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A novel domain of fibronectin revealed by epitope mapping of a monoclonal antibody which inhibits fibroblasts-mediated collagen gel contraction

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Abstract The ability of cells to organize collagen fibrils is fundamental to a variety of processes found in embryogenesis, wound healing, fibrosis, and scar formation. We previously isolated a monoclonal antibody (mAb A3A5) which inhibits human fibroblast-mediated collagen gel contraction, an in vitro model producing the process of collagen morphogenesis. Human fibronectin (FN) has been shown to be the antigen of A3A5. The present study aimed at identifying the A3A5 epitope to reveal the mode of binding between collagen, FN, and fibroblasts in the process of gel contraction. The epitope was sought in FN fragments obtained by pepsin digestion and in recombinant FN fragments expressed in *Escherichia coli* by determining their immunological reactivity with A3A5, and was identified as a short segment consisting of the fourth through the amino half of the fifth FN type III. We propose a new functional domain of FN which plays a crucial role in the binding of fibroblasts to collagen fibrils and is involved in collagen morphogenesis.

Key words: Collagen morphogenesis; Epitope mapping; Fibronectin; Human fibroblast; Type III module

1. Introduction

Fibroblasts populated in 3-dimensional lattices of collagen bind collagen fibrils and organize them into more dense and compact structures [1–4]. This cellular process is thought to be an in vitro model of the collagen morphogenesis found in a wide variety of biological processes such as embryogenesis, organogenesis, and wound healing [5,6]. However, the mechanism of cell-mediated gel contraction has been largely unknown.

Binding between fibroblasts and collagen fibrils is evidently the first and key step of the consecutive events taking place in gel contraction. Among such binding processes, the direct interaction of cell surface integrins with collagens appears to be important [7]. In fact, a recent work showed that the binding of $\alpha_2\beta_1$-integrin receptors to type I collagen is involved in the contraction of hydrated collagen lattices [8]. Indirect interactions of cells with collagen mediated by fibronectin (FN) might also be crucial for gel contraction because FN possesses the ability to bind both collagen and cell surface [7]. However, the role of FN in collagen gel contraction has been controversial. Some studies showed that the FN-mediated reaction is not involved in gel contraction [9,10], while other studies suggested the involvement of FN in the process and proposed that FN links cell surface molecules to the surrounding collagen fibrils [11–13].

Previously, we obtained a monoclonal antibody (mAb) called A3A5 that blocks the initial phase of human fibroblasts-mediated collagen gel contraction [13]. This mAb was found to be reactive with both plasma (pFN) and cellular FN (cFN) in human, indicating the involvement of FN in collagen gel contraction. Therefore, determination of the epitope of this unique mAb appeared to be useful and important to gain an insight into the mechanism of gel contraction. Especially, epitope identification is pivotal to know which domain of an FN molecule is responsible for mediating the binding between collagen and fibroblasts.

FN is a high-molecular-mass heterodimeric glycoprotein consisting of three types of modules (type I, type II, and type III repeat) and plays an important role in diverse phenomena including cell attachment, cell spreading, cell migration, wound healing, and tumor metastasis (for reviews, see [7,14]). The present study located the A3A5 epitope on a human FN molecule. Identification of A3A5-reactive fragments in the pepsin digests of FN revealed that the epitope is included in the region ranging from III_4 to III_6 modules. This epitope region was further shortened to a 122-amino-acid sequence in the III_4 repeat through the amino half of III_5 repeat by testing the A3A5 reactivity of polymerase chain reaction (PCR)-generated FN fragments. These results suggest that this specific short segment of FN has a biologically significant role in complex interactions between cells and collagen fibrils taking place on the cell surface which lead to collagen morphogenesis.

2. Material and methods

2.1. Materials

Materials and chemicals were obtained as follow: human pFN from BioMedical Technology, Inc. (Boston, MA); immobilized pepsin from Pierce (Rockford, IL); ECL detection kits from Amersham Japan (Tokyo); peroxidase conjugated with second antibody from Dainihon Pharmaceutical Co. Ltd. (Tokyo); *Bal*31 exonuclease, and *malE* gene fusion vector pMAL-c2 and restriction endonucleases of *Xmn*I, *Sal*I, *Bam*HI, and *Pst*I from New England Biolabs (Beverly, MA); Taq polymerase from Perkin Elmer (Norwalk, CT); *Escherichia coli* (*E. coli*) cells (JM109) from Toyobo (Tokyo); NuSieve GTG low-melting agarose and β-agarose from FMC BioProducts (Rockland, ME);

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pCRII vectors from Invitrogen (San Diego, CA); Immobilon polyvinylidene difluoride (PVDF) transfer membranes from Millipore (Bedford, MA); CHROMA SPIN-200 columns from Clontech (Palo Alto, CA). Reagents not specified otherwise were of analytical grade and purchased from Wako Pure Chemicals Inc. (Tokyo).

2.2. Western immunoblot analysis of FN fragments

Human pFN (2 mg/ml) was digested with immobilized pepsin in 0.15 M NaCl and 25 mM glycine buffer (pH 2.0) at 37°C for 2 h at a protease/substrate ratio of 1:4. The enzyme was then removed by centrifugation, and the supernatants which contained fibronection fragments were recovered. The fragments were precipitated with 5% trichloroacetic acid and solubilized in electrophoresis sample buffer containing 2% sodium dodecyl sulfate (SDS) and 0.1 M dithiothreitol. After neutralized with 0.1 N NaOH, peptides in the solution were electrophoresed in wide slots (30 mm) of 9×10 cm 4–20% gradient polyacrylamide-SDS gels (SDS-PAGE) and transferred to Immobilon PVDF membranes. The PVDF blots were washed with the solution of 0.15 M NaCl, 50 mM Tris-HCl (pH 7.6), and 0.05% Tween-20 (TBST), dipped in 1% bovine serum albumin in TBST for 30 min, and then reacted for 1 h with the hybridoma culture supernatants of A3A5 mAb [13]. The blots were extensively washed with TBST, incubated for 1 h in TBST containing horseradish peroxidase-conjugated second antibodies, and washed extensively with TBST. A3A5-reactive fragments were visualized by a ECL kit according to the manufacture's instruction.

2.3. Amino acid sequencing

FN was digested with pepsin, separated on gels as described above, and visualized with Coomassie blue. Peptide bands were cut out with a scalpel and sequenced by a gas-phased protein sequencer of Applied Biosystems (Foster City, CA).

2.4. Construction of cDNAs for FN segments

A cDNA clone (pFH100) coding for the full length of FN except ED domains was generously provided by Dr. A. Kornblihtt (INGEBI-CONICET, Argentina). All nucleotide sequences are numbered according to GenBank file HUMFNMC of Kornblihtt [15]. Two different cDNA segments covering A3A5-reactive fragments of FN were constructed as follow: pFH100 cDNA was linearized by digesting with restriction enzyme *Sal*I (at 1592 bp) and then digested with *Bal*31 exonuclease. The resulting cDNA fragments were further digested with either *Pst*I (at 2657 bp) or *Bam*HI (at 4086 bp). These DNA fragments were inserted into pMAL-c2 plasmid vectors coding for maltose-binding protein (MBP) which had been treated with either *Xmn*I and *Pst*I, or *Xmn*I and *Bam*HI. The correct coding frame of pMAL-c2 recombinant clones was verified by nucleotide sequencing of the insert DNA. The unidirectional expression of the recombinants was carried out as described by Maina et al. [16].

The fourth to sixth type III repeats of FN which include the two A3A5-positive fragments (P2 and P3) were amplified by the polymerase chain reaction (PCR) using pFH100 DNA as template and two different *Bam*H1 site-franked primers: 5′ sense primer, Fn3N (CCGGATCCGTGCCCTCTCCCAGGGACCT, *Bam*H1 site underlined, 28-mer), and 3′ antisense primer, Fn7C (CCGGATCCCACCACTTTGTTTACAATTG). These oligonucleotides were synthesized by an Applied Biosystems 391 DNA synthesizer. The PCR reaction was carried out in 25 μl of the solution consisting of 20 mM Tris-HCl (pH 8.4), 50 mM KCl, 1 mM dNTP mix (dATP, dGTP, dCTP, and dTTP), 2.5 ng pFH 100, 1 pmol of each of primers, and 1 U of Taq polymerase.

Amplification was performed for 25 cycles of a consecutive incubation at 94°C for 30 s, 55°C for 30 s, and 72°C for 30 s, and finally at 72 for 10 min. The PCR product (805 bp in length) was first subcloned into the pCRII vector following the manufacture's protocol. The insert DNA was removed from the vector by digesting with *Bam*HI, then electrophoretically purified on a low-melting agarose gel. A gel band containing the insert was excised and digested with β-agarose to release the insert DNA. The DNA was further purified by passing through a CHROMA SPIN-200 column, then subcloned into *Bam*HI site of pMAL-c2 plasmid for *E. coli* expression. This clone was named as PCR(X)-18.

The proteins fused with MBP were prepared as recommended by the manufacturer and were analyzed by 4–20% gradient SDS-PAGE, or Western immunoblot analysis as described above, except that the visualization of antigen with antibodies was performed by color detection using 3,3′-diaminobenzidine (DAB) [17].

2.5. Preparation of deletion mutants of clone PCR(X)-18 by site-directed PCR mutagenesis

The clone PCR(X)-18 was deleted from its 5′- or 3′-terminus by site-directed PCR mutagenesis and four deletion mutants were obtained: clone 3/6 cover a region from nucleotide 2633 to 3175 of the GenBank sequence HUMFNMC, clone 3/25 from 2633 to 3110, clone 3/24 from 2633 to 2998, and clone 9/7 from 3176 to 3438. Primers utilized are described for each clone in the sequence of 5′ sense primer and 3′ antisense primer, respectively: Fn3N (see Section 2.4 describing the construction of cDNAs for FN segments) and Fn6C (CCGGATCCGGTAAAGACTCCAGTGGCTT, *Bam*H1 site underlined, 28-mer) for clone 3/6; Fn3N and Fn25C (CCGGATCCAGGCTGCAGATTCCTCAGGG, 28-mer) for clone 3/25; Fn3N and Fn24C (CCGGATCCTATCTGGGCCCGAGGTGGAG, 28-mer) for clone 3/24; and Fn9N (CCGGATCCGGGAGCTCTATTCCACCTTA, 28-mer) and Fn7C (CCGGATCCCACCACTTTGTTTACAATTG, 28-mer) for clone 9/7.

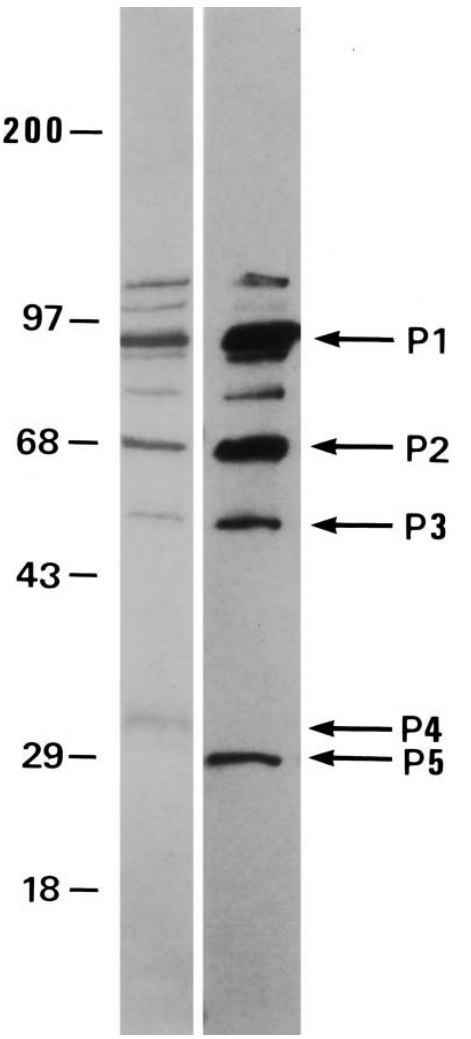

Fig. 1. Reactivity of fragments of human pFN to the mAb A3A5. FN was digested with pepsin, electorphoretically separated, and subjected to the Western blotting. Left: Coomassie blue staining. Right: Western immunoblot analysis by the ECL film detection system. Molecular weights were determined by the migration of standard proteins and are shown in kDa at the left side. Five bands detected on the Coomassie blue-stained gel or the immunoblots were designated as P1 through P5 and subjected to amino acid sequencing.

3. Results

3.1. Confinement of the epitope of mAb A3A5 in a region spanning III_4 to III_6

Human pFN was partially cleaved by pepsin, separated on a SDS-PAGE gel, and was subjected to the Western blotting with mAb A3A5 (Fig. 1). Five fragments (P1–P5) were selected for amino acid sequencing as shown in Fig. 1. P2 and P3, but not P4, were found to be immunologically reactive with mAb A3A5. P2, P3, and P4 gave their amino terminal sequences as follows: P2, ETTCTRSDTV (E at amino acid 870 or nucleotide 2609); P3, SVTEITASS (S at 700 or 2099); and P4, VTTIPAPTDLKPTQVT (V at 1602 or 4805). Their carboxyl end was estimated from their molecular weights (65 kDa for P2, 50 kDa for P3, and 30 kDa for P4). Positive fragments of P2 and P3 shared the region spanning III_4–III_6 domain, while a negative fragment of P4 located in a region containing III_{11}–III_{14} (Fig. 2A) which is a carboxyl terminal side of the cell-binding sequence (RGD) in III_{10}. Thus, it is clear that the immunological recognition of mAb A3A5 is sequence-specific and its epitope locates within the III_4–III_6 region.

To confirm this conclusion on the epitope location, FN cDNA segments coding for III_4–III_6 repeats were expressed as proteins fused with MBP in *E. coli* and their reactivity to the mAb was tested by Western immunoblotting. As schematically shown in Fig. 2A, three clones were prepared by deleting appropriate region of pFH100 clones: clone #18 covered a region from Val^{587} (or nucleotide bp 1760) to Leu^{886} (2657); clone #160 from Glu^{857} (2570) to Ile^{1363} (4088); clone PCR(x)-18 from Asp^{878} (2633) to Leu^{1058} (3173). Thus, clone #18 included III_1 through III_3, clone #160 III_3 through most III_9, and clone PCR(X)-18 III_4 through III_6. The fusion proteins with expected sizes were efficiently expressed as shown in Fig. 2Bc in which gels were stained with anti-MBP antibodies. A number of polypeptides whose molecular weights were lower than expected ones were also detected by antibody binding. These might be degradation products due to endogenous proteases present in *E. coli*. Western immunoblotting using mAb A3A5 is shown in Fig. 2Bb, which clearly demonstrates that the mAb recognizes products of both #160 and PCR(X)-18 clones, but not that of clone #18. As expected, mAb A3A5 bound to plasma FN but not MBP. These results indicate that reactivity of the mAb is FN-segment specific, and confirm the

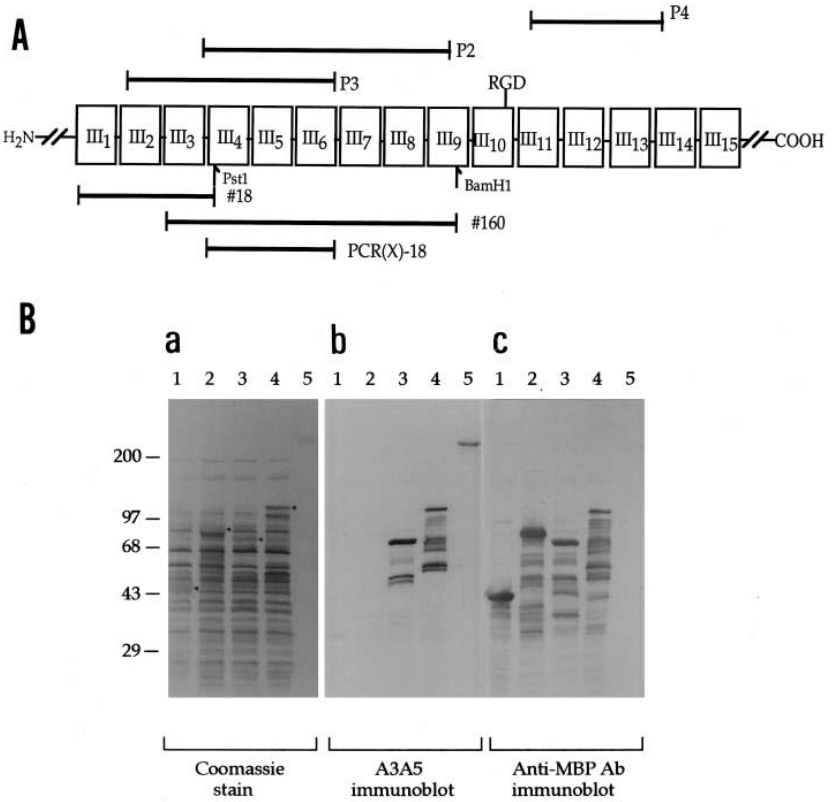

Fig. 2. The A3A5 epitope is located in the 4th to 6th type III repeat of human FN. A: Schematic representation of locations of peptides tested on human FN. A central part of a subunit of a dimeric FN molecule is presented. Three vertical lines above the FN molecule represent the location of three pepsin digests, the upper, middle, and lower line being P4, P2 and P3, respectively, and those below the molecule represent the location of peptides encoded by clone #18, #160, and PCR(X)-18, respectively. Clones #18, #160, and PCR(X)-18 code for FN sequences of bp 1760–2657 (amino acid 587–886), bp 2570–4088 (amino acid 857–1363), and bp 2633–3173 (amino acid 878–1058), respectively. These sequences were defined according to the GenBank sequence HUMFNMC. B: A3A5 reactivity of products encoded by recombinant cDNAs of FN fragments. The FN segments were expressed in *E. coli* as MBP fusion proteins. *E. coli* lysates were separated on a 4–20% gradient gel of SDS-PAGE and were stained with Coomassie blue (a), or processed for immunoblot analysis probed with either mAb A3A5 (b) or polyclonal anti-MBP antibodies (c). Lane 1: *E. coli* lysates containing vectors without the insert; 2, clone #18; 3, clone PCR(X)-18; 4, clone #160; 5, human pFN (1 μg). Open triangles marked at the right side of each lane in (a) indicate the polypeptides of MBP (lane 1) and MBP fusion proteins (lanes 2–4) with the expected sizes. The number on the left side represents molecular weights in kDa. Lower molecular mass polypeptides detected by the antibody-binding might be degradation products due to *E. coli* proteases.

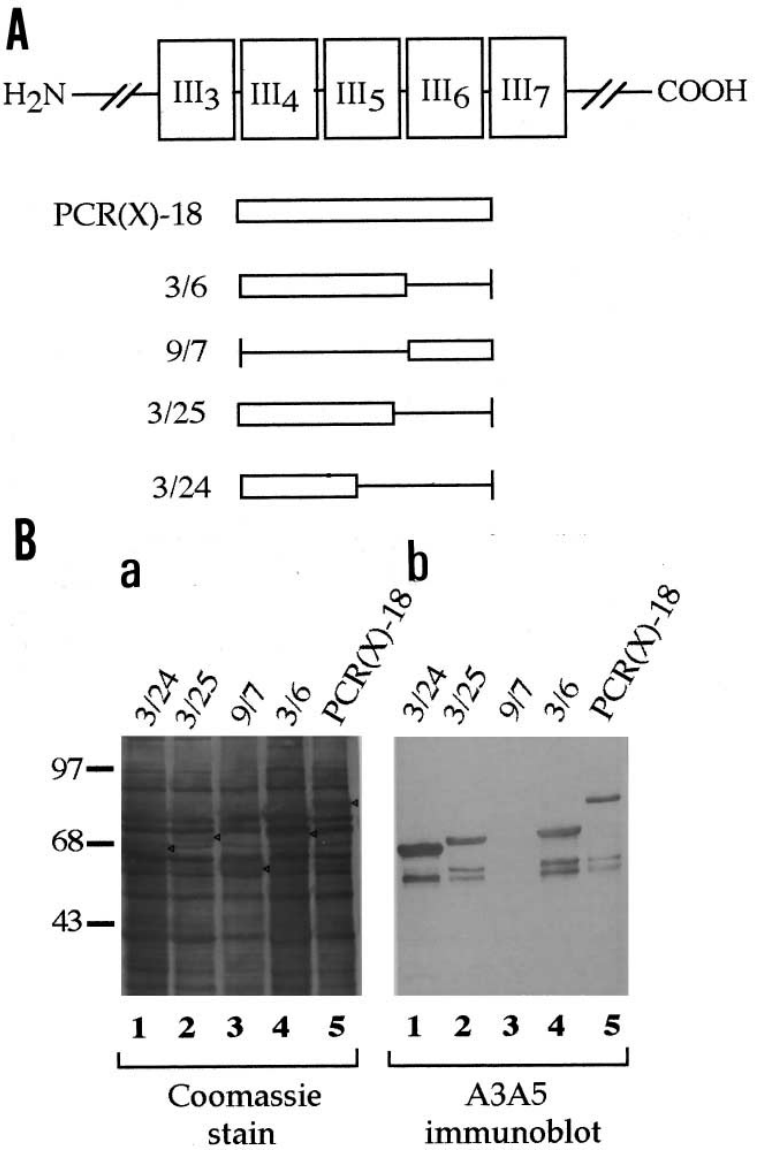

Fig. 3. Effect of mutations in the epitope region on their reactivity to A3A5. A: Schematic representation of amino- or carboxyl-terminal deletion of the epitope expressed by PCR(X)-18. B: Western blotting of mutants. Immunoblottings in (b) were carried out as in Fig. 2B. Mutants were constructed by the site-directed PCR mutagenesis. Proteins were stained by Coomassie blue (a). Open triangles marked at the right side of the panel indicate MBP fusion proteins with the expected sizes. Arabic numerals at the left represent molecular weights in kDa. Lanes 1–5: *E. coli* cell lysates of clone 3/24, clone 3/25, clone 9/7, clone 3/6, and clone PCR(X)-18.

conclusion obtained by the pepsin-digestion experiment that the epitope of the mAb is within the III_4–III_6 domain (amino acid 878–1058 or, nucleotide 2633–3173). In addition, it is clear that the sugar moieties present in this region [7] are not involved in the antigenic determinant for the mAb, since bacterial cells lack the machinery for adding carbohydrate chains to proteins.

3.2. *Further confinement of the epitope region by recombinant PCR mutagenesis*

To shorten the epitope region further, PCR(X)-18 clones were deleted from their 5′- or 3′-end by PCR mutagenesis. Deletion mutants were cloned into pMAL-c2 vectors and expressed as MBP fusion proteins in *E. coli*. The reactivity of the expressed proteins to mAb A3A5 was checked by the Western immunoblotting (Fig. 3B). The proteins produced by mutant clones of 3/6, 3/24, and 3/25 were positive as that of the original clone PCR(X)-18, whereas the III_6 repeat expressed by clone 9/7 was negative. Clone 3/24 codes for the whole III_4 (amino acid 878–968) and the amino-terminal one-third of III_5 (969–999) and was the shortest positive clone we could obtain. Further narrowing the epitope region by this method was not successful because there were difficulties in expressing MBP fusion proteins reactive to mAb A3A5 when the size of inserts was smaller than that of clone 3/24 (data not shown). It appears that there is a lower limitation of insert DNA size to be expressed in *E. coli* with pMAL-c2 vector. Therefore, the minimal epitope region we could identify by the methods presently adopted was a region expressed by clone 3/24 which codes for a FN segment composed of 122 amino acid residues (amino acid 878–999 or nucleotide 2633–2998).

4. Discussion

Previously we succeeded in screening a unique mAb A3A5 from hybridomas obtained from mouse cells immunized with human skin fibroblasts. The mAb recognizes human FN as its antigen and inhibits fibroblasts-mediated collagen gel contraction [13]. A3A5 seems to interrupt an interaction step between fibroblasts and collagen fibrils because mAb A3A5 immunologically stains fibroblasts cultured on gelatin but not those on collagen fibrils [13]. Therefore, elucidation of an epitope recognized by the antibody was expected to contribute to the understanding of the mode of interactions among fibroblasts, FN, and collagen fibrils which take place in 3-dimensional architecture.

The present study was undertaken to determine the A3A5 epitope in the FN molecule. We first attempted to identify A3A5-reactive fragments in pepsin digests of FN. As a result, a region of the III_4–III_6 modules was obtained as an overlapping sequence between two reactive fragments. The presence of the epitope within the III_4–III_6 modules was confirmed by producing an A3A5-positive fusion protein in *E. coli* which bears plasmids containing cDNAs coding for the module as an insert. This recombinant fusion protein methodology further confined the epitope in the region containing about 300 amino acid residues. The recombinant PCR mutagenesis successfully produced A3A5-reactive deletion mutant cDNAs and could shortened the epitope region to a sequence composed of 122 amino acid residues in the III_4–III_5 region.

The actual mechanism of inhibition of collagen gel contraction by mAb A3A5 is not known at present. It has been shown that cFN but not pFN participates in gel contraction [18]. Morla and Ruoslahti [19] previously demonstrated an important role of the III_1 module of FN in the polymerization of FN and/or its assembly with other matrix components which take place on the cell surface. The interaction of the III_1 module with amino-terminal regions of FN is necessary for FN self-assembly and is a conformation-dependent process [20]. The epitope of A3A5 (III_4–III_5 repeat) is located near the III_1 module and, therefore, antibody binding to the epitope may cause a steric change in the region containing III_1 and hinder III_1 module-dependent process of FN self-assembly. The hindrance might explain the inhibitory action of A3A5 on cell-mediated collagen gel contraction. It is also plausible that binding of the mAb to cell surface FN inhibits a direct binding of FN to collagen fibrils which is mediated through an unknown site of FN as well as a well-characterized gelatin-binding domain [7,14]. There also remains the possibility that the epitope itself can specifically bind to collagen fibrils.

We succeeded in demonstrating that the novel domain consisting of the III_4–III_5 repeat of FN is involved in collagen gel contraction. Since this domain is neither located at the integrin-binding domain nor at the classical gelatin-binding domain [7,14], the new mode of interaction is strongly suggested among FN, cells and collagen fibrils. We are attempting to identify a protein(s) which directly interacts with the domain of FN. This might be a fragment of collagen molecule or some specific cell surface protein.

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