Fibronectin is a cell-adhesive protein comprised of three types of repeating homologous sequences, I, II, and III (Petersen, T. E., Thogersen, H. C., Skorsten-gaard, K., Vibe-Pedersen, K., Sahi, P., Sottrup-Jensen, L., and Magnusson, S. (1983) Proc. Natl. Acad. Sci. U.S.A. 80, 137-141). The amino-terminal portion of fibronectin is comprised of five type I modules and mediates assembly of dimeric soluble fibronectin into insoluble fibrils by cultured fibroblasts, binding and cross-linking of fibronectin to Staphylococcus aureus, and binding and cross-linking of fibronectin to fibrin. It is not known whether these binding activities require individual type I modules, several modules, or all five modules. To answer this question, we generated recombinant truncated fibronectin molecules with deletions of or mutations in the amino-terminal type I modules. Binding to cellular fibronectin assembly sites and S. aureus required all five type I modules. In contrast, proteins with deletions of type I modules interacted well with fibrin.

Fibronectin is a 500-kDa disulfide-bonded dimer that contains three types of repeating homologous sequences, types I, II, and III (1, 2). There are 12 type I modules per 250-kDa subunit, each contains approximately 45 residues with two consensus disulfides and conserved tyrosine and tryptophans (2). Type I sequences of fibronectin, except for the 12th (1-12), are encoded by separate exons (2, 3). It is thought that such protein modules fold independently and represent individual structural and binding domains (4). NMR studies of the 7th type I module of fibronectin (1-7), expressed in yeast, are consistent with this idea (5). 1-7 forms a compact structure with two anti-parallel β-sheets and a hydrophobic core consisting of the conserved tyrosine and tryptophans and the two consensus disulfides (5). Both the amino- and carboxyl-terminal residues of the module lie in β-sheets, however, raising the possibility that adjacent modules, for example, 1-2/1-3, 1-3/1-4, and 1-4/1-5, link together through common β-strands (5).

The amino-terminal portion of fibronectin contains 5 type I modules (2) and mediates binding to fibrin (6, 7), Staphylo-
coccus aureus (8), and fibroblasts (9, 10). To determine which type I modules participate in these interactions, we generated a series of recombinant 70-kDa (70 kDa) fibronectin molecules with mutations in the first 5 type I modules. We found that binding to fibroblasts and S. aureus requires all 5 type I modules, whereas binding to fibrin appears to be a general property of type I modules.

EXPERIMENTAL PROCEDURES

In Vitro Mutagenesis—FN 571, encoding the amino-terminal 70-kDa region of fibronectin (11), was cloned into M13mp18 and used as a template for oligonucleotide-directed mutagenesis (12). Oligonucleotides that contained an altered base to change a tyrosine to a serine (YS series) or spanned DNA to be deleted (Gap series) were synthesized using an Applied Biosystems DNA synthesizer: YS 1, GGGAAACATTCTCAATCTGACAGAAAGGCATC (317-337, Tyr32) ; YS 2, GGCACTGCTCCAACTGTTGTGCTGTCAGTCAGAGCAAG-GTTCTACAGAG (320, Tyr32); YS 3, GGCACGTCTTCCAACTGTTGTGCTGTCAGTCAGAGCAAG-GTTCTACAGAG (320, Tyr32); YS 4, GGGACTCTCCCTCGTGGG (557, Tyr554); YS 5, AGGACCTTCCCTGATAGGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAG
electrophoresis in sodium dodecyl sulfate (SDS-PAGE) (16) (5% stacking and 8% separating gels were used) showed that r70-kDa protein and the Gap series mutants were of the predicted size and represented 90-95% of gelatin-bound protein. The contaminating protein was endogenous COS cell fibronectin. Protein production was quantified by comparing the yield of purified 35S-labeled mutant protein with the yield of r70-kDa protein from control cultures transfected with nonmutated DNA. SDS-PAGE showed that r70-kDa or mutant protein was low or undetectable in cell lysates and in the fraction of secreted proteins that did not bind to gelatin-agarose.

Binding Assays—Binding of labeled protein to monolayer cultures of human diploid fibroblasts seeded into 24-well cluster dishes (9, 11) or S. aureus after incubation in suspension (8, 11, 17) was determined using previously described assays. Bound protein in both assays was solubilized with sample buffer (50 mM Tris-HCl, pH 6.8, 1% SDS, 10% glycerol, and 0.01% bromphenol blue) containing 10% β-mercaptoethanol and separated by SDS-PAGE. Gels were analyzed by autoradiography so that bound proteins could be identified by size (see Fig. 2), and the label in recombinant protein was quantified by scintillation counting of the appropriate gel sectors. Of labeled r70-kDa protein added in binding assays, 20-40% bound to S. aureus, and 2-3% bound to fibroblasts. Nonspecific binding was determined by the addition of 20-50 μg/ml unlabeled r70-kDa protein (prepared using a baculovirus expression system (11)) to the binding mixtures. For the proteins that bound well, nonspecific binding was 10-30% of total binding in the bacteria binding assay and 15-40% in the fibroblast binding assay. The normalized numbers in the figure were calculated using specific binding, that is total binding minus nonspecific binding.

Factor XIIIa Cross-linking—Cross-linking of labeled proteins to fibrin by Factor XIIIa was carried out for 1 h at 37 °C as described (7). Control incubations lacked Factor XIIIa. Proteins were solubilized in sample buffer containing 10% β-mercaptoethanol and analyzed by SDS-PAGE. The amount of cross-linking was ascertained by the increase in labeled protein that migrated in the stacking gel as determined by scintillation counting. Similar results were obtained when the amount of cross-linking was ascertained by the loss of label from the position of the noncross-linked protein. The maximum amount of cross-linking seen in the assay was approximately 60% of the total labeled protein in the presence of Factor XIIIa and <3% in its absence.

RESULTS AND DISCUSSION

DNA encoding for the signal and propeptide sequences, the five amino-terminal type I modules, and the adjacent gelatin-binding region of rat fibronectin (3) was subjected to oligonucleotide-directed mutagenesis as described above. Some mutant molecules lacked one or more of the amino-terminal type I modules (Gap series). Other mutant molecules were made in which the conserved tyrosine was changed to a serine (YS series). Proteins were expressed in COS cells and labeled with 35S-containing methionine and cysteine. The non-mutant r70-kDa protein and mutant proteins all contained the gelatin-binding region of fibronectin and could be purified efficiently from conditioned medium by affinity chromatography on gelatin-agarose (18). In addition, all contained the glutamine near the amino terminus that is a site for protein-protein cross-linking (via -(γ-glutamyl)lysyl cross-links) catalyzed by activated blood coagulation Factor XIII or plasma transglutaminase (2).

The Gap 1–3 and 4–5 mutants were produced at levels comparable with or higher than non-mutant r70-kDa protein (Fig. 1). Mutants with deletions of single modules, especially Gap 5 and 6, were produced at lower levels. The YS mutants were all expressed at levels of r70-kDa protein from control cultures transfected with nonmutant r70-kDa protein. We could detect no trace of a double mutant in which consensus tyrosines were changed to serines in the first and fifth type I modules. Although differences in transfection efficiencies could contribute to differences in protein production among the various mutants, the low production (0-40% of non-mutant) of many of the YS mutants suggests that mutation of the conserved tyrosine is more disruptive for protein processing and secretion than en bloc deletion of modules.

Soluble fibronectin becomes insolubilized into fibrils after binding to matrix assembly sites on surfaces of fibroblasts in monolayer culture (19-21). The region of fibronectin responsible for binding to these sites is not the Arg-Gly-Asp-containing cell adhesion part but the type I sequences near the amino terminus (9, 10). Thus, although amino-terminal 70-kDa protein does not assemble into fibrils (9), it binds specifically to fibroblasts in monolayer culture, and blocks the binding and assembly of intact fibronectin (9, 11). Most of the binding activity is in the 27-kDa amino-terminal portion (9, 10). Binding of the Gap mutants to fibroblast monolayers was decreased 7-100-fold compared with non-mutant r70-kDa protein (Figs. 1 and 2). The YS mutants also bound less well, although the decrease was not as pronounced (2-3-fold).

Binding of bacteria to fibronectin is important for bacterial attachment to host tissues (22). In suspension binding assays, the 27-kDa amino-terminal fragment of fibronectin binds to S. aureus as effectively as intact fibronectin and can be cross-linked on the bacteria by Factor XIIIa (8). Non-mutant r70-kDa protein binds to S. aureus and blocks binding of the proteolytically derived 70-kDa fragment (11). The effects of the mutations on binding to S. aureus in suspension were similar to the effects on binding to fibroblasts in monolayers.

### FIG. 1. Production of recombinant truncated fibronectin and interaction with fibroblasts, S. aureus, and fibrin.

At the top is a schematic representation of the non-mutant r70-kDa truncated fibronectin secreted by COS cells. The amino terminus is to the left. Type I modules, type II modules, and nonhomologous sequences are depicted as numbered rectangles, unnumbered ovals, and lines, respectively. The x indicates the site of Factor XIII-mediated cross-linking. Mutant 35S-labeled proteins containing point (YS) or deletion (Gap) mutations were isolated from conditioned medium after transfection and tested for their ability to interact with S. aureus, fibroblasts in monolayer culture, and fibrin. Sites of mutations are shown schematically on the left; modules with point mutations are shaded black, and deletions are indicated by broken lines.

The gelatin-binding region of fibronectin, comprised of 1-6 through 5-12 modules, respectively. The x indicates the site of Factor XIII-mediated cross-linking. Mutant 35S-labeled proteins containing point (YS) or deletion (Gap) mutations were isolated from conditioned medium after transfection and tested for their ability to interact with S. aureus, fibroblasts in monolayer culture, and fibrin. Sites of mutations are shown schematically on the left; modules with point mutations are shaded black, and deletions are indicated by broken lines.

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counts (approximately 110,000 cpm/dish) of "S-labeled

described (1) weight standards are indicated by

bovine serum albumin

artifact of this gel, inasmuch anhydrase

was solubilized with sample buffer. Samples were analyzed by SDS-

PAGE as described under "Experimental Procedures." Molecular

weight standards are indicated by dashes. Standards were from (top
to bottom): "C-labeled myosin (200 kDa), phosphorylase b (92.5 kDa),

bovine serum albumin (69 kDa), ovalbumin (46 kDa), and carbonic

anhdrase (30 kDa). The arrow points to the position of the non-

mutant r70-kDa protein. The arrowhead points to endogenous COS

cell fibronectin. The anomalous migration of the YS 1 protein is an

artifact of this gel, inasmuch as YS 1 comigrated with the r70-kDa

protein in other gels. YS 2, however, consistently migrated more

slowly than the r70-kDa protein.

(Fig. 1). Binding of each of the Gap mutants was decreased to

less than 5% of binding of r70-kDa protein. The YS mutants

also bound less well, but the decrease was less pronounced.

When Factor XIIIa was added to the binding assay and cross-

linking was assayed by the appearance of higher molecular

weight bands in polyacrylamide gels, cross-linking of mutant

proteins was decreased in proportion to the decrease in binding

(results not shown).

Covalent incorporation of fibronectin into the fibrin clot

by Factor XIIIa is thought to promote the adhesion and migration of cells into wounds (23, 24). When Factor XIIIa

catalyzed cross-linking of r70-kDa and mutant proteins to fibrin at 37 °C was quantified by a gel electrophoretic assay,
cross-linking was either unchanged or only slightly decreased for all of the mutants tested except for Gap 1–3 and 1–5 (Fig.
1). In affinity chromatographic assays done at 4 °C, however, the Gap 1–3 and 1–5 mutants bound as well to fibrin as non-

mutant r70-kDa protein (Fig. 3). Decreased cross-linking of Gap 1–3 to fibrin compared with Gap 4–5 suggests specificity

in the interaction of mutant proteins with fibrin and/or Factor

XIIIa at physiological temperature. Binding to fibrin in the
cold, however, appears to be a general property of type I

sequences. Others have shown that the 31-kDa carboxyl-
terminal region of fibronectin (25–27), which contains three
type I modules; the gelatin-binding region of fibronectin (27,

28), which contains four; and the single type I module of
tissue plasminogen activator (29) interact with fibrin.

Our results indicate that all five amino-terminal type I

modules of fibronectin are required for binding to fibroblasts

in monolayer culture or to S. aureus. One explanation for

these results is that the five modules form a single functional

unit, so that deletion of one module or disruption of its

structure by mutation of the consensus tyrosine disrupts the

structure and function of the entire unit. A second explanation

is that binding to fibroblasts or S. aureus requires that re-

peating structures in fibronectin interact with repeating struc-

tures on the surfaces of the cell or bacteria. A bacterial protein

with a repeating motif has been identified that interacts with the

amino-terminal portion of fibronectin (30). We, nevertheless,

favor the first explanation. Each of the type I modules of

fibronectin has a characteristic and unique sequence that

is conserved among species (2), and there is no common

feature that distinguishes the five amino-terminal type I

modules from the other seven type I modules. The unimpaired

secretion but grossly impaired binding activities of several of the

Gap mutants suggests that individual modules fold cor-

rectly during biosynthesis but cannot function cooperatively.

This view is compatible with the suggestion by Baron et al.

(5) that the carboxyl-terminal segment of a type I module and the

amino-terminal segment of an adjacent type I module interact
to form a common β-strand between the two modules.

The common β-strand puts constraints on the order of type I

modules within fibronectin; for example, I-1 would not be

expected to substitute for I-2 to form a β-strand with I-3.

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