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A 31-kDa Recombinant Fibronectin Cell-Binding Domain Fragment: Its Binding to Receptor, Cell Adhesive Activity, and Fusion Proteins

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The binding of fibronectin to fibronectin receptor was studied using a recombinant 31-kDa cell-binding domain fragment of fibronectin (C279), which consisted of three type III repeats (III_8-III_9-III_{10}). Fibronectin receptor in several cell lysates was bound to a column of C279-immobilized Sepharose HP and obtained in a highly purified form by elution with a synthetic peptide, GRGDSP. $\alpha_5\beta_1$-Integrin was detected in the GRGDSP-eluted fraction by immunoblotting. The cell-adhesive activity of C279 was inhibited by GRGDSP peptide, an anti-integrin α_5 subunit antibody, and an anti-integrin β_1 subunit antibody. The cell adhesion of fusion proteins of the 31-kDa fragment with biologically interesting polypeptides (heparin-binding domain of fibronectin, and basic fibroblast growth factor) was also studied. In the presence of an anti-integrin α_5 subunit antibody, human fibrosarcoma HT-1080 cells attached to the fusion protein containing fibroblast growth factor, giving rise to changes the morphology of the attached cells. The cell adhesion of C279 was inhibited by GRGDSP peptide but that of the fusion protein with the heparin-binding domain of fibronectin was not completely inhibited by the peptide. These results suggest that these biologically interesting polypeptides contribute to the cell adhesion of the fusion proteins.

Key words: cell adhesion, fibroblast growth factor, fibronectin, fibronectin receptor, integrin.

Fibronectin (FN) is a multifunctional glycoprotein which is abundant in extracellular matrix and plasma and plays important roles in cell-matrix interaction, including cell-to-substrate adhesion (reviewed in Ref. *1*). Cells adhere to extracellular matrices through the cell surface receptors. FN receptor has been isolated and characterized as well as other receptors. The receptor was isolated from cultured osteosarcoma cells and fibroblasts by affinity chromatography on a column of immobilized 120-kDa chymotryptic peptide, a cell attachment-promoting fragment of FN (*2*). The receptor was bound to the column and eluted with a small synthetic peptide containing the Arg-Gly-Asp (RGD) sequence. It has also been demonstrated that when the FN receptor was incorporated into liposomes, it provided the liposome surface with the ability to bind FN, namely, an activity consistent with the function of a receptor (*2*). On the other hand, Pytela *et al.* (*3*) reported that vitronectin (VN) receptor, but not FN receptor, was bound to Gly-Arg-Gly-Asp-Ser (GRGDS) peptide immobilized on agarose gel beads. It was shown that very late antigen 5 (VLA-5) on lymphocytes is identical to FN receptor, and VLA-5-dependent adhesion of lymphocytes to FN can be blocked by a peptide containing the RGD sequence (*4*).

Other studies (*5-8*) have shown that proteolytic fragments of 70-120 kDa derived from the cell-binding domain of FN retained the full activity in the adhesion assay, but a 11.5-kDa fragment from the domain or small RGD-containing synthetic peptides lost 95-99% of the activity. Recent studies (*9-11*) have suggested that not only the RGDS signal but also some additional information is required for the full adhesive function of FN, or that a second adhesive recognition site cooperates with the RGDS sequence to mediate the full adhesive activity of FN. In addition, when an artificial cell adhesive protein was engineered by grafting the RGD sequence to a calpastatin segment by *in vitro* mutagenesis, the grafted RGD signal was recognized by VN receptor but not FN receptor (*12*).

Recently, a 31-kDa fragment corresponding to the FN cell-binding domain was used for construction of fusion proteins of the fragment and other biologically interesting polypeptides such as the heparin-binding domain of FN (*13*), epidermal growth factor (*14*), and basic fibroblast growth factor (FGF) (*15*). For example, Hashi *et al.* (*15*) demonstrated that the 31-kDa fragment-FGF fusion protein (FN-FGF) had a cell-adhesive activity on Swiss mouse 3T3 cells and stimulated the DNA synthesis of the cells in serum-starved culture. The 31-kDa fragment is a recombinant polypeptide expressed in *Escherichia coli*, which is much smaller than the native FN (*9*). Detailed analysis of the fragment on cell recognition has not been performed yet. Further analysis of the function of these fragments could contribute not only to the studies on the cell-binding

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Abbreviations: EIA, enzyme linked immunosorbent assay; FBS, fetal bovine serum; FGF, fibroblast growth factor; FN, fibronectin; FN-FGF, fusion protein of cell-binding domain of fibronectin and basic fibroblast growth factor; MEM, minimal essential medium; PBS, phosphate-buffered saline; VLA, very late antigen; VN, vitronectin.

domain of fibronectin itself but also to those on the fusion proteins described above.

In this paper, we investigated the binding to FN receptor and the cell adhesion of the 31-kDa fragment described above. We also studied the interaction of the fusion proteins containing heparin-binding domain of FN or FGF to cells by cell-adhesion assay.

MATERIALS AND METHODS

Materials—A 31-kDa fragment corresponding to the FN cell-binding domain (C279) and a 60-kDa fusion protein (CH271) containing the cell- and heparin-binding domains of FN were prepared as described by Kimizuka *et al.* (*9, 13*). FN-FGF, which is a fusion protein of FN cell-binding domain and basic FGF, was prepared according to the method of Hashi *et al.* (*15*). A schematic diagram of the structures of these proteins is shown in Fig. 1. NHS-activated Sepharose HP (HiTrap NHS-activated) was purchased from Pharmacia-LKB (Uppsala, Sweden). Synthetic peptides, Gly-Arg-Gly-Asp-Ser-Pro (GRGDSP), Gly-Arg-Gly-Glu-Ser-Pro (GRGESP), and FN receptor (Integrin β_1) enzyme linked immunosorbent assay (EIA) kit were products of Takara Shuzo (Kyoto). Anti-FN receptor ($\alpha_5\beta_1$-integrin) polyclonal antibody, anti-VN receptor ($\alpha_v\beta_3$-integrin) polyclonal antibody, anti-integrin α_3 subunit monoclonal antibody (clone No. P1B5), anti-integrin α_5 subunit monoclonal antibody (clone No. P1D6), anti-integrin β_1 subunit monoclonal antibody (clone No. P4C10), and anti-integrin β_2 subunit monoclonal antibody (clone No. P4H9) were purchased from Telios Pharmaceuticals (San Diego, CA). Other chemicals were purchased from Nacalai Tesque (Kyoto).

Cells—Human osteosarcoma MG-63 and fibrosarcoma HT-1080 cells were cultivated in minimal essential medium (MEM)-Earle supplemented with non-essential amino acids and 10% fetal bovine serum (FBS). Baby hamster kidney (BHK) cells were cultivated in Dulbecco's MEM supplemented with 10% FBS.

Preparation of C279-Immobilized Column—C279 (8.3 mg) was dissolved in 1 ml of 0.2 M $NaHCO_3$ (pH 8.3) containing 0.5 M NaCl. Coupling of the fragment to NHS-activated Sepharose HP (1 ml) was carried out at room temperature for 1 h, then the gel was washed with 6 ml of 0.5 M ethanolamine-HCl buffer (pH 8.3) containing 0.5 M NaCl (buffer A) and 6 ml of 0.1 M sodium acetate buffer (pH 4.0) containing 0.5 M NaCl (buffer B). Residual active groups of the gel were blocked with buffer A. After blocking, the gel was washed with buffer B, then with buffer A (6 ml of each buffer). The gel was washed again with 6 ml of buffer B. The column was stored in phosphate buffered saline (PBS) containing 0.02% sodium azide before use.

Assay of FN Receptor—The grown cells were washed twice with a basal buffer (PBS containing 1 mM $CaCl_2$, 1 mM $MgCl_2$, and 1 mM phenylmethanesulfonyl fluoride), then lysed with 100 mM *n*-octyl-β-D-thioglucoside in the basal buffer (1.5 ml/10-cm dish). The lysed cell suspension was centrifuged and the supernatant was filtered through a 0.8-μm membrane filter. The cell extract was applied on a C279-immobilized Sepharose 4B column equilibrated with the basal buffer containing 50 mM *n*-octyl-β-D-thioglucoside (starting buffer). The column was washed with 10 column volumes of starting buffer, then with the starting buffer containing 1 mg/ml of synthetic GRGESP peptide. Elution was carried out with the starting buffer containing 1 mg/ml of synthetic GRGDSP peptide. The β_1-integrin was detected with FN receptor EIA kit. Horseradish peroxidase-conjugated antibody was assayed by use of *o*-phenylenediamine and H_2O_2 as substrate and the absorbance at 490 nm was measured.

Immunoblotting—Samples were spotted on nitrocellulose membranes (100 μl/spot). The spotted membranes were treated with 1% bovine serum albumin (BSA) at 37°C for 1 h, then with anti-FN receptor antibody or anti-VN receptor antibody at 37°C for 1 h. The antibodies were used at a dilution of 1 : 500 (v/v). The membranes were washed with PBS containing 0.01% Tween 20 (three times). The washed membranes were treated with horseradish peroxidase-conjugated anti-rabbit IgG antibody [1 : 500 (v/v)] at 37°C for 1 h, then washed three times with PBS containing 0.01% Tween 20 and twice with PBS. Spots were visualized by use of 4-chloronaphthol and H_2O_2 as substrate of peroxidase.

SDS-Polyacrylamide Gel Electrophoresis and Silver Staining—SDS-polyacrylamide gel electrophoresis (SDS-PAGE) was performed according to the method of Laemmli (*16*). Protein bands were visualized by staining with Silver Stain KANTO II (Kanto Chemicals, Tokyo).

Cell Adhesion Assay—Cell adhesive activity of C279 was assayed using human fibrosarcoma HT-1080 cells. Ninety-six-well polystyrene plates (Immunoplate; Nunc, Roskilde, Denmark) were coated with the fragment by appropriate dilutions in PBS at 4°C overnight and then blocked with 1% BSA in PBS for 2 h. The HT-1080 cells were detached from the culture dish with PBS containing 0.25% trypsin and 0.02% EDTA, washed once with the adhesion medium (MEM-Earle supplemented with nonessential amino acids) containing 0.01% soybean trypsin inhibitor, and then once with the adhesion medium without trypsin inhibitor. Finally, the detached cells were resuspended in the adhesion medium. The cells were incubated on the precoated polystyrene plates at 1×10^5 cells/well for 90 min at 37°C in a CO_2 incubator. The plates were then gently washed with PBS. The attached cells were fixed with 4% formaldehyde in PBS, then photographed under a phase-contrast microscope. In the case of BHK cells, Dulbecco's MEM was used instead of MEM-Earle supplemented with nonessential amino acids. The amount of proteins adsorbed on plastic substrate were determined by the method of Kimizuka *et al.* (*9*). Horseradish peroxidase-conjugated antibody was assayed by use of *o*-phenylenediamine and H_2O_2 as substrate and the absorbance at 492 nm was measured.

RESULTS AND DISCUSSION

Binding of C279 to FN Receptor—The extract of human osteosarcoma MG-63 cells was applied to a column of the C279-immobilized Sepharose 4B. Elution was carried out with synthetic GRGDSP peptide. FN receptor was detected in GRGDSP-eluted fractions by EIA with anti-integrin β_1 subunit antibody as shown in Fig. 2a. As shown in Fig. 3, α and β subunits of the receptor were only identified on SDS-PAGE under nonreduced conditions, indicating that the receptor was highly purified. The results show that FN receptor is easily purified on a C279-immobilized column and detected without radioisotopic reagents (for example, labeling with ^{125}I-sodium iodide). They also show that

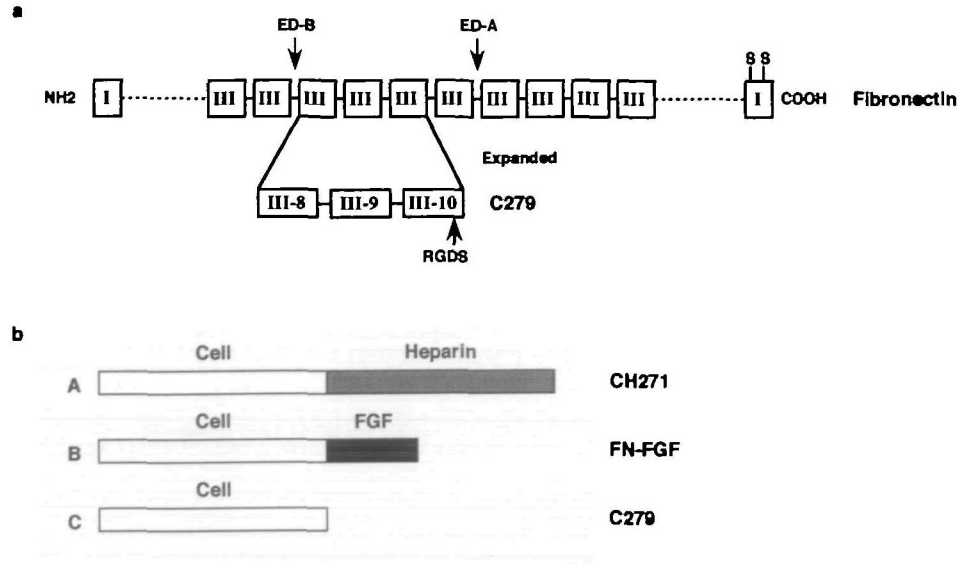

Fig 1 **Schematic diagram of the structures of C279 and the fusion proteins.** (a) Module structure of FN and C279 around the cell-binding domain. Type III repeats are numbered as described by Kornblihtt *et al* (*20*). The location of the RGDS sequence and the extra domains (ED-A and ED-B) are indicated by arrows (b) Domain structure of fusion proteins containing a 31-kDa fragment of FN cell-binding domain. A: CH271, B. FN-FGF, C: C279. The two domains of FN are marked as "Cell" for cell-binding domain I (III_8-III_9-III_{10}), and "Heparin" for heparin-binding domain II (III_{12}-III_{13}-III_{14})

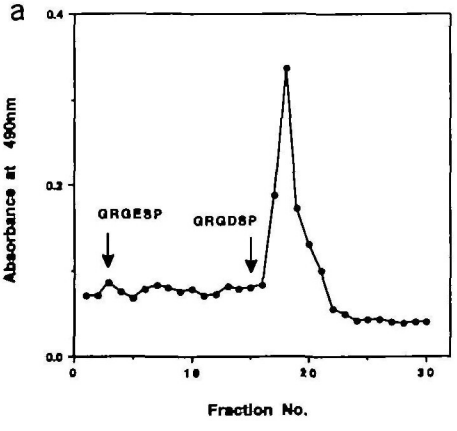

Fig 2. **Isolation of FN receptor of human osteosarcoma MG-63 cell lysate from a column of C279-immobilized Sepharose HP with GRGDSP peptide.** (a) Elution profile of β_1-integrin from a C279-immobilized column. The cell lysate was applied to a C279-immobilized Sepharose HP column. The column was washed with the starting buffer, then with the same buffer containing GRGESP peptide. Elution was carried out with the starting buffer containing GRGDSP peptide Fractions of 300 μl were collected. β_1-Integrin was detected by EIA as described in "EXPERIMENTAL PROCEDURES " Fraction number in this profile begins with the fraction before the last washing fraction. (b) Immunoreactivity of GRGDSP-eluted fraction. The GRGDSP-eluted fraction, in which β_1-integrin was detected, was analyzed by immunoblotting with anti-FN receptor ($\alpha_5\beta_1$-integrin) and anti-VN receptor ($\alpha_v\beta_3$-integrin) antibody on nitrocellulose membranes.

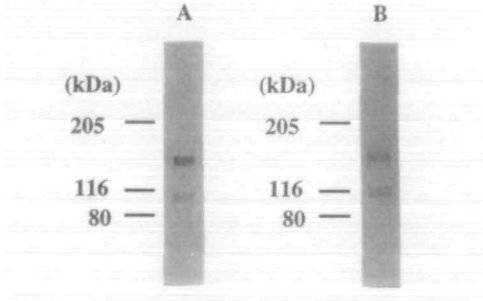

Fig. 3. **SDS-PAGE of FN receptor purified from MG-63 and HT-1080 cells.** FN receptor of human osteosarcoma MG-63 cells from a column of C279-immobilized Sepharose HP with GRGDSP peptide was analyzed by SDS-PAGE (lane A). A GRGDSP-eluted fraction (fraction No 18 in Fig. 2) was subjected to electrophoresis on 6% gel under nonreduced conditions. Protein bands were visualized by silver staining. FN receptor was purified from HT-1080 cells and analyzed by the same procedure as that used for MG-63 cells (lane B).

β_1-integrin bound to C279 in an RGD-dependent manner. The GRGDSP-eluted fraction in which β_1-integrin was detected was further analyzed by immunoblotting. As shown in Fig. 2b, $\alpha_5\beta_1$-integrin was detected in the fraction.

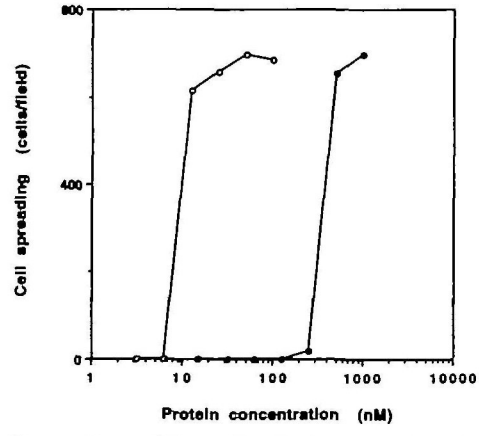

Fig. 4. **Comparison of the cell adhesive activity of C279 and the native FN.** Trypsinized human fibrosarcoma HT-1080 cells were incubated on plastic substrates precoated with increasing concentrations of either C279 (filled circles) or native FN (open circles), then subjected to the cell adhesion assay as described under "MATERIALS AND METHODS." The concentration of FN is expressed as that of the fibronectin monomer in order to compare the cell adhesive activity on a molar basis.

Interaction of the cell-binding domain of FN with the receptor was studied in detail with osteosarcoma MG-63 cells by Pytela *et al.* (*2*). They indicated that the receptor binds to a 110-kDa cell-binding fragment of FN and is eluted by GRGDSP peptide. FN receptor was also identified on T-lymphocytes with a 120-kDa chymotryptic cell-binding fragment of FN (*17*). By use of an anti-FN receptor antibody, Wayner *et al.* showed that fibronectin receptor exists on monocytes and promonocytes, *i.e.*, human promyelocyte HL-60 cells (*18*). We also isolated RGD-dependent and β_1-integrin from HL-60 cells with the C279-immobilized column (data not shown). Therefore, our results in this study suggests that C279 binds to FN receptor as well as the 110-kDa fragment (or the 120-kDa fragment). We also demonstrated that the fragment might be a useful tool for isolation of the receptor. Use of the obtained receptor would allow further analysis, such as re-binding of the receptor to C279 and its comparison with the binding to other fragments of cell-binding domain of FN.

Adhesion of Human Fibrosarcoma HT-1080 Cells on Plastic Substrates Coated with C279—Since the cell adhesion was highly dependent on FN receptor, *i.e.*, $\alpha_5\beta_1$-integrin (*19*), human fibrosarcoma HT-1080 cells should be suitable for this examination. C279 was capable of mediating the cell adhesion when coated on an inert plastic surface, and the maximal level of the cell adhesion achieved was as high as that achieved by FN (Fig. 4). As shown in Fig. 5a, the cell adhesion to the substrate coated with C279 was completely inhibited by the hexapeptide GRGDSP, but not by GRGESP. These results indicate that the cell adhesion to

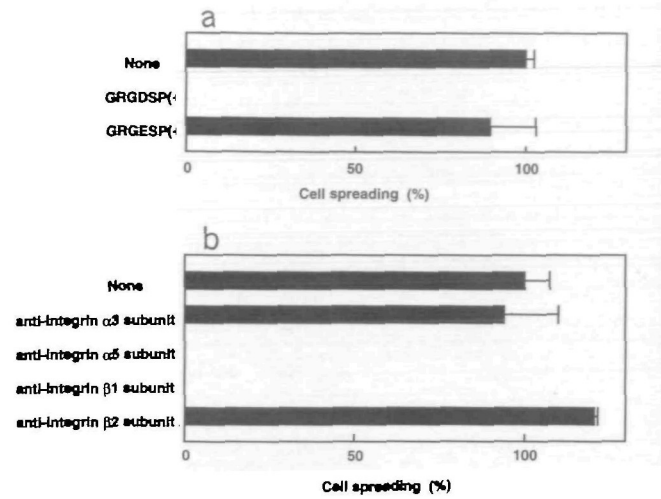

Fig 5 **Cell adhesive activity of C279.** Trypsinized human fibrosarcoma HT-1080 cells were incubated on plastic substrates precoated with C279 (500 nM). (a) One of the following peptides was included in the medium to examine its inhibitory effect on the cell-adhesion to the substrates precoated with the fragment: GRGDSP peptide (100 μg/ml), and GRGESP peptide (100 μg/ml). (b) One of the following antibodies was included in the medium to examine the inhibitory effect on the cell-adhesion to the substrates precoated with the fragment. anti-integrin α_3 subunit antibody (clone No P1B5), anti-integrin α_5 subunit antibody (clone No P1D6), anti-integrin β_1 subunit monoclonal antibody (clone No. P4C10), and anti-integrin β_2 subunit monoclonal antibody (clone No P4H9) Antibodies were used at a dilution of 1:25 (v/v).

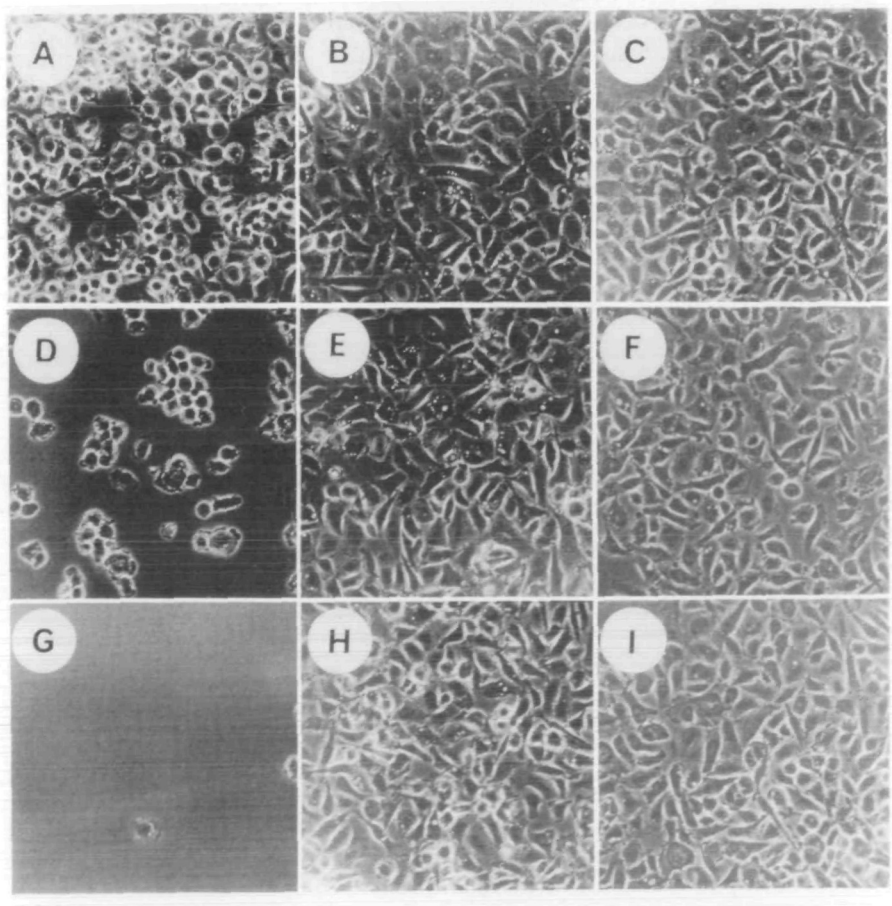

Fig 6. **Cell adhesive activity of FN-FGF fusion protein.** Trypsinized human fibrosarcoma HT-1080 cells were incubated on plastic substrates precoated with the following proteins. FN-FGF (panel A, B, C, 500 nM); CH271 (panel D, E, F; 500 nM); C279 (panel G, H, I, 500 nM). The anti-integrin α_5 subunit antibody (panel A, D, G; clone No. P1D6, diluted 1:50) or anti-integrin α_3 subunit antibody (panel B, E, H; clone No. P1B5, diluted 1:50) was included in the medium to examine the inhibitory effect on the cell adhesion to the substrates precoated with each protein described above.

C279 depends on the RGD signal. Figure 5b shows effects of several antibodies on the cell adhesion of RGD-dependent integrins to C279. The cell adhesion was inhibited by anti-integrin α_5 subunit antibody and anti-integrin β_1 subunit antibody, but not by anti-integrin α_3 subunit or anti-integrin β_2 subunit antibody. In addition, FN receptor of HT-1080 cells was also bound to a C279-immobilized column and eluted from the column with GRGDSP peptide (Fig. 3). $\alpha_5\beta_1$-Integrin was detected in the GRGDSP-eluted fraction in which β_1-integrin was detected as in MG-63 cells (data not shown). These results suggest that C279 promotes cell-adhesion by $\alpha_5\beta_1$-integrin and the integrin can be isolated with a C279-immobilized column.

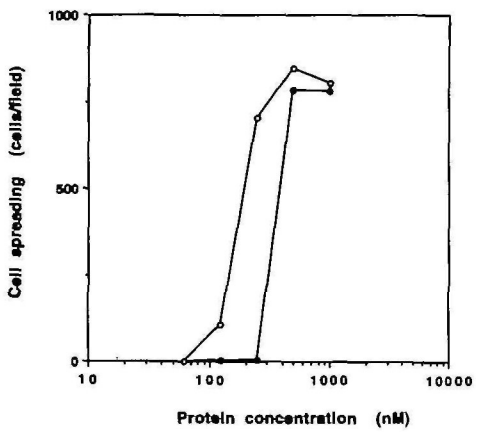

Fig. 7 **Comparison of the cell adhesive activity of FN-FGF and C279.** Trypsinized human fibrosarcoma HT-1080 cells were incubated on plastic substrates precoated with increasing concentration of either FN-FGF (filled circles) or C279 (open circles), then subjected to the cell adhesion assay as described under "MATERIALS AND METHODS."

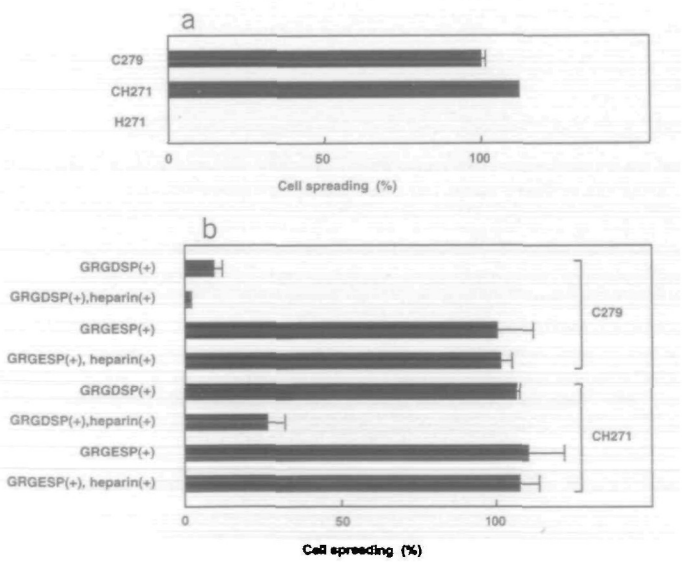

Fig. 8. **Effects of GRGDSP peptide on cell adhesion on CH271.** (a) Trypsinized baby hamster kidney BHK cells were incubated on plastic substrates precoated with C279, CH271, or H271 (500 nM). (b) Synthetic peptide (GRGDSP or GRGESP; 100 μg/ml) or heparin (500 μg/ml) was included in the medium to examine the inhibitory effect on adhesion of BHK cells to the substrates precoated with C279 or CH271 (500 nM)

In the previous paper, we reported that various combinations of recombinant type III repeats within the cell-binding domain of FN differ in their relative contribution to the cell adhesive activity depending on the order and length from the RGDS site in III_{10} repeat, and in particular, the location of III_9 repeat is crucial (*9*). Nagai *et al.* (*11*) showed that a monoclonal antibody directed to III_9 repeat inhibits the cell adhesion to FN, but antibodies to any of other repeats within the domain do not; and they proposed that a second adhesive recognition site may exist in III_9 repeat. On the other hand, we showed that RGD-grafted calpastatin segment, an artificial cell-adhesive protein, mediates the adhesion of MG-63 or BHK fibroblast cells but not HT-1080 cells. The adhesion of MG-63 cells to the segment was inhibited by an anti-VN receptor antibody, indicating that the RGD sequence grafted to the calpastatin segment is recognized as the cell adhesion signal by VN receptor (*12*). These results indicate that the RGD sequence alone is not sufficient as the cell adhesive signal for the recognition by FN receptor ($\alpha_5\beta_1$-integrin), and contrivances of the receptor-recognition should exist in the cell-binding domain of FN. Although C279 is a recombinant fragment much smaller than the native FN, it has at least enough capability to promote cell adhesion by FN receptor.

Cell Adhesion of Fusion Proteins—We analyzed the cell-adhesive activity of fusion proteins which contained the 31-kDa fragment of FN cell-binding domain (Fig. 1b). Firstly, FN-FGF, a fusion protein containing the 31-kDa fragment and basic FGF, was analyzed. Both attachment and spreading of HT-1080 cells on C279 were inhibited by an anti-integrin α_5-subunit antibody. In contrast, the cells attached to but did not spread on the substrate coated with FN-FGF in the presence of the antibody (Fig. 6, panel A). In the case of FN-FGF, the number of spread cells decreased, but the total number of spread and attached cells was the same level as that of the spread cells without the antibody (panel C). As shown in Fig. 7, cell-spreading on FN-FGF (panel C) and C279 (panel I) was the same level under the coating conditions used in Fig. 6. On the other hand, anti-integrin α_5 subunit antibody inhibited the attachment and spreading of the cells on CH271, composed of the cell- and heparin-binding domains of FN (Fig. 6,

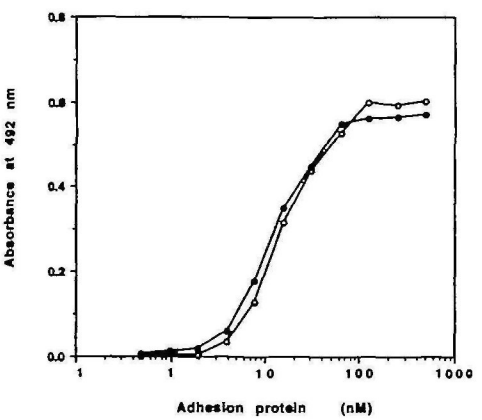

Fig. 9. **Protein adsorption on the plastic substrate.** The amount of proteins adsorbed on the plastic substrate was determined by ELISA (*9*): CH271 (open circles), C279 (filled circles).

panel D). The number of attached cells to CH271 decreased in the presence of the antibody. The effect of the antibody on cell adhesion to FN-FGF appears to differ from that on the adhesion to C279 and CH271. The difference is presumably caused by the interaction of FGF domain with FGF receptor, because the interaction is independent of the FN receptor and is not inhibited by the anti-integrin α_5 subunit antibody. Further analysis of this phenomenon is needed.

Subsequently, the effect of GRGDSP peptide on the cell adhesion to CH271 was analyzed. As shown in Fig. 8b, BHK cells adhered to both of the substrates coated with C279 and CH271. GRGDSP peptide, however, inhibited the cell adhesion to C279 but not that to CH271. As shown in Fig. 9, no significant difference was found in the amount of the proteins adsorbed on plastic substrate. The cell adhesion to the chimeric protein was completely inhibited in the presence of both GRGDSP peptide and heparin. On the other hand, a recombinant fragment corresponding to the heparin-binding domain of FN (referred to as H271) alone barely mediated the cell-adhesion (Fig. 8a). When CH271 was used, it appeared that the domain might indirectly contribute to the cell adhesion to CH271. We assume that the interaction between the domain and the cell surface increases the probability of the interaction between the cell-binding domain and FN receptor.

In conclusion, the results described above suggest that these fusion proteins express multiple functions of their component domains. Such the fusion proteins should be useful tools for studies on the function of biologically interesting peptides.

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