Identification of Two Amino Acids within the EIIIA (ED-A) Segment of Fibronectin Constituting the Epitope for Two Function-blocking Monoclonal Antibodies

Yung-Feng Liao, Kenneth G. Wieder, Jeanne M. Classen, and Livingston Van De Water

From the Center for Engineering in Medicine and Surgical Services, Massachusetts General Hospital and Harvard Medical School, the Shriners Burns Hospital, Boston, Massachusetts 02114

Alternative splicing of the fibronectin gene transcript gives rise to a group of adhesive glycoproteins showing restricted spatial and temporal expression during embryonic development, tumor growth, and tissue repair. Alternative splicing occurs in three segments termed EIIIB, EIIIA, and V. The EIIIA (or ED-A) segment of fibronectin is expressed prominently but transiently in healing wounds coincident with fibroblast expression of an activation marker, smooth muscle cell α-actin. A monoclonal antibody (IST-9) to the EIIIA segment blocks transforming growth factor-β-mediated smooth muscle cell α-actin expression by fibroblasts in culture. A second monoclonal antibody (DH1) blocks chondrocyte condensation in chicken embryos. We find that IST-9 and DH1 react with human, rat, and chicken but not with mouse or frog EIIIA, suggesting that His44 may be important for antibody binding. A series of deletion mutants of rat EIIIA, constructed as glutathione S-transferase fusion proteins, do not react with either IST-9, DH1, or a third monoclonal antibody (3E2). Mutations of pairs of amino acids to alanine have little effect, except for either (Val34 Thr35) or (Tyr36 Ser37), which are located in a β strand upstream from His44. For these double mutants, the binding to all three monoclonal antibodies is markedly reduced. By contrast, single mutants at Thr35, Tyr36, or Ser37 retain full activity, suggesting that the epitope for these antibodies is determined in part by conformation. Alanine-scanning mutagenesis of rat EIIIA demonstrates the importance of Ile43 and His44 for binding. Mutation of frog EIIIA (normally Val43 Lys44) to rat (Ile43 His44) is sufficient to restore fully IST-9 binding and much of the activity of DH1 and 3E2. Our findings demonstrate that the function-blocking antibodies, IST-9 and DH1, bind to the Ile43 and His44 residues in a conformationally dependent fashion, implicating the loop region encompassing both residues as critical for mediating EIIIA function.

The fibronectins (FNs) comprise a group of extracellular matrix proteins that mediate cell adhesion, migration, proliferation, and differentiation (1). FNs play significant roles in embryonic development and are prominent components of the provisional matrix following tissue injury in adults (2, 3). The fundamental importance of the FNs is substantiated by the observation that homozygous mutations in either the FN gene or in the α5 integrin, a FN-specific receptor, are lethal (4, 5). The FNs are disulfide-linked, dimeric glycoproteins with structural domains that bind cells, collagen, proteoglycans, and fibrin. Each FN consists of homologous repeats, either type I, II, or III. Individual type III repeats within FN exhibit high sequence similarity between species (greater than 90% identity (6)). Despite variations in protein sequence identity between different type III repeats within FN (20–40% (6)), these repeats show a high degree of structural homology (7–11). X-ray crystallographic studies demonstrate that each type III repeat consists of two β sheets, made up of four strands (G, F, C, C’) and three strands (A, B, E) respectively, folded into a β sandwich (7). This structural arrangement is also conserved in other proteins, including growth hormone (12), tenascin (13), neuroglian (14), tissue factor (15), and chitinases (16).

Diversity in the FNs occurs by alternative splicing in two type III repeats termed EIIIA (or ED-A) and EIIIB (or ED-B) and one non-homologous repeat called V (or IIICS) (1). The EIIIA and EIIIB segments are either entirely included or excluded, whereas the V region may be included, excluded, or partially included in FN. An additional splicing variant lacking the V region, the 10th type I repeat (I10), and the 15th type III repeat (III15) has recently been reported (17). Despite the present understanding of the structures within type III repeats and the mechanisms controlling the alternative splicing of FN mRNA (18–26), the roles of the alternatively spliced domains on overall FN protein structure and cellular function remain unclear. Alterations in FN structure, which occur as a consequence of splicing, may influence the function of adjacent domains. For example, insertion of an alternatively spliced domain may change the conformation of the adjacent type III domains by re-adjusting interdomain rotations and tilts (7, 13). This is supported by the observation that a small rotation occurs between FN-III9 and FN-III10 and places the RGD loop of FN-III10 and the synergy site of FN-III9 on the same surface of the FN molecules (7). Alternatively, the inclusion of an extra type III domain could have longer range effects on the overall conformation of the FN molecule by rotating the N-terminal portion of the FN molecule relative to the C-terminus. Such a structural change could enhance the accessibility of a functional domain, such as the RGD loop, in the cell-binding domain or of sites involved in fibril assembly (8, 27, 28). Finally, an alternatively spliced segment may interact directly with cells.

The FNs display a wide range of physiological functions, which have been mapped to specific segments of FN. In some instances, the reacting sequences have been localized to short
stretches of amino acids. For example, synthetic peptides that include the Arg-Gly-Asp (RGD) sequence from the FN-III α block interactions between FN and integrins (29, 30). Although identified first in FN, the RGD sequence has been identified in numerous proteins and mediates cell adhesion (31). However, key peptide sequences often function best in the context of the whole type III repeat. For example, a short stretch of amino acid residues (Pro-His-Ser-Arg-Aasn, PHSRN) in the 9th type III domain (FN-III α3), termed the synergy site, of FN has been found to enhance cell adhesion to the RGD sequence in FN (32–35).

The function of the EIIIA and EIIIB sequences is largely unknown. These extra domains are present at specific stages of embryonic development and organogenesis (36–41), whereas most normal adult tissues express much lower amounts of EIIIA and EIIIB (42). However, during specific pathological conditions, such as wound healing (43, 44), lung, liver, and kidney fibrosis (45–47), vascular intimal proliferation (48, 49) and cardiac transplantation (50), the expression of EIIIA and EIIIB domains is significantly up-regulated. Several lines of evidence, including studies utilizing the mAb IST-9 to block function, have shown that the EIIIA segment may play roles in promoting cell adhesion, regulating cell proliferation, and in promoting the differentiation of lipocytes and fibroblasts into myofibroblasts (27, 47, 51–54). Another EIIIA-specific mAb, DH1, has recently been shown to block chondrogenesis in chicken embryos (55). The differentiation of myofibroblasts is observed during morphogenetic processes, wound healing, organ fibrosis, and the stromal reaction to carcinomas (56–58). Uncontrolled myofibroblast differentiation has been suggested to be the leading cause of several fibrotic diseases as well (59).

Under pathological conditions, TGF-β1 has been shown to be a potent inducer of the myofibroblast phenotype and can increase the expression of collagen, FN, and certain integrins by fibroblasts (60–62). Tissue fibrosis may result from the disregulation of the expression of collagen, FN, and certain integrins by fibroblasts (60–62). Tissue fibrosis may result from the disregulation of TGF-β expression (63). Recent data demonstrate a potential role for the EIIIA segment of FN in the regulation of TGF-β/s action on dermal fibroblasts (53).

The observation that an EIIIA-specific mAb (IST-9) and a soluble form of the EIIIA segment markedly reduce TGF-β-mediated myofibroblast differentiation suggests that structural features of EIIIA are important in fibrosis. Here, we present an epitope map of EIIIA for two function-blocking mAbs, IST-9 and DH1, and another EIIIA-specific mAb, 3E2. To do so, we carried out systematic mutation of bacterial EIIIA fusion proteins, including deletion mapping and alanine-scanning mutagenesis. Our results clearly identify two amino acid residues of the EIIIA domain that are located at a loop region between two β strands and constitute the IST-9 and DH1 epitopes. These data provide the foundation for the identification of a functional motif in the EIIIA domain.

**EXPERIMENTAL PROCEDURES**

**Materials**—Restriction enzymes were obtained from Promega (Madison, WI). Roche Molecular Biochemicals, and Amersham Pharmacia Biotech. The anti-EIIIA mAbs, IST-9 (64), DH1 (65), and 3E2, were purchased from Harlan Bioproducts (Indianapolis, IN), Locus Genex (Helsinki, Finland), and Sigma, respectively. BCA Protein Assay Kit, Gel Code Blue, SuperBlock, Super Signal, and horseradish peroxidase-conjugated goat anti-mouse IgG were from Pierce. GST gene fusion vectors and U.S.E. Mutagenesis kit were obtained from Amersham Pharmacia Biotech. Qiagen Plasmid Maxi Kit, QIAprep 8 Miniprep kit, QIAprep 8 M13 kit, and QIAquick Gel Extraction kit were from QIAGEN Inc. (Valencia, CA). Glutathione (reduced form) and glutathione-immobilized agarose were obtained from Sigma. AEBSP hydrochloride was from Calbiochem. Synthesized oligonucleotides were purchased from Life Technologies, Inc. All other reagents were at least reagent grade and obtained from standard suppliers.

**Polymerase Chain Reaction (PCR) and Subcloning of the EIIIA cDNAs**—Full-length wild type rat EIIIA cDNA was amplified using PCR in a 50-μl reaction mixture containing 1 ng of a rat cDNA (clone 74T, kindly provided by R. O. Hynes, Massachusetts Institute of Technology) (6), 50 μM KCl, 10 mM Tris-HCl, pH 8.3, 1.5 mM MgCl2, and 0.2 μg of μg/ml gelatin, 200 μ units each dNTP, 0.5 μ units of (EIIIA-sense and EIIIA-antisense, Table I). Amplified DNA purified from the gel, digested with EcoRI, and subcloned into the pGEX-2T vector. The recombinant plasmid (EIIIA-pGEX-2T) was isolated from liquid bacterial cultures using QIAfilter Plasmid Maxi kit or QIAPrep Spin Miniprep kit and subjected to DNA sequencing.

Various deletion constructs of rat EIIIA cDNA were generated by using PCR. The amplification was performed in a 25-μl reaction volume containing 1 ng of EIIIA-pGEX-2T plasmid DNA, 10 mM Tris-HCl, pH 9.0, 50 μM KCl, 0.1% Triton X-100, 1.5 μM MgCl2, 200 μ units each dNTP, 0.5 μM sense strand and antisense strand primer, and 2.5 units of Taq DNA polymerase (Promega, Madison, WI). The sequences of 5’ and 3’ primers are listed in Table I. Constructs were then subcloned and propagated as described above. Deletion of EIIIA cDNA was confirmed by DNA sequencing analysis.

**Site-directed Mutagenesis of the EIIIA Expression Plasmid**—Point mutations were selectively introduced into the wild type rat EIIIA expression construct, EIIIA-pGEX-2T, by a procedure called unique site elimination (66) using the U.S.E. Mutagenesis kit. Synthesized oligonucleotides (300 pmol each; Table I) were phosphorylated in a 30-μl reaction mixture containing One-Phor-All Buffer PLUS (10 mM Tris acetate, 10 mM magnesium acetate, 50 mM potassium acetate, pH 7.5), 1 mM ATP, and 10 units of T4 polynucleotide kinase. Phosphorylation reactions were incubated (37 °C, 30 min), terminated by heating to 65 °C for 10 min, and used directly in the mutagenesis reactions. Mutagenesis mixtures consisted of 0.025 pmol of plasmid DNA, 1.25 pmol of U.S.E. selection primer (Table I), 1.25 pmol of target mutagenic primer, and One-Phor-All Buffer PLUS in a total volume of 20 μl. Following incubation at 100 °C for 5 min, the reaction mixtures were cooled in ice for 5 min and then incubated at room temperature for 30 min. Subsequently, 7 μl of Nucleotide Mix (2.86 mM each dATP, dCTP, dGTP, and dTTP, 4.34 mM ATP, 1.43 × One-Phor-All Buffer PLUS) and 3 μl of Reaction Mix (0.83–1.17 kilounits/ml FPL T4 DNA ligase, 0.83–1.67 kilounits/ml FPL T4 DNA polymerase, and 0.2–0.28 mg/ml T4 DNA ligase) were added into the reaction mixtures. The reaction mixtures were further incubated at 37 °C for 1 h, followed by heating at 85 °C for 15 min. Before being transformed into bacterial host cells, the mutagenesis reaction mixture was digested with 10 units of PstI in a final volume of 50 μl, and transformation was carried out as described by the manufacturer. Transformed cells were incubated in 4 ml of L-broth with 100 μg/ml ampicillin at 37 °C overnight with shaking at 200 rpm. Plasmid DNAs were prepared from the overnight cultures using QIAPrep 8 Miniprep kit (Qiagen) and were subjected to a second round of restriction enzyme selection was transformed into Escherichia coli competent cells, followed by plating of the transformed cells onto LB plates containing 100 μg/ml ampicillin. The plates were incubated at 37 °C overnight, and individual transformant colonies were selected to prepare plasmids for sequencing to verify the presence of the desired point mutations.

DNA sequencing was performed on subcloned fragments in multi-copy plasmids, PCR amplifier fragments using Taq polymerase in a dideoxy dye-terminator reaction (67). The sequencing reactions were resolved on an Applied Biosystems ABI 377 Sequencer (DNA Sequencing Core Facility, Department of Molecular Biology, Massachusetts General Hospital). The sequencing results were assembled and analyzed using the GCG Software Package (version 9.0, the University of Wisconsin and Genetics Computer Group), which includes “Pileup,” “Bestfit,” and “Pretty.”

**Production and Purification of Bacterially Expressed EIIIA Proteins**—A 500-ml LB-broth culture containing 100 μg/ml ampicillin was incubated at 37 °C overnight with shaking at 200 rpm. Plasmid DNAs were prepared from the overnight cultures using Qiagen Plasmid Maxi kit (Qiagen) and transformed into E. coli strain and grown at 37 °C for 2 h. Protein expression was then induced by the addition of isopropyl-β-D-thiogalactoside to a final concentration of 1 mM. A protease inhibitor, AEBSF, was included in the induction culture medium with a final concentration of 1 mM. After 4 h induction, cells were harvested by centrifugation at 5,000 × g for 30 min. The cell pellets were washed with PBS and used for protein purification or
Purified recombinant EIIIA proteins (10 μg or as specified elsewhere) were mixed with an equal volume of 2× SDS sample buffer (125 mM Tris-HCl, pH 6.8, 4% SDS, 0.005% bromophenol blue, 20% glycerol, 2% dithiothreitol, and 5% β-mercaptoethanol) and boiled at 100 °C for 5 min. Samples were then loaded onto a pre-cast Tris glycine polyacrylamide gel (10%) (Novex, San Diego, CA) and resolved with 25 mA/gel in a Mini-Cell system (Novex) containing SDS running buffer (24 mM Tris, 192 mM glycine, and 0.1% SDS) (68). Gels were then stained using Gel Code Blue for 1 h at room temperature, followed by extensive washes in distilled water.

Enzyme-linked Immunosorbent Assays—The reactivity of mutant EIIIA-GST fusion proteins was tested by enzyme-linked immunosorbent assays (ELISA). Microtiter plates (96-well) were coated with 100 μl/well of 10 μg/ml purified EIIIA-GST proteins in coating buffer (100 mM NaHCO₃, pH 8.6) at 4 °C in a humidified chamber overnight. The plates were briefly rinsed in washing buffer (0.1% Tween 20 in PBS), blocked with 100 μl/well of 5% bovine serum albumin in coating buffer at 37 °C for 1 h, and rinsed again in washing buffer. Serial dilutions (1:10–1:10⁵) of monoclonal antibodies (mAbs) were prepared in washing buffer. Diluted mAbs (100 μl) were added to the wells, and the reactions were incubated at room temperature for 1 h. Wells were washed as above, incubated (1 h, room temperature) with horseradish peroxidase-conjugated goat anti-mouse IgG (1:5000 in SuperBlock), followed by three washes in PBS. The immunoblot was then incubated with Supersignal chemiluminescence substrate for 10 min and exposed to a phosphor cassette. Images of the blots and gels were processed with the Molecular Image System GS-525 and the Fluor-S MultiImager, to a phosphor cassette. Images of the blots and gels were processed with the Molecular Image System GS-525 and the Fluor-S MultiImager, to a phosphor cassette. Images of the blots and gels were processed with the Molecular Image System GS-525 and the Fluor-S MultiImager.
room temperature) with substrate solution (200 μl/well, 0.4 mg/ml o-phenylenediamine dihydrochloride, 0.4 mg/ml urea hydrogen peroxide, and 50 mM phosphate-citrate buffer). The absorbance of individual reactions was then measured (420 nm, ThermoMax microplate reader, Molecular Devices). Titer evaluations of antibody dilutions were done by logarithmic curve fitting. The x value corresponding to 50% of the highest absorbance along the sigmoidal curve is defined as titer for the reactivity of mAbs to specific EIIIA proteins.

RESULTS

Protein Sequence Comparison and Antibody Reactivities Suggest That the C-C_9-E Segment of EIIIA Encompassing the His_44 Residue Constitutes Epitopes—Protein sequences, derived from mRNAs, for human, mouse, rat, chicken, and frog EIIIA segments show extensive sequence similarity (Fig. 1A). All are 90 amino acids in length, and the consensus sequence for these five species is 70% conserved. The EIIIA protein sequences for mouse, rat, chicken, and frog display 96.7, 94.4, 85.6, and 80% identity, respectively, to the human EIIIA protein (Fig. 1B). All sequences conform to a domain structure in which six β-strands (denoted by A, B, C, C’ E, F, and G) are conserved in the type III repeat crystal structure (7).

The mAb IST-9, raised against human cellular fibronectin (cFN), specifically recognizes the EIIIA segment in rat and human cFN (64). This mAb exhibits function-blocking activities in these species (47, 53). We tested the reactivity of IST-9 and 3E2 against the EIIIA segment in chicken, frog, and mouse FN by either immunofluorescence, immunoblotting, or ELISA. IST-9 reacted with chicken cFN (data not shown), but not appreciably with either mouse or frog EIIIA (see below). Likewise DH1, which binds chicken FN (55), also does not react with mouse or frog EIIIA (see below). 3E2 reacts with chicken (data not shown) and mouse (41) but not frog EIIIA (see below). Comparisons of these reactivities with the protein sequences (Fig. 1) revealed that an amino acid residue (His_44) within the EIIIA segment is conserved in rat, human, and chicken but is not in either mouse or frog (arrow, Fig. 1). As a preliminary test of reactivity with mouse EIIIA, we prepared a mutation that replaces the conserved His_44 residue of rat EIIIA segment with the arginine found in mouse EIIIA. This resulted in a significant reduction of IST-9 reactivity (Fig. 2). A potential polymorphism at residue Glu_54 suggests that this residue might also play a role. Based on the sequence comparison and our immunological evidence, we hypothesized that the conformational domain C-C_9-E of rat EIIIA encompassing the His_44 residue is crucial for constituting the IST-9 epitope.

The “Native” Conformation of Full-length Rat EIIIA Protein Is Required for Its IST-9 Reactivity—To generate sufficient material for mapping the IST-9 binding epitope, the wild type sequence of rat EIIIA was cloned into a bacterial expression
vector, pGEX-2T, and then used to generate various deletion constructs of rat EIIIA by PCR (Fig. 3). All of these deletion mutants retain the hypothesized epitope sequence of domain C-C-E. Wild type and deletion mutant constructs were expressed as GST fusion proteins in E. coli and purified by glutathione-affinity chromatography.

Antibody reactivities of wild type rat EIIIA and the derived deletion mutants were tested by ELISA, and the dilution yielding 50% binding (titer) for each mAb was determined. Three EIIIA-specific mAbs, IST-9, 3E2, and DH1, were included in these analyses. When reacted with wild type rat EIIIA, the titers of IST-9, 3E2, and DH1 were 5 \times 10^4, 4 \times 10^4, and 1 \times 10^4, respectively (Fig. 4). However, unlike the strong reactivity exhibited by rat EIIIA toward these mAbs, none of the six deletion mutants displayed any detectable antibody reactivity (insets, Fig. 4). These results ruled out the possibility that the C-C-E domain alone of the EIIIA segment was sufficient to constitute the antibody recognition epitope. Nevertheless, the amino acid sequence of C-C-E domain could still be crucial for the reactivity with mAbs and may need to be maintained in a specific conformation. This antibody-reactive conformation may only react with these mAbs when the full-length EIIIA polypeptide sequence is intact.

Thr35, Tyr36, Ser37, Glu40, and Asp41 Residues of Residues of Rat EIIIA Are Important for Maintaining an Optimal Conformation for Antibody Binding—to test whether and to what extent the C-C-E domain of EIIIA protein is crucial for antibody activity, we generated a series of rat EIIIA double mutants in which two adjacent amino acids were simultaneously replaced by alanines and then tested for reactivity to mAbs by ELISA. In some preliminary screenings, antibody reactivities were determined by Western blotting. We found that most of the double mutants retain some or all of the antibody reactivity of the wild type rat EIIIA protein (Table II). Among these was a double mutant, rat EIIIA-D53A/E54A, representing a potential polymorphism in mouse EIIIA (Fig. 1 and Table II). No loss of antibody binding were observed (Fig. 5, Table II), indicating that His44 as it did to H44R. When the adjacent isoleucine residue was mutated to alanine (I43A), IST-9 reactivity was markedly reduced. These results indicated that Ile43 and His44 were required for IST-9 and DH1 binding. These data also indicated that IST-9, 3E2, and DH1 recognized distinct but overlapping epitopes.

Ile43 and His44 Substitution in Ile43 and His44 Substitution in Frog EIIIA Protein Restore Ile43 and His44 Substitution in Frog EIIIA Protein Restore Antibody Binding—to establish whether or not Ile43 and His44 are sufficient to constitute the IST-9 binding epitope, we sought to introduce these two residues into comparable positions of a homologous type III repeat that does not react with IST-9. We found that neither IST-9, 3E2, nor DH1 reacted with frog EIIIA (Fig. 7B). Although frog EIIIA does not react with these mAbs, it displays an 80% homology in protein sequence to both human and rat EIIIA (Figs. 1B and 7A). Moreover, it is well established that type III repeats are highly related structurally (7). We prepared a double (V43I/K44H) and a single (K44H) mutant and observed that IST-9 reacted with these mutants at levels comparable to, or higher than, wild type rat EIIIA (solid bar, Fig. 7B). Thus, either Ile43 and His44 together or frog Val43 with His44 are sufficient to reconstitute IST-9 reactivity. These results taken with those for rat I43A (Fig. 6) indicate that specific amino acids at position 43 in conjunction with His at position 44 are required for IST-9 binding. On the other hand, both the double (V43I/K44H) and single (K44H) mutants exhibited about 50% of the binding to 3E2 and DH1 relative to wild type rat EIIIA (gray bar and striped bar, Fig. 7B).
summary, Ile43 and His44 in conjunction with the conformation of the EIIIA segment, likely involving the C domain, are critical to the IST-9 epitope and contribute to the epitopes for 3E2 and DH1 (Fig. 8).

DISCUSSION

We report here that two key amino acids, Ile43 and His44, are a necessary part of the epitope for a function-blocking monoclonal antibody (IST-9) that reacts with the EIIIA segment of human, rat, and chicken FN. The Ile43 and His44 residues lie in a loop between two β strands (C and C'). The overall conformation of the EIIIA segment, and particularly that conferred by the C domain, exerts important effects on the IST-9 epitope (Fig. 8). The epitopes for DH1 and 3E2 are also conformationally dependent and appear to reside in the same loop. The key residues for DH1 and 3E2 binding overlap with, and yet are distinct from, those for IST-9. We infer that the loop between the C and C' β strands is critical to the mechanism by which the EIIIA segment regulates cell function.

Emerging evidence suggests that alternatively spliced FNs, prominent in embryogenesis, are important in the adult tissue response to injury. The expression of FNs that include the EIIIA or EIIIB segments are present in rather low amounts in many adult tissues (42). A stereotypical pattern of response to injury is evident in which FNs that lack the EIIIA and EIIIB segments (i.e. plasma FN) are deposited first. Following tissue injury, the EIIIA and EIIIB segments are included in the FN mRNAs synthesized by wound cells. This occurs in skin (43, 44), arteries (48, 49, 69), kidneys (70, 71), liver (47), and heart (50, 72, 73). Such up-regulation occurs both as a consequence of increases in total FN and increases in the ratios of inclusion of segments into FN (50, 74). Moreover, the temporal pattern of appearance of EIIIA and EIIIB differ, suggesting distinct roles for each segment (47, 50). Splicing in a nonhomologous repeat, the V region, also occurs following injury. The V95 segment is included in most FNs, but variations occur in the inclusion of the cell adhesive portion, termed CS-1, during regeneration of peripheral nerves (75) and following cardiac transplantation (50).

In addition to data on expression, a growing body of evidence supports a functional role for the EIIIA segment in modulating the phenotype of cells. A mAb, IST-9, blocks the conversion of lipocytes to myofibroblasts (47). Another mAb, DH1, has been shown to block chondrogenesis (55). Recent studies also demonstrate that IST-9 blocks the stimulatory activity of TGF-β on smooth muscle cell α-actin expression during myofibroblast differentiation (53) and that TGF-β controls the expression of extracellular matrix molecules and certain integrins (60, 61).

FIG. 3. Deletion mutants of rat EIIIA protein. Map of various N-terminal, C-terminal, or internal peptides (arrows) derived from wild type (WT) rat EIIIA (rEIIIAWT, 90 amino acids). Deletion constructs were generated by PCR and subcloned into the pGEX-2T vector as described under “Experimental Procedures.” Arrows indicate the length of individual deletion constructs relative to the wild type sequence that is shown on the top of the figure. A solid circle above the peptide sequence of rEIIIA-WT highlights the conserved His44. The amino acids included in truncated mutant rat EIIIA proteins are numbered.

FIG. 4. The reactivity of rat EIIIA-GST deletion mutants with three monoclonal antibodies. The deletion mutants were tested for reactivity in ELISAs with monoclonal antibodies as described under “Experimental Procedures.” The sigmoid plot in each panel represents the reactivity of wild type rat EIIIA (rEIIIA-WT) with IST-9 (A), 3E2 (B), or DH1 (C). Dilution folds are expressed in log scale. Bar graph insets in each panel illustrate the titer (in percentage) of each deletion protein relative to rEIIIA-WT. The points of OD420 at various dilution folds represent the average of quadruples; standard deviations greater than 0.1 OD are shown as bars.
Thus, disregulation in the TGF-β signaling pathway may result in tissue fibrosis (63, 77, 78). Because the EIIIA segment appears to be involved in the TGF-β signaling mechanism in myofibroblasts, identification of the IST-9 epitope on EIIIA provides an important first step to probing this mechanism.

In this report we observe that the Ile43 and His44 residues of EIIIA are necessary for IST-9 binding and partially constitute the reactive epitopes for DH1 and 3E2. We have shown that none of the three mAbs react with frog EIIIA unless the Lys44 is replaced by a His residue found in human, rat, and chicken FN (Fig. 7). Replacement of the His44 with Arg by site-directed mutagenesis in rat EIIIA provides an important first step to probing this mechanism.

In this report we observe that the Ile43 and His44 residues of EIIIA are necessary for IST-9 binding and partially constitute the reactive epitopes for DH1 and 3E2. We have shown that none of the three mAbs react with frog EIIIA unless the Lys44 is replaced by a His residue found in human, rat, and chicken FN (Fig. 7). Replacement of the His44 with Arg by site-directed mutagenesis in rat EIIIA (Fig. 6) or Lys which occurs in frog

---

**TABLE II**

Epitope mapping of three rat EIIIA-specific monoclonal antibodies using deleted and mutagenic rat EIIIA proteins

| EIIIA Constructs | Antibody Reactivity | Domain |
|------------------|---------------------|--------|
| EIIIA-WT         | IST-9: +, 3E2: +, DH1: + | C |
| EIIIA-33A        | IST-9: +, 3E2: +, DH1: nd | C |
| EIIIA-V34A-T35A  | IST-9: - , 3E2: -, DH1: nd | C |
| EIIIA-T35A       | IST-9: - , 3E2: nd, DH1: nd | C |
| EIIIA-Y36A-S37A  | IST-9: - , 3E2: nd, DH1: nd | C |
| EIIIA-Y36A       | IST-9: - , 3E2: nd, DH1: nd | C |
| EIIIA-S37A       | IST-9: - , 3E2: nd, DH1: nd | C |
| EIIIA-P39A       | IST-9: +, 3E2: +, DH1: + | C |
| EIIIA-E40A-D41A  | IST-9: +/-, 3E2: +/-, DH1: +/- | C |
| EIIIA-E40A       | IST-9: +, 3E2: +, DH1: + | C |
| EIIIA-D41A       | IST-9: +, 3E2: +, DH1: + | C |
| EIIIA-G42A       | IST-9: +, 3E2: +, DH1: + | C |
| EIIIA-H43A       | IST-9: +, 3E2: +, DH1: + | C |
| EIIIA-H44A       | IST-9: +, 3E2: +, DH1: + | C |
| EIIIA-H45A       | IST-9: +, 3E2: +, DH1: + | C |
| EIIIA-E45A       | IST-9: +, 3E2: +, DH1: + | C |
| EIIIA-P47A       | IST-9: +, 3E2: +, DH1: + | C |
| EIIIA-P48A       | IST-9: +, 3E2: +, DH1: + | C |
| EIIIA-P50A-D51A  | IST-9: +, 3E2: +, DH1: + | C |
| EIIIA-P53A-E54A  | IST-9: +, 3E2: +, DH1: + | C |
| EIIIA-P55A-T56A  | IST-9: +, 3E2: +, DH1: + | C |
| EIIIA-E58A-H60A  | IST-9: +, 3E2: +, DH1: + | C |

1 The antibody reactivities of mutant EIIIA proteins were determined by Western blotting and ELISA. Reactivity of each mutant is shown relative to the reactivity of EIIIA-WT protein (100%). +, >20% reactivity; +/-, 1–20% reactivity; -, <1% reactivity; nd, not determined.

2 The domains are defined by the crystallography of a human FN fragment (FN-IIIc,10) elucidated by Leahy et al. (7).

* Deletion constructs containing the indicated amino acids.
FN (Fig. 7) does not suffice for either IST-9 or DH1 binding. In contrast, mutation at His44 made to either Arg or Ala does not significantly affect 3E2 binding (Fig. 6). Although full activity is restored to IST-9 by His44, partial reactivity is regained with DH1 and 3E2. The adjacent residue, Ile43, is important to all three mAb epitopes as well. Substitution of an Ala for Ile43 blocks IST-9 binding completely and markedly reduces 3E2 and DH1 binding (Fig. 6). Reduction in binding was also observed when Asp41 and Gly42 were mutagenized to Ala. Gly42 appeared to be quite important for the epitope for DH1. Taken together, these results indicate that the loop between the C and C\textsubscript{9} strands encompasses the epitope for these mAbs.

Interestingly, when either the Pro39 or the Glu45 is replaced by alanine, the IST-9 reactivity is enhanced. Replacement with alanine at Pro39 may relieve the steric restriction imposed by the proline residue and hence make the conformation of the His44-containing C-C\textsubscript{9} loop more reactive. On the other hand, the charged Glu45 may provide electrochemical interaction with the adjacent His44 residue, and this charge-charge interaction could be disrupted by the replacement of an uncharged residue like alanine, potentially making the Ile43 and His44 residues more accessible to IST-9. However, the enhancement of IST-9 reactivity due to mutations made to Pro39 and Glu45 residues was not observed with either 3E2 or DH1, indicating that rat EIIIA presents distinctive epitopes for each mAb.

The reactivities of rat EIIIA deletion constructs to the three mAbs demonstrate that these epitopes are only active in an intact polypeptide (Fig. 4). The GST moiety in the purified EIIIA fusion proteins does not appear to alter the conformation of rat EIIIA because comparable reactivities are obtained when the GST and EIIIA moieties are separated by thrombin cleavage (data not shown). The influence of molecular conformation on rat EIIIA reactivity is further demonstrated by the antibody reactivities of rat EIIIA double mutants. Mutation of pairs of amino acids to Ala, either Val34Thr35 or Tyr36Ser37, located in a \(\beta\)-strand upstream from His44, markedly reduces antibody binding, while these as single mutants or other mutant pairs tested remain active (Fig. 5, Fig. 6, and Table II). It is likely that the double mutants at Val34Thr35 or Tyr36Ser37 have a greater impact on the conformation of the \(\beta\)-strand C, which in turn would result in a significant change in the conformation and antibody reactivity of the downstream C-C\textsubscript{9} loop.

The loop regions between \(\beta\)-strands of type III repeats often serve functional roles, such as cell adhesion and antibody recognition. Indeed, the type III repeat structure is highly conserved in tenascin (13), neuroglian (14), human growth hormone receptor (12), and the isolated FN-III\textsubscript{10} of FN (10, 79), despite relatively low levels of sequence identity among FN-III repeats. The most notable sequence differences are observed when comparing the loop regions of different type III repeats, suggesting that these loops may mediate functions as well as antibody epitopes (10, 80).

In conclusion, we have demonstrated that the critical residues mediating antibody recognition by two function-blocking mAbs do not suffice for either IST-9 or DH1 binding. In contrast, mutation at His44 made to either Arg or Ala does not significantly affect 3E2 binding. Although full activity is restored to IST-9 by His44, partial reactivity is regained with DH1 and 3E2. The adjacent residue, Ile43, is important to all three mAb epitopes as well. Substitution of an Ala for Ile43 blocks IST-9 binding completely and markedly reduces 3E2 and DH1 binding. Reduction in binding was also observed when Asp41 and Gly42 were mutagenized to Ala. Gly42 appeared to be quite important for the epitope for DH1. Taken together, these results indicate that the loop between the C and C\textsubscript{9} strands encompasses the epitope for these mAbs.
mAbs are located at the C-C' loop of the EIIIA domain. Because IST-9 is a function-blocking mAb, these data support a model in which the functional motif resides within the rat EIIIA domain in the proximity of the Ile43His44. It is not known yet if either the EIIIA segment interacts directly with a cell-surface receptor (53) or influences indirectly the activity of another segment of FN (27). In either case, the EIIIA+FN-triggered signals may converge onto the TGF-β-mediated signaling pathway and thus modulate the stimulatory activity of TGF-β. The detailed characterization of the EIIIA structure will provide a basis for developing therapeutic strategies for modulating excessive scarring and tissue fibrosis.

Acknowledgments—We are grateful to Dr. Douglas DeSimone (University of Virginia) for providing us with the Xenopus EIIIA cDNA construct, to Dr. Richard Hynes (Massachusetts Institute of Technology) for a rat FN cDNA plasmid, and to Dr. Luisa Iruela-Arispe (University of California, Los Angeles) for a gift of chicken embryo fibroblasts. Thanks also to Drs. Jeffrey Morgan and Donald Senger for critically reading the manuscript. We are thankful to Robert Crowther, Elizabeth Hudson, and the Morphology Core Facility of the Shriners Burns Hospital for assistance in immunocytochemistry.

REFERENCES

1. Hynes, R. O. (1990) Fibroconnectin. Springer-Verlag Inc., New York

2. Yamada, K. M., and Clark, R. A. F. (1990) In The Molecular and Cellular Biology of Wound Repair (Clark, R. A. F., ed) pp. 51–93, Plenum Publishing Corp., New York

3. Clark, R. A. F. (1990) In The Molecular and Cellular Biology of Wound Repair (Clark, R. A. F., ed) pp. 3–50, Plenum Publishing Corp., New York

4. George, L. E., Georges-Labouesse, E. N., Patel-King, R. S., Rayburn, H., and Hynes, R. O. (1990) Development 119, 1093–1105

5. Yang, J. T., Rayburn, H., and Hynes, R. O. (1993) Development 119, 1093–1105

6. Schwartzberg, J. E., Patel, R. S., Fonda, D., and Hynes, R. O. (1987) EMBO J. 6, 2573–2580

7. Leahy, D. J., Asaki, I., and Erickson, H. P. (1996) Cell 84, 155–164

8. Copie, V., Tomita, Y., Akiyama, S. K., Aota, S., Yamada, K. M., Venable, R. M., Pastor, R. W., Krueger, S., and Torchia, D. A. (1998) J. Biol. Chem. 273, 127–148

9. Baron, M., Main, A. L., Driscoll, P. C., Mardon, H. J., Boyd, J., and Campbell, I. D. (1992) Biochemistry 31, 2068–2073

10. Main, A. L., Harvey, T. S., Baron, M., Boyd, J., and Campbell, I. D. (1992) J. Cell Biol. 119, 1059–1091

11. Muller, Y. A., Ultsch, M. H., and de Vos, A. M. (1996) J. Mol. Biol. 258, 2573–2580

12. de Vos, A. M., Ultsch, M., and Kossiakoff, A. A. (1992) J. Mol. Biol. 235, 306–312

13. Leahy, D. J., Hendrickson, W. A., Asaki, I., and Erickson, H. P. (1992) Science 258, 887–901

14. Huber, A. H., Wang, Y. M., Bieber, A. J., and Bjorkman, P. I. (1994) Neuron 12, 717–731

15. Mercola, N., Utretch, M. H., and de Vos, A. M. (1996) J. Mol. Biol. 265, 144–159

16. Perrakis, A., Ouzounis, C., and Wilson, K. S. (1997) EMBO J. 16, 291–294

17. MacLeod, J. N., Burton-Wurster, N., Gu, D. N., and Lust, G. (1996) J. Biol. Chem. 271, 18954–18960

18. Huh, G. S., and Hynes, R. O. (1993) Cell 74, 671–678

19. Blatztzen, C., Grant, R. P., Mardon, H. J., and Campbell, I. D. (1997) J. Mol. Biol. 265, 565–579

20. de Vos, A. M., Utretch, M., and Koossiakoff, A. A. (1992) Science 255, 306–312

21. Leahy, D. J., Hendrickson, W. A., Asaki, I., and Erickson, H. P. (1992) Science 258, 887–901

22. Hake, I., Wang, Y. M., Bauer, K. M., and Bumgarner, J. R. (1994) Neuron 12, 717–731

23. Mercola, N., Utretch, M. H., and de Vos, A. M. (1996) J. Mol. Biol. 265, 144–159

24. Perrakis, A., Ouzounis, C., and Wilson, K. S. (1997) EMBO J. 16, 291–294

25. MacLeod, J. N., Burton-Wurster, N., Gu, D. N., and Lust, G. (1996) J. Biol. Chem. 271, 18954–18960

26. Huh, G. S., and Hynes, R. O. (1993) Nature 365, 579–582

27. Mardon, H. J., and Sebastian, G. (1992) J. Cell Sci. 105, 423–433

28. Caputi, M., Casari, G., Guenzi, S., Tagliahue, R., Sidoli, A., Melo, C. A., and Baralle, F. E. (1994) Nucleic Acids Res. 22, 1018–1022

29. Leviauge, A., La Branche, H., Korahibhi, A. R., and Chabot, B. (1993) Genes Dev. 7, 2405–2417

30. Mardon, H. J., Sebastian, G., and Baralle, F. E. (1987) Nucleic Acids Res. 15, 7725–7735

31. Lin, L. P., and Sharpe, P. A. (1998) Mol. Cell. Biol. 18, 3900–3906

32. Norton, P. A., Uporova, T., and Bennett, V. D. (1995) Biochim. Biophys. Acta 1297, 145–150

33. Manabe, R., Nohe, T., and Yamada, K. M. (1991) J. Biol. Chem. 266, 15938–15943

34. Aota, S., Nomizu, M., and Yamada, K. M. (1994) J. Biol. Chem. 269, 24756–24761

35. Bowditch, R. D., Halloran, C. E., Aota, S., Obara, M., Plow, E. F., Yamada...