

## Effects of Fibronectin-Type V Collagen Recombinant Fusion Protein on Cell Adhesion and Cell Proliferation

Mika Hatai<sup>1</sup>, Kazuhiko Takahara<sup>2</sup>, Hidetaka Hashi<sup>2</sup>, Ikunoshin Kato<sup>2</sup> and Yoshihito Yaoi<sup>1\*</sup>

<sup>1</sup>Biology Division, National Cancer Center Research Institute, Tsukiji 5-1-1, Chuo-ku, Tokyo 104, and

<sup>2</sup>Biotechnology Research Laboratories, Takara Shuzo Co., Seta 3-4-1, Ohtsu, Shiga 520-21, Japan

**Key words:** fibronectin/type V collagen/cell adhesion/insulin

**ABSTRACT.** An expression vector pTF7520-Col-V-In, which encodes a fusion protein of the cell-binding domain of fibronectin (C277) and the insulin- and heparin-binding domain of the  $\alpha 1$  chain of human type V collagen, was constructed. *E. coli* transfected with this plasmid synthesized a 50-kDa fusion protein. This fusion protein, C277-V, was purified from the crude extract by a single step heparin HPLC. Similar amounts of insulin bound to purified C277-V and to the  $\alpha 1$  chain of type V collagen as judged by the binding of peroxidase-conjugated insulin. Cell-adhesive activity of C277-V was lower than that of the original fibronectin fragment C274, but similar numbers of cells adhered to both protein substrates when the culture dishes were coated with 1 mM of each protein. Insulin bound to the C277-V substratum stimulated the growth of mouse mammary tumor MTD cells in serum-free culture medium.

A variety of endothelial cell growth factors, such as fibroblast growth factors, bind to cell surface heparan sulfate proteoglycans (9). These interactions of growth factors with extracellular matrix are important in the compartmentalization, storage, stabilization, and modulation of the activities of growth factors. We studied the interaction of insulin with several extracellular matrix proteins, and reported that type V collagen binds to insulin most strongly (15). The insulin-binding site of type V collagen is in a 30-kDa CNBr fragment of the  $\alpha 1(V)$  chain. This region is rich in basic amino acids, and also binds to heparin (14). We determined the complete primary structure of the cDNA of human collagen  $\alpha 1(V)$  chain, and found that the insulin- and heparin-binding region is located in the N-terminal half of the  $\alpha 1$  chain (12).

Insulin bound to the type V collagen substratum stimulates the DNA synthesis of mouse mammary tumor MTD cells (15). Type V collagen is therefore expected to be a useful cell adhesion protein for cell culture, because insulin is an important growth factor for a variety of cultured cells. However, many types of cell cannot adhere to type V collagen, and type V collagen even inhibits the adhesion of endothelial cells or fibroblasts to fibronectin (1, 3). This antiadhesive activity of type V collagen is mainly found in the  $\alpha 1(V)$  chain, but the activity was abolished after treatment of type V collagen

with CNBr (3). We therefore thought that the 30-kDa CNBr fragment of the  $\alpha 1(V)$  chain, which binds to insulin and heparin, has little antiadhesive activity.

Kimizuka *et al.* (6, 7) prepared several recombinant fibronectin fragments that contained the cell binding Arg-Gly-Asp-Ser sequence, and reported that these recombinant fragments had the cell-adhesive activity. One of the active fragments, C277, contains three pieces of type III repeats of fibronectin, and one Arg-Gly-Asp-Ser sequence in the C-terminal part of the peptide (7). In the present study, we constructed a plasmid encoding the insulin- and heparin-binding region of human  $\alpha 1(V)$  chain, and attempted to make a fusion protein of this peptide with the fibronectin fragment C277. Such a fusion protein is expected to have both cell-adhesive and insulin-binding activities. Here, we report the construction of the fusion gene, its expression in *E. coli*, purification of the fusion protein, and the insulin-binding and cell-adhesive properties of the purified fusion protein.

### MATERIALS AND METHODS

**Materials.** Restriction enzymes, cloning vectors M13mp18, M13MP19, pTV119N, pUC118, and pUC119, isopropyl- $\beta$ -D-thio-galactopyranoside (IPTG), and cell-adhesive fibronectin peptide C274, were the products of Takara Shuzo Co. (Kyoto). Aprotinin, leupeptin, phenylmethyl-sulfonyl fluoride (PMSF), ampicillin, bovine crystalline insulin, and bovine serum albumin (BSA, RIA grade) were purchased from Sigma (St. Louis, MO).

\* To whom reprint requests and correspondence should be addressed.

**Construction of plasmid.** A cDNA fragment that encodes the insulin-binding region of the  $\alpha 1$  chain of human type V collagen was amplified by polymerase chain reaction (PCR) on the basis of the previously reported DNA sequence of the  $\alpha 1(V)$  (12). A template cDNA was synthesized from 1  $\mu$ g of human placental mRNA (Clontech) using Rous associated virus-2 reverse transcriptase (Takara Shuzo) (2). The PCR was done with 100 pmol each of primers:

5'-GATCCCATGGGCATCCGTGGTCTGAAG-3', and  
5'-  
GATCGGATCCTTAAGTCTCGCCTCTCTGTCC-3',

and one-tenth of the synthesized template by the use of a DNA amplification kit (GeneAmp™, Cetus), with 30 repeats a cycle of 0.5 min at 95°C for denaturation, 0.5 min at 55°C for annealing, and 1 min at 72°C for elongation. The amplified fragment had additional *Nco*I site, stop codon, and *Bam*HI site for cloning and termination of translation. The fragment was digested with *Nco*I and *Bam*HI, and then ligated into *Nco*I-*Bam*HI site of plasmid pTV118N. A DNA fragment was prepared from this plasmid by treatment with *Nco*I-*Hind*III, and ligated with a plasmid pTF7520, which encodes the cell binding domain of human fibronectin (Fig. 1). The resulting plasmid that encodes the fusion protein of fibronectin and type V collagen was termed pTF7520-Col-V-In. Nucleotide sequence of the plasmid was confirmed by the dideoxy chain termination method (11) using suitable cloning vectors with the enzyme Sequenase ver. 2.0 (U.S. Biochemical).

**Purification of fusion protein.** *E. coli* strain JM109 having the plasmid pTF7520-Col-V-In was grown to late log phase in 100 ml of ampicillin-containing LB medium at 37°C. IPTG (1 mM) was added to the medium and cultivation was continued overnight. The cells were harvested by centrifuga-

tion, washed with phosphate buffered saline (PBS), and then disrupted by sonication in 5 ml of lysis buffer (PBS that contained 1 mM EDTA, 0.05% Nonidet P40, 10  $\mu$ g/ml aprotinin, 10  $\mu$ g/ml leupeptin, and 2 mM PMSF). The extract was centrifuged at 12,000 r.p.m. for 15 min, and 1 ml of the supernatant was put on a HPLC column of Heparin 5PW (Tosoh, Tokyo). After the column was washed with PBS, the bound material was eluted with PBS that contained 0.5 M NaCl at a flow rate of 1 ml/min. Purity of the proteins was tested by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) in 4–20% gradient gels in Laemmli's buffer system (8).

**Amino acid sequencing of fusion protein.** The fusion protein isolated by a heparin column was separated by SDS-PAGE. Peptides were electrophoretically transferred to polyvinylidene difluoride membranes (Millipore) (13), and sequenced with a protein sequencer (477A, Applied Biosystems). The phenylthiohydantoin derivatives of amino acids were identified with a separate analyzer (120A, Applied Biosystems) connected to the sequencer.

**Binding of insulin-peroxidase.** Proteins were separated by SDS-PAGE and then transferred to nitrocellulose membranes (BA 85, Schleicher & Schuell, Keene, NH) by the method of Towbin *et al.* (13). After the membranes were washed with PBS, they were overlaid with PBS that contained 5  $\mu$ g/ml insulin-peroxidase (1.2 mol horseradish peroxidase/mol bovine insulin; Sigma) and 0.005% Tween 20 at room temperature for 2 hr. The bound insulin-peroxidase was detected with 4-chloro-1-naphthol (Merck, Darmstadt) as the substrate (15).

**Cell adhesion assay.** Swiss mouse 3T3 cells and mouse melanoma B16-F10 cells were cultivated in Dulbecco's modified Eagle's minimal essential medium (DMEM, Nissui Pharmaceutical Co., Tokyo) that contained 10% calf serum (GIBCO,

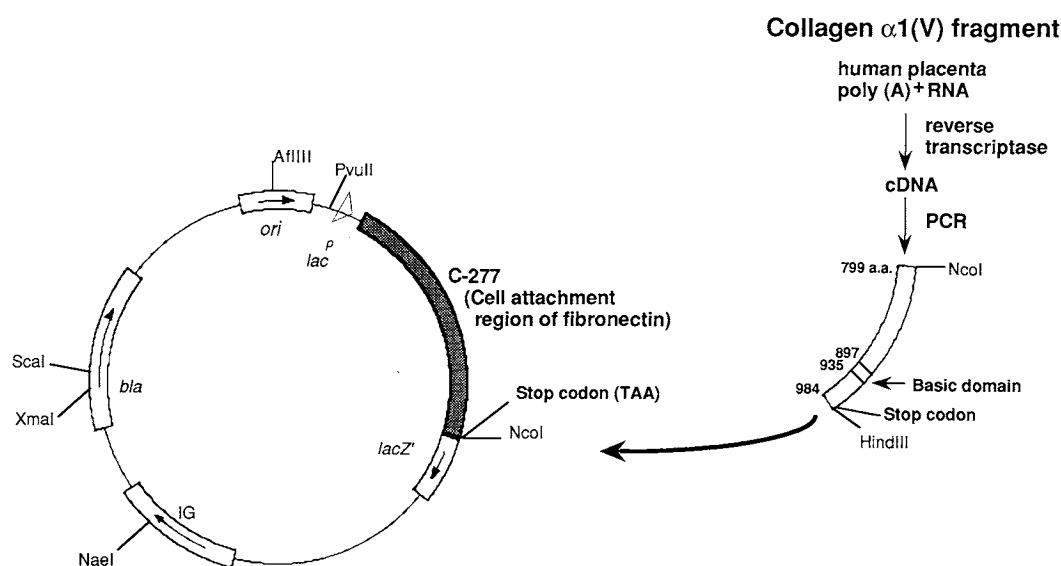


Fig. 1. Construction of a plasmid pTF7520-Col-V-In for the expression of fibronectin-type V collagen fusion protein.

Grand Island, NY). Nunclon 24-well multiplates (Nunc, Roskilde) were coated with C274 or fusion protein at room temperature for 2 hr, and any uncoated surface of the wells were blocked with 2% BSA at 37°C for 2 hr. Cells were trypsinized and suspended in a small amount of DMEM containing 0.5 mg/ml soybean trypsin inhibitor (Sigma), and then diluted with serum-free DMEM. Approximately  $10^5$  cells were put in each well, and the wells were incubated at 37°C for 2 hr. The wells were then washed with PBS, and the cells were dispersed by trypsinization. The numbers of the cells were counted by a Coulter Counter Model D.

**Growth of MTD cells.** A mouse mammary tumor cell line, MTD (supplied by Dr. J. Enami, Dokkyo University) was maintained in Eagle's MEM supplemented with 10% fetal bovine serum. The serum-free culture medium used for the assay of the growth-stimulating activity of insulin was a mixture of DMEM and Ham's F10 medium (Nissui) that contained 10  $\mu$ g/ml transferrin (human, Sigma) and 5 mg/ml BSA (tissue culture grade, Nitta Gelatin, Osaka).

## RESULTS

**PCR and expression of fusion gene.** The PCR yielded a DNA fragment of about 600 base pairs (data not shown), which encoded amino acids number 799 to 984 of human procollagen  $\alpha 1(V)$ . The DNA sequence of the fragment was confirmed by the dideoxy chain termination method. An expression vector pTF7520-Col-V-In, that encodes the fusion protein of fibronectin and  $\alpha 1(V)$ , was constructed as described in Fig. 1. *E. coli* strain JM109 was transfected with the expression vector and was grown in 100 ml of LB medium at 37°C. IPTG (1 mM) was added to the medium when the OD 600 of the culture was 0.3. Cultivation was continued until the OD600 reached 1.0, and the cells were harvested and then disrupted by sonication. The insoluble materials were removed by centrifugation.

SDS-PAGE analysis of the total cellular extract showed that a new protein component of about 50-kDa was induced by IPTG in JM109-pTF7520-Col-V-In cells (Fig. 2, lane 3), which was not found in the control *E. coli* strain JM109 (Fig. 2, lane 1) or in the transfected cells before induction by IPTG (Fig. 2, lane 2). The size of this component agreed with the calculated molecular weight (48, 160) of the fusion protein (7, 12).

**Purification of fusion protein.** The extract (1 ml) was put on a column of Heparin 5PW. After the column was washed with PBS, the bound materials were eluted by addition of 0.5 M NaCl (Fig. 3A). The heparin-bound fraction contained one major (50-kDa) and one minor (48-kDa) components (Fig. 3B, lane 3). Analysis of the N-terminal amino acid sequences of these two components indicated that they had the same N-terminal structure, Pro-Thr-Asp-Leu-Arg-Phe-Thr-Asn-Ile, which agreed with that of C277. The N-termi-

nal methionine residue predicted from the DNA sequence was probably cleaved off after the peptides were synthesized in *E. coli*. The 48-kDa fragment seemed to be produced by the proteolytic degradation of the C-terminal part of the 50-kDa fusion protein. If the proteinase inhibitors were omitted from the extraction buffer, a number of broken-down products of various sizes appeared (data not shown). We call this 50-kDa fusion protein C277-V. About 2 mg of C277-V was obtained from 100 ml culture.

**Insulin-binding and cell-adhesive properties of C277-V.** Figure 4 shows the binding of peroxidase-conjugated insulin to the *E. coli* extract and to purified C277-V on nitrocellulose membranes. As a control, we used the recombinant fibronectin fragment C274, which lacks three amino acid residues of the C-terminus of C277 but has similar cell-adhesive activity as C277 (7). Insulin-peroxidase bound to a 30-kDa protein present in the extract in the control *E. coli* strain JM109, which

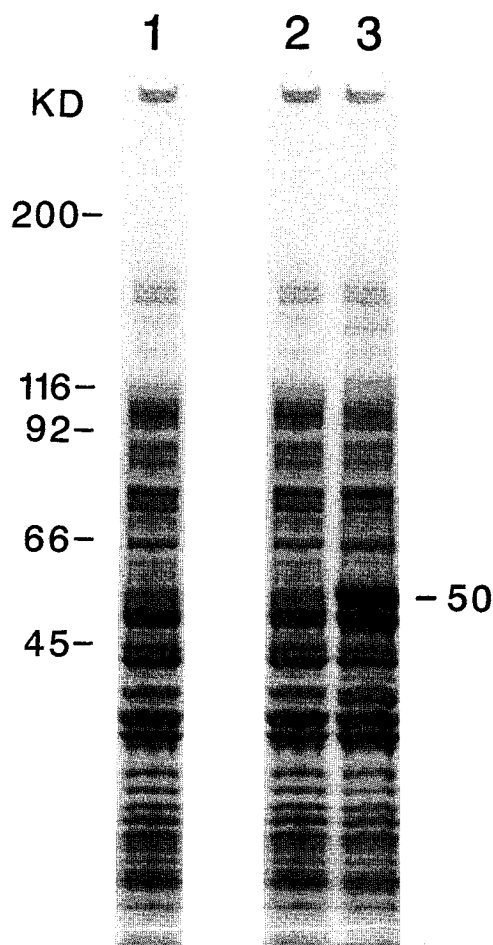
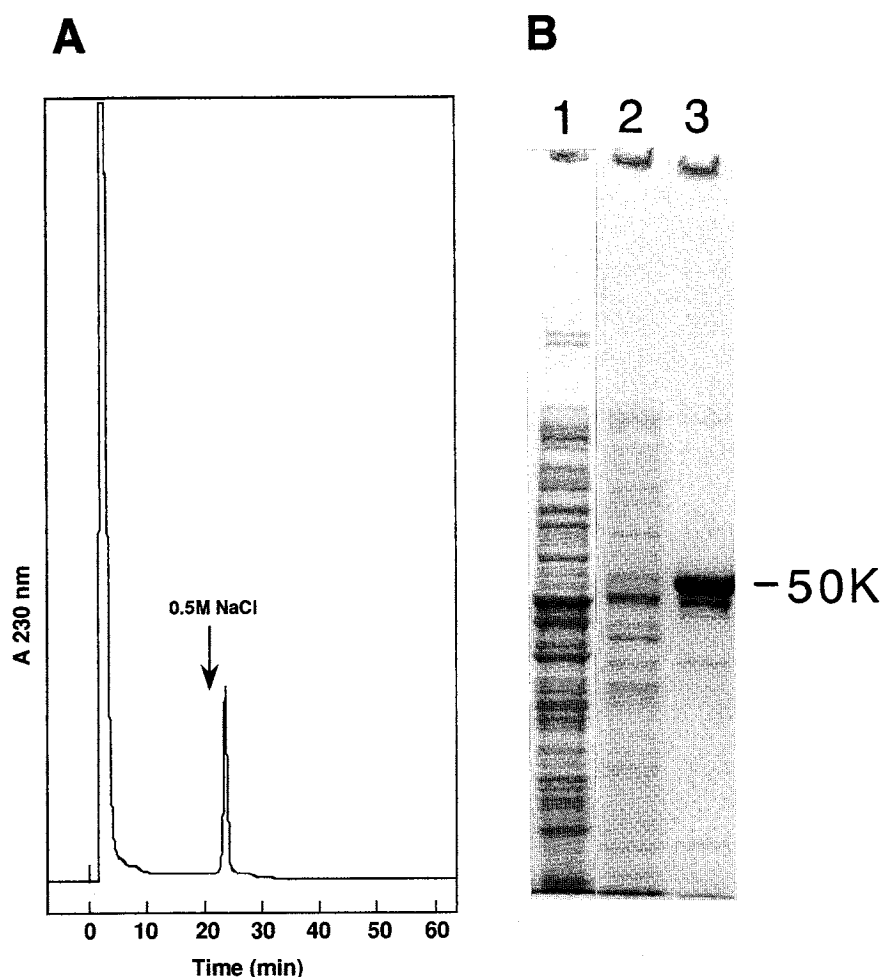


Fig. 2. SDS-PAGE of *E. coli* proteins in 4–20% gradient gels. Lane 1, total cellular extract of JM109; lane 2, JM109-pTF7520-Col-V-In before induction; and lane 3, after induction by IPTG.



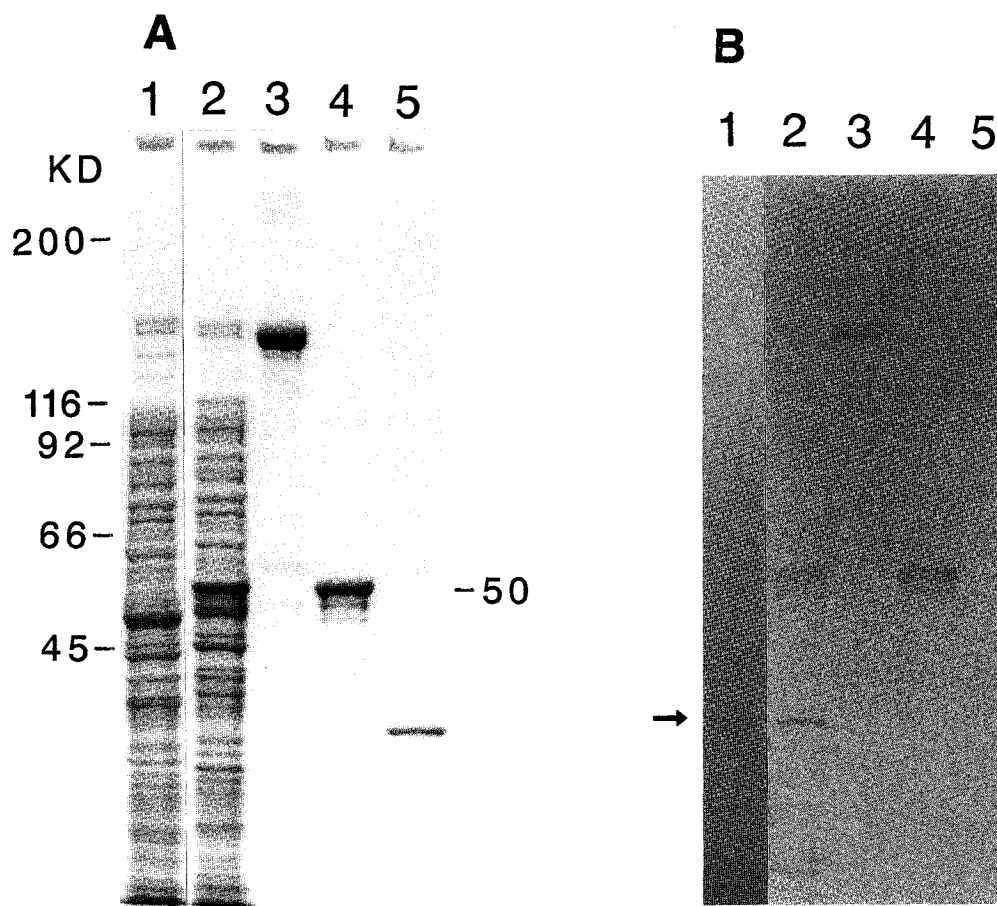
**Fig. 3.** Purification of the fusion protein by heparin affinity chromatography. A, crude extract of *E. coli* was put on a heparin 5PW column, and the column was washed with PBS. Bound materials were eluted by addition of NaCl at a final concentration of 0.5 M. B, SDS-PAGE of the proteins in 4–20% gradient gels. Lane 1, crude extract; lane 2, heparin-unbound, and lane 3, heparin-bound fractions.

did not contain the plasmid (Fig. 4B, lane 1, indicated by an arrow). The nature of this insulin-binding protein produced by *E. coli* is not known. Insulin-peroxidase bound to the 50-kDa fusion protein in the crude extract of JM109-pTF7520-Col-V-In (lane 2), the  $\alpha 1$  chain of type V collagen (lane 3), and the purified fusion protein C277-V (lane 4), but did not bind to the fibronectin fragment C274 (lane 5). As judged from the staining intensity, the insulin-binding activity of C277-V seemed to be similar to that of  $\alpha 1(V)$ .

The cell-adhesive activity of C277-V was determined using Swiss mouse 3T3 (Fig. 5A) and mouse melanoma B16-F10 cells (Fig. 5B). In the both cases, the fusion protein showed cell-adhesive activity, but the concentration required for the half-maximal activity for C277-V was approximately 10-times higher than that of C274. In the case of 3T3 cells, similar numbers of cells adhered to the dishes and spread well when the dishes were

coated with 1  $\mu$ M of each protein. Subsequent experiments were therefore carried out using 1  $\mu$ M C277-V.

**Growth of mouse MTD cells on fusion protein.** Mouse mammary tumor MTD cells grow well in serum-free culture medium with insulin as the only growth factor supplement (4). We used this cell line to examine the mitogenic activity of substrate-bound insulin. Tissue culture wells were coated with 1  $\mu$ M C274 or C277-V, respectively, and then blocked with BSA. Insulin (10  $\mu$ g/ml in PBS) was added to the wells and left at room temperature overnight. Unadsorbed insulin was then removed by repeated washing with PBS. MTD cells were inoculated into the wells in serum-free medium without insulin, and the growth rate of the cells was determined (Fig. 6). The cell numbers at day 0 were determined at 4 hr after inoculation. On the C277-V substratum pretreated with 10  $\mu$ g/ml insulin, significant stimulation of cellular growth was observed on day 2 (Fig. 6A), but no



**Fig. 4.** Binding of insulin-peroxidase to the fusion protein on nitrocellulose membranes. Proteins were separated by SDS-PAGE in 4–20% gradient gels, and electrophoretically transferred to nitrocellulose membranes. A, Coomassie blue staining, and B, binding of insulin-peroxidase. Lane 1, total extract of *E. coli* JM109; lane 2, total extract of JM109-pTF7520-Col-V-In; lane 3,  $\alpha 1$  chain of human type V collagen, lane 4, purified fusion protein C277-V; and lane 5, recombinant fibronectin peptide C274.

such a stimulating effect was observed on C274 (Fig. 6B). Attachment of the cells to the dishes on day 0 was also enhanced by the pretreatment of the C277-V substratum with insulin, but the cell numbers in the insulin-treated dishes began to decrease after day 3.

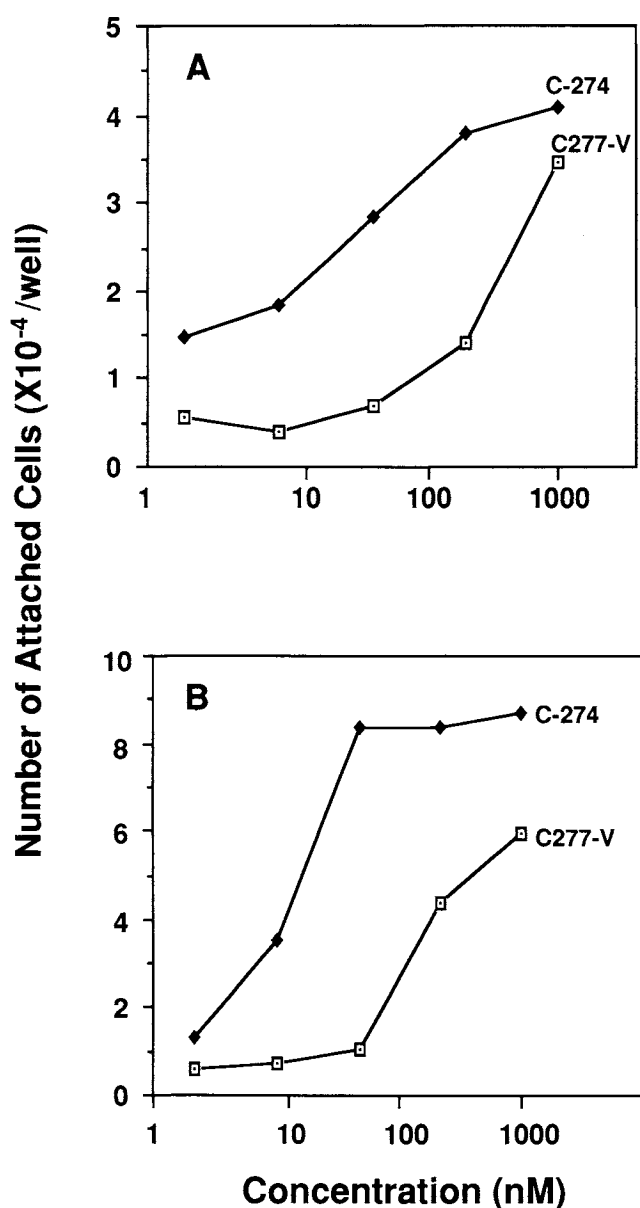
Table I shows the effects of different concentrations of insulin on the growth of MTD cells on the C277-V and C274 substrates. Maximum stimulation of cellular growth was obtained when the C277-V substratum was pretreated with 10 to 100  $\mu\text{g}/\text{ml}$  insulin. This agreed with the previous results obtained using type V collagen as an adsorbent of insulin (15).

MTD cells were cultivated on C274 or C277-V substrates in serum-free medium that contained various concentrations of insulin (Fig. 7). In this experiment, the substrates were not pretreated with insulin. The growth of the cells were stimulated by insulin in a dose-dependent manner on the both substrates during the first two days, but the cell number also decreased, on

day 4, probably due to the detachment of the cells from the dishes.

#### DISCUSSION

Fusion protein C277-V, which contains the cell-binding region of human fibronectin and the heparin- and insulin-binding regions of the  $\alpha 1$  chain of human type V collagen, was expressed in *E. coli* and purified by heparin affinity chromatography. The collagen region in this fusion protein contained 186 amino acids (amino acid residue 799 to 984 in the  $\alpha 1$  chain), which contained a cluster of basic amino acids (12, 14). The basic amino acids in this region are thought to be important in the interaction of type V collagen with heparin and insulin (14). The size of the collagen fragment incorporated into the fusion protein was much smaller in size than the heparin- and insulin-binding CNBr peptide (30-kDa), which we have previously isolated (14), but the



**Fig. 5.** Attachment of Swiss mouse 3T3 cells and mouse melanoma B16-F10 cells to the fusion protein. Nunclon 24-well plates were coated with various concentrations of C274 or C277-V, and the wells were then blocked with 2% BSA. Swiss mouse 3T3 cells (A) or melanoma B16-F10 cells (B) were suspended in serum-free DMEM at  $1$  to  $2 \times 10^5$  cells/ml, and 1 ml of the cell suspension was put in each well. After incubation at  $37^\circ\text{C}$  for 1.5 hr, the wells were washed twice with PBS. The attached cells were then dispersed by trypsinization and the cell number was counted by means of a Coulter Counter Model D.

present results demonstrated that the peptide of 186 amino acids was sufficient for the binding of heparin and insulin. Furthermore, the fusion protein expressed in *E. coli* does not contain hydroxyprolines, hydroxylysines, or glycosylated amino acids, that are commonly present in intact collagen molecules, indicating that such post-

**Table I.** DOSE-DEPENDENT EFFECT OF SUBSTRATE-BOUND INSULIN ON THE GROWTH OF MTD CELLS.

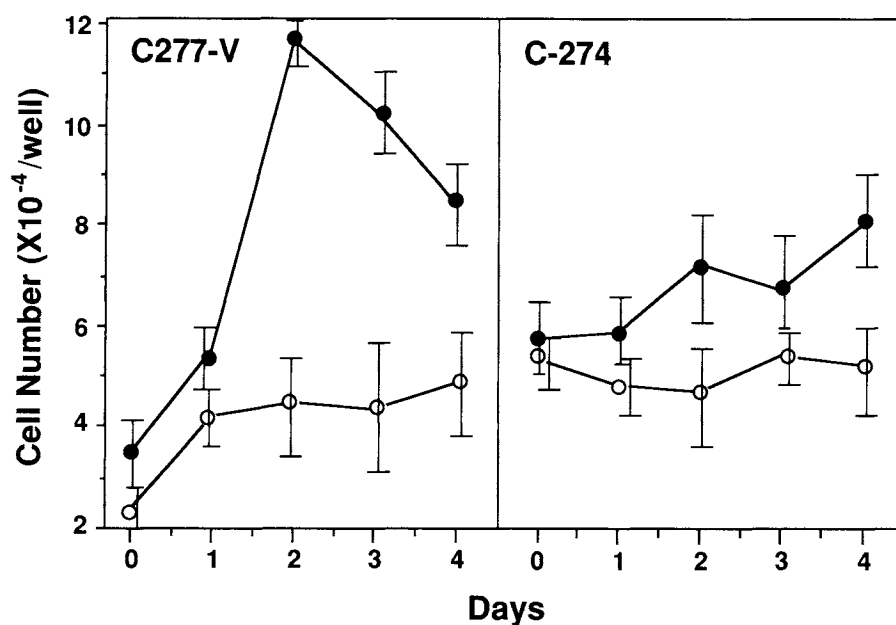
Concentration of insulin	Cell number $\times 10^{-4}$ /well		
	Day 0	Day 2	Day 2/Day 0
C277-V			
0 $\mu\text{g/ml}$	$2.97 \pm 0.49$	$3.34 \pm 0.29$	1.13
10	$4.48 \pm 0.43$	$13.47 \pm 0.83$	2.93
100	$5.13 \pm 0.28$	$16.83 \pm 0.95$	3.28
C274			
0	$5.92 \pm 0.27$	$5.10 \pm 0.31$	0.86
10	$5.90 \pm 0.23$	$7.41 \pm 0.29$	1.26
100	$5.23 \pm 0.44$	$11.15 \pm 0.42$	1.79

Nunclon 24-well culture plates were coated with  $1 \mu\text{M}$  C274 or C277-V, respectively, and preincubated with the indicated concentrations of insulin. The growth rate of MTD cells was determined as described in the legend to Fig. 6.

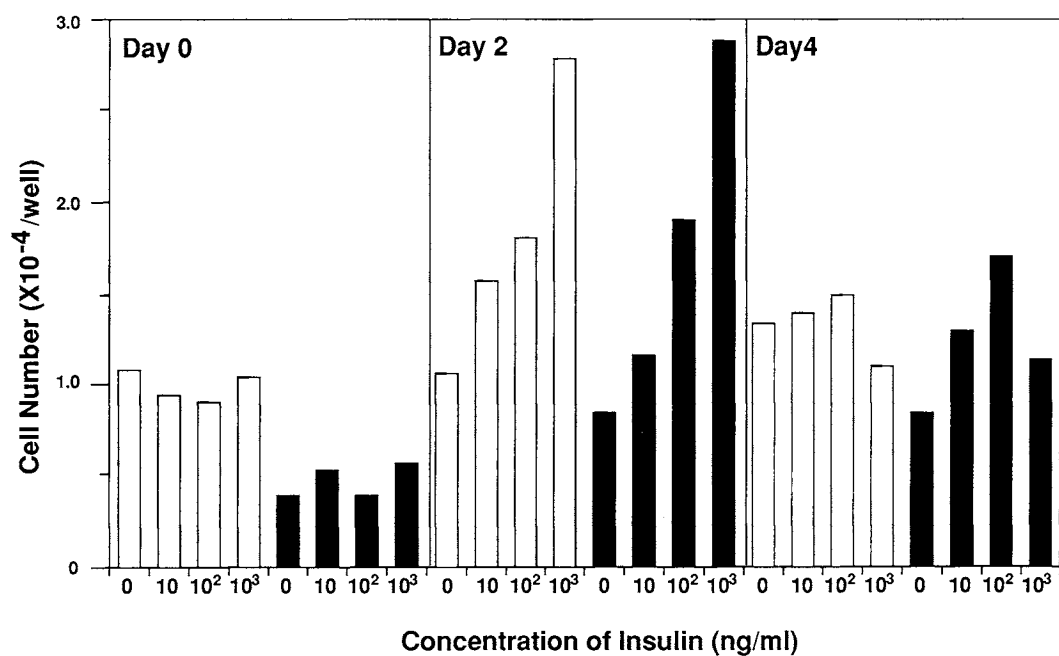
translational modifications are not necessary for these binding activities.

The fusion protein retained cell-adhesive and cell-spreading activities of the original fibronectin fragment, but approximately 10-times higher concentration of C277-V was required for the half-maximal cell adhesion as compared with C274. The cell binding RGDS sequence is present in the very C-terminal region of C277, so we suspect that the insertion of another peptide fragment to the C-terminus of C277 might cause some decrease in the cell-adhesive activity by interfering with the interaction of the fusion protein with the cell surface adhesion receptors.

Insulin bound to the fusion protein substrates enhanced the adhesion and growth of mouse mammary tumor MTD cells. Larger number of cells usually adhered to the dishes on day 0 when C277-V was pretreated with insulin (Fig. 6 and Table I). Such stimulation of cell adhesion might be explained by the binding of the cells to substrate-bound insulin by means of insulin receptors on the cell surface. Maximum stimulation of the growth was observed when the substrates were coated with 10 to  $100 \mu\text{g/ml}$  insulin. These growth-promoting effects of the fusion protein were comparable with those obtained for native type V collagen as we reported previously (15). Because no such stimulating effect was observed on the C274 substrates, this growth enhancement must be due to the increased affinity of the fusion protein for insulin. The stimulation of growth was most distinct during the first two days of cultivation, but the cell number tended to decrease thereafter. The cell numbers also tended to decrease after prolonged cultivation on the C274 or C277-V substrates even when  $1 \mu\text{g/ml}$  insulin, which was sufficient to sustain the growth of MTD cells (4, 15), was added to the culture medium (Fig. 7). The decrease in the cell numbers therefore seems to be due to the limited cell adhesive activity of the recombi-



**Fig. 6.** Growth of mouse mammary tumor MTD cells on fusion protein substrates. Nunclon 24-well plates were coated with 1  $\mu$ M C277-V or C274, respectively, at room temperature for 2 hr, and then blocked with 2% BSA. The wells were treated with 10  $\mu$ g/ml insulin (closed circle) at room temperature overnight. Control wells (open circle) were treated with PBS without insulin. Mouse MTD cells were inoculated to the wells in 1 ml of serum-free medium without insulin, and the wells were incubated at 37°C. The cell numbers on day 0 were determined at 4 hr after inoculation.



**Fig. 7.** Effects of soluble insulin on the growth rate of MTD cells. Nunclon 24-well plates were coated with 1  $\mu$ M C274 (closed bar) or C277-V (open bar), respectively. MTD cells were grown in the serum-free medium that contained various concentrations of insulin.

nant fibronectin molecules, and further improvement of this point would be required.

The present results showed that a new biological function could be added to the cell-binding peptide of fibronectin using gene technological methods. We have recently found that a fusion protein of fibronectin with epidermal growth factor (EGF) retained the growth enhancing activity of EGF (5). These bifunctional fusion proteins, that have both cell-adhesive and growth enhancing activities, would probably be effective in the concentration of growth factors in the vicinity of cell surfaces or in the stabilization of their activities. The recombinant techniques described here seem to be useful to construct the cell-adhesive proteins that are more advantageous for the proliferation of the cells in culture. We also attempt to apply these recombinant proteins for clinical uses such as wound healing.

#### REFERENCES

1. FUKUDA, K., KOSHIHARA, Y., ODA, H., OHYAMA, M., and OYAMA, T. (1988). Type V collagen selectively inhibits human endothelial cell proliferation. *Biochem. Biophys. Res. Commun.*, **151**: 1060–1068.
2. GUBLER, U. and HOFFMAN, B.J. (1983). A simple and very efficient method for generating cDNA libraries. *Gene*, **25**: 263–269.
3. HASHIMOTO, K., HATAI, M., and YAOI, Y. (1991). Inhibition of cell adhesion by type V collagen. *Cell Struct. Funct.*, **16**: 391–397.
4. KAWAMURA, K., ENAMI, J., KOHMO, K., and KOGA, M. (1985). Growth of an established line of mouse mammary tumor cells under serum-free conditions. *Dokkyo J. Med. Sci.*, **12**: 167–180.
5. KAWASE, Y., OHDATE, Y., SHIMOJO, T., TAGUCHI, Y., KIMIZUKA, F., and KATO, I. (1992). Construction and characterization of a fusion protein with epidermal growth factor and the cell-binding domain of fibronectin. *FEBS Letters*, **298**: 126–128.
6. KIMIZUKA, F., OHDATE, Y., KAWASE, Y., SHIMOJO, T., TAGUCHI, Y., HASHINO, K., GOTO, S., HASHI, H., KATO, I., SEKIGUCHI, K., and TITANI, K. (1991). Role of type III homology repeats in cell adhesive function within the cell-binding domain of fibronectin. *J. Biol. Chem.*, **266**: 3045–3051.
7. KIMIZUKA, F., TAGUCHI, Y., OHDATE, Y., KAWASE, Y., SHIMOJO, T., HASHINO, K., KATO, I., SEKIGUCHI, K., and TITANI, K. (1991). Production and characterization of functional domains of human fibronectin expressed in *E. coli*. *J. Biochem.*, **110**: 284–291.
8. LAEMMLI, U.K. (1970). Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature*, **227**: 680–685.
9. LOBB, R.R., HARPER, J.W., and FETT, J.W. (1986). Purification of heparin-binding growth factors. *Anal. Biochem.*, **154**: 1–14.
10. MATSUDAIRA, P. (1987). Sequence from picomole quantities of proteins electroblotted onto polyvinylidene difluoride membranes. *J. Biol. Chem.*, **262**: 10035–10038.
11. SANGER, F., NICKLEN, S., and COULSON, A.R. (1977). DNA sequencing with chain-terminating inhibitors. *Proc. Natl. Acad. Sci. USA*, **74**: 5463–5467.
12. TAKAHARA, K., SATO, Y., OKAZAWA, K., OKAMOTO, N., NODA, A., YAOI, Y., and KATO, I. (1991). Complete primary structure of human collagen  $\alpha 1(V)$  chain. *J. Biol. Chem.*, **266**: 13124–13129.
13. TOWBIN, H., STAEBLIN, T., and GORDON, J. (1979). Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: Procedure and some applications. *Proc. Natl. Acad. Sci. USA*, **76**: 4350–4354.
14. YAOI, Y., HASHIMOTO, K., KOITABASHI, H., TAKAHARA, K., ITO, M., and KATO, I. (1990). Primary structure of the heparin-binding site of type V collagen. *Biochim. Biophys. Acta.*, **1035**: 139–145.
15. YAOI, Y., HASHIMOTO, K., TAKAHARA, K., and KATO, I. (1991). Insulin binds to type V collagen with retention of mitogenic activity. *Exp. Cell Res.*, **194**: 180–185.

(Received for publication, June 22, 1992

and in revised form, September 2, 1992)