Assembly of Fibronectin Molecules with Mutations or Deletions of the Carboxyl-Terminal Type I Modules†

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ABSTRACT: Fibronectin is a large modular protein that is assembled into fibrils in a stepwise process that involves the binding of soluble fibronectin to the cell surface and formation of fibronectin multimers that are stabilized by disulfides. Fibronectin contains two types of disulfide-containing repeat modules, types I and II. The type I modules form units that mediate binding to assembly sites (I-1 through I-5), mediate binding to gelatin (I-6 through I-9 plus the type II modules), or have no known function other than fibrin binding (I-10 through I-12). All type I modules contain four cysteines that are disulfide-linked in a 1–3, 2–4 arrangement, except for I-12 that contains six cysteines disulfide-bonded in an unknown arrangement. I-12 contains the consensus sequence Cys-Xaa-Yaa-Cys found in a number of proteins involved in disulfide exchange reactions [Holmgren, A. (1985) *Annu. Rev. Biochem. 54*, 237; Boniface, J. J., & Reichert, L. E., Jr. (1990) *Science 247*, 61]. We explored the role of I-12 and adjacent type I modules of fibronectin in matrix assembly. We generated mutant fibronectins in which the second and sixth or fifth and sixth cysteine residues in I-12 were changed to serines (*CS* mutants) or that contained deletions of the 12th (Δ12) or 10th through 12th (Δ10–12) type I modules. Expression of I-12 as a fusion protein with the gelatin binding part of fibronectin indicated that this module folds independently and that the most likely disulfide pairing is 1–4, 2–6, 3–5. Full-length fibronectins lacking I-12 or I-10 through I-12 were produced and secreted by COS cells. These mutant fibronectins were assembled into fibrils and formed high molecular weight multimers that resist dissociation with sodium dodecyl sulfate. Thus, these results indicate that I-12 has no essential role in assembly or stabilization of fibronectin multimers.

Fibronectin is a modular protein comprised of three types of repeating structural units, types I, II, and III (Petersen et al., 1983). The 12 type I modules of fibronectin are clustered at the amino- and carboxyl-terminal regions of the molecule (Petersen et al., 1989). Each type I module contains 4 cysteine residues, except for the 12th (I-12), which contains 6 (Petersen et al., 1983). Where known, the cysteine pairing is 1–3, 2–4 (Petersen et al., 1989). The structure of the seventh type I module (I-7) has been determined by NMR (Baron et al., 1990). It consists of two antiparallel β-sheets, which are stacked on top of one another and which enclose a hydrophobic core containing conserved tyrosine and tryptophan residues and the disulfide bonds. One of the disulfide bonds (1–3) links the two β-sheets, while the other (2–4) connects two strands on the same β-sheet.

Fibronectin is a dimeric molecule linked by disulfide bonds near the carboxyl terminus. Fibronectin exists in a soluble form in plasma, and in an insoluble form in the extracellular matrix. Assembly of fibronectin into high molecular weight disulfide-stabilized multimers follows the binding of soluble fibronectin to the surface of cells. Several regions of fibronectin are involved in matrix assembly. The first five amino-terminal type I modules (I-1 through I-5) mediate the binding of soluble fibronectin to the cell surface (McKeown-Longo & Mosher, 1985; Quade & McDonald, 1988; Sottile et al., 1991). The ninth type I and the first type III modules are thought to be involved in fibronectin–fibronectin interactions important in fiber elongation (Chernousov et al., 1991). The cell attachment region, located in the tenth type III and adjacent modules, has also been implicated in matrix assembly (McDonald et al., 1987; Akiyama et al., 1989: Fogerty et al., 1990), though its exact role remains unclear (Schwarzbauer, 1991a). Finally, fibronectin must be dimeric to be incorporated into the extracellular matrix (Schwarzbauer et al., 1991a).

The multimerization of fibronectin is thought to involve disulfide exchange between type I or type II modules (McKeown-Longo & Mosher, 1984). A number of proteins involved in disulfide exchange reactions contain the consensus sequence Cys-Xaa-Yaa-Cys, including protein disulfide isomerase (PDI),[1] thioredoxin, lutropin, follitropin, and von Willebrand factor (Holmgren, 1985; Edman et al., 1985; Boniface & Reichert, 1990; Mayadas & Wagner, 1992). Mutation of the vicinal cysteines or alteration in the spacing of the cysteines abolishes the isomerase activity of PDI (Vuori et al., 1992) and the ability of von Willebrand factor to multimerize (Mayadas & Wagner, 1992). The extra pair of cysteine residues in I-12 are found in a similar arrangement: Cys-Asp-Asn-Cys (Petersen et al., 1989). These extra cysteines are encoded by a separate exon; the other type I modules are all encoded by a single exon (Patel et al., 1987). This suggests that the disulfide pairing in I-12 may be 1–3, 2–4, 5–6 (Figure 1A). Homology considerations with other type I modules, however, suggest a 1–4, 2–6, 3–5 pairing, as described (Fogerty & Mosher, 1990).

The role of the three type I modules (I-10 through I-12) adjacent to the intersubunit disulfides is controversial. Frag-

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[1] Abbreviations: DMEM, Dulbecco's modified Eagle's medium; PCR, polymerase chain reaction; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; PDI, protein disulfide isomerase; TBS, Tris-buffered saline, pH 7.4.

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ments or expressed portions of fibronectin containing these modules have been reported to bind to cell layers (McKeown-Longo & Mosher, 1985; Ichihara-Tanaka et al., 1992). However, fragments containing these modules do not inhibit binding of intact fibronectin (Quade & McDonald, 1988). We undertook this study to ask whether I-12 and its idiosyncratic disulfides participate in matrix assembly. In addition, we tested the ability of fibronectin lacking I-12 or I-10 through I-12 to become incorporated into the extracellular matrix.

EXPERIMENTAL PROCEDURES

Construction of Gelatin Expression Vector, GE-1. A 1.8-kb 5′ cDNA clone from rat fibronectin, FN571 (Sottile et al., 1990), was subcloned into M13mp18 and used as a template for the first round of mutagenesis experiments. Oligonucleotide-directed mutagenesis was performed according to Kunkel (1987). Oligonucleotides were synthesized using an Applied Biosystems automated DNA synthesizer. An oligonucleotide designated GAP1-3, TGGCTGTCAGTCAGAGCAAGGAGAAATGTTTTGATCACG, was used to delete the first through third type I modules of fibronectin (bases 151–552; Sottile et al., 1991). Nucleotides are numbered from the A in the initiation codon (EMBL Accession No. X15906). The fourth through fifth type I modules (bases 553–822) were deleted from GAP1-3/M13 template DNA using the primer GGTGGCTGTCAGTCAGAGCAAGGTTCTACAGAGTGCTTCAGC. The resulting Gap1-5 phage was used to prepare single-stranded DNA for the last round of mutagenesis. A 34mer, CGTGCAGCCTCCATCCCCGTGGGCTGTCAGTCAG, was designed to introduce a *Bst*XI site after position 128. Underlined bases represent substitutions of the wild-type sequence. In addition to creation of the *Bst*XI site, the codon for Val-12 was changed to a tryptophan codon. The amino acids are numbered from the amino-terminal pyroglutamic acid in the mature protein (Petersen et al., 1989). Single-stranded DNA was sequenced (Sanger et al., 1977) through the *Acc*I site at base 944 to confirm the mutations, and to verify that no other base changes were introduced. A 295 bp *Hin*dIII–*Acc*I fragment was subcloned into Fn571/pGEM7Zf that was partially digested with *Hin*dIII/*Acc*I to remove 5′ fibronectin sequences through the *Acc*I site at base 944. The resulting construct, GE-1/pGEM7Zf, encodes a 32 amino acid preprosequence, the first 18 amino acids of mature fibronectin, and contains a 672 bp deletion (bases 151–822) encoding the first through fifth type I homology units (amino acids Pro-19 through His-242). The protein resumes with Val-243 and ends with Leu-571, and thus retains the intact coding region for the gelatin binding region of fibronectin. The sequence of GE-1 fusion proteins after loss of signal and prosequences is Glu-Ala-Gln-Gln-Ile-Val-Gln-Pro-Pro-Ser-Pro-Trp-XXX-Pro-Ser-Pro-Trp-Ala-Val-Ser-Gln-Ser-Lys-Val-Leu-Gln-Ser-Ala-Ser-Ala-Gly-Ser-Gly-Ser-Phe-Thr-Asp-Val-Arg. The X's represent the sequence of the foreign protein. Underlined sequences represent amino acids added by the *Bst*XI linkers. **Lys-Val** is the junction between natural sequences after the deletion of I-1 to I-5. Following digestion with *Bam*HI, GE-1 was subcloned into pGEM4 (Promega, Madison, WI), which contains no *Bst*XI restriction enzyme sites.

Construction of I-12/GE-1. Polymerase chain reaction (PCR) primers were synthesized to amplify fibronectin cDNA encoding the 12th type I homology unit (I-12): primer A (antisense) is 5′-GGCCACGGGGATGGGCCAGTGGTACCATCGGG-3′; primer B (sense) is 5′-GGCCATCCCCGTGGGCAACGTGTTATGACGAC-3′. Underlined sequences represent bases introduced at the 5′ ends of the fibronectin DNA to create a *Bst*XI site for in-frame cloning into GE-1/pGEM4. PCR was performed according to established procedures (Sambrook et al., 1989) using Taq polymerase. A 3′ fibronectin cDNA clone which extends from the *Hin*dIII site at 3815 to the *Sac*II site at 7605 (a gift from J. Schwarzbauer, Princeton University, Princeton, NJ) was used as a template for amplification of nonmutant DNA. Full-length fibronectin DNA with mutations in I-12 (see below) was used to amplify mutant I-12 modules. Amplified DNA was gel-purified, digested with *Bst*XI, and then subcloned into the *Bst*XI cloning site of GE-1/pGEM4. PCR-amplified DNA was sequenced to verify that no base changes had been introduced. The resulting I-12/GE-1 construct was subcloned into the COS cell expression vector pSVL (Pharmacia, Piscataway, NJ) and the baculovirus expression vector pACYM1 (a gift from D. Bishop, Oxford, England; Matsuura et al., 1987).

Construction of Mutant Full-Length Fibronectins. Full-length rat fibronectin cDNA was cloned into pGEM7Zf (Fn7535/pGEM7Zf). This fibronectin cDNA includes sequences encoding 1 of the alternatively spliced type III modules, ED-A, and the entire 120 aa variable (V) region. Digestion with *Sac*I generated a 1641 bp fragment containing fibronectin sequences 5948 through 7535 and part of the multiple cloning site from pGEM7Zf. This 1641 bp fragment was subcloned into M13mp18 and used as a template for mutagenesis experiments. Oligonucleotides contained altered bases to change cysteine to serine (CS mutants) or spanned the DNA to be deleted. Point mutations were generated using the following oligonucleotides: CS(6), CGCTGTGACAACAGCCGCAGACC (7267, Cys-2393); CS(5,6), GGGCTGGCGCAGTGACAACAGCCGCAGACC (7258, 7267; Cys-2390 and Cys-2393); CS(2), CGGAGCCATTAGCTCCTGCACG (7219, Cys-2377). The CS(2,6) mutant was generated with the CS(2) oligonucleotide using CS(6)/M13 DNA as a template. Deletion mutations were generated using the following oligonucleotides: Δ12, CAAATGCGATCCCCATGAA-AGACCTGGGGCTGCTGAACC (7147–7272, Ala-2352 to Arg-2394); Δ10–12, GCCTGAACCAGCCTACGGAT-AGACCTGGGGCTGCTGAACC (6880–7272, Asp-2264 to Arg-2394). For the point mutations, the change from the wild-type sequence is underlined. The number of bases mutated and the amino acids changed are shown in parentheses. For the deletion mutations, the site of the deletion is shown by a dash, and the bases and amino acids deleted are shown in parentheses. Phage containing point mutations were identified by hybridization to ^{32}P-labeled oligonucleotides under permissive and nonpermissive temperatures (Zoller & Smith, 1989). Deletion mutants were identified by analysis of M13 replicative-form DNA on agarose gels. The entire *Sac*I fragment containing mutations of the carboxyl-terminal type I modules was sequenced to confirm the mutations and to ensure that no other base changes had been introduced.

Full-length mutant fibronectins were generated by subcloning the mutant fibronectin *Sac*I fragments from M13 into Fn7535/pGEM7Zf that had been digested with *Sac*I to remove fibronectin bases 5948–7535. Clones with the DNA inserted in the correct orientation were subcloned into pSVL.

COS Cell Expression and Purification. COS cell expression experiments were performed as described (Sottile et al., 1991a). Two days after transfection, cells were labeled with 50 μCi/mL Tran^{35}S-label (ICN, Irvine, CA) in serum-free Dulbecco's modified Eagle's medium (DMEM; Gibco, Gaithersburg, MD) lacking methionine and cysteine. For production of the proteins used in immunofluorescence assays, cells were labelled

with 10 μCi/mL Tran^{35}S-label. After 20–24 h, conditioned medium was collected and applied to a gelatin–agarose column. In some experiments, the conditioned medium was first applied to a LabMab–agarose column to remove monkey fibronectin. LabMab (Chernousov et al., 1991) is a monoclonal antibody that recognizes monkey and human but not rat fibronectin (data not shown). Proteins were eluted from the gelatin column with 3 M guanidine/Tris-buffered saline, pH 7.4 (TBS), and then dialyzed against TBS and DMEM. Purity and composition of the proteins were assessed by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE; Laemmli, 1970) with and without reduction followed by fluorography. The percentage of dimeric fibronectin was determined by scintillation counting of gel slices (Harlow & Lane, 1988).

Competitive enzyme-linked immunosorbent assays showed that there was 0.6–1 μg/mL monkey fibronectin, and approximately 10–12 μg/mL total fibronectin, after LabMab– and gelatin–agarose chromatography. Formation of extensive fibronectin fibrils, visualized by immunofluorescence (see Figure 4), required the addition of >2.5 μg/mL exogenous fibronectin as assessed by immunofluorescence of fibroblasts incubated with various amounts of rat fibronectin purified from serum.

Baculovirus Expression. Recombinant viruses were prepared as described (Sottile et al., 1990) using established methods (Summers & Smith, 1987; Miller et al., 1986). SF-21 cells (Vaughn et al., 1977) were infected with recombinant viruses at a multiplicity of infection of 5–10 as described (Sottile et al., 1990). Recombinant proteins were purified from cell culture medium (lacking serum) on columns of gelatin–agarose.

Binding Assay. Binding assays were previously described (McKeown-Longo & Mosher, 1985). Briefly, human fibroblasts were seeded at 1.4 × 10^5 cells/well in 24-well cluster dishes in DMEM with 10% fetal calf serum. One hour after being seeded, cells were washed and then incubated for 1 h with serum-free DMEM containing 20 μg/mL cycloheximide. After cells were washed, they were incubated with ^{35}S-labeled fibronectins in DMEM containing 20 μg/mL cycloheximide for 24 h. Bound proteins were solubilized with sample buffer (50 mM Tris-HCl, pH 6.8, 1% sodium dodecyl sulfate, 10% glycerol, and 0.01% bromphenol blue) and analyzed by SDS–PAGE. Approximately 2–5% of the added counts were cross-linked into high molecular weight multimers in the Δ10–12 mutant, 5–8% in the Δ12 mutant, and 12–14% in the nonmutant.

Immunofluorescence. Human fibroblasts were seeded into 12-well cluster dishes onto 18-mm glass coverslips at 2.1 × 10^5 cells/well. In some experiments, cells were seeded in medium containing fibronectin-depleted serum. One hour after being seeded, cells were washed and then incubated for 1 h in serum-free DMEM containing 20 μg/mL cycloheximide. After 60 min, cells were washed and then incubated with equal cpm of recombinant fibronectins in DMEM with 20 μg/mL cycloheximide. Cells were incubated for 24 h and then processed for immunofluorescence. Cells were incubated with VT-1, a monoclonal antibody that recognizes rat but not human, bovine, or monkey fibronectins [prepared by K. Fukuda, University of Vermont; see also Schwarzbauer (1991a)]; 9D2, a monoclonal antibody that recognizes monkey and human fibronectins, but not rat fibronectin (Chernousov et al., 1991); and/or polyclonal antiserum to fibronectin that recognizes fibronectins from all three species. Following a 60-min incubation, cells were washed and then incubated with a combination of fluorescein-conjugated anti-mouse and rhodamine-conjugated anti-rabbit IgGs for 30 min. Cells were washed, mounted, and then examined using a Nikon microscope equipped with epifluorescence and phase contrast. For the data shown in Figure 7, three independent observers scored the slides by assessing the amount of fibrillar staining and the colocalization or noncolocalization of polyclonal and monoclonal antibody staining.

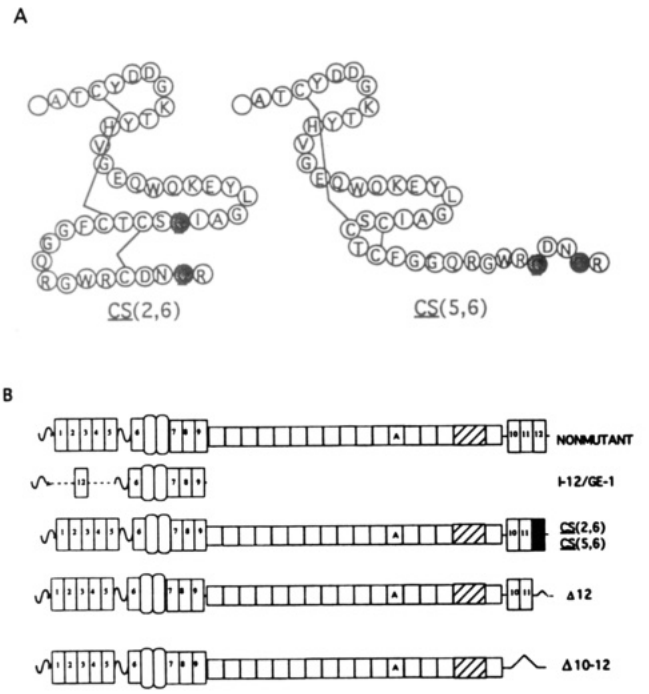

FIGURE 1: (A) Models of the 12th type I (I-12) module of fibronectin. The model on the left is drawn to indicate a β-sheet structure as shown for I-7 by Baron et al. (1990). The model on the right is drawn so that the fifth and sixth cysteines can form a disulfide. Cysteines mutated to serine in CS(2,6) and CS(5,6) are shaded. The solid lines indicate disulfides that would persist in the mutated modules, if the proposed structures were correct. (B) Mutant fibronectin constructs. At the top is a model showing one subunit of nonmutant fibronectin. The amino terminus is to the left. Type I modules (numbered rectangles), type II modules (unnumbered ovals), and type III modules (squares) are shown. "A" indicates an alternatively spliced type III module, and the shaded rectangle indicates the 120 amino acid nonhomologous variable spliced region present in our protein. The cysteines involved in interchain disulfide-bonding follow I-12. Mutant proteins contain point (CS) or deletion (Δ12 and Δ10–12) mutations. The gelatin binding region of fibronectin, comprised of I-6 through I-9 including the type II modules, was present in all mutants. I-12/GE-1 was constructed by removal of I-1 through I-5 from a truncated molecule (Sottile et al., 1991a) and insertion of DNA encoding I-12.

RESULTS

To ask whether the extra pair of cysteines in I-12 are involved in multimerization of fibronectin, mutant rat fibronectin molecules were generated in which cysteines were mutated to serines. Since the disulfide pairing in I-12 is not known, two sets of mutant molecules were produced: one with the second and sixth cysteines changed to serines, CS(2,6), and one with the fifth and sixth cysteines changed to serines, CS5,6) (Figure 1A).

Before attempting expression of full-length fibronectin with mutations in I-12, we did experiments to test whether the mutations would interfere with folding and secretion of the module in a construct that contains other type I modules and which we knew was processed efficiently by cells. cDNA encoding the amino-terminal 571 amino acids of rat fibronectin (Sottile et al., 1991) was altered so that the sequence encoding the first five type I modules could be replaced with PCR-

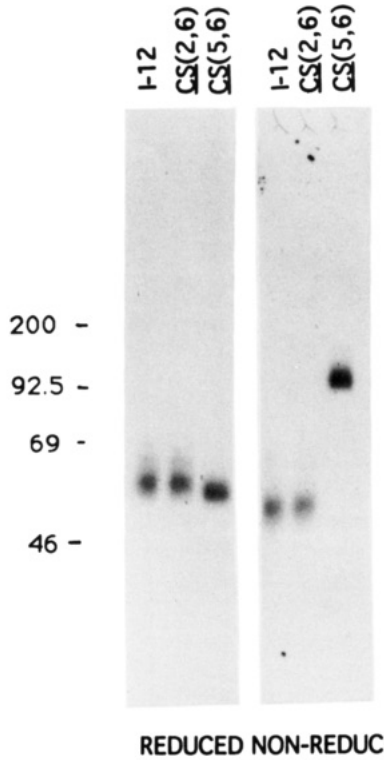

FIGURE 2: Mutant and nonmutant I-12/GE-1 fusion proteins. I-12 was made as a fusion protein with the gelatin binding region of fibronectin in the expression vector GE-1/pSVL. ^{35}S-Labeled nonmutant and mutant CS(2,6) and CS(5,6) proteins from COS cells were analyzed by SDS–PAGE in the presence (reduced) or absence (nonreduced) of 5% β-mercaptoethanol. Reduced molecular weight markers were ^{14}C-labeled: myosin (200K); phosphorylase *b* (92.5K); bovine serum albumin (69K); ovalbumin (46K). The slightly faster migration of the CS(5,6) mutant in comparison to the CS(2,6) and nonmutant fusion proteins under reducing conditions is an artifact of this gel; on other gels, these proteins comigrate.

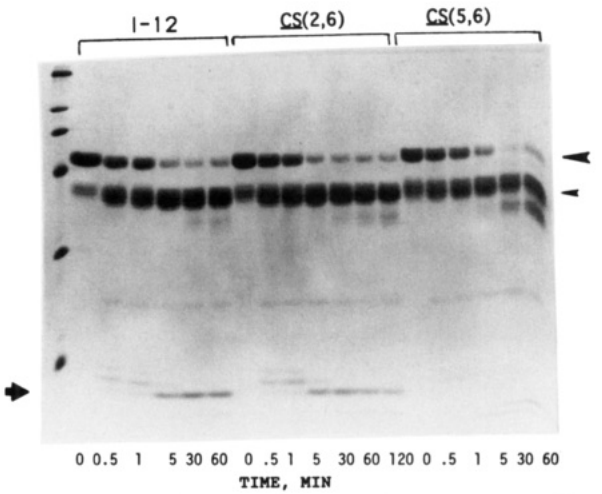

FIGURE 3: Trypsin digestion of mutant and nonmutant I-12/GE-1 proteins. I-12/GE-1 fusion proteins were produced in SF-21 cells using recombinant baculoviruses. Purified proteins (10 μg in 48 μL) were subjected to trypsin digestion (2 μg/mL final concentration) for various lengths of time at room temperature. Reactions were stopped by the addition of soybean trypsin inhibitor to a final concentration of 10 μg/mL. The large arrowhead shows the nondigested fusion proteins. All starting material (0) had some 40K breakdown product (small arrowhead) generated during purification: this represents the gelatin binding part of the fusion protein. Undegraded I-12 modules (arrow) were released from the nonmutant and CS(2,6) mutant fusion proteins but not from the CS(5,6) mutant fusion protein. The band migrating between the 16.5- and 24.5-kDa markers is soybean trypsin inhibitor. Molecular weight standards are fibronectin (250K), phosphorylase *b* (92K), bovine serum albumin (68K), ovalbumin (46K), chymotrypsinogen (24.5K), and hemoglobin (16.5K).

amplified DNA encoding other modules. Nonmutant I-12, I-12 CS(2,6), and CS(5,6) were produced as fusion proteins with the gelatin binding portion of fibronectin using this expression system, GE-1 (Figure 1B). COS cells were transfected with mutant or nonmutant rat cDNA and then labeled with [^{35}S]methionine. Secreted proteins were fractionated on gelatin–agarose and then analyzed by SDS–PAGE. Figure 2 shows that all three fusion proteins were secreted by COS cells. The CS(5,6) mutant migrated as a dimer under nonreducing conditions. The fusion proteins were also secreted efficiently by SF-21 insect cells (Figure 3); I-12 modules were released by limited trypsin digestion from the nonmutant and the CS(2,6) mutant proteins, but not from the CS(5,6) mutant (Figure 3). The formation of disulfide-bonded dimers by CS(5,6) and its susceptibility to trypsinization suggest that the cysteines in this mutant cannot form disulfides to stabilize the module fully; i.e., the proposed 1–3, 2–4, 5–6 pairing shown in Figure 1A is incorrect.

We then asked whether full-length fibronectin molecules containing CS(2,6) and CS(5,6) mutations could be assembled into a fibronectin matrix and incorporated into disulfide-stabilized multimers. Recombinant fibronectins were isolated from COS cell conditioned medium by gelatin–agarose chromatography. Multiple attempts to produce full-length fibronectin in insect cells were unsuccessful. The mutant proteins from COS cells were 35% dimeric when analyzed by SDS–PAGE without reduction (data not shown). Mutant and nonmutant recombinant fibronectins were added to monolayers of cycloheximide-treated human fibroblasts for 24 h, and fibronectin fibrils were visualized by indirect immunofluorescence using the rat-specific antibody VT-1. Both the CS(2,6) and CS(5,6) mutants were incorporated into fibronectin fibrils (Figure 4). Little staining was detected when the COS cell-derived fibronectin was not incubated with the fibroblasts (not shown; also see Figure 7). The CS(2,6) and CS(5,6) proteins formed high molecular weight multimers that resisted dissociation with SDS unless reducing agent was added (Figure 5).

The mutant CS fibronectin molecules contain four cysteine residues in I-12 instead of six. To eliminate the possibility that these four remaining cysteine residues were involved in disulfide exchange reactions, we generated full-length fibronectin in which the entire I-12 module (Δ12) was deleted. To test the possible role of the other carboxyl-terminal type I modules in assembly, we also generated mutant fibronectin in which I-10 through I-12 was deleted (Δ10–12). These molecules were all produced by COS cells (Figure 6). Quantitation of mutant fibronectins showed that approximately 60% of the fibronectin produced in cells transfected with Δ12 and nonmutant fibronectins was dimeric. Endogenous COS cell fibronectin was also approximately 60% dimeric (data not shown). In contrast, only 30% of the Δ10–12 fibronectin was dimeric.

To determine whether the deletion mutants are incorporated into the extracellular matrix, purified recombinant fibronectins were added to monolayers of human fibroblasts. Following incubation with the rat-specific antibody VT-1, rat fibronectin was clearly visualized in a fibrillar staining pattern by indirect immunofluorescence (Figure 7, panels A1, B1, and C1). Since COS cells produce a small amount of endogenous monkey fibronectin, some of the staining could be due to monkey fibronectin or heterodimers of rat and monkey fibronectins. To minimize the amount of monkey fibronectin present, COS cell proteins were absorbed to a LabMab monoclonal antibody

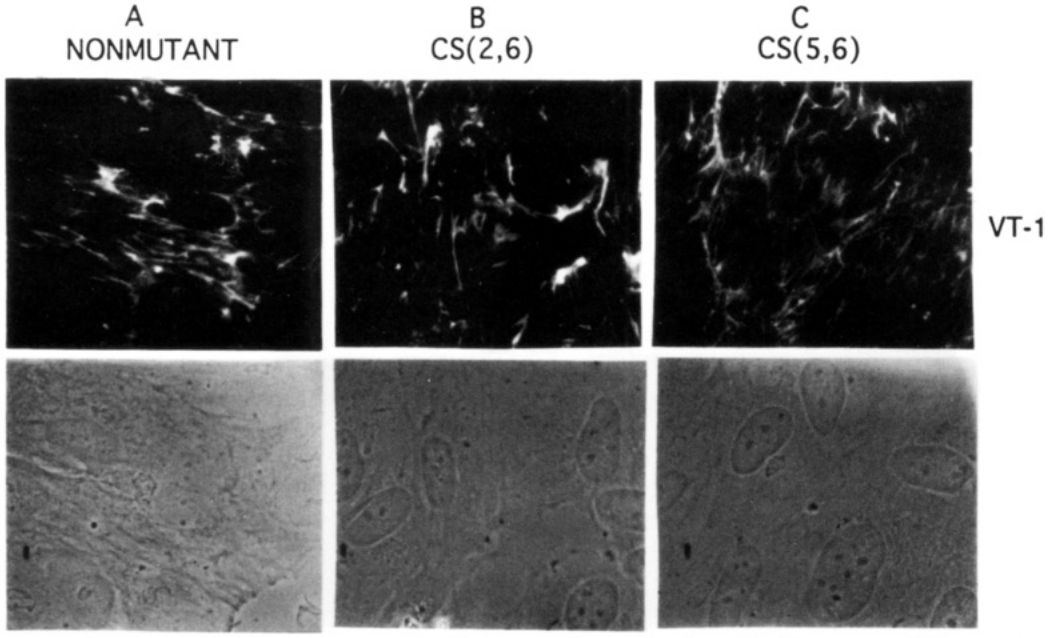

FIGURE 4: Incorporation of full-length nonmutant and CS mutant fibronectins into fibroblast extracellular matrix as assessed by immunofluorescence. Recombinant fibronectins were purified from ^{35}S-labeled COS cell-conditioned medium by passage over a LabMab–agarose column to remove monkey fibronectins, and then over a gelatin–agarose column. Equal cpm of ^{35}S-labeled nonmutant (A) and CS(2,6) (B) and CS(5,6) (C) mutant fibronectins were incubated with monolayers of human fibroblasts as described under Experimental Procedures. After 24 h, monolayers were washed and then incubated with VT-1, a monoclonal antibody that recognizes rat fibronectin. Cells were photographed at 250× magnification. Fluorescent (VT-1) and corresponding phase fields are shown.

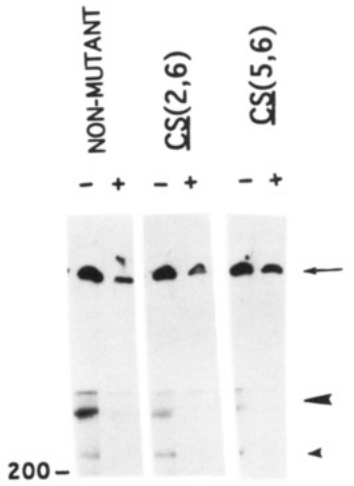

FIGURE 5: Incorporation of ^{35}S-labeled fibronectins into fibroblast extracellular matrix. Equal cpm of nonmutant and CS(2,6) and CS(5,6) mutant fibronectins were added to monolayers of human fibroblasts in the presence (+) and absence (–) of unlabeled nonmutant human fibronectin (300 μg/mL). After 24 h, cells were washed and then solubilized in SDS–PAGE sample buffer without reducing agent. Samples were analyzed by SDS–PAGE followed by fluorography. The arrow indicates high molecular weight disulfide-bonded multimers at the top of the stacking gel. Small amounts of dimeric (large arrowhead) and monomeric (small arrowhead) fibronectins are also present. Migration of ^{14}C-labeled myosin (200K) is shown at the left.

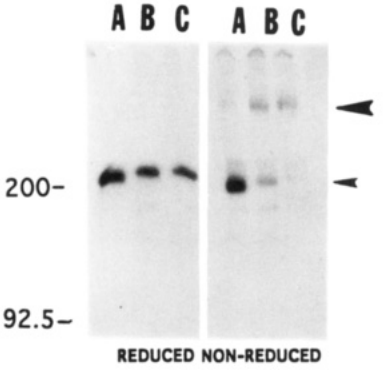

FIGURE 6: Production of Δ12 and Δ10–12 full-length fibronectins. COS cells were transfected with Δ10–12 (A), Δ12 (B), and nonmutant (C) fibronectin cDNAs. Equal cpm of ^{35}S-labeled fibronectins purified from COS cell-conditioned medium were analyzed by SDS–PAGE in the presence (reduced) or absence (nonreduced) of 5% β-mercaptoethanol. Dimeric (large arrowhead) and monomeric (small arrowhead) fibronectins are indicated. Molecular weight standards are ^{14}C-labeled myosin (200K) and phosphorylase *b* (92.5K).

column before purification on gelatin–agarose. LabMab recognizes monkey but not rat fibronectin. Further, although the human fibroblasts were treated with cycloheximide, it is likely that the cells secrete some human fibronectin during the 24-h incubation. Therefore, we also stained for human and monkey fibronectins. After incubation with purified rat fibronectin, cells were double-labeled with 9D2, a monoclonal antibody that recognizes monkey and human fibronectins but not rat fibronectin, and with a polyclonal antibody that recognizes fibronectins of all three species. Panel D3 shows faint fluorescent fibrils in cultures of fibroblasts that were not exposed to recombinant fibronectins. A comparison of rows 3 and 4 of Figure 7 shows that the staining patterns with 9D2 (to monkey and human) and the polyclonal (to monkey, human, and rat) antibodies are different. In cells incubated with the nonmutant and the Δ12 mutant rat fibronectins, there were large areas where antibody staining did not colocalize, indicating that monkey (or human) and rat fibronectins are located on distinct fibrils.

The staining patterns with the Δ10–12 mutant were more difficult to interpret. The original slides were surveyed and scored by colleagues who did not know the identities of the slides. The observers agreed that there were less fibrils and a greater degree of colocalization of monkey/human and rat fibronectins with the Δ10–12 mutant than with the nonmutant and Δ12 mutant fibronectins; i.e., faint fibrils stained with 9D2 appeared to be present in many areas where polyclonal antibody staining was present. However, there were areas of the slides where antibody staining did not colocalize as shown in panels C3 and C4.

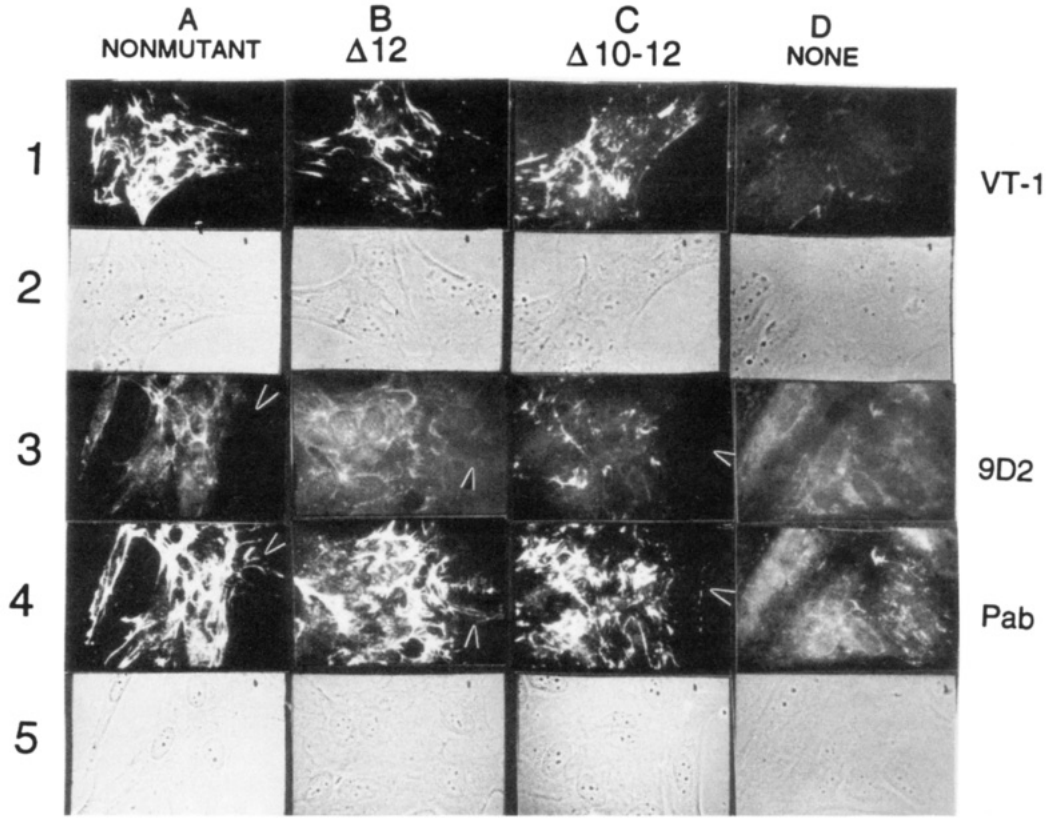

FIGURE 7: Incorporation of Δ12 and Δ10–12 fibronectins into fibroblast extracellular matrix. ^{35}S-Labeled COS cell-conditioned medium was passed over a LabMab–agarose column to remove monkey fibronectins before purification of fibronectin on gelatin–agarose columns. Equal cpm of ^{35}S-labeled nonmutant (A), Δ12 (B), and Δ10–12 (C) fibronectins were added to monolayers of human fibroblasts. Control cells received no exogenous fibronectin (NONE, D). After being fixed, cells were double-labeled with VTI (specific for rat) and rabbit polyclonal (specific for rat, monkey, and human) anti-fibronectin antibodies (1) or with 9D2 (specific for monkey and human) and polyclonal anti-fibronectin antibodies (3, 4). After cells were washed, they were incubated with fluorescein-conjugated anti-mouse IgG and rhodamine-conjugated anti-rabbit IgG. 9D2 (3) and VT-1 (1) antibodies were visualized with fluorescein-conjugated anti-mouse IgG (3), and rabbit polyclonal antibodies (4) by rhodamine-conjugated anti-rabbit IgG. Arrowheads show areas where polyclonal and monoclonal antibodies do not colocalize. Panel 5 represents the same field shown in panels 3 and 4. Cells were photographed at 250× magnification.

Δ10–12 and Δ12 fibronectins were cross-linked into large disulfide-stabilized aggregates (Figure 8). Cross-linking was decreased approximately 40% in the Δ12 mutant and 60% in the Δ10–12 mutant in comparison with nonmutant fibronectin.

DISCUSSION

We produced a number of recombinant fibronectins with mutations in the carboxyl-terminal type I modules (I-10 through I-12) to assess the importance of these modules in fibronectin matrix assembly. We also expressed nonmutant and mutant I-12 as fusion proteins with the gelatin binding part of fibronectin to determine whether mutations in I-12 would destabilize the module. Mutant and nonmutant fusion proteins were produced and secreted by insect cells in amounts (10–20 μg/mL) comparable to a nonmutant 70 K amino-terminal fragment of fibronectin. Intact I-12 modules could be released from the nonmutant and CS(2,6) mutant molecules by trypsin digestion. These data suggest that I-12 folds independently. Expression of I-12 modules in GE-1 indicates that the most likely pairing for the cysteines in this module is 1–4, 2–6, 3–5 (see Figure 1A) inasmuch as the CS(5,6) mutant module was sensitive to trypsin digestion and formed dimers under nonreducing conditions by SDS–PAGE. Although other arrangements are not ruled out, homology considerations with other type I modules would also suggest that this is the correct pairing (Petersen et al., 1983, 1989). Thus, in contrast to thioredoxin, the vicinal cysteines (5 and 6) in I-12 are probably not disulfide-bonded to each other.

Full-length fibronectins containing CS mutations or deletion of type I modules were secreted by COS cells at levels comparable to the nonmutant molecule. Attempts to produce full-length fibronectin in insect cells were unsuccessful. For some of the mutants secreted by COS cells, the majority of the fibronectin was monomeric, either because the molecules do not dimerize efficiently in the secretory pathway or because they are more susceptible to proteolysis. This has been a problem in other laboratories as well. Schwarzbauer et al. (1989) showed that sequences in the variable region (present in all of our constructs) were required for dimerization. Guan et al. (1990) showed that full-length recombinant fibronectins lacking the alternatively spliced type III modules ED-A or ED-B, or the variable region, dimerize poorly in lymphoid cells. Full-length fibronectins containing CS mutations were incorporated into the extracellular matrix, indicating that the extra cysteine residues in I-12 are not necessary for this process (Figures 4 and 5). Further, fibronectins lacking the entire 12th type I module (Δ12) were also assembled into the matrix, as shown in Figures 7 and 8. Thus, the C-terminal type I modules must not function as a unit in assembly, as do the five amino-terminal type I modules (Sottile et al., 1991a). It is difficult to assess the importance of I-10 and I-11. Fibronectin lacking I-10 through I-12 was assembled into fibrils (Figure 7) and cross-linked into high molecular weight multimers (Figure 8), but less robustly than Δ12 or nonmutant molecules. Because the double immunofluorescence was difficult to interpret, we cannot exclude the possibility that the deposition observed was of heterodimers of Δ12 rat subunits and COS cell subunits. Further analysis of these molecules will require expression in cells that lack endogenous fibronectin.

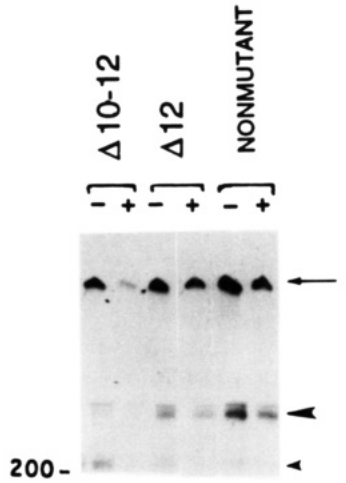

FIGURE 8: Incorporation of ^{35}S-labeled Δ12 and Δ10–12 fibronectins into fibroblast extracellular matrix. ^{35}S-Labeled COS cell-conditioned medium was passed over a LabMab–agarose column to remove monkey fibronectins before purification of fibronectin on gelatin–agarose columns. Equal cpm of nonmutant and Δ12 and Δ10–12 mutant fibronectins were added to monolayers of human fibroblasts in the presence (+) and absence (–) of unlabeled nonmutant human fibronectin (300 μg/mL). After 24 h, cells were washed and then solubilized in SDS–PAGE sample buffer without reducing agent. Samples were analyzed by SDS–PAGE followed by autoradiography. The arrow indicates high molecular weight disulfide-bonded multimers at the top of the stacking gel. Small amounts of dimeric (large arrowhead) and monomeric (small arrowhead) fibronectins are also present. Migration of ^{14}C-labeled myosin (200 kDa) is shown at the left.

Other studies have shown that fibronectins lacking type III modules III-1 through III-7 (Schwarzbauer, 1991) or III-1 through III-14 (Ichihara-Tanaka et al., 1992) can be assembled into fibrils. Although it was not ruled out that the mutant subunits were incorporated into the matrix as heterodimers with endogenous subunits or alongside nonmutant endogenous fibronectin (Schwarzbauer, 1991b), these data, along with the data presented in our paper, suggest that the minimum requirements for assembly are modules I-1 through I-5 (McKeown-Longo & Mosher, 1985; Quade & McDonald, 1988; Schwarzbauer, 1991a; Sottile et al., 1991) plus the cysteines involved in fibronectin dimerization (Schwarzbauer, 1991a). Other regions of fibronectin implicated in assembly such as I-9/III-1 (Chernousov et al., 1991) and III-10 and adjacent modules (McDonald et al., 1987; Akiyama et al., 1989; Fogerty et al., 1990) may be important for nucleation of matrix assembly in the absence of endogenous fibronectin (McDonald et al., 1987; Schwarzbauer, 1991b; Ichichara-Tanaka, 1992), may allow for efficient dimerization, may alter the length or thickness of the fibrils, or may allow fibronectin to interact with other extracellular matrix molecules.

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REFERENCES

Akiyama, S. K., Yamada, S. S., Chen, W.-T., & Yamada, K. M. (1989) *J. Cell Biol. 109*, 863–875.

Baron, M., Norman, D., Willis, A., & Campbell, I. D. (1990) *Nature 345*, 642–646.

Boniface, J. J., & Reichert, L. E., Jr. (1990) *Science 247*, 61–64.

Chernousov, M. A., Fogerty, F. J., Koteliansky, V. E., & Mosher, D. F. (1991) *J. Biol. Chem. 266*, 10851–10858.

Edman, J. C., Ellis, L., Blacher, R. W., Roth, R. A., & Rutter, W. J. (1985) *Nature 317*, 267–273.

Ehrismann, R., Roth, D. E., Eppenberger, H. M., & Turner, D. C. (1982) *J. Biol. Chem. 257*, 7381–7387.

Fogerty, F. J., & Mosher, D. F. (1990) *Cell Differ. Dev. 32*, 439–450.

Fogerty, F. J., Akiyama, S. K., Yamada, K. M., & Mosher, D. F. (1990) *J. Cell Biol. 111*, 699–708.

Guan, J.-L., Trevithick, J. E., & Hynes, R. O. (1990) *J. Cell Biol. 110*, 833–847.

Harlow, E., & Lane, D. (1988) *Antibodies: A Laboratory Manual*, p 648, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.

Holmgren, A. (1985) *Annu. Rev. Biochem. 54*, 237–271.

Ichihara-Tanaka, K., Maeda, T., Titani, K., & Sekiguchi, K. (1992) *FEBS Lett. 299*, 155–158.

Kunkel, T. A., Roberts, J. D., & Zabour, R. A. (1987) *Methods Enzymol. 154*, 367–382.

Laemmli, U. K. (1970) *Nature (London) 227*, 680–685.

Matsuura, Y., Possee, R. D., Overton, H. A., & Bishop, D. H. L. (1987) *J. Gen. Virol. 68*, 1233–1250.

Mayadas, T. N., & Wagner, D. D. (1992) *Proc. Natl. Acad. Sci. U.S.A. 89*, 3531–3535.

McDonald, J. A., Quade, B. J., Broekelmann, T. J., LaChance, R., Forsman, K., Hasegawa, E., & Akiyama, S. (1987) *J. Biol. Chem. 262*, 2957–2967.

McKeown-Longo, P. J., & Mosher, D. F. (1983) *J. Cell Biol. 97*, 466–472.

McKeown-Longo, P. J., & Mosher, D. F. (1984) *J. Biol. Chem. 259*, 12210–12215.

McKeown-Longo, P. J., & Mosher, D. F. (1985) *J. Cell Biol. 100*, 364–374.

Miller, D. W., Safer, P., & Miller, L. K. (1986) in *Genetic Engineering: Principles and Methods* (Setlow, J. A., & Hollaender, A., Eds.) Vol. 8, pp 277–289, Plenum Press, New York.

Patel, R. S., Odermatt, E., Schwarzbauer, J. E., & Hynes, R. O. (1987) *EMBO J. 6*, 2565–2572.

Petersen, T. E., Thogersen, H. C., Skorstengaard, K., Vibe-Pedersen, K., Sahl, P., Sottrup-Jensen, L., & Magnusson, S. (1983) *Proc. Natl. Acad. Sci. U.S.A. 80*, 137–141.

Petersen, T. E., Skorstengaard, K., & Vibe-Pedersen, K. (1989) in *Fibronectin* (Mosher, D. F., Ed.) pp 1–24, Academic Press, New York.

Quade, B. J., & McDonald, J. A. (1988) *J. Biol. Chem. 263*, 19602–19609.

Sambrook, J., Fritsch, E. F., & Maniatis, T. (1989) *Molecular Cloning: A Laboratory Manual*, 2nd ed., Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.

Sanger, F., Miklen, S., & Coulson, A. R. (1977) *Proc. Natl. Acad. Sci. U.S.A. 74*, 5463–5467.

Schwarzbauer, J. E. (1991a) *J. Cell Biol. 113*, 1463–1473.

Schwarzbauer, J. E. (1991b) *Curr. Opin. Cell Biol. 3*, 786–791.

Schwarzbauer, J. E., Spencer, C. S., & Wilson, C. L. (1989) *J. Cell Biol. 109*, 3445–3453.

Sottile, J., Selegue, J., & Mosher, D. F. (1990) *Protein Expression Purification 1*, 104–110.

Sottile, J, Schwarzbauer, J., Selegue, J., & Mosher, D. F. (1991) *J. Biol. Chem. 266*, 12840–12843.

Summers, M. D., & Smith, G. E. (1987) *Tex., Agric. Exp. Stn. Bull. No. 1555*, 1–55.

Vaughn, J. L., Goodwin, R. H., Tomkins, G. J., & McCawley, P. (1977) *In Vitro 13*, 213–217.

Vuori, K., Myllyla, R., Pihlajaniemi, T., & Kivirikko, K. I. (1992) *J. Biol. Chem. 267*, 7211–7214.

Zoller, M. J., & Smith, M. (1989) in *Recombinant DNA Methodology* (Wu, R., Grossman, L., & Moldave, K., Eds.) pp 537–568, Academic Press, San Diego.