CELL STRUCTURE AND FUNCTION 16: 149–156 (1991)

Manganese Ion Elicits a Binding Activity of Placenta Vitronectin Receptor to Fibronectin Cell-Binding Domain

Takanori Yanai†, Tadashi Shimo-Oka and Ichio Ii

Tissue Culture Laboratory, Iwaki Glass Company, Funabashi, Chiba 273, Japan

Key words: integrin/ligand specificity/vitronectin receptor/RGD sequence/divalent cation/manganese ion

ABSTRACT. **Some members of the integrin family recognize the RGD sequence which is common to cell adhesive proteins in a divalent cation-dependent manner. In the presence of Ca^{2+} and Mg^{2+}, the fibronectin receptor of placenta recognizes the RGD sequence of fibronectin, but not that of vitronectin, while the vitronectin receptor of placenta recognizes the RGD sequence of vitronectin, but not that of fibronectin, although both receptors recognize the same RGD sequence. We have found by performing an enzyme-linked immunosorbent assay (ELISA) using receptor-specific monoclonal antibodies and by electrophoretic analysis that in the presence of Mn^{2+} a vitronectin receptor of placenta binds to an affinity column coupled with the cell-binding domain of fibronectin. By replacing divalent cations from Mn^{2+} to Ca^{2+} and Mg^{2+}, the vitronectin receptor was completely eluted from the column. When the synthetic peptides GRGDSP and GRGESP were applied to the column as competitors, the Mn^{2+}-dependent binding was inhibited by both peptides. These results suggest that Mn^{2+} elicits a binding activity of the placenta vitronectin receptor to the RGD site of fibronectin. The modulation of ligand specificity by Mn^{2+} will provide an important clue in the elucidation of the cause of individual ligand specificity of RGD-recognizing integrins.**

Interaction between cells and cell adhesive proteins is essential in many cellular functions including growth, migration and differentiation of cells. Many kinds of cell adhesive proteins including fibronectin (FN), laminin and vitronectin (VN) have been discovered. They have been shown to contain the RGD sequence responsible for cell binding (21, 22). The RGD sequence is recognized by a class of divalent cation-dependent cell surface receptors designated as an integrin superfamily (9). The RGD-recognition system between integrins and cell adhesive proteins seems to be simple, but has a complex nature since each integrin must have individual ligand specificity using the common RGD-recognition system. A fibronectin receptor ($\alpha_5\beta_1$ integrin, FNR) purified from MG 63 human osteosarcoma cells specifically recognizes the RGD sequence of FN (17, 19). On the other hand, a vitronectin receptor ($\alpha_v\beta_3$ integrin, VNR) purified from the same cells recognizes only the RGD sequence of VN (17, 18). In contrast to these monospecific integrins, platelet gp IIb/IIIa recognizes the RGD sequence of FN, fibrinogen, VN and von Willebrand factor (18). The mechanism that causes these specificities of the RGD-recognition system still remains unclear.

Recently, Gailit and Ruoslahti (6) and Hautanen *et al.* (8) reported that Mn^{2+} eminently enhances the binding of FNR to FN, as compared to the abilities of Ca^{2+} and Mg^{2+}. Some cell types such as endothelial cells or melanoma cells have been shown to adhere to VN, fibrinogen, and von Willebrand factor in a way that is fully inhibitable by monoclonal antibodies directed to the $\alpha_v\beta_3$ integrin, suggesting that the $\alpha_v\beta_3$ integrin interacts with these proteins (1, 3, 4). Lawler and Hynes have shown that the $\alpha_v\beta_3$ integrin from platelets binds to both VN and thrombospondin (13). These findings suggest that the ligand specificity of RGD-recognizing integrins could be modified by ionic conditions and cell types where they are expressed. Therefore, these phenomena might give important clues to the mechanism of ligand specificity of integrins from the viewpoint of the structure of the integrins.

We show here that Mn^{2+} elicits a binding activity of the placenta $\alpha_v\beta_3$ integrin to the FN cell-binding domain. On the basis of the results, we discuss the cause of the ligand specificity of RGD-recognizing integrins.

MATERIALS AND METHODS

Reagents. The following reagents were purchased from the Wako Chemical Co.: phenylmethylsulfonyl fluoride

† To whom correspondence should be addressed.

Abbreviations: FN, fibronectin; VN, vitronectin; FNR, fibronectin receptor; VNR, vitronectin receptor; PMSF, phenylmethylsulfonyl fluoride; DTT, dithiothreitol; FN 110 KDa-column, 110 K dalton fibronectin fragment-Sepharose column; OTG, n-octyl-β-D-thiogluco-pyranoside; TBS, Tris-buffered saline.

(PMSF), tris (hydroxymethyl) aminomethane, n-octyl-β-D-thioglucopyranoside (OTG), NaCl, $MnCl_2 \cdot 4H_2O$, $MgCl_2 \cdot 6H_2O$, $CaCl_2 \cdot 2H_2O$, ethylenediaminetetraacetic acid (EDTA), sodium dodecylsulfate (SDS), dithiothreitol (DTT), and glycine. The CNBr-activated Sepharose 4B was obtained from Pharmacia Fine Chemicals. The GRGDSP and GRGESP were purchased from the Iwaki Glass Co. The GRGDSPK was a kind gift from the Asahi Glass Co.

Preparation of affinity columns. The 110 K dalton fibronectin cell-binding fragment (FN 110 KDa) was prepared by chymotryptic digestion followed by fractionation on gelatin-Sepharose and heparin-Sepharose, as described by Ruoslahti *et al.* (20). The fragment contained the RGD sequence and the synergistic cell-binding region which are responsible for the cell attachment to FN (15). The FN 110KDa and synthetic peptide GRGDSPK were coupled to cyanogen bromide-activated Sepharose 4B (Pharmacia), according to the manufacturer's instructions.

Isolation of the fibronectin and vitronectin receptors. Full-term human placenta was stored at −80°C until use. The FNR and VNR of the human placenta were isolated by essentially the same method as that reported by Gailit and Ruoslahti (6). Homogenized placental tissue was washed once with 2 volumes of cold Tris-buffered saline (TBS; 0.15 M NaCl, 0.05 M Tris-HCl, pH 7.4) containing 1 mM $MgCl_2$, 1 mM $CaCl_2$ and 1 mM PMSF, and was extracted with 1 volume of cold TBS containing 50 mM OTG, 1 mM $MgCl_2$, 1 mM $CaCl_2$, and 1 mM PMSF. After 1-hr extraction on ice, the extract was clarified by centrifugation at 10,000 g for 30 min. To isolate FNR, the clear extract was slowly applied to a FN 110 KDa column at 4°C. The column was washed at 25°C with 6 volumes of TBS containing 25 mM OTG, 1 mM $MgCl_2$, 1 mM $CaCl_2$, and 1 mM PMSF. The bound receptor was eluted with the TBS containing 25 mM OTG, 20 mM EDTA, and 1 mM PMSF. In order to isolate VNR, a GRGDSPK-Sepharose column (GRGDSPK column) was substituted for a FN 110 KDa column. Both receptors were further purified by a wheat germ agglutinin-agarose column. The purified receptors were used as immunogens for monoclonal antibody production and as antigens of ELISA for characterization of the monoclonal antibodies. The FNR and VNR were also purified by the same procedure from human diploid fibroblasts (TIG-3, JCRB 0506) (14).

Monoclonal antibodies to FNR and VNR. Monoclonal antibodies were prepared essentially according to the procedure described by Iwasaki *et al.* (10) and Izumi *et al.* (11). Briefly, purified FNR and VNR from placenta were mixed with complete Freund's adjuvant and injected intraperitoneally into 8-week-old BALB/c female mice. Three weeks later, the receptors with incomplete Freund's adjuvant were injected intraperitoneally. After two weeks, the receptors in phosphate-buffered saline (PBS) were injected intravenously. Three days later, the spleen cells of the immunized mice were fused with the mouse myeloma cell line X63-Ag 8.653 using polyethyleneglycol. To select hybridoma producing anti-FNR and anti-VNR antibodies, respectively, the hybridoma cultures were screened by ELISA. Cloning was carried out several times by limiting dilution until monoclonality was evidenced. Monoclonal antibodies were purified from ascites with a protein A agarose column. Isotyping of the antibodies was performed with an isotyping kit (Nippon Bio-Rad Lab., Tokyo).

Detection of VNR and FNR. Each receptor was detected by ELISA using monoclonal antibodies to VNR or FNR. Each fraction from the affinity columns was diluted 50~200-times with PBS and was incubated with ELISA plates (Corning, New York) overnight at 25°C. As a control, some wells of the plates were coated with PBS. The plates were washed twice with PBS, and coated with PBS containing 10% fetal bovine serum (PBS-FBS) for 60 min at 25°C. After the plates were washed twice with PBS, each monoclonal antibody (0.5 μg/ml in PBS-FBS) was added to the wells and was incubated for 60 min at 25°C. Unbound antibodies were washed away by washing the plates three times with PBS containing 0.1% Tween 20 (PBS-Tween). Bound antibodies were reacted with a goat anti mouse IgG antibody coupled to horseradish peroxidase for 60 min at 25°C. After washing four times with PBS-Tween, the bound enzymatic activity was measured by use of o-phenylenediamine and H_2O_2 as substrates. The absorbance at 492 nm was measured with Uniskan II (Labsystems). Duplicate determinations were performed for each assay. Detection of receptors was also performed by SDS polyacrylamide gel electrophoresis in the method reported by Laemmli (12), by use of silver staining (Kanto Chemical Co., Tokyo).

RESULTS

Characterization of monoclonal antibodies. Several hybridoma cell lines were established and expanded as ascites tumors in mice. The best two lines of the hybridomas were selected. One line produced antibodies (S6) that reacted with purified FNR from placenta and human fibroblasts (TIG-3), but which did not react at all with purified VNR from the same sources in ELISA. Another line produced antibodies (H4) which reacted with purified VNR from placenta and human fibroblasts, but which did not react at all with purified FNR from the same sources in ELISA. Subclass analysis revealed that both S6 and H4 were of the IgG_1 subclass containing κ light chains. Immunoblotting showed that S6 reacted with the β_1 subunit of FNR under nonreducing conditions (Fig. 1a). Since H4 did not react with VNR in immunoblotting, specificity of H4 was confirmed by H4-Sepharose column chromatography. When a placental extract was applied to the column, the α_v and β_3 subunits of VNR predominantly bound to the column, although the binding of other proteins was also observed (Fig. 1b). These results showed that S6 and H4 were specific for FNR and VNR, respectively. Hence, the antibodies were used in ELISA for the detec-

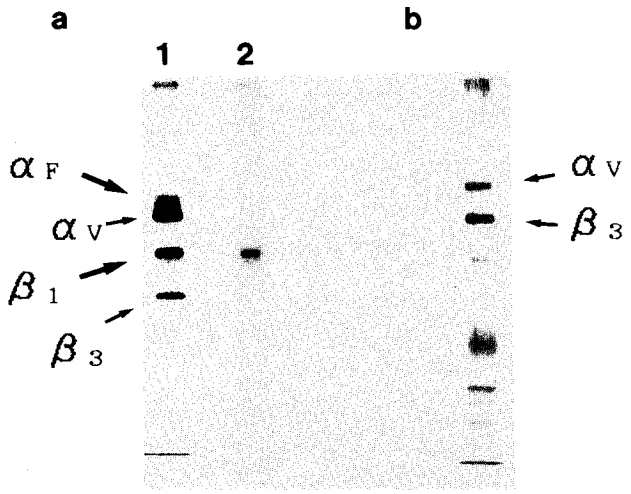

Fig. 1. Specificity of monoclonal antibodies. (a) Immunoblotting with anti-FNR (S6). Purified FNR and VNR were mixed and electrophoresed on a 6% gel under nonreducing conditions. The gel was stained with silver (lane 1) or immunoblotted with S6 (lane 2). (b) Affinity chromatography with an anti-VNR (H4)-Sepharose column. Placental extract (10 ml) was applied to an H4-Sepharose column equilibrated with PBS (pH 7.2) containing 25 mM OTG, 1 mM $CaCl_2$, 1 mM $MgCl_2$ and 1 mM PMSF. After washing with the same buffer, the bound materials were eluted with 50 mM diethylamine (pH 11.5) containing 25 mM OTG, 1 mM $CaCl_2$, 1 mM $MgCl_2$ and 1 mM PMSF. The eluate was electrophoresed on a 7.5% gel under reducing conditions and protein bands were made visible by silver staining.

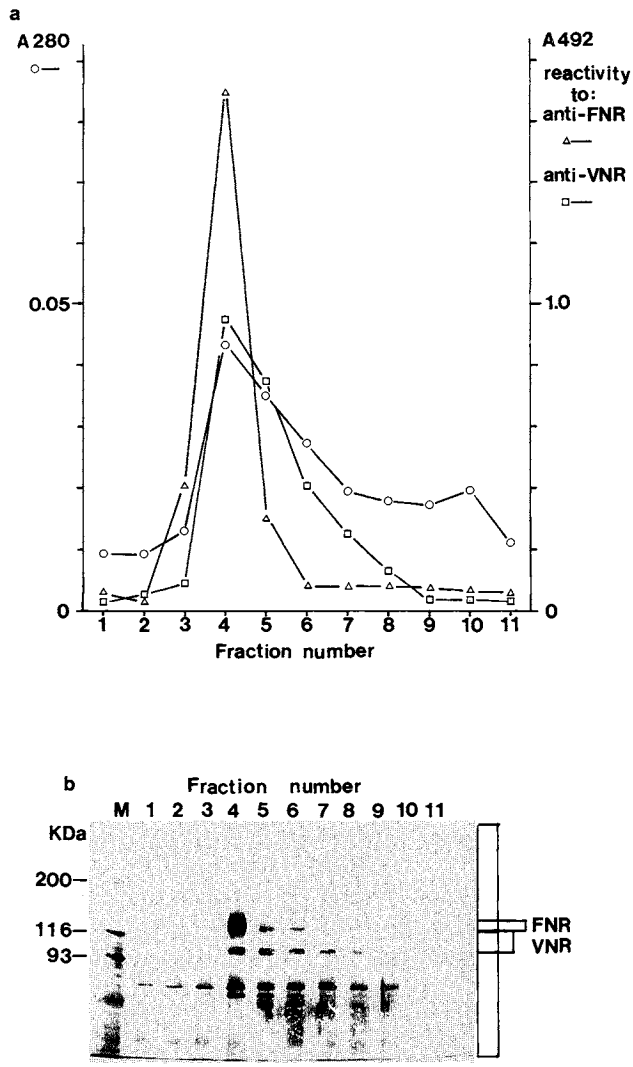

Fig. 2. Affinity chromatography of placental extract with a FN 110 KDa column in the presence of Mn^{2+}. Placental tissue was washed, extracted and applied to a FN 110 KDa column as described in "Materials and Methods", except that the buffers used contained 1 mM $MnCl_2$ in place of 1 mM $CaCl_2$ and 1 mM $MgCl_2$. Ten ml of the clear extract from 10 g of wet tissue was applied to 1 ml of the FN 110 KDa column containing 5 mg of the FN fragment. The column was washed with 15 ml of the equilibration buffer and eluted with the buffer containing 20 mM EDTA in place of divalent cations. Fractions of 1.2 ml were collected. The fractions were analyzed by ELISA (a) and by SDS-PAGE on a 6% gel under reducing conditions (b). M, molecular weight markers; lanes 1~11, fractions 1~11. Electrophoretic patterns of FNR and VNR are shown schematically on the right side.

tion of FNR and VNR in the following experiments.

VNR bound to the FN 110 KDa fragment in the presence of Mn^{2+}. Gailit and Ruoslahti (6) reported that the yield of FNR with FN 110 KDa column chromatography was substantially increased in the presence of Mn^{2+}, as opposed to Ca^{2+} and Mg^{2+}. We have repeated their experiments with the unexpected results that in the presence of Mn^{2+}, not only the yield of FNR increased, but VNR was also retained in the FN 110 KDa column and eluted with EDTA (Fig. 2). The result was inconsistent with that reported by Gailit and Ruoslahti (6) since they did not find any significant binding of VNR to the FN 110 KDa column. In the presence of Ca^{2+} and Mg^{2+} instead of Mn^{2+}, the FN 110 KDa column bound FNR but not VNR (Fig. 3). The result was consistent with that which was reported previously (16). A GRGDSPK column is another affinity column which is used to isolate RGD-recognizing integrins including VNR and platelet glycoprotein gp IIb/IIIa. Therefore, we examined the affinity of the receptors to a GRGDSPK column in the presence of Mn^{2+}. About the same amount of VNR bound to the GRGDSPK column in the presence of Mn^{2+}, as compared to Ca^{2+} and Mg^{2+} (Fig. 4). The FNR did not bind to the column even in the presence of Mn^{2+}. The electrophoretic mobilities of FNR and VNR have been shown to change in a characteristic fashion under reducing and nonreducing conditions (17). By electrophoresis of eluted proteins from affinity columns under these conditions, we definitely confirmed that both FNR and VNR bound to the FN 110 KDa column (Fig. 5).

Purified VNR bound to a FN 110 KDa column in the

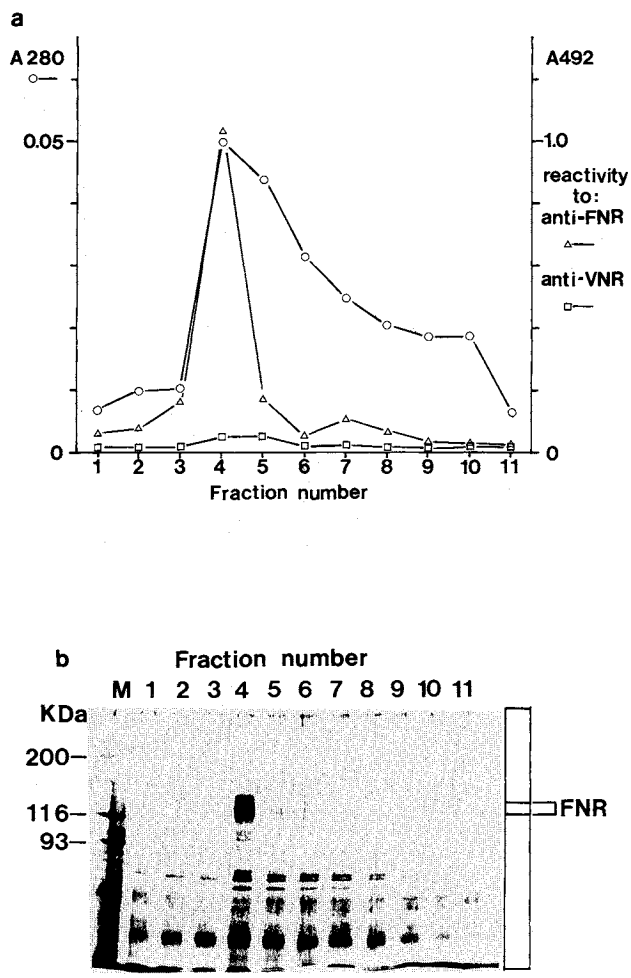

Fig. 3. Affinity chromatography of placental extract with a FN 110 KDa column in the presence of Ca^{2+} and Mg^{2+}. Placental tissue (10 g) was extracted and applied to a FN 110 KDa column as described in Fig. 2, except that the buffers used contained 1 mM $CaCl_2$ and $MgCl_2$ in place of 1 mM $MnCl_2$. Fractions of 1.2 ml were collected. The fractions were analyzed by ELISA (a) and by SDS-PAGE on a 6% gel under reducing conditions (b). M, molecular weight markers; lanes 1~11, fractions 1~11. Electrophoretic pattern of FNR is shown on the right side.

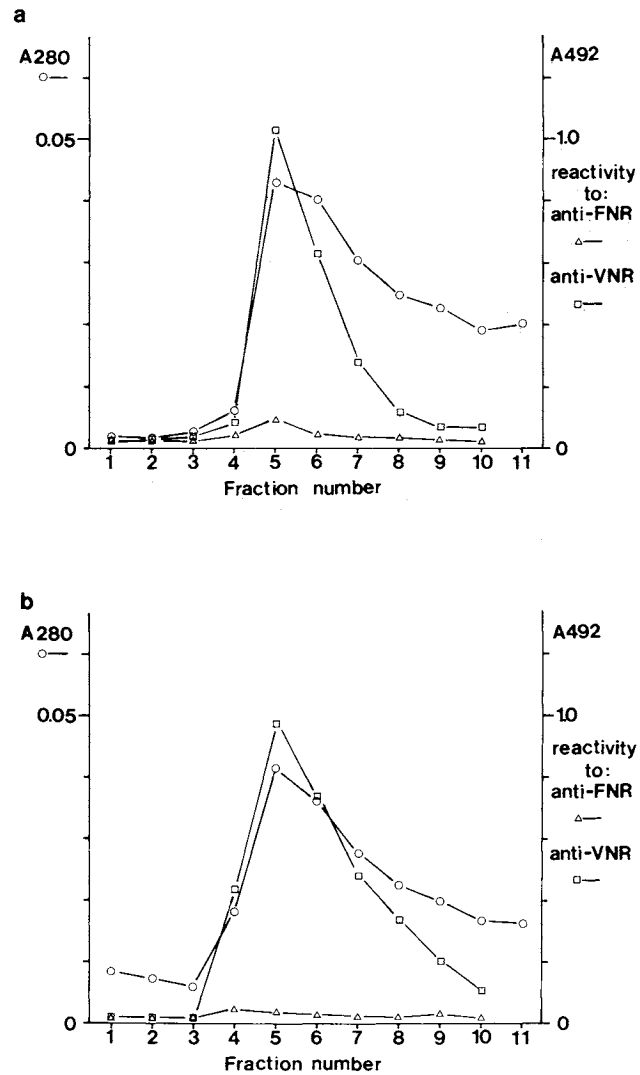

Fig. 4. Affinity chromatography of placental extract with a GRGDSPK column. The preparation of the samples and the isolation conditions were the same as described in Fig. 2 for (a) and in Fig. 3 for (b), except that a GRGDSPK column was used instead of a FN 110 KDa column.

presnce of Mn^{2+}. In the experiments described above, we applied the crude extract of human placenta to a FN 110 KDa column. Under the existing conditions, there was a possibility that Mn^{2+} could cause the association of VNR with FNR and VNR bound indirectly to the FN 110 KDa column through FNR. We therefore applied purified VNR (Fig. 6a) to the FN 110 KDa column. The purified VNR also bound to the FN 110 KDa column in the presence of Mn^{2+} (Fig. 6b), but not in the presence of Ca^{2+} and Mg^{2+} (Fig. 6c). It was thus concluded that placenta VNR bound directly to the FN 110 KDa column in the presence of Mn^{2+}.

Effect of changing divalent cations on the affinity of FNR and VNR to a FN 110 KDa column. In the presence of Mn^{2+}, both receptors bound to a FN 110 KDa column. The change of the buffer containing Mn^{2+} to that containing Ca^{2+} and Mg^{2+} eluted all the VNR and the greater part of FNR (Fig. 7). The rest of the FNR remaining in the column was eluted with the buffer containing EDTA.

Effect of GRGDSP and GRGESP on the affinity of each receptor to a FN 110 KDa column. The FNR can be eluted from a FN 110 KDa column by the competitive peptide GRGDSP, but not by the control peptide GRGESP in the presence of Ca^{2+} and Mg^{2+} (19). When the same experiments were carried out in the presence

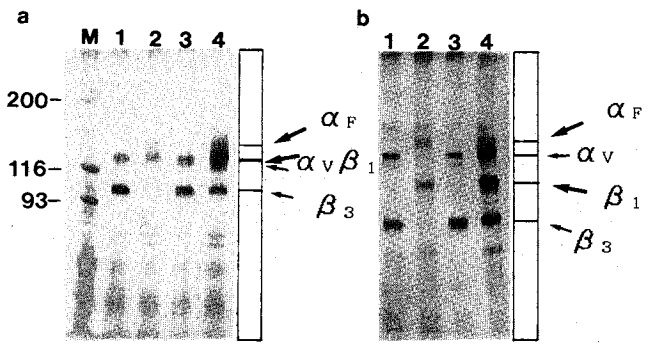

Fig. 5. Electrophoretic analysis of fractions of affinity chromatography by SDS-PAGE under reducing (a) and nonreducing (b) conditions. Peak fractions in Figs. 2, 3 and 4 were run on a 6% gel, and protein bands were made visible by silver staining. M, molecular weight markers; lane 1, a peak fraction in Fig. 4b; lane 2, a peak fraction in Fig. 3; lane 3, a peak fraction in Fig. 4a; lane 4, a peak fraction in Fig. 2. Electrophoretic patterns of FNR and VNR are shown schematically on the right sides in (a) and (b).

of Mn^{2+} in which both FNR and VNR bound to the column, the greater part of VNR was eluted from the column with GRGESP (Fig. 8a). On the other hand, FNR was retained in the column by GRGESP elution and was washed out by subsequent elution with GRGDSP (Fig. 8a). When the column was eluted first with GRGDSP and then with GRGESP, both FNR and VNR were completely eluted with the first GRGDSP (Fig. 8b).

DISCUSSION

The VNR of human placenta was shown to bind to the cell-binding domain of FN in the presence of Mn^{2+} by ELISA, using receptor specific monoclonal antibodies and electrophoretic analyses. By the replacement of divalent cations from Mn^{2+} to Ca^{2+} and Mg^{2+}, VNR binding to the FN 110 KDa column was completely eluted from the column (Fig. 7). This suggests that Mn^{2+} does not merely increase the affinity of VNR to FN 110 KDa, but additionally elicits the binding activity of VNR to FN 110 KDa. On the other hand, a part of FNR was retained on the FN 110 KDa column by the same replacement of divalent cations and was eluted with a buffer containing EDTA (Fig. 7). The result was explained by the fact that Mn^{2+} enhanced the binding of FNR to FN 3~10-times as compared to Ca^{2+} and Mg^{2+} (6, 8). In the report of Gailit and Ruoslahti (6), they did not find the binding of VNR to the FN 110 KDa column in the presence of Mn^{2+} under the same experimental conditions described here. The reason for this inconsistency is not known. Recently, Charo *et al.* (2) reported that the placenta $\alpha_v\beta_3$ integrin, which was immobilized in microtiter wells, bound FN with high affinity and in an RGD-dependent manner in the presence of 2 mM $CaCl_2$. Their results are inconsistent with our results since we did not detect the binding of VNR to the FN 110 KDa column in the presence of Ca^{2+} and Mg^{2+}. Some differences in the experimental conditions, including the purification methods of placenta VNR,

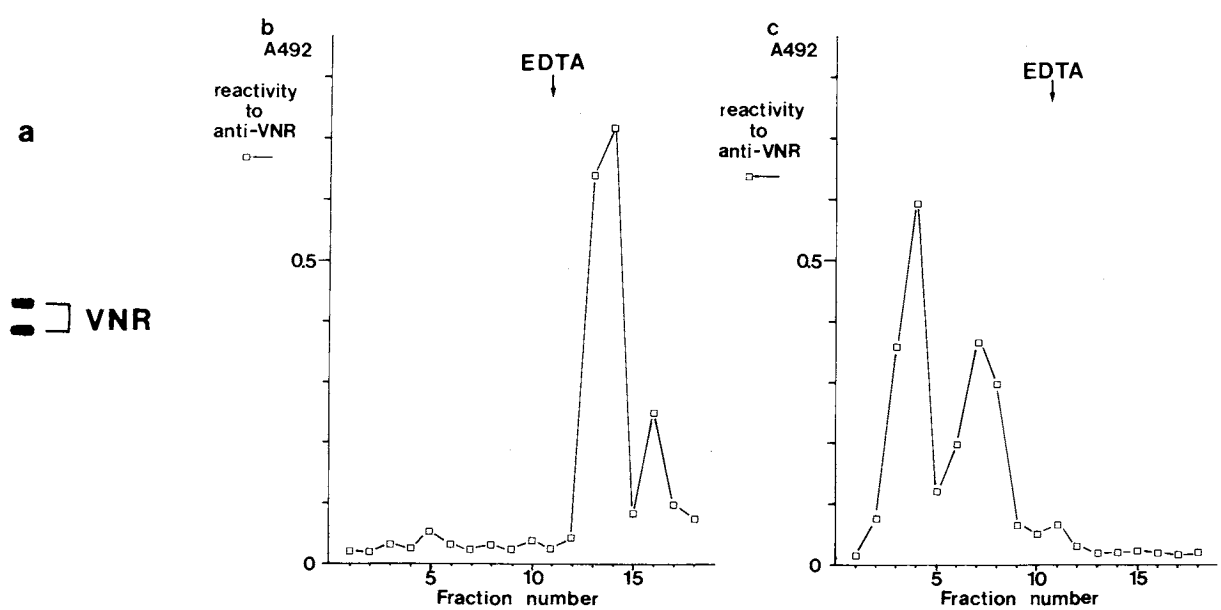

Fig. 6. Affinity chromatography of purified VNR with a FN 110 KDa column. One ml of purified VNR fraction (50 μg/ml) (a) was applied to a FN 110 KDa column in the presence of 1 mM $MnCl_2$ (b) or in the presence of 1 mM $CaCl_2$ and 1 mM $MgCl_2$ (c). The experimental conditions were the same as described in Fig. 2 for (b) and in Fig. 3 for (c).

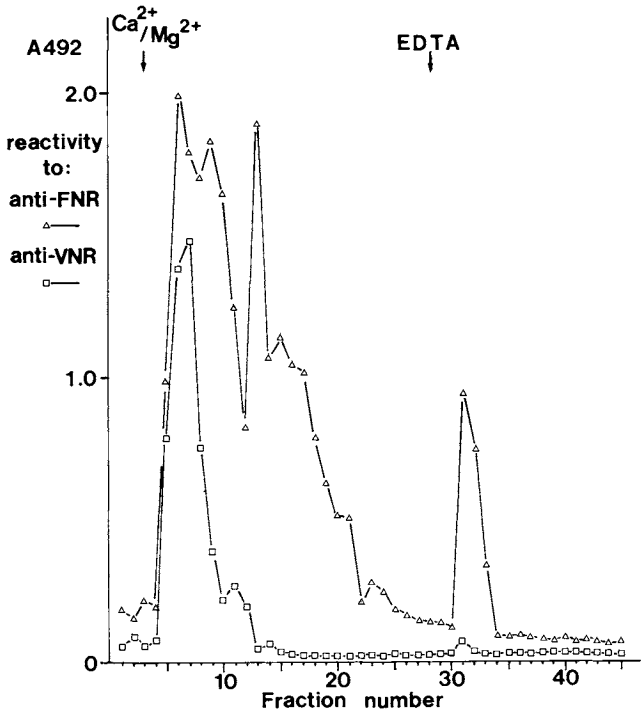

Fig. 7. Effect of changing cations on the binding properties of FNR and VNR to an FN 110 KDa column. Ten ml of the placental extract was applied to a FN 110 KDa column equilibrated with the buffer containing 1 mM Mn^{2+} as described in Fig. 2. After the column was washed with 20 ml of the same buffer, bound materials were eluted with 15 ml of the buffer containing 1 mM Ca^{2+} and Mg^{2+} in place of Mn^{2+} and then eluted with 20 ml of the buffer containing 20 mM EDTA. Fractions of 1.2 ml were collected. The fractions were analyzed by ELISA.

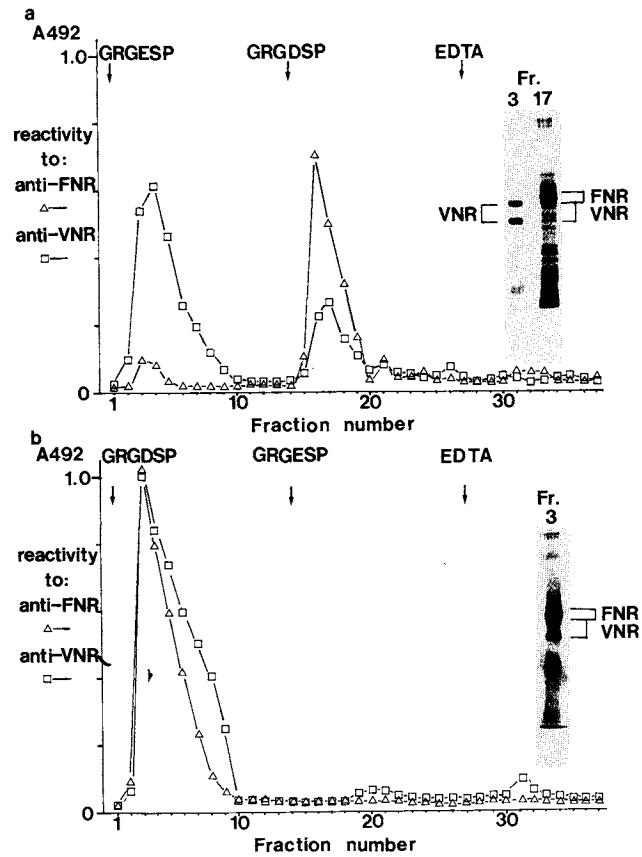

Fig. 8. Effect of GRGDSP and GRGESP on the binding properties of FNR and VNR to a FN 110 KDa column in the presence of Mn^{2+}. Ten ml of placental extract was applied to a FN 110KDa column as described in Fig. 2. After washing, bound materials were eluted as follows. (a) Successive application of 15 ml of TBS containing 3 mM GRGESP, 25 mM OTG, 1 mM $MnCl_2$ and 1 mM PMSF, 15 ml of TBS containing 3 mM GRGDSP, 25 mM OTG, 1 mM $MnCl_2$ and 1 mM PMSF, and 20 ml of TBS containing 20 mM EDTA, 25 mM OTG and 1 mM PMSF. (b) Successive application of 15 ml of TBS containing 3 mM GRGDSP, 25 mM OTG, 1 mM $MnCl_2$ and 1 mM PMSF, 15 ml of TBS containing 3 mM GRGESP, 25 mM OTG, 1 mM $MnCl_2$ and 1 mM PMSF and 20 ml of TBS containing 20 mM EDTA, 25 mM OTG and 1 mM PMSF. Fractions of 1.2 ml were collected. The fractions were analyzed by ELISA. Peak fractions were analyzed by SDS-PAGE (inlets).

the components in binding solutions and the conditions for binding assays, may explain the inconsistency. However, elucidation of the precise causes of the inconsistency needs further studies.

The VNR bound to the FN 110 KDa column in the presence of Mn^{2+} was detached from the column by a buffer containing GRGDSP or GRGESP. This result indicates that the RGD-recognition site of VNR is responsible for the binding to FN 110 KDa. It is surprising that GRGESP acts as a competitor since GRGESP has been used as a control peptide to study the action of RGD-containing peptides. An exception to this premise was reported by Yamada and Kennedy (25), who found that GRGES at higher concentrations acted as an inhibitor for cell spreading of chick embryo fibroblasts on VN, laminin or three-dimensional native type I collagen gels. GRGDSP, however, seems to be a stronger competitor than GRGESP since GRGESP could not elute VNR completely from the FN 110 KDa column, while GRGDSP almost completely eluted the receptor (Fig. 8). In order to interpret this RGE sensitivity of VNR, a possibility can be considered that in the presence of Mn^{2+}, the requirement of VNR for D in the RGD sequence becomes loose and only an RG sequence becomes the key sequence for the recognition. The result that GRGESP is an effective competitor for VNR in the presence of Mn^{2+} may be applied to the interpretation of previous works on the Mn^{2+} effect in cell attachment assays. Mn^{2+} enhanced cell attachment to surfaces coated with a variety of usually nonadhesive proteins, such as bovine serum albumin (BSA) and ovalbumin (7, 24). In preliminary studies, we observed that cell attachment of human fibroblasts (TIG-3) to BSA in the pres-

ence of Mn^{2+} was inhibited by both GRGDSP and GRGESP (data not shown). Since BSA contains an RGV sequence, the recognition of the RG sequence by the VNR of TIG-3 cells might account for the attachment. The details will be reported elsewhere.

FNR does not bind to a GRGDSPK column or the RGD sequence of VN. This phenomenon can be explained by the fact that both the RGD sequence and the synergistic binding site of FN are required for the binding of FNR to FN (15). On the other hand, in the presence of Ca^{2+} and Mg^{2+}, VNR does not bind to the FN 110 KDa column, *i.e.*, the RGD sequence of FN, although it does bind to the GRGDSPK column (see Fig. 3, 4b and 6, and refer to the manuscripts of Pytela *et al.* (16) for placenta VNR and Shimo-Oka *et al.* (23) for VNR of placenta and human fibroblasts). This phenomenon can not be explained in this situation by the existence of a supposed synergistic binding site in VN, since VNR exhibits strong affinity to the GRGDSP peptide that originates from FN itself. It is therefore concluded that the accessibility of VNR to the RGD sites of FN and VN, *i.e.*, the conformation around the RGD sites of FN and VN, must be different.

Which is the target of the Mn^{2+} ion: FN 110 KDa or the VNR? So far, the binding of divalent cations to FN 110 KDa has not been evidenced. Analysis of the FN primary sequence also does not predict overt divalent cation binding sites. On the other hand, VNR as well as other integrins have the predicted divalent cation binding sites in the α subunit. It has been shown that Mn^{2+} can compete with radioactive Ca^{2+} for the same site of an FNR α subunit (6). Therefore, it appears that the target of the Mn^{2+} ion is the α subunit of VNR and its binding to the Mn^{2+} can change VNR from a fibronectin-nonbinding to a fibronectin-binding type. Recently, Conforti *et al.* (5) found that placenta $\alpha_v\beta_3$ integrin reconstituted into phosphatidylcholine vesicles bound only vitronectin, in agreement with the earlier work of Pytela *et al.* (17), and when a mixture of phosphatidylcholine and phosphatidylethanolamine or a membrane composed of five kinds of membrane lipids was used to form the vesicles, the $\alpha_v\beta_3$ integrin also bound fibronectin and von Willebrand factor. A conformational change of the $\alpha_v\beta_3$ integrin was suggested from a difference in the intrinsic emission fluorescence spectrum of the receptor in the different membranes. Under these circumstances, we also consider that the conformational change of VNR when induced by Mn^{2+} might enable the receptor to approach the RGD site of FN. We therefore propose the idea that the individual specificity of RGD-recognizing integrins is preserved not only by the difference in the conformation around the RGD region of each cell-adhesive protein, but also by the difference of conformation at the RGD-recognition site of each integrin itself.

REFERENCES

1. Charo, I.F., Bekeart, L.S., and Phillips, D.R. (1987). Platelet glycoprotein IIb-IIIa-like proteins mediate endothelial cell attachment to adhesive proteins and the extracellular matrix. *J. Biol. Chem.*, **262**: 9935–9938.
2. Charo, I.F., Nannizzi, L., Smith, J.W., and Cheresh, D.A. (1990). The vitronectin receptor $\alpha_v\beta_3$ binds fibronectin and acts in concert with $\alpha_5\beta_1$ in promoting cellular attachment and spreading on fibronectin. *J. Cell Biol.*, **111**: 2795–2800.
3. Cheresh, D.A. (1987). Human endothelial cells synthesize and express an Arg-Gly-Asp-directed adhesion receptor involved in attachment to fibronectin and von Willebrand factor. *Proc. Natl. Acad. Sci. USA*, **84**: 6471–6475.
4. Cheresh, D.A. and Spiro, R.C. (1987). Biosynthetic and functional properties of an Arg-Gly-Asp-directed receptor involved in human melanoma cell attachment to vitronectin, fibrinogen and von Willebrand factor. *J. Biol. Chem.*, **262**: 17703–17711.
5. Conforti, G., Zanetti, A., Pasquali-Ronchetti, I., Quaglino, Jr., D., Neyroz, P., and Dejana, E. (1990). Modulation of vitronectin receptor binding by membrane lipid composition. *J. Biol. Chem.*, **265**: 4011–4019.
6. Gailit, J. and Ruoslahti, E. (1988). Regulation of the fibronectin receptor affinity by divalent cations. *J. Biol. Chem.*, **263**: 12927–12932.
7. Grinnell, F. (1984). Manganese-dependent cell-substratum. *J. Cell Sci.*, **65**: 61–72.
8. Hautanen, A., Gailit, G., Mann, D.M., and Ruoslahti, E. (1989). Effects of modifications of the RGD sequence and its context on recognition by the fibronectin receptor. *J. Biol. Chem.*, **264**: 1437–1442.
9. Hynes, R.O. (1987). Integrins: A family of cell surface receptors. *Cell*, **48**: 549–554.
10. Iwasaki, T., Ando, T., Ichikawa, K., and Yasui, K. (1982). Monoclonal Antibody (in Japanese), Kodansha Scientific, Tokyo.
11. Izumi, M., Shimo-Oka, T., Morishita, N., Ii, I., and Hayashi, M. (1988). Identification of the collagen-binding domain of vitronectin using monoclonal antibodies. *Cell Struct. Funct.*, **13**: 217–225.
12. Laemmli, U.K. (1970). Cleavage of structural proteins during the assemlty of the head of bacteriophage T4. *Nature*, **227**: 680–685.
13. Lawler, J.L. and Hynes, R.O. (1989). An integrin receptor on normal and thrombasthenic platelets that binds thrombospondin. *Blood*, **74**: 2022–2027.
14. Matsuo, M., Kaji, M., Utakoji, T., and Hosoda, K. (1982). Ploidy of human embryonic fibroblasts during in vitro aging. *J. Gerontol.*, **37**: 33–37.
15. Obara, M., Kang, M.S., and Yamada, K.M. (1988). Site-derected mutagenesis of the cell-binding domain of human fibronectin: Separable, synergistic sites mediate adhesive function. *Cell*, **53**: 649–657.
16. Pytela, R., Pierschbacher, M.D., Argraves, S., Suzuki, S., and Ruoslahti, E. (1987). Arginine-glycine-aspartic acid adhesion receptors. *Methods in Enzymol.*, **144**: 475–489.
17. Pytela, R., Pierschbacher, M.D., Ginsberg, M.H., Plow, E.F., and Ruoslahti, E. (1986). Platelet membrane glycoprotein IIb/IIIa: member of a family of Arg-Gly-Asp-specific adhesion receptors. *Science*, **231**: 1559–1562.
18. Pytela, R., Pierschbacher, M.D., and Ruoslahti, E. (1985). A 125/115-kDa cell surface receptor specific for vitronectin interacts with the arginine-glycine-aspartic acid adhe-

sion sequence derived from fibronectin. *Proc. Natl. Acad. Sci. USA,* **82**: 5766–5770.
19. Pytela, R., Pierschbacher, M.D., and Ruoslahti, E. (1985). Identification and isolation of a 140 kd cell surface glycoprotein with properties expected of a fibronectin receptor. *Cell,* **40**: 191–198.
20. Ruoslahti, E., Hayman, E.G., and Engvall, E. (1981). Alignment of biologically active domains in the fibronectin molecule. *J. Biol. Chem.,* **256**: 7277–7281.
21. Ruoslahti, E. and Pierschbacher, M.D. (1986). Arg-Gly-Asp: A versatile cell recognition signal. *Cell,* **44**: 517–518.
22. Ruoslahti, E. and Pierschbacher, M.D. (1987). New perspectives cell adhesion: RGD and Integrins. *Science,* **238**: 491–497.
23. Shimo-Oka, T., Hasegawa, Y., and Ii, I. (1988). Differential properties of attachment of human fibroblasts to various extracellular matrix proteins. *Cell Struct. Funct.,* **13**: 515–524.
24. Stenn, K.S. and Core, N.G. (1986). Cation dependence of Guinea Pig epidermal cell spreading. *In Vitro Cellular and Developmental Biology.,* **22**: 217–222.
25. Yamada, K.M. and Kennedy, D.W. (1987). Peptide inhibitors of fibronectin, laminin, and other adhesion molecules: unique and shared features. *J. Cellular Physiol.,* **130**: 21–28.

(Received for publication, December 11, 1990
and in revised form, February 4, 1991)