6 was more problematic to localize; in fact, the minimum requirement for a positive reaction with the mAb IST-6 for a FN-β-galactosidase fusion protein was the simultaneous presence of the complete type III homology repeats 7 and 8 (Fig. 5).

The main observations described here are that 1) the mAb BC-1 is specific for ED-B-containing FN molecules even though the epitope recognized by this mAb is not localized within the ED-B sequence and 2) the mAb IST-6 is strictly specific for FN molecules lacking the ED-B sequence. Thus, these two mAbs represent useful reagents to study the distribution of different FN isoforms in different tissues. Furthermore, these data demonstrate that the presence of the ED-B sequence within the FN molecules induces conformational modification in the central part of the molecule which, in turn, leads to the unmasking of a previously cryptic sequence within the type III repeat 7 and to the masking of the epitope recognized by the mAb IST-6. The fact that these structures are detectable also in immunoblotting experiments, after electrophoresis in presence of SDS, indicates that strong interactions between different FN regions take place. It has been reported that the free sulphhydril group present in the repeat 7 is cryptic when plasma FN is in solution, while it is unmasked in FN bound to solid phase substrates (Narasimhan et al., 1988; Narasimhan and Lai, 1991). However, the mAb BC-1 recognized an epitope in which this cysteine is not involved because, in FN molecules lacking the ED-B, it is cryptic when FN is in solution or when it is bound to solid phase substrates. Furthermore, in the β-galactosidase-FN fusion protein AF7.8.9/2 (see Fig. 5), we have substituted the cysteine present in the repeat 7 with a serine; the reactivity of this mutant with mAbs BC-1 and IST-6 did not show any modification (data not shown). At present, we have no evidence of whether these conformational modifications may influence the biological functions of FN. However, it has been reported that the cell binding site GRGD needs two synergistic regions in order to express its activity, and these regions have been localized within type III homology repeats 8 and 9, respectively (Ohara et al., 1988); Nagai et al., 1991). Further studies are needed in order to clarify the structural conformation of this crucial region of the FN molecule and the possible functional modifications induced by the expression of the ED-B sequence.

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