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# Role of Type III Homology Repeats in Cell Adhesive Function within the Cell-binding Domain of Fibronectin*

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**Recombinant fibronectin (FN) fragments and their mutant proteins were produced to elucidate the role of type III homology repeats in cell adhesive activity within the cell-binding domain of FN. Cell adhesive activity of the 11.5-kDa fragment, the cell attachment site of the cell-binding domain, was <0.1% that of native FN despite the presence of the Arg-Gly-Asp-Ser sequence. The activity increased as type III homology repeats were added to the N terminus of the 11.5-kDa fragment, and a 52-kDa fragment with four additional type III repeats had almost the same activity of native FN. Deletion of Arg-Gly-Asp from the fully active fragments completely abolished the cell adhesive activity. Deletion of one or two repeats from the 52-kDa fragment affected the extent of the cell adhesive activity, the degree of the effect being inversely correlated with the distance of the deletion from the type III repeat containing Arg-Gly-Asp-Ser. Rearrangement of type III repeats caused much loss of activity. These results suggest that the number and kinds of type III repeats and their correct alignment rather than the putative synergistic site decide the extent of the specific cell adhesive activity.**

Fibronectins (FNs)[1] are multifunctional cell adhesive glycoproteins present in the extracellular matrix and plasma. FN is important in a variety of cellular processes, including cell-to-substrate adhesion, cell migration, and regulation of cell morphology (Yamada, 1983; Thiery *et al.*, 1985; Hynes, 1986; Mosher, 1988). FN interacts with cells by means of a cell-binding domain that contains the tetrapeptide Arg-Gly-Asp-Ser (RGDS) sequence (Pierschbacher and Ruoslahti, 1984a, 1984b; Ruoslahti and Pierschbacher, 1986). The RGDS sequence serves as a recognition signal for the interaction with integrins, a family of cell-surface receptor molecules (Buck and Horwitz, 1987; Ruoslahti and Pierschbacher, 1987; Ruoslahti, 1988).

Other studies suggest that more than this sequence is needed for sufficient cell adhesive activity. Proteolytic fragments of 75–120 kDa of the cell-binding domain retain full activity in adhesion assays, but an 11.5-kDa fragment and smaller synthetic peptides lose 95–99% activity (Akiyama and Yamada, 1985; Akiyama *et al.*, 1985; Pierschbacher and Ruoslahti, 1984a; Streeter and Rees, 1987). Similar results were obtained by use of fusion proteins of the cell-binding domain of FN with β-galactosidase (Obara *et al.*, 1987).

Obara *et al.* (1988) have described a second adhesive recognition site that cooperates with the RGDS sequence to produce full adhesive activity. Deletion mutagenesis in these experiments identified a peptide sequence crucial for cell adhesive activity in an area more than two type III homology repeats away from the RGDS sequence, possibly at the type III-8 repeat. This second site seems to function synergistically with the first RGDS site; its precise location has not been established.

Another explanation of why additional information is required for full activity could be that the 75-kDa fragment contains information that specifies the correct folding and conformation of the RGDS sequence during its interaction with cell-surface receptor(s) (Pierschbacher and Ruoslahti, 1987).

Here, we attempted to assess both of these explanations of the mechanism of the cell adhesive signal and to elucidate the role of type III repeats for the cell adhesive function. We constructed several recombinant polypeptides in unfused form corresponding to the cell-binding domain of FN with different numbers of type III repeats and also constructed their mutant forms at the RGDS site or the putative second site. The relationship between the primary structure and cell adhesive activity was then studied with fibroblasts as the test cells. We found that the deletion of RGD or serine from the polypeptides containing both the putative second site and the RGDS site resulted in the complete loss of adhesive activity. Deletion of the second site alone from the polypeptide did not cause much loss of activity. Type III repeats were different in the extent to which they promoted cell adhesive activity, the order of which was related to their linear distance from the repeat containing the RGDS sequence (type III-10). Change in the relative position of type III repeats caused a great loss of activity compared with that of the original arrangement.

## EXPERIMENTAL PROCEDURES[2]

## RESULTS

*Relative Cell Adhesive Activity of Recombinant Fragments*—The 11.5-kDa cell adhesive fragment reported by Pierschbacher and Ruoslahti (1984a) was expressed in the periplasm of recombinant *Escherichia coli* HB101 cells harboring

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[1] The abbreviations used are: FNs, fibronectins; kb, kilobase(s).

[2] Portions of this paper (including "Experimental Procedures" and Figs. 1, 3, 4, 6, and 8) are presented in miniprint at the end of this paper. Miniprint is easily read with the aid of a standard magnifying glass. Full size photocopies are included in the microfilm edition of the Journal that is available from Waverly Press.

pTF102. The fragment, termed C-108, was purified by affinity chromatography. Two 5′-terminal deletion mutant plasmids, pTFD707 and pTFD1171, which expressed fragments C-279 and C-195, respectively, were constructed from plasmid pTF301 as described under "Experimental Procedures." In a similar way, two mutants, pTFB800 and pTFB900, which expressed C-385 and C-478, respectively, were constructed from pTF1101. Mutant proteins with deletions or a substitution at the RGDS site were also prepared by mutagenesis of the corresponding plasmids.

The locations of these polypeptides within the cell-binding domain and the profile by sodium dodecyl sulfate-polyacrylamide gel electrophoresis of affinity-purified polypeptides are shown in Figs. 2 and 3, respectively. The cell adhesive activities of the recombinant fragments were assayed as described under "Experimental Procedures." Typical results obtained by phase-contrast microscopy and relative activity by $ED_{50}$ are shown in Fig. 4 and Table I, respectively. The degree of spreading cells achieved with the 11.5-kDa polypeptide, C-108, was low even at a high concentration (40 μg/ml); and the relative activity of C-108 was <0.1% that of native FN (Table I). The 21-kDa polypeptide, C-195, which had an additional 87 amino acids but not the putative synergistic site at the N terminus of C-108, had ~0.5% of the activity of native FN. The 31-kDa polypeptide, C-279, which had two additional type III homology repeats and which contained the second site, was >80-fold as active as C-108, although its $ED_{50}$ was 16-fold that of native FN. When a high concentration was used, C-279 caused as much spreading as native FN (Fig. 4). C-385, with four type III repeats, was five times as active as C-279. C-478, with five type III repeats, caused as much spreading as native FN. The difference in cell adhesive activity among these proteins was not due to the differences in the amount of the proteins adsorbed on the plastic substrate; no significant difference in adsorption was found at the concentrations that gave a significant dose response (Fig. 8). These results are consistent with those obtained by Obara *et al.* (1988) and suggest that there is either a required sequence or a minimum size for maximum activity of the cell-binding domain.

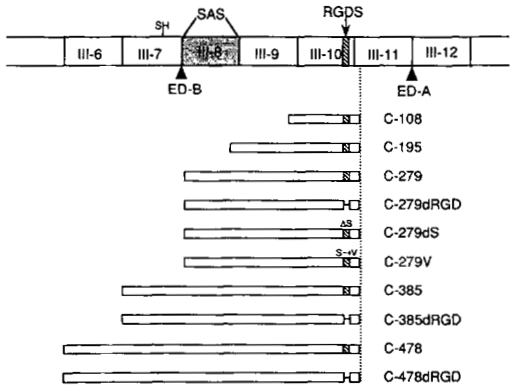


FIG. 2. **Diagram of recombinant FN fragments.** The *boxes* at the top show type III homology repeats. Type III repeats are numbered as described elsewhere (Kornblihtt *et al.*, 1985). The *shaded box* indicates the putative synergistic adhesion site (*SAS*). The *hatched boxes* indicate the RGDS sequence, and the *solid lines* in place of the *hatched boxes* indicate the absence of the RGD sequence. Deletion of serine from the RGDS sequence is indicated (Δ*S*), as is a change of serine to valine (*S* → *V*). The locations of the extra domains (*ED-A* and *ED-B*) are indicated with *closed triangles*.

TABLE I

*Cell spreading activity of FN fragments and their RGDS mutant forms*

The location of each fragment within the cell-binding domain is shown in Fig. 1. $ED_{50}$ is the concentration that gives 50% of the maximum number of spreading cells achieved with native human FN (Green Cross Corp., Osaka, Japan) as described under "Experimental Procedures."

| Polypeptides | Sequence at RGDS site | Cell spreading activity $ED_{50}$ |
|---|---|---|
| | | *nM* |
| FN | -GRGDSP- | 3 |
| C-108 | -GRGDSP- | >4000[a] |
| C-195 | -GRGDSP- | 1400 |
| C-279 | -GRGDSP- | 48 |
| C-279dRGD | -G---SP- | Inactive[b] |
| C-279dS | -GRGD-P- | Inactive[b] |
| C-279V | -GRGDVP- | 90 |
| C-385 | -GRGDSP- | 10 |
| C-385dRGD | -G---SP- | Inactive[b] |
| C-478 | -GRGDSP- | 4 |
| C-478dRGD | -G---SP- | Inactive[b] |

[a] Maximum number of spreading cells did not reach 50% of that obtained with FN even at 4000 nM.

[b] No cell adhesion was found at any concentration tested (≤10 μM).

*Mutational Analysis of RGDS Site*—Mutational analysis of the RGDS site was then performed to determine more about the sequences essential for the cell adhesive signal. Deletion of RGD or serine from the fragments containing the putative synergistic site, giving C-279dRGD, C-279dS, C-385dRGD, and C-478dRGD, resulted in a complete loss of activity even at 10 μM (Table I). The original fragment and the mutant forms adsorbed in equal amounts to the well (data not shown). When RGDS was converted to RGDV, which is the cell-binding site of vitronectin, giving C-279V, the activity of C-279 decreased slightly. Thus, the RGD sequence was essential for the cell adhesive signal, and the putative synergistic site alone did not have cell adhesive activity.

*Deletion Analysis of Type III Homology Repeats*—Several mutant proteins with deletions at the type III homology repeats were designed and produced to determine if there was a synergistic site. The deletion sites of the mutant proteins and their profile by sodium dodecyl sulfate-polyacrylamide gel electrophoresis are shown in Figs. 5 and 6, respectively. When the type III-8 repeat was removed from C-385, cell adhesive activity decreased to a level nearly equal to that of C-279, which was produced by the deletion of the type III-7 repeat from C-385 (C-385dIII-8; Fig. 7*a*, ◇). Cell adhesive activity decreased significantly after deletion of the type III-9 repeat from C-385 (C-385dIII-9; Fig. 7*a*, □). The number of spreading cells did not reach the level of C-385 even when a high concentration of C-385dIII-9 was used.

Type III deletion proteins derived from C-478 were examined in a similar way. When the type III-7 repeat was removed, cell adhesive activity decreased to a level lower than that of

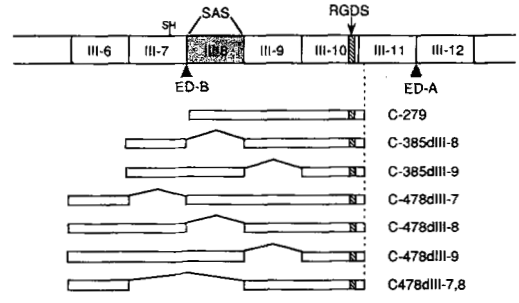


FIG. 5. **Diagram of deletion mutant proteins of type III homology repeats.** The entire sequence of the type III homology repeats was deleted as described under "Experimental Procedures." The *boxes* at the top are the same as those described in the legend of Fig. 2. Below, deleted regions are indicated by *angled solid lines*.

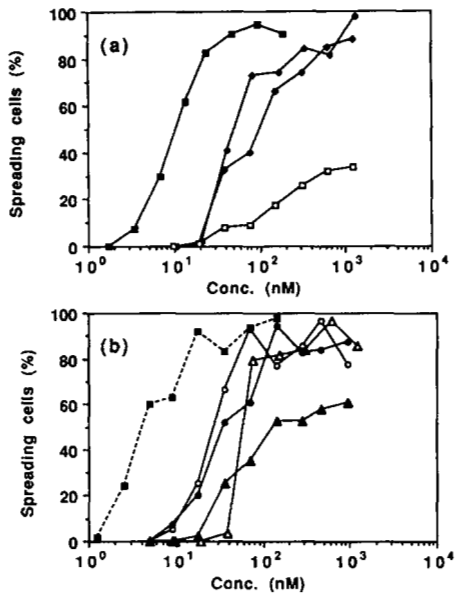


FIG. 7. **Effects on cell adhesive activity of deletion of type III homology repeats from C-385 and C-478.** Cell spreading activity was assayed as described under "Experimental Procedures." *a*, the activities of two deletion mutant forms of C-385, C-385dIII-8 (◇) and C-385dIII-9 (□), were compared with the activities of C-385 (■) and C-279 (♦). *b*, the activities of four deletion mutant forms of C-478 were compared with the activity of C-478. ■, C-478; ○, C-478dIII-7; ●, C-478dIII-8; ▲, C-478dIII-9; △, C-478dIII-7,8.

C-385, which was produced by the deletion of the type III-6 repeat from C-478 (C-478dIII-7; Fig. 7*b*, ○). The cell adhesive activity of C-478dIII-8 (Fig. 7*b*, ●), which was produced by the deletion of the type III-8 repeat from C-478, was nearly equal to that of C-478dIII-7. Deletion of the type III-9 repeat (C-478dIII-9; Fig. 7*b*, ▲), caused more loss of activity than that caused by other type III repeats. The activity of C-478dIII-7,8, which was produced by deletion of both type III-7 and III-8 repeats from C-478, was higher than that of C-478dIII-9, but lower than that of C-478dIII-8. Compared with C-279, which was produced by the deletion of both type III-6 and III-7 repeats from C-478, the activity of C-478dIII-7,8 was lower. These results suggest that there are differences in the contribution to cell adhesive activity among these type III homology repeats. The degree of the contribution seems to be related to their distance from type III-10, which contains the RGDS site.

*Effects of Rearrangement of Type III Homology Repeats*—A mutant form of C-478 in which the type III-9 repeat was placed between the type III-6 and III-7 repeats was constructed to determine whether the order of the repeats was crucial for achievement of the maximal cell adhesive activity. The procedures for the construction of the mutant protein are described in Table II. Cell adhesive activity of the mutant protein was one-sixtieth that of unmodified C-478 (Table II). These results show that not only the number of type III repeats but also their correct alignment is needed for full cell adhesive activity.

## DISCUSSION

In this study, we assessed the current two models for the cell adhesive signal within the cell-binding domain of FN. One model stresses the role of the conformation of the RGDS sequence (Pierschbacher and Ruoslahti, 1987), and the other postulates that there are two distinct binding sites (Akiyama and Yamada, 1987; Obara *et al.*, 1988). We produced more than 20 recombinant polypeptides in unfused forms with different numbers and combinations of type III homology repeats. Of them, C-108 had one type III homology repeat, (III-10), and its amino acid sequence was identical to that of the 11.5-kDa pepsin fragment containing the RGDS site characterized by Pierschbacher and Ruoslahti (1984a). Other polypeptides (C-195, C-279, C-385, and C-478) contained additional type III repeat(s) at the N terminus of C-108.

As would be expected from either model, cell adhesive activity increased as type III repeats were added to the N terminus of C-108. The 52-kDa fragment, C-478, which contained four additional type III homology repeats at the N terminus of C-108, had almost the same activity as that of native FN. These results were consistent with those reported previously (Obara *et al.*, 1988). In contrast, the deletion of RGD from C-279, C-385, or C-478 resulted in complete loss of activity even at high concentrations. The same result was obtained by the deletion of serine from the RGDS site of C-279. Replacement of serine by valine did not cause much loss of activity. These results were different from those obtained by Obara *et al.* (1988), who found very low but detectable activity when the RGDS sequence was removed from a fully active fragment. The reason for this discrepancy is not clear. It should be noted, however, that our results were obtained by use of four independent, unfused RGDS mutants with natives sequences of FN, but that Obara *et al.* used β-galactosidase fusion proteins for the assay.

Deletion analysis showed that there was a difference among the type III repeats in the extent to which they promoted cell adhesive activity. For full understanding of the results, changes in cell adhesive activity after the deletion of one of the type III repeats are summarized in Table III. Cell adhesive activity of C-478 and C-385 decreased with the deletion of one of the type III repeats in the order of types III-6, -7, and -8, and -9. In particular, deletion of the type III-9 repeat caused more loss of activity than that of other type III repeats. This does not necessarily mean that the type III-9 repeat served as the putative synergistic site because C-195, consisting of type III-9 and III-10 repeats, had a very low activity. These results indicate that the type III repeats were functionally distinguishable and suggest that the extent of their contribution to cell adhesive activity was inversely correlated to their distance from the RGDS site. In other words, our results show that cell adhesion-promoting activity of the type III repeats decreased depending on their distance from the RGDS site. These findings also suggest that a distinct second site is not present within the cell-binding domain, although the putative second site is reported to be at least two type III repeats away from the RGDS site (Obara *et al.*, 1988; Dufour *et al.*, 1988). This notion is supported by no significant loss of activity being observed after the deletion of both type III-7 and III-8 repeats from C-478. A fully active domain can be assembled by connection of the type III homology repeats in the correct order, despite the weak contribution of each type III repeat alone.

The distinct function of the type III-9 repeat may have to do with its being located immediately before the III-10 repeat, which contains the RGDS sequence, and with its containing more basic amino acids than the other type III repeats. After the attachment of a cell-surface receptor to the RGDS site, the positively charged type III-9 repeat adjacent to the RGDS site may well interact with other target(s), including heparan sulfate proteoglycan on the cell surface, thereby mediating cell spreading (Laterra *et al.*, 1983; Izzard *et al.*, 1986; Wood *et al.*, 1986; Saunders and Bernfield, 1988). This interaction could be enhanced by the presence of other type III repeats, which bring about proper folding and conformation of the

TABLE II

*Effects of rearrangement of type III homology repeats within the molecule of C-478*

A mutant form of C-478 in which the type III-9 repeat was placed between the type III-6 and III-7 repeats was constructed as follows. An *Apa*I site was introduced by site-directed mutagenesis into the position corresponding to the junction of type III-6 and III-7 repeats within plasmid pTFD0235, which expressed C-478dIII-9. In a similar way, restriction sites were introduced into the positions corresponding to the N and C termini of the type III-9 repeat within plasmid pTFB900. A 0.27-kilobase *Apa*I fragment was isolated from the modified pTFB900 plasmid and inserted into the *Apa*I site of the modified pTFD0235 plasmid. Two *Apa*I sites formed by the ligation were removed to generate the original sequence. Plasmid pTFD0236 thus obtained had the sequence for the rearranged form of C-478. The mutant protein was purified, and the cell adhesive activity was compared with that of C-478 and C-478dIII-9.

| Proteins | Alignment of type III repeats | $ED_{50}$ |
|---|---|---|
| | | *nM* |
| C-478 | III-6-III-7-III-8-III-9-III-10 | 4 |
| C-478dIII-9 | III-6-III-7-III-8- - -III-10 | 110 |
| Rearranged form of C-478 | III-6-III-9-III-7-III-8-III-10 | 250 |

TABLE III

*Changes in cell adhesive activity by the deletion of type III homology repeats*

| Polypeptide | No. of type III repeats | Activity as $ED_{50}$ | | | | |
|---|---|---|---|---|---|---|
| | | Before deletion[a] | After deletion of[b]: | | | |
| | | | III-6 | III-7 | III-8 | III-9 |
| | | *nM* | | | | |
| C-195 | 2 | 1400 | | | | >4000 |
| C-279 | 3 | 48 | | | 1400 | ND[c] |
| C-385 | 4 | 9 | | 48 | 70 | >1000 |
| C-478 | 5 | 4 | 9 | 25 | 30 | 110 |

[a] Values are taken from Table I.

[b] Values were estimated from the dose-response curve shown in Fig. 7 (*a* and *b*).

[c] ND, not done.

positively charged region of the type III-9 repeat and RGDS site. From these points of view, it is of interest that the positively charged type III-14 repeat is adjacent to the type III connecting segment because B16 melanoma cells preferentially adhere to the CS1 region of this segment (Humphries *et al.*, 1987). Also, the type III-14 repeat is a C-terminal repeat of the heparin-binding domain, suggesting that this domain is responsible for the promotion of the cell spreading activity of B16 melanoma cells.

The importance of the type III-9 repeat is also suggested by the dislocation of the type III-9 repeat of C-478 between type III-6 and III-7 repeats, resulting in a large decrease in the cell adhesive activity, whereas the mutant protein contains the same set of type III repeats as the unmodified C-478. These results indicate that the type III-9 repeat can enhance the effects of the type III-10 repeat, which contains RGDS, only when the type III-9 repeat is located immediately before the N terminus of the type III-10 repeat, irrespective of the overall number of type III repeats preceding the type III-10 repeat. The reason why correct alignment of type III repeats is required for full activity is unclear. Such alignment may be responsible for the proper folding of the cell-binding domain, after which the RGDS sequence is exposed to the surface of the molecule, enhancing affinity to the integrin receptor(s). Conformational changes could be another explanation for the reduced activity caused by the deletion of type III repeats.

Previously, we found that monoclonal antibody FN30-8, which binds to a locus ~150-230 amino acids upstream of the RGDS sequence, inhibits cell attachment to a substrate coated with FN more strongly than monoclonal antibody FN12-8, which binds to the RGDS signal (Katayama *et al.*, 1989). This finding is not contradictory to those described above because, despite binding to a locus far from the RGD signal, the FN30-8 antibody may be able to interfere sterically with the interaction of the signal with its receptor(s) on the cell surface.

We have also reported that cell adhesive function can be conferred to any protein by the introduction of the RGDS sequence by use of recombinant DNA technology (Maeda *et al.*, 1989). This suggests that another adhesion site besides the RGDS sequence is not essential for cell adhesive activity, although additional information is required for full adhesive function.

In conclusion, 1) relative cell adhesive activity seems to depend mostly on the number of type III repeats, rather than on the putative synergistic site; 2) the RGD sequence, not the putative synergistic site, is essential for the cell adhesive signal; and 3) type III repeats differ in their relative contribution to cell adhesive activity, the order of which is related to their distance from the RGDS site. In particular, the type III-9 repeat, which is closest to the type III-10 repeat, which contains the RGDS site, is crucial. Correct alignment of type III repeats within the cell-binding domain is also needed for full cell adhesive activity.

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Supplementary Material to:

**Role of type III homology repeats in cell adhesive function within the cell-binding domain of fibronectin**

Fusao Kimizuka, Yoichi Ohdate, Yasutoshi Kawase, Tomoko Shimojo, Yuki Taguchi, Kimikazu Hashino, Shoichi Goto, Hidetaka Hashi, Ikunoshin Kato, Kiyotoshi Sekiguchi, and Koichi Titani

## Experimental Procedures

All protein sequences are numbered by the system of Kornblihtt *et al.* (1985) and all nucleotide sequences are numbered as in the EMBL data bank file HUMFNMC of Kornblihtt.

cDNA clones

Two cDNA clones, pLF5 (Sekiguchi *et al.*, 1986) and pUFN74, were used for the construction of expression plasmids. Plasmid pLF5 is a derivative of pUC13, and it contains a 1.4-kb insert corresponding to Thr1218-Thr1778, but lacking the extra domain A (Asn1600-Thr1689). The plasmid pUFN74 was constructed from human liver mRNA by the method of Gubler and Hoffman (1983), giving cDNA that covered the region upstream from the cell-binding domain. A cDNA library constructed by primer extension was screened by plaque hybridization with use of a 0.43-kb *Pvu*II-*Eco*RI fragment of pLF5 as the probe. One clone among the 24 positive clones contained a 1.1-kb insert (G2990 to A4105), which was subcloned into pUC118 and termed pUFN74.

Construction of an expression plasmid for the 11.5-kDa fragment

A 0.76-kb *Ba*II fragment was isolated from pLF5 and digested with *Fok*I. The two fragments obtained (0.1 and 0.2 kb) were ligated to each other to yield a 0.3-kb fragment. Deficient sequences at both ends were filled with two synthetic double-stranded DNAs so as to create cloning sites (*Eco*RI sites) at both end and to express the entire, unfused sequence of the 11.5-kDa fragment. The 0.34-kb fragment thus obtained was inserted into the *Eco*RI site of a secretion vector, pIN-III-*ompA*-1 (Ghrayeb *et al.*, 1984) to yield the expression plasmid pTF101 (Fig. 1a). The extra sequence derived from the vector was removed by site-directed mutagenesis as described below. The plasmid obtained, pTF102, expressed the 11.5-kDa polypeptide with 108 amino acids (Ile1410-Met1517, referred to as C-108 here), and some of these polypeptides were secreted into the periplasm of *E. coli* cells, to judge from results of fractionation done by published procedures (Neu and Hepel, 1965) followed by immunoblotting as described below.

Construction of an expression plasmid for a 31-kDa fragment

A 0.25-kb *Pvu*II-*Eco*RI fragment from pTF101 was ligated with a 0.6-kb *Pvu*II fragment from pLF5 previously modified with methylase *Eco*RI. The resultant 0.86-kb fragment was inserted into the *Eco*RI site of pIN-III-*ompA*-1 by use of an *Eco*RI linker. The plasmid pTF301 (Fig. 1a) thus obtained expressed a 31-kDa fragment with 283 amino acids (Ala1235-Met1517, termed C-283) that had an additional 175 amino acids at the N-terminus of C-108; its expression level was very low. This plasmid was used for 5'-terminal deletions.

Construction of an expression plasmid for a 52-kDa fragment

A 0.5-kb *Bam*HI-*Hin*dIII fragment isolated from pTF301 previously modified with methylase *Eco*RI was ligated with a 1.0-kb fragment from pUFN74 to construct a 1.5-kb fragment corresponding to a 52-kDa fragment with 504 amino acids (Gly1014-Met1517, termed C-504) that had an extension at the N-terminus of C-108. The 1.5-kb fragment was subcloned into the *Hin*dIII-*Eco*RI site of pUC19, followed by partial digestion with *Eco*RI. The fragment obtained was cloned into the *Eco*RI site of pIN-III-*ompA*-1 to yield pTF1101 (Fig. 1a), which expressed a very low level of the 52-kDa fragment C-504. This plasmid was also used for 5'-terminal deletions.

Construction of 5'-terminal deletion mutant forms

The plasmids pTF301 and pTF1101 were used for the construction of a series of 5'-terminal deletion mutant forms. Each plasmid was linearized with *Xba*I, partially digested with Bal 31 nuclease, and digested with *Hin*dIII. The 5'-terminal deletion fragments thus obtained were cloned into the *Nco*I-*Hin*dIII site of the expression vector pUC119N (Maki *et al.*, 1987) by use of an *Nco*I linker (Fig. 1b). The 13 immunologically positive clones were examined for expression in the cytoplasm of *E. coli* HB101 by immunoblotting, and the base sequence of the 5'-end of the inserts was identified. The coded sequences were 478, 385, 279, 258, 219, 213, 207, 206, 198, 195, 190, 186, and 178 amino acids long, counting from Met1517 as the C-terminus. The N-terminal sequences of the purified proteins were also identified by use of a protein sequencer (model 477A/120A, Applied Biosystems, Foster City, CA). Four polypeptides with 195, 279, 385, and 478 amino acids (termed respectively C-195, C-279, C-385, and C-478) were used for the assay of cell adhesive activity. Three plasmids, pTFD707, pTFB800, and pTFB900, corresponding to C-279, C-385, and C-478, respectively, were used for further mutagenesis.

Site-directed mutagenesis

Oligonucleotide-directed mutagenesis was done with a commercially available mutagenesis kit (Mutan-K, Takara Shuzo, Kyoto, Japan) constructed by the method of Kunkel (1985). Single-stranded DNAs for mutagenesis were isolated from the 5'-terminal deletion clones pTFD707, pTFB800, and pTFB900 by infection by the helper phage M13 KO7. Oligonucleotide primers for the introduction of a mutation at the desired position were designed and synthesized by use of an automatic DNA synthesizer (model 380A, Applied Biosystems). Procedures used for mutagenesis were those in the kit instructions, except that long-chain oligonucleotide primers were used for the deletion of the entire sequence of type III homology repeat(s). As a simple example, a 30mer primer, 5'-TGGGGAATCAAGACCTGGGATGATGGTATC-3', designed for the deletion of the type III-8 repeat, was made up of the 3' half immediately upstream of the type III-8 repeat plus the 5' half immediately downstream.

Immunoblotting and enzyme-linked immunosorbent assay (ELISA)

Two monoclonal antibodies FN12-8 and FN30-8 were used for immunoblotting and ELISA as described below (Katayama *et al.*, 1989).

Protein purification

General procedures for the purification of recombinant polypeptides were as follows. *E. coli* cells transformed with a recombinant plasmid were grown in L-broth and harvested. Cells were suspended in 20 mM Tris-HCl, pH 8.0, containing 5 mM EDTA, 5 mM 2-mercaptoethanol, and 4 μM phenylmethylsulfonyl fluoride, and sonicated. The sonicated extract was put on a column of DEAE-Toyopearl (Tosoh, Osaka, Japan) and eluted with a linear gradient of NaCl. The immunologically positive fractions were pooled and put on an affinity column of Sepharose 4B bound with FN12-8. After the column was washed with 20 mM Tris-HCl, pH 8.0, containing 100 mM NaCl, and then with 20 mM ammonium acetate, the bound protein was eluted with 40 mM acetic acid. Fractions that gave a single band in SDS-PAGE were neutralized with aqueous ammonia and lyophilized.

Cell adhesion assay

The cell adhesive activity of the purified polypeptides was assayed basically as described elsewhere (Yamada and Kennedy, 1984; Humphries *et al.*, 1986). A 96-well immunoplate (Nunc, Roskilde, Denmark) was coated with 50 μl per well of a recombinant polypeptide at the concentration of $5 \times 10^3$ nM and also serially diluted with phosphate-buffered saline (PBS), pH 7.4, and the plate was kept at 4°C overnight. After blocking of the plate with 3% bovine serum albumin in PBS for 1 hr, the plate was washed twice with PBS and incubated with 0.1 ml of trypsinized baby hamster kidney (BHK) cells suspended in serum-free Dulbecco's modified Eagle's medium ($5 \times 10^5$ cells/ml). After incubation at 37°C for 1 hr, unattached cells were removed and the plate was washed twice with PBS. The attached cells were fixed with 4% formaldehyde and photographed by phase-contrast microscopy. The percentage of cell spreading was calculated for each dilution and the concentration required to produce 50% spreading (i. e., the $ED_{50}$) was estimated from a dose-response curve. The $ED_{50}$ was defined as the concentration that gave 50% of the maximum number of spreading cells achieved with native FN.

Protein adsorption assay

The amount of proteins adsorbed on the plastic substrate in the cell adhesion assay was determined by ELISA. The immunoplate was coated with 50 μl of a solution of a recombinant polypeptide as described above. After blocking of the plate, FN12-8 monoclonal antibody was added and the plate was incubated at room temperature for 60 min. The plate was washed twice with PBS and incubated with a second antibody labeled with peroxidase. After washing of the plate, the peroxidase activity remaining was measured by use of *o*-phenylenediamine as the chromogen substrate. The amount of proteins adsorbed on the well was expressed as the absorbance at 492 nm. The relationship between the absorbance and the amount of proteins was obtained with C-279 as one example by sandwich ELISA with use of another monoclonal antibody, FN30-8, and peroxidase-labeled FN12-8. A PBS solution of C-279 was added to the well coated with FN30-8. After incubation, the well was washed twice with PBS and then incubated with the enzyme-labeled FN12-8. Peroxidase activity was measured as described above.

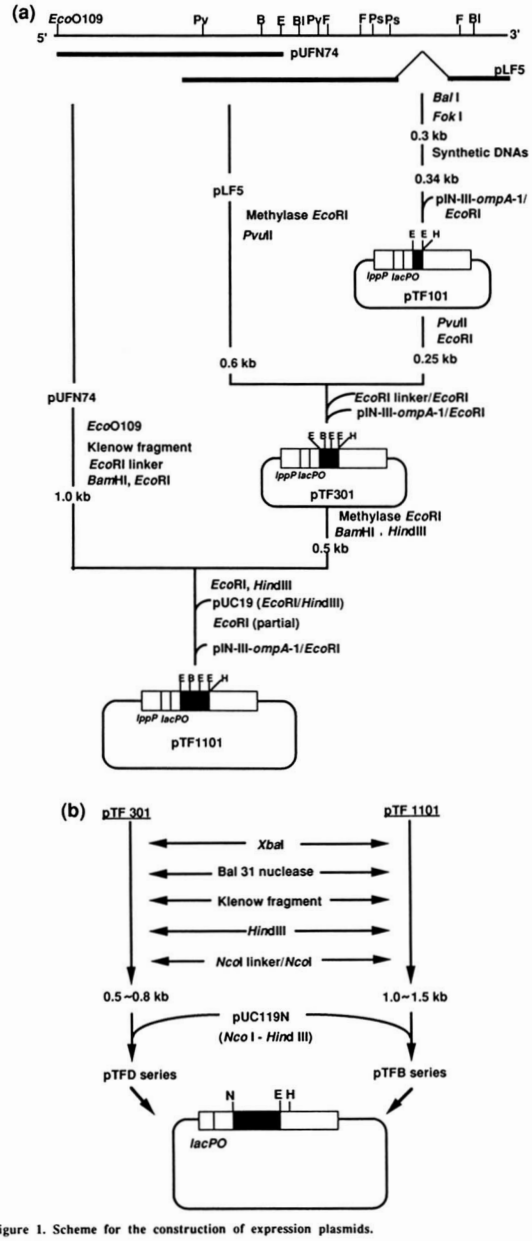


Figure 1. Scheme for the construction of expression plasmids.

(a) The solid line at the top represents the restriction map of the coding region of two cDNA clones, pLF5 and pUFN74, which are shown below it by solid bars. pTF10L was constructed from pLF5, a secretion vector pIN-III-*ompA*-1, and synthetic DNAs for the expression of the 11.5-kDa fragment (Ruoslahti and Pierschbacher, 1984) containing 108 amino acids. pTF301 was constructed from pTF101, pLF5, and the secretion vector for the expression of a 31-kDa fragment, and used for 5'-terminal deletion of the coding region. pTF1101 was constructed from pTF301, pUFN74, and the secretion vector for the expression of a 52-kDa fragment. It was also used for 5'-terminal deletion of the coding region. Details of procedures are described in Experimental Procedures. Closed boxes in the plasmids indicate coding regions. These fragments are expressed under the control of an *lpp* promoter (*lppP*) and *lac* promoter (*lacPO*). Abbreviations for restriction sites: BI, *Bal*I; B, *Bam*HI; E, *Eco*RI; F, *Fok*I; H, *Hin*dIII; N, *Nco*I; Ps, *Pst*I; Pv, *Pvu*II.
(b) Two sets of 5'-terminal deletion fragments were isolated from linearized plasmids pTF301 and pTF1101 by partial digestion with Bal 31 nuclease, followed by *Hin*dIII digestion. The fragments were cloned into the *Nco*I-*Hin*dIII site of an expression vector pUC119N that contained an initiation codon at the cloning site. The deletion clones, the pTFD series from pTF301 and the pTFB series from pTF1101, were immunologically screened by use of a monoclonal antibody, FN12-8.

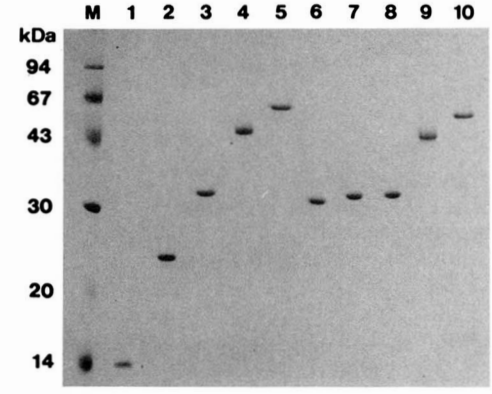


Figure 3. Profile of affinity-purified recombinant fragments and their RGDS mutant forms by SDS-PAGE.

Affinity-purified recombinant fragments and their RGDS mutant forms were put on a 15% gel (2 μg per lane) and stained with Coomassie Brilliant Blue. Lane M shows molecular weight markers; lane 1, polypeptide C-108; lane 2, C-195; lane 3, C-279; lane 4, C-385; lane 5, C-478; lane 6, C-279dRGD; lane 7, C-279dS; lane 8, C-279V; lane 9, C-385dRGD; and lane 10, C-478dRGD.

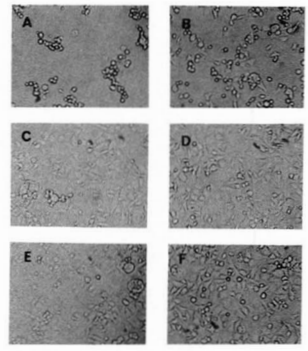


Figure 4. Attachment and spreading of BHK cells on a substrate coated with recombinant FN fragments.

Trypsinized BHK cells were incubated on the plastic substrate precoated with the following proteins at 40 μg/ml: A, C-108; B, C-195; C, C-279; D, C-385; E, C-478; F, native FN.

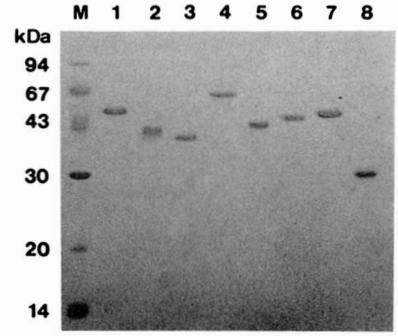


Figure 6. Profile of affinity-purified deletion mutant proteins of type III homology repeats by SDS-PAGE.

Affinity-purified mutant forms were put on a 15% gel (2 μg per lane) and stained with Coomassie Brilliant Blue. Lane M shows molecular weight markers; lane 1, polypeptide C-385; lane 2, C385dIII-8; lane 3, C-385dIII-9; lane 4, C-478; lane 5, C-478dIII-7; lane 6, C-478dIII-8; lane 7, C-478dIII-9; and lane 8, C-478dIII-7,8.

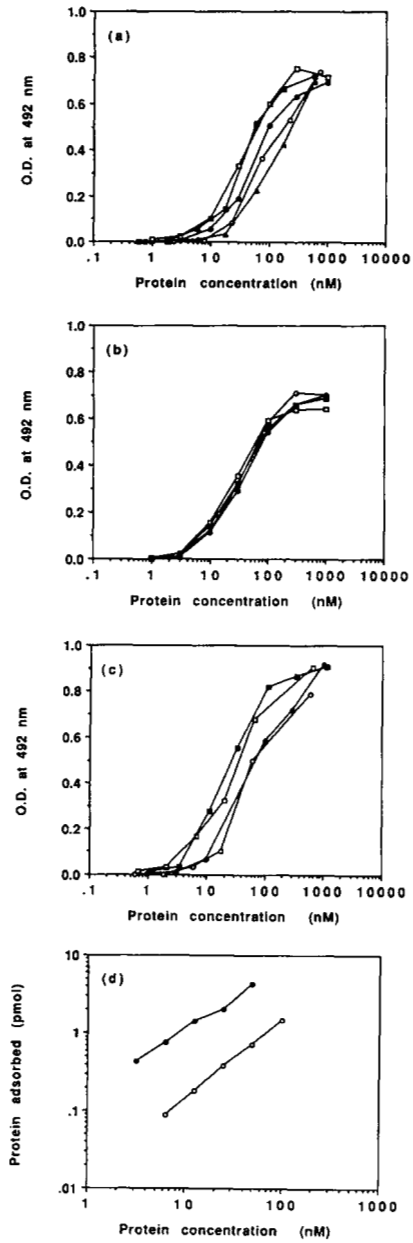


Figure 8. Protein adsorbed on the well at different concentrations.

(a) Effects of chain length on the adsorption of recombinant proteins. Symbols: open circles, C-108; closed circles, C-195; open squares, C-279; closed squares, C-385; open triangles, C-478.
(b) Effects of type III alignment on the adsorption of recombinant proteins with three repeats. Symbols: open circles, C-279; closed circles, C-385dIII-8; open squares, C-385dIII-9; closed squares, C-478dIII-7,8.
(c) Effect of type III alignment on the adsorption of recombinant proteins with four repeats. Symbols: open circles, C-385; closed circles, C-478dIII-7; open squares, C-478dIII-8; closed squares, C-478dIII-9.
(d) Relationship between the protein concentration and the protein adsorption. C-274 and FN were used as examples and sandwich ELISA was done to measure the amount of protein adsorbed on the wells by calculation from the value of absorbance at 492 nm. Symbols: open circles, C-279; closed circles, FN.