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Nucleotide sequence of the gene for a fibronectin-binding protein from *Staphylococcus aureus*: Use of this peptide sequence in the synthesis of biologically active peptides

(fibronectin/bacterial adhesion/synthetic peptides)

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ABSTRACT **Binding of cells of *Staphylococcus aureus* to fibronectin, which may represent a mechanism of host tissue adherence, involves a fibronectin-receptor protein present on the bacterial surface. Cloning of a gene coding for a staphylococcal fibronectin-binding protein and construction of a fusion protein with fibronectin-binding properties was previously reported from our laboratory. We have now sequenced the gene and deduced a primary sequence of the fibronectin-binding protein. The protein resembles other cell-wall-associated proteins on Gram-positive bacteria in that it (*i*) appears to be anchored in the cell membrane via its C-terminal end, (*ii*) contains a proline-rich repeating unit outside the membrane anchor, and (*iii*) contains a long (36-amino acid) signal sequence at the N terminus. The fibronectin-binding activity has been localized to a domain composed of a 38-amino acid unit repeated completely three times and partially a fourth time; the identity between the three 38-amino acid sequences varies from 42 to 87%. Three synthetic peptides mimicking the structure of each 38-amino acid unit were constructed. All three peptides interacted with fibronectin, as indicated by their ability to inhibit binding of fibronectin to staphylococcal cells, whereas an unrelated 37-amino acid peptide showed no inhibitory activity.**

Fibronectin is a disulfide-linked dimeric glycoprotein ($M_r \simeq$ 450,000) present in a soluble form in blood plasma and other body fluids and as fibrils in extracellular matrices. The major function of fibronectin is probably related to its ability to mediate substrate adhesion of eukaryotic cells, a process that involves the binding of specific cell-surface receptors to discrete domains in the fibronectin molecule (1–4). Fibronectin also interacts with several species of bacteria including Gram-positive bacteria, such as *Staphylococcus aureus* (5–7), coagulase-negative staphylococci (8), and beta-hemolytic (group A, C, and G) streptococci (9–12). Also, certain species of Gram-negative bacteria such as *Escherichia coli* (13), *Salmonella enteritidis* (14), and *Treponema pallidum* (15) have been reported to bind fibronectin.

Studies on bacterial fibronectin-binding proteins (FnBPs) have so far mostly dealt with the FnBP of *S. aureus*, the first bacterial species described to bind fibronectin (16). In an earlier report from our laboratories a protein with a M_r of 18,000 present in lysostaphin lysates of *S. aureus* was implicated as a FnBP (6). On the other hand, Espersen and Clemmensen (5) isolated by affinity chromatography of sonicated staphylococci a FnBP with a M_r of ≈200,000. Recently Fröman *et al.* (7) also reported that the native FnBP isolated from lysostaphin lysates of *S. aureus* strain Newman migrates as a protein of M_r 210,000 on sodium dodecyl sulfate (SDS)/PAGE. Bacterial proteases present in the lysate can degrade the large FnBP, generating smaller fragments that retain fibronectin-binding activity.

We have earlier reported on the molecular cloning in *E. coli* of the gene for a FnBP from *S. aureus* strain 8325-4 (17). In this paper we report the complete nucleotide sequence of the gene.§ The fibronectin binding domain of the protein has been identified as a 38–amino acid homologous unit that is repeated three times and partly repeated a fourth time. Synthetic peptides mimicking the deduced amino acid sequences of the three repeats interacted with fibronectin, as indicated by their ability to inhibit fibronectin binding to bacteria.

MATERIALS AND METHODS

Bacterial Strains and Plasmids. *E. coli* K-12 strains HB101 (18) and TG1 (19) were used as bacterial hosts. The plasmid vectors were pBR322 (20) and pUC18 (21). The isolation of an *E. coli* clone containing the gene for a FnBP from *S. aureus* strain 8325-4 was described (17).

Assay of FnBP. Fusion proteins or synthetic peptides were analyzed for fibronectin-binding activity by measuring their ability to compete with staphylococcal cells for binding of ^{125}I-labeled intact fibronectin or the labeled 29-kDa N-terminal fragment. Bacterial cells (5×10^8) were suspended in phosphate-buffered saline (PBS) supplemented with 0.02% sodium azide, 0.1% bovine serum albumin, and 0.1% Tween 80. After addition of the FnBP analogs, followed by 5×10^4 cpm of ^{125}I-labeled intact fibronectin or labeled N-terminal 29-kDa fragment prepared as described (13), the volume was adjusted to 0.5 ml, and the mixtures were incubated for 1 hr at room temperature. At the end of the incubation, 3 ml of ice-cold 0.1% Tween 80 in PBS was added, and the tubes were centrifuged to pellet the bacteria. The supernatants were aspirated, and radiolabeled protein bound to the bacterial pellets was quantitated in an LKB γ counter.

DNA Preparation and Sequencing. Isolation of plasmid DNA was done as described (22). Enzymes were purchased from International Biotechnologies (New Haven, CT) or from New England Biolabs and used according to recommendations of the manufacturers. Other methods for handling DNA were essentially as described (23). DNA fragments were sequenced by the method of Maxam and Gilbert (24) or by the dideoxy chain-termination method of Sanger *et al.* (25). In the latter case suitable subclones were generated using the Erase-a-base system (26) from Promega Biotec

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Abbreviations: FnBP, fibronectin-binding protein; Boc, *t*-butyloxycarbonyl; SDS, sodium dodecyl sulfate.

§The sequence reported in this paper is being deposited in the EMBL/GenBank data base (accession no. J04151).

(Madison, WI). The sequencing samples were analyzed by PAGE on thin 8% or 20% acrylamide gels containing 8.3 M urea (27). Computer programs were used to record and analyze the sequence data (28).

Synthesis of Peptides. Peptides were synthesized on an Applied Biosystems automatic peptide synthesizer at the University of Alabama Cancer Center core facility by use of the solid-phase method of peptide synthesis on a benzhydrylamine resin support. t-Butyloxycarbonyl (Boc)-Gly was coupled to the resin as a Boc-Gly-OCH_2-C_6H_4-CH_2-COOH derivative (29) with dicyclohexylcarbodiimide/1-hydroxybenzotriazole. The following protecting groups were used for the side chains of bifunctional Boc-amino acids: 2-chlorocarbobenzoxy for lysine, benzyl ether for serine and threonine, benzyl ester for aspartic acid and glutamic acid, 2,4-dinitrophenyl for histidine, and 2,6-dichlorobenzyl ether for tyrosine.

After synthesis, dinitrophenyl groups from the side chain of histidine were removed by treatment of the peptide resin with thiophenol in dimethylformamide (30). Release of peptides from the solid support was achieved with the modified hydrogen fluoride procedure (31). After thorough washing with ether, the released peptides were extracted by stirring the resin with trifluoroacetic acid and filtering. Peptides were purified on a preparative column (Vydac C_{18} TP 510; The Separation Group, Hesperia, CA) fitted on an LKB HPLC system. To verify purity and sequence the isolated peptides were subjected to amino acid analyses, amino acid sequencing, and analytical HPLC.

RESULTS

DNA Sequence Strategy. We had earlier reported that the gene for a FnBP from *S. aureus* strain 8325-4 was located on a 6.5-kb insert of staphylococcal DNA cloned in the plasmid pBR322 (17). The recombinant plasmid was designated pFR001, and the approximate location of the gene within the 6.5-kb insert was also established.

A 3342-base-pair fragment from pFR001 has now been sequenced (Figs. 1 and 2). This fragment contained the complete gene for FnBP. The sequence of both DNA strands was determined for most of the gene by use of the Maxam and Gilbert method (24). However, in an 800-base-pair region, reliable sequences from both strands were not obtained due to the lack of usable restriction fragments. This region was subcloned into the pUC18 vector, and a series of overlapping clones of different lengths were constructed by *Exo* III digestion for different periods and religation of the DNA. These clones were sequenced using the dideoxynucleotide chain-termination method, and thus an unequivocal sequence throughout the whole fragment was obtained.

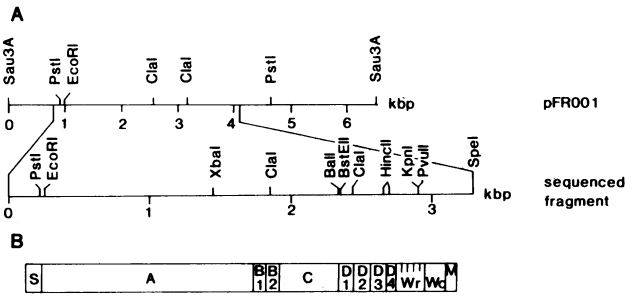

FIG. 1. (*A*) Restriction map of pFR001. The sequenced fragment of the insert is indicated. (*B*) Schematic diagram of the different regions of the gene for FnBP. S, signal sequence; A and C, spacer regions; B and D, different repeat regions outside the cell wall domain; W_r, repetitive region of the cell wall-spanning domain; W_c, nonrepetitive region of the same domain; and M, membrane-spanning domain.

Features of the Established Sequence. The sequence (Fig. 2) contains one open reading frame encoding a polypeptide of 1018 amino acids starting from a GTG codon at nucleotide 118 and terminating in a TAA stop codon at nucleotide 3172. Although GUG (GTG) is a fairly uncommon start codon, GUG has been proposed to have this function in the synthesis of several other bacterial proteins (32, 33). Two possible initiation signals for transcription are seen, as well as a potential ribosome-binding site. Downstream of the coding sequence is a possible hairpin loop that may serve as a transcriptional terminator. The presence of these signals suggests that the FnBP is translated from a monocistronic mRNA. When the deduced amino acid sequence was compared with a partial N-terminal sequence of native FnBP prepared from *S. aureus* strain Newman (G. Fröman, personal communication), the cleavage of the signal peptide could be defined to a point between the second and third alanine in a row of three alanine residues (marked in Fig. 2). Thus, the signal peptide is 36 amino acids long. The amino acid composition of the deduced sequence minus the signal peptide fits well with that previously determined for the native protein isolated from *S. aureus* strain Newman (compare ref. 7).

Three different regions of repetition were identified by computer analysis comparing the sequence with itself in a dot-blot matrix (see Figs. 1 and 2). Near the middle of the polypeptide is a stretch of 30 amino acids directly repeated once (B1–B2). About 140 amino acids further downstream is another repetitive region that consists of a 38-amino acid sequence repeated three times and partly repeated a fourth time (D1–D4). Directly after this region is a third repeat region consisting of a 14-amino acid unit repeated five times (W_r1–W_r5). Remarkably, 6 of these 14 amino acids are prolines. Downstream from this region is a 45-amino acid stretch with no special features followed by a hydrophobic domain, which may represent a transmembrane segment. The C-terminal domain of the polypeptide ends with a stretch of mainly basic amino acids, probably located on the cytoplasmic side of the membrane.

The Fibronectin-Binding Region. In a previous report from our laboratories (17), the region coding for the fibronectin-binding activity was located at the middle of the original 6.5-kb insert. Furthermore, a fusion protein (ZZ–FR) containing a 200-amino acid segment coded for by a *Bal* I–*Pvu* II fragment (Fig. 1), covalently linked to a truncated form of protein A, was shown to have fibronectin-binding activity (17). These results place the fibronectin-binding activity in the region containing the three D sequences. To further analyze the fibronectin-binding activity of this domain, we constructed synthetic peptides corresponding to the D1, D2, and D3 sequences (Fig. 3). These three peptides were subsequently tested for their ability to interfere with the bacterial binding of intact fibronectin or a 29-kDa fibronectin fragment. When bacteria were incubated with a ^{125}I-labeled 29-kDa N-terminal fragment of fibronectin in the presence of the synthetic peptides, the amount of radiolabeled protein bound to the cells was inversely proportional to concentration of the peptides (Fig. 4). The three peptides had inhibitory activity, whereas peptides digested with proteinase K were not inhibitory, demonstrating the importance of peptide structure for activity.

All three peptides also inhibited binding of the intact ^{125}I-labeled fibronectin to bacterial cells (Fig. 4). However, surprisingly, peptide D3 was substantially more active than D1 and D2, causing 50% inhibition at 2 μg/ml compared with 90 μg/ml and 230 μg/ml for D2 and D1, respectively, to obtain similar inhibition. Hydrophobicity plots showed that D3 exhibits a different hydrophobicity profile than do D1 and

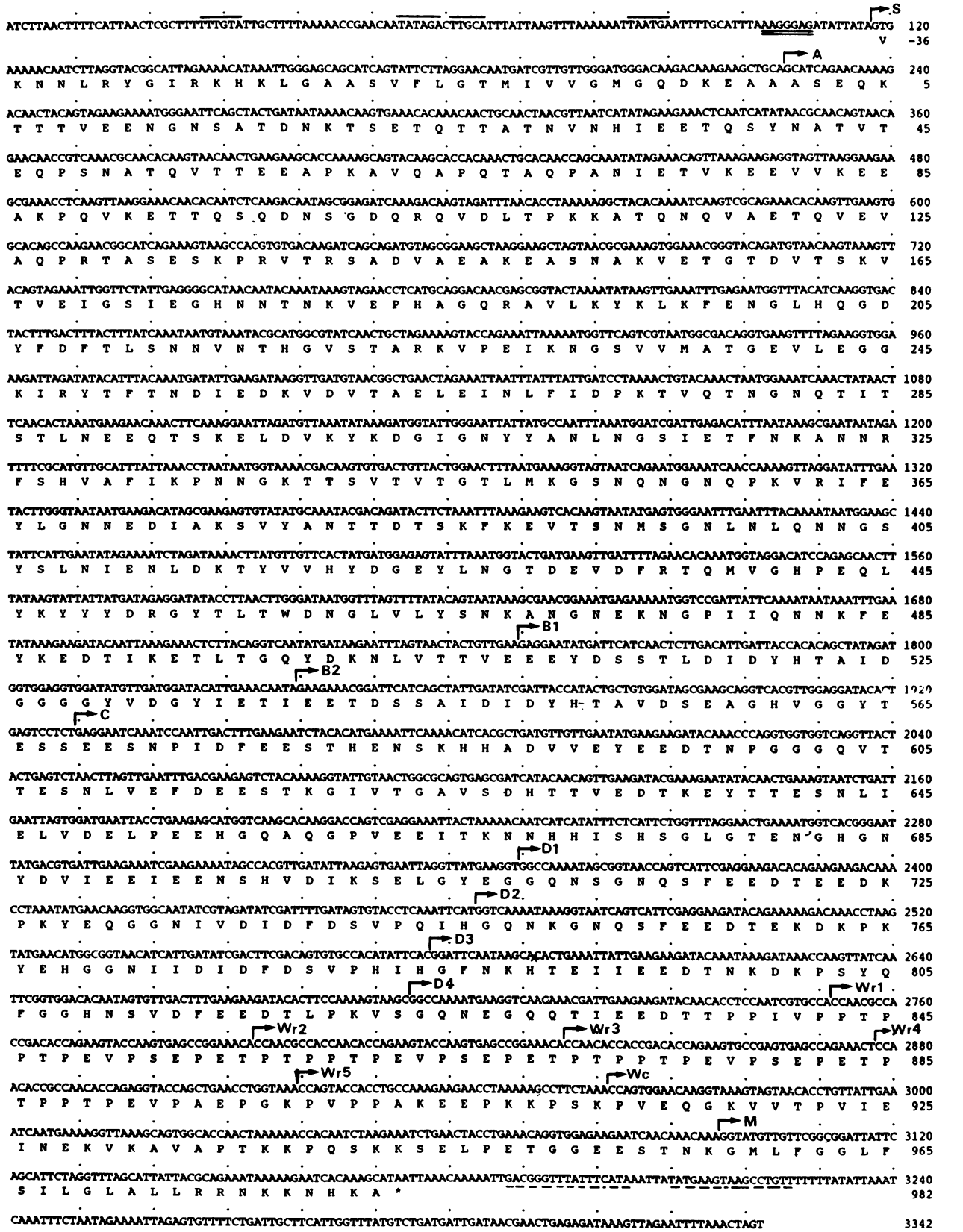

FIG. 2. Complete nucleotide and deduced amino acid sequence of the gene for FnBP from *S. aureus* strain 8325-4. Numbering of the amino acids starts at the N terminus of the mature protein, and the names of the amino acids are given in standard one-letter code. Amino acids −1 to −36 correspond to the signal peptide. Overbar, possible promoter signals; double underbar =, ribosome binding site; and single underbar ---, transcription termination hairpin loop. Different regions of the protein are shown as defined in the legend for Fig. 1. Dots mark every 10th base, and the asterisk marks a stop codon.

D2 (data not shown). Possibly D3 fits intact fibronectin better than do either D1 or D2. On the other hand, binding of the 29-kDa fragment to bacteria may not involve the same hypothetical hydrophobic fit; the three D peptides inhibit binding of this ligand comparably. Peptides digested with proteinase K (Fig. 4) and a synthetic peptide of similar length

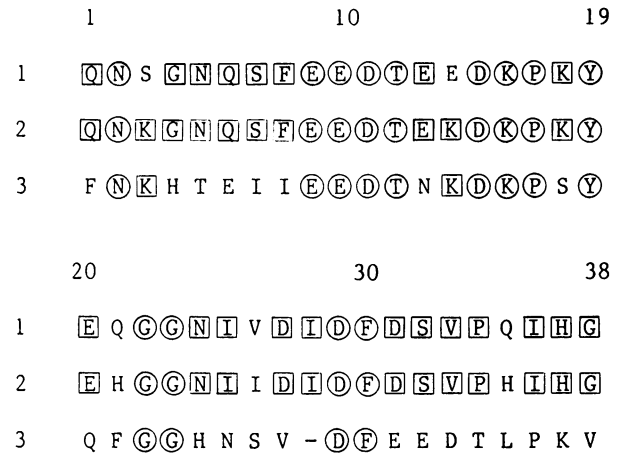

FIG. 3. Sequence of active synthetic peptides (reported in one-letter amino acid code). The sequences of the synthetic peptides D1, D2, and D3, which together constitute the active site of the FnBP, are shown. Circled residues are conserved in all three peptides, whereas residues in squares are found in two of three sequences.

(37 amino acids) but with an unrelated sequence (Fig. 5) did not affect binding of ^{125}I-labeled fibronectin to bacteria.

Because the inhibitory activity of the individual synthetic peptides appears substantially lower than that of the native receptor (see refs. 7 and 17), we explored the hypothesis that a mixture of the three peptides is required for full activity. When the three synthetic peptides were mixed in a 1:1:1 ratio, resulting inhibitory activity was not dramatically improved over that of individual peptides. The fusion protein ZZ–FR, which is composed of all D repeats and most of the W_r region, linked to a truncated form of protein A (17), was substantially more active (Fig. 5). On a molar basis, the ZZ–FR fusion protein caused 50% inhibition of bacterial binding of fibronectin and the 29-kDa fragment at 280 pM and 240 pM, respectively, whereas similar inhibition was obtained by the mixture of synthetic peptides at 170 mM and 6 mM, respectively.

DISCUSSION

The ability of bacteria to bind fibronectin has been proposed as a virulence factor enabling bacteria to colonize wound tissues and blood clots (6, 35). Fibronectin has also been shown to increase adherence of bacteria to neutrophils (36,

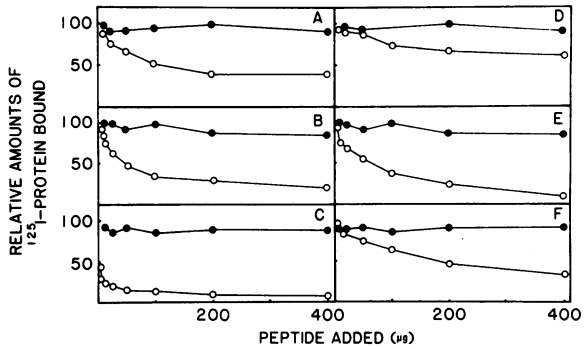

FIG. 4. Inhibition of binding of ^{125}I-labeled intact fibronectin or the labeled 29-kDa N-terminal fragment to bacterial cells by synthetic peptides. Cells of *S. aureus* strain 8325-4 were incubated with ^{125}I-labeled fibronectin (*A*, *B*, and *C*) or its labeled N-terminal fragment (*D*, *E*, and *F*) in the presence of indicated amounts of D1 (*A* and *D*), D2 (*B* and *E*), and D3 (*C* and *F*) before (○) or after digestion with proteinase K (●). Labeled protein bound in the absence of peptide was set to 100%.

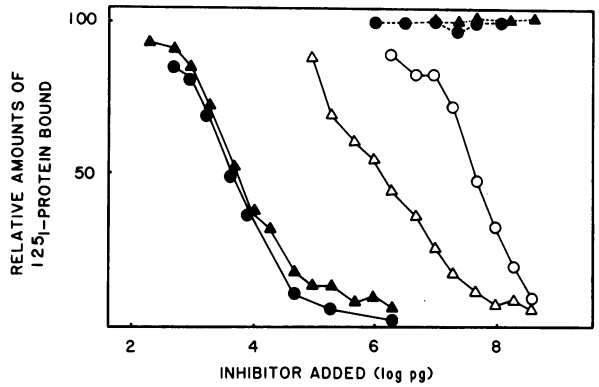

FIG. 5. Inhibition of binding of ^{125}I-labeled intact fibronectin or the labeled 29-kDa N-terminal fragment to bacterial cells by the ZZ–FR fusion protein or a synthetic peptide mixture. Cells of *S. aureus* strain 8325-4 were incubated with ^{125}I-labeled fibronectin (△, ▲) or the labeled N-terminal fragment (○, ●), in a total volume of 0.5 ml containing the indicated amounts of the ZZ–FR fusion protein (●—●, ▲—▲), D1, D2, and D3 mixed at equal weight ratio (○, △), protein A (●—●), and a control unrelated 37-amino acid peptide mimicking a domain in the human apoprotein A (DWLKAFYDKVAEKLEAF-PDWLKAFYDKVAEKLKEAF; see ref. 34) (▲—▲).

37), although whether bacterial uptake by neutrophils is facilitated by fibronectin remains unclear (36–39). However, regardless of opsonic activity, binding of plasma fibronectin to the bacterial surface might block adhesion receptors on *S. aureus*, thus representing an important defense mechanism against tissue invasion (35, 40–42).

The native FnBP of *S. aureus* strain 8325-4 released by lysostaphin treatment has a M_r of 210,000 as determined by Western blot (immunoblot) analyses (data not shown), which coincides with the M_r of the corresponding protein from *S. aureus* strain Newman (7). We earlier had reported that *E. coli* containing the cloned gene for the FnBP from *S. aureus* 8325-4 produces two FnBPs with M_r values of 165,000 and 87,000, respectively, as determined by SDS/PAGE. The discrepancy between the 165-kDa FnBP produced in *E. coli* and the native 210-kDa FnBP from *S. aureus* may be explained by the presence of cell-wall components associated with the FnBP released by lysostaphin digestion. Furthermore, the polypeptide deduced from the nucleotide sequence of the cloned gene is only 108,000 in M_r. The difference between 165,000 and 108,000 is not due to deletions created during the cloning work because Southern blot hybridizations of DNA from *S. aureus* strain 8325-4 and plasmid pFR001 revealed restriction fragments of the same size. The high proline content within the W_r region (Fig. 4), which can cause abnormal migration in SDS/PAGE (compare ref. 43) may contribute to the big difference between deduced and determined molecular weights.

The structural gene (Figs. 1 and 2) is preceded by sequences that have common features with promoter and ribosome-binding sites reported for other staphylococcal genes (44, 45). The structural gene encodes an N-terminal signal peptide sequence of 36 amino acids. This signal sequence is longer than is typical for *E. coli* signal sequences, but this sequence is typical of signal sequences in Gram-positive bacteria, especially those of staphylococcal and streptococcal proteins (44, 46, 47). The FnBP signal peptide is apparently recognized by the *E. coli* membranes (17). In the C-terminal end the stop codon is followed by a palindromic sequence indicating a transcription termination signal. Repeat sequences such as B1–B2, D1–D4, and W_r1–W_r5 are present in this protein as well as in other cell-wall proteins from Gram-positive bacteria (43, 44, 46, 48).

We identified the D repeats as the fibronectin-binding domain. A *Bal* I–*Hin*cII clone (Fig. 1) contains the first 104 amino acids of the D region, which is equal to D1, D2, and 70% of D3 (Fig. 3). This clone has fibronectin-binding activity (17) as do synthetic peptides corresponding to the D1, D2, and D3 sequences. Furthermore, a rabbit antibody raised against the D2 peptide recognizes the native receptor and inhibits the binding of ^{125}I-labeled fibronectin to the isolated receptor protein (G.R. and M.H., unpublished work). These data help locate the fibronectin-binding domain to the D repeats. Because D1 and D3 are both active, but show only 42% similarity, a considerable variability in the amino acid sequence is clearly allowed without loss of inhibitory activity. Possibly the active site is located in a smaller conserved region of the three D sequences. The D peptides appear to have a much lower affinity for fibronectin than that of the ZZ–FR fusion protein. Perhaps this difference is due to conformational restrictions for optimal binding exhibited by the fusion protein but not by the individual synthetic peptides. An alternative explanation may be related to binding valency. The ZZ–FR fusion protein contains three binding sites (D1, D2, and D3) per molecule. Because the N-terminal region of fibronectin, which contains the staphylococcal binding site, consists of five repeats of type I homology, the binding of the fusion protein to this domain could involve three sites of ligand–receptor interactions. If this were true, the ZZ–FR fusion protein should theoretically have a substantially higher affinity for the fibronectin molecule compared with that of the individual synthetic peptides.

The extreme C-terminal end of FnBP, called region M, probably anchors the protein in the cell membrane. The M region has a hydrophobic structure followed by some positively charged amino acids. This structure exhibits a certain degree of similarity to the corresponding regions of staphylococcal protein A (44) and streptococcal proteins G and M (43, 46).

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