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The Binding Ability to Matrix Proteins and the Inhibitory Effects on Cell Adhesion of Synthetic Peptides Derived from a Conserved Sequence of Integrins

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The β peptide (113-125), derived from a conserved sequence of the β subunit of integrins, was synthesized to investigate its adhesive properties to matrix proteins and the effects on cell adhesion to immobilized fibronectin. In this study, we observed that the biotinylated β peptide was able to bind efficiently to immobilized fibronectin, fibrinogen, collagen Type I and vitronectin with different degrees of affinity. It was also demonstrated that biotinylated fibronectin or fibrinogen could bind to the coated β peptide. This kind of binding, which might be non-covalent linkage, was partially blocked by coincubation with the peptide GRGDS or EDTA, but not by SDGRG. Cell adhesion experiments were performed to study the effect of the β peptide. The data showed that the β peptide partially inhibited both fibroblast L929 and MC3T3-E1 osteoblastic cells from adhering to immobilized fibronectin in a dosage-dependent manner. In the presence of 100 μM concentration of the β peptide, the inhibition rate of cell adhesion was 34% for fibroblast L929 cells and 54.1% for MC 3T3-E1 osteoblastic cells. This research suggests that the β peptide might act independently as an adhesive region of the β subunit of integrins and may occupy the cell-binding site within fibronectin.

Key words: cell adhesion, fibrinogen, fibronectin, inhibitory effect, integrin.

The integrins expressed on a wide variety of cells constitute a superfamily of non-covalently linked heterodimeric cell surface receptors for extracellular matrix molecules and are involved in cell-cell interaction and/or cell-matrix attachment, performing cell recognition and cell adhesion. Integrins play a very important role in extracellular signal transduction, cell proliferation, and cell differentiation, as well as wound healing and tumor metastasis (*1, 2*).

All integrins are composed of an α and a β subunit, which are the products of different genes and share similar structures: *i.e.*, a large extracellular part, a transmembrane region, and a short cytoplasmic domain (*2-4*). To date, this family has been found to have 14α and 8β subunits, which associate to produce probably more than 20 transmembrane glycoproteins with different ligand specificities, generating diversity and versatility in integrin functions (*3, 5*).

The integrin superfamily can be classified into three major subfamilies based on the kinds of β subunits. The β_1 subunits can associate with at least eight various α subunits, including α_1 and α_v (*3*), and contain receptors for fibronectin (*6-8*), collagen (*9, 10*), laminin (*11-14*), tenascin (*15*), and so on. The β_2 subfamily consists of leukocyte receptors with common immunoglobulin-related cell-surface molecules, such as ICAMs (*16, 17*). In the β_3 subfamily, the multispecific receptors are known to contain $\alpha_{IIb}\beta_3$ on the surface of platelets and $\alpha_v\beta_3$ for vitronectin, osteopontin and others (*18-21*). All integrin β subunits are almost identical with respect to their amino acid sequence (*4*).

The ligands for some integrins are known to be adhesive extracellular or matrix macromolecules, such as fibronectin, vitronectin, fibrinogen, collagen, and laminin. Many integrins recognize the sequence of the tripeptide Arg-Gly-Asp (RGD) in fibronectin and other adhesive proteins, but not all integrins recognize this sequence, and other peptide-binding motifs have also been identified. It is clear that one kind of integrin has multiple ligands, and that a given ligand can bind to various integrins with different degrees of affinity (*3*).

Since RGD-containing peptides inhibit the binding of these adhesive proteins to GPIIb-IIIa (*e.g.*, $\alpha_{IIb}\beta_3$) (*1*), the potential binding domains (to adhesive matrix molecules) in several integrin subunits—especially the β subunits—have also been investigated, using immunological, biochemical, and mutational approaches.

In the early studies, data from the $\alpha_{IIb}\beta_3$ indicated that the ligand-binding fragment was located in the N-terminal part for both α and β subunits (*22-30*). D'Souza *et al.* (*26*) found a 72-residue sequence in β_3 (Arg^{109}-Glu^{171}) which was thought to bind the ligand, by the method of chemical cross-linking of the RGD peptide to $\alpha_{IIb}\beta_3$, followed by proteolytic degradation and micro-amino acid sequencing

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Abbreviations: α-MEM, alpha minimum essential medium; BSA, bovine serum albumin; EDTA, ethylenediaminetetraacetic acid; PBS, phosphate buffered saline; TFA, trifluoroacetic acid.

of relevant fragments. This finding also proved the existence of an overlapping region in the β_3 (Glu^{65}-Glu^{220}) by photoaffinity cross-linking of the RGD peptide to $\alpha_v\beta_3$ (*25*). The region in the β_3 from amino acid residues 113 to 128 has been identified by several experiments: (1) point mutations of this region, especially in Asp^{119}, Ser^{201}, and Ser^{203}, abrogate the ligand-binding functions (*31, 32*); (2) antibodies against this region inhibit ligand-binding (*33-35*). This fragment within β_3 is also highly conserved among all the β subunits and some α subunits, suggesting that this is a common ligand-contacting site. This conserved region is expressed as D$$$$$DXSXS$KDDL ($, any hydrophobic residue; X, any residue). In the major β subfamilies, the sequence is DLYYLMDLSYSMKDDL. This region has a hydrophobic pentapeptide sequence and probably contains a ligand-binding motif, and it provides coordinating ligands for divalent cations. A second highly conserved ligand-recognition site in the β_3 subunit has been identified as residues 211-222 by Charo *et al.* (*36*). This group synthesized several polypeptides corresponding to the short sequences in the β_3 subunit and concluded that this sequence is a highly conserved recognition site by studying the inhibition of fibrinogen binding by the peptide. However, they did not carry out inhibition experiments on the region (113-128), because of experimental difficulty.

In this study, we designed and synthesized a polypeptide with the sequence Asp-Leu-Tyr-Tyr-Leu-Met-Asp-Leu-Ser-Tyr-Ser-Met-Lys (DLYYLMDLSYSMK), which fit the conserved sequence (Asp^{113}-Lys^{125}) of the β subunits of integrins. Then we observed the polypeptide's binding abilities to several matrix proteins and its inhibitory effects on the adhesion of fibroblast cells and MC3T3-E1 osteoblastic cells to immobilized fibronectin. We found that the β peptide might act independently as an adhesive region of the β subunits and may mainly occupy the cell-binding site within fibronectin.

MATERIALS AND METHODS

Materials—A polypeptide with the sequence DLYYLMDLSYSMK, referred to below as the β peptide, was synthesized by the Peptide Institute in Osaka, Japan. The purity (HPLC) was calculated to be 95.1% by analyzing the synthetic product on a YMC Pkk ODS-AM column with a gradient elution of 20-27% CH_3CN in 0.1% TFA.

Peptides Gly-Arg-Gly-Asp-Ser (GRGDS) and Ser-Asp-Gly-Arg-Gly (SDGRG), rat fibronectin, rat fibrinogen, BSA, monoclonal anti-biotin alkaline phosphatase conjugate, monensin, γ-globulin, and *N*-succinimido-biotin were purchased from Sigma, St. Louis, MO, USA. Bovine plasma vitronectin was from the Yagai Research Center, Yamagata. Porcine skin collagen Type I was from Wako, Osaka. Alkaline phosphatase substrate kit was from Bio-Rad, Richmond CA, USA. *N*-Succinimidyl 3-(2-pyridyldithio)propionate (SPDP) was from Pierce, Rockford, Illinois, USA. Methylene blue was from Schmid. GmbH. Other reagents and chemicals were obtained commercially, with analytical purities. Sephadex G-25 and CNBr Sepharose 4B were from Pharmacia, and Bio-gel P-2 was from Bio-Rad. Lidded microtiter plates with 96 flat-bottomed wells (well diameter 6.4 mm) were obtained from Corning Glass Works, Corning, N.Y., USA.

Affinity Chromatography of Fibrinogen and Fibronectin on the Immobilized β Peptide—The β peptide was coupled to CNBr-Sepharose 4B according to the manufacturer's instructions. The coupled Sepharose was then packed into a 2 ml column and equilibrated with 20 mM Tris-HCl, pH 7.0, containing 0.1% Triton X100, 150 mM NaCl, 1 mM $CaCl_2$, 1 mM $MgCl_2$, and 0.05% NaN_3. Then 0.4 mg of fibrinogen or fibronectin was applied to the column. The column was washed with 80 ml of the above buffer, then bound proteins were then eluted by washing with 4 ml of column buffer containing 1.5 mg of the β peptide, followed by another 10 ml of column buffer. The fractions were analyzed by electrophoresis on 12.5% sodium dodecyl sulfate-polyacrylamide gels. Protein bands were detected by staining with Coomassie Blue.

Biotinylation of the β Peptide and Proteins—The β peptide (2 mg) was dissolved in 0.75 ml of a solution of 0.1 M $NaHCO_3$+0.1 M NaCl, pH 8.2; then 3 mg of *N*-hydrosuccinimido-biotin in 150 μl of fresh dimethyl sulfoxide was added. The reactants were gently mixed and allowed to stand at room temperature for 2 h.

The uncoupled *N*-hydrosuccinimido-biotin was removed by gel filtration on a Sephadex G-25 or a Bio-gel P-2 column (40×1.2 cm) at a flow rate of 1 ml/1.5 min. The effluence was monitored at wavelengths of 220 and 280 nm. The peak in the void volume was collected and stored at −20°C for later use.

For biotinylation, 1 mg of rat fibronectin or fibrinogen was dissolved in a small amount of distilled water and dialyzed against 0.1 M $NaHCO_3$+0.1 M NaCl at 4°C overnight. Then 0.2 mg of *N*-hydrosuccinimido-biotin in 40 μl of dimethyl sulfoxide was added. The reaction conditions and the removal of free *N*-hydrosuccinimido-biotin were as described above.

Solid Phase Binding Assay of the Biotinylated β Peptide to Adhesive Proteins—The solid-phase binding assay of the biotinylated β peptide was performed as described in a previous study (*37*), with some modifications. Microtiter plates were coated with fibronectin (50 μg/ml), fibrinogen (1 mg/ml), vitronectin (30 μg/ml), or collagen Type I (500 μg/ml) dissolved in buffer A (20 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1 mM $CaCl_2$, 1 mM $MgCl_2$, and 1 mM $MnCl_2$). Then, 0.1 ml of this solution was added to each well, and the plates were incubated overnight at 4°C. Each plate was washed once with buffer B (50 mM Tris-HCl pH 7.4, 100 mM NaCl, 2 mM $CaCl_2$, 1 mM $MgCl_2$, and 1 mM $MnCl_2$) simply by filling and then emptying by aspiration. The non-specific binding sites were blocked with buffer B+35 mg/ml BSA and the plates were incubated for an additional 2 h at 30°C with gentle shaking. Then the plates were washed twice with the incubation buffer (buffer B+1 mg/ml BSA). The biotinylated β peptide dissolved in the incubation buffer was added to the indicated concentrations, and the plates were further incubated for 2-3 h at 30°C. Each well was then rinsed once with 300 μl of buffer B, and the bound β peptide was quantitated by the addition to each well of 0.1 ml of anti-biotin antibody conjugated to alkaline phosphatase (1:1,000 dilution in buffer B) and incubation at 37°C for 1 h. Then the wells were washed twice with buffer B and all the solvents in the wells were completely discharged by inverting the plate and pressing it upside down on a sheet of filter paper. Then 0.1 ml of the substrate *p*-nitrophenol phosphate, prepared daily according to the manufacturer's instructions, was added and

incubated until the development of a light yellow color (for approximately 15-30 min). Then 0.1 ml of 0.4 N NaOH was added to stop the reactions, and optical density at a wavelength of 415 nm was read by use of an MTP-32 (PC-9801) microplate reader (Corona Electric). All reaction rates were read within the linear range, and all data were presented as means of triplicate determination. Standard deviation was always less than 15%, and usually less than 10%, of the mean. Nonspecific binding was indicated in every experiment by determining the binding of the biotinylated β peptide to the BSA-coated wells, as well as in the presence of excess unlabeled β peptide.

For the competitive assay, a GRGDS or a SDGRG peptide at the desired concentration was coincubated with the β peptide or biotinylated proteins. Experiments were carried out as described above.

Biotinylated Fibronectin and Fibrinogen Binding to the Immobilized β Peptide—Wells were coated with 0.1 ml of 20 μg/ml rat γ-globulin in buffer B overnight at 4°C. Then the wells were washed three times with buffer B and incubated with 0.1 ml of 10 μg/ml *N*-succinimidyl 3-(2-pyridyldithio)propionate (SPDP) in buffer B for 30 min at room temperature. After three washes with buffer B, the β peptide was added to each well at a concentration of 20 μg/ml and incubation was continued for 2 h at room temperature. Finally, the wells were washed three times with buffer B. The indicated biotinylated fibrinogen or fibronectin was added and incubated. The bound biotinylated fibrinogen and fibronectin were quantitated as described above.

Cells and Cell Culture—MC3T3-E1, an osteoblastic cell line derived from C57BL/6 mouse calvaria was kindly provided by Dr. Takashi Ushida. It was then cultured in α-MEM with 10% fetal calf serum at 37°C and 5% CO_2 in air, and subcultured every two days. The cells were incubated to the confluence stage before use. Fibroblast L929 cells were incubated under normal laboratory conditions.

Cell Adhesion—Ninety-six-well microtiter plates were coated with 0.1 ml of fibronectin (10 μg/ml) at 4°C overnight. The non-specific binding sites were saturated with an adhesion buffer (10 mM Hepes, pH 7.4, 140 mM NaCl, 5.4 mM KCl, 5.56 mM glucose, 3% BSA, 2 mM $CaCl_2$, 1 mM $MgCl_2$, 1 mM $MnCl_2$) and incubated for 2 h at 37°C. The cells were pretreated with 0.7 ng/ml monensin (for 1 h in RPMI-1640+10% fetal calf serum) to inhibit endogenous protein synthesis and secretion, and then resuspended in the adhesion buffer as single cells and adjusted to a cell density of $1\text{-}5 \times 10^5$/ml. Cells were either coincubated with the indicated concentration of the β peptide, or allowed to attach to fibronectin-coated wells in the absence of the peptide, and incubated at 30°C for 3 h. After washing the wells twice with the adhesion buffer, 0.1 ml of cells was placed in each well, and incubation was continued at 30°C for 2 h.

For pre-incubation, the cells were allowed to attach to coated fibronectin for 2 h at 37°C. After washing the plates twice with buffer B, the desired concentration of the β peptide was added to the wells, and incubation was continued for 2 h at 37°C.

Bound cells were quantitated by the method described in a previous paper (*38*). The adherent cells were fixed with 3% paraformaldehyde in PBS for 20 min at room temperature. Then the cells were rinsed once with a 0.1 M borate buffer, pH 8.5, and stained with 0.1 ml of 1% methylene blue (in 0.1 M borate buffer, pH 8.5) for 20 min at room temperature. The wells were washed four times with 0.1 M borate buffer, pH 8.5; then 0.1 ml of 1 M HCl was applied to each well to release the specific cell-incorporated dye, followed by incubation for 40 min at 37°C. Absorbance was determined at a wavelength of 600 nm. The uptake of methylene blue has a linear correlation to the number of viable cells (*39*). Each data point was the average of the results for three wells. Each experiment was performed at least three times, yielding comparable results.

RESULTS

Binding Properties of the β Peptide with Matrix Proteins—Interaction between macromolecules usually involves a two-step process: initial substrate recognition and binding (*37*). This process requires conformational changes that lead to binding between the contacting molecules (*36*).

The interaction between the β peptide and matrix proteins was investigated by measuring the peptide's binding ability, using affinity chromatography and solid phase binding assays as described in the experimental procedures. Two matrix proteins were chosen for the present study: fibronectin, the main adhesion protein that can bind to several different integrins in most tissues; and fibrinogen, existing in plasma and acting as a ligand to the platelet receptor $GP_{IIa}GP_{III}$ (integrin $\alpha_{IIa}\beta_3$) (*1*).

To verify that the β peptide can bind to fibrinogen or fibronectin, an affinity matrix was conjugated with the β peptide. The commercial fibrinogen or fibronectin was applied to the affinity column and then eluted with the β peptide solution. The major species eluted from the affinity column was fibrinogen or fibronectin, as shown in Fig. 1. Both fibrinogen and fibronectin could bind specifically to the β peptide.

In most research of this kind, microtiter plates are coated with the purified integrin proteins, then labeled ligand proteins are coincubated with the peptide. Then the inhibitory effects are determined. In the present research, we developed an experiment in which the contacting peptides and proteins interacted directly with each other. Two approaches were used in the binding assays. One consisted of the biotinylated β peptide binding to immobilized matrix

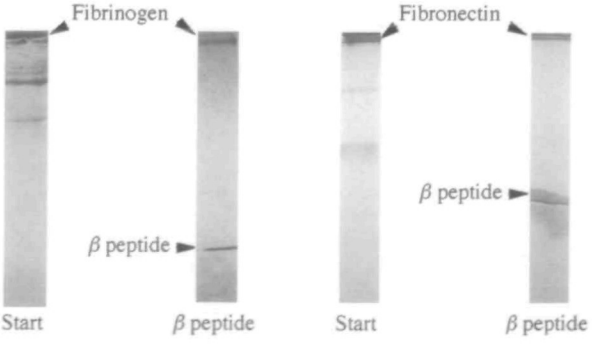

Fig. 1. **Elution of bound fibrinogen and fibronectin by the β peptide.** Fibrinogen (0.4 mg) or fibronectin (0.4 mg) was applied to a β peptide-Sephadex 4B column. After washing, the column was eluted with the β peptide. The elute was dialyzed against 20 mM triethylamine-acetate buffer, pH 8.3, then lyophilized and analyzed on 12.5% sodium dodecyl sulfate-polyacrylamide gels.

proteins. Figure 2 shows that the biotinylated β peptide could bind to both fibronectin and fibrinogen, but not to BSA. All of these bindings were dosage-dependent. The specific binding of the biotinylated β peptide to the proteins was confirmed by coincubation with excess unlabeled β peptide, which had the effect of reducing the binding to a certain degree (not shown). The non-specific binding of the biotinylated β peptide was monitored by the same assay with BSA (10 mg/ml)-coated wells, as a comparative indicator. Because of the lower solubility of the β peptide, the maximal concentration was only 100 μM. The data revealed that the concentration required for half of the maximal binding of the biotinylated β peptide to fibrinogen was 3.5-4.0 μM, and that required for fibronectin was approximately 15 μM. This may indicate that the binding ability of biotinylated β peptides to fibrinogen is greater than that to fibronectin.

The other approach used in the binding assays was coating the microtiter plate with the β peptide for the immobilized phase, incubating with biotinylated fibronectin and fibrinogen, and thereafter quantifying the amount of bound biotinylated proteins. Figure 3 indicates that these two biotinylated proteins could bind efficiently to the β peptide and that this binding was also dosage-dependent. The dosage-dependent binding curve was similar to that

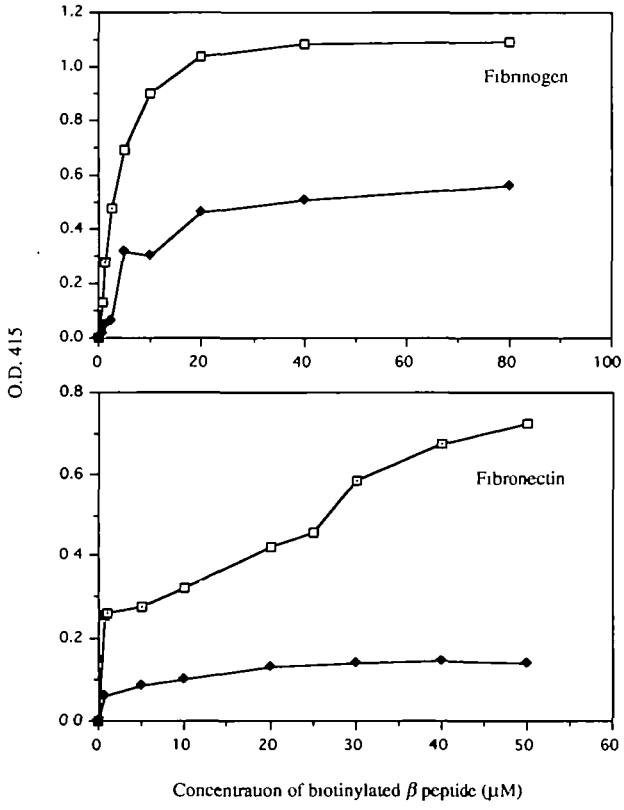

Fig. 2. **Binding of the biotinylated β peptide to immobilized fibrinogen and fibronectin.** The microtiter plates were coated with fibrinogen (1 mg/ml) and fibronectin (50 mg/ml), respectively. Then the biotinylated β peptide was added to each well to indicated concentrations. After incubation at 30°C (for 1 h for binding, plates were washed once or twice) with buffer B. The anti-biotin antibody conjugated with alkaline phosphatase was added; then the substrate solution was applied into each well. The time for color development for the β peptide binding to fibrinogen was 10 min, and that for binding to fibronectin was 25 min. The results were expressed as optical density at 415 nm and the means for three wells. Non-specific binding was monitored by the same assay in BSA-coated wells or in the presence of excess unlabeled β peptide. Each experiment was performed at least four times, yielding comparable results. Total binding (–□–). Non-specific binding (–◆–).

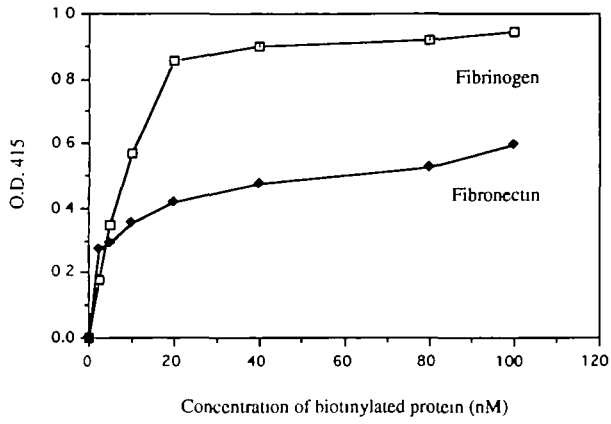

Fig. 3. **Binding of biotinylated fibrinogen and fibronectin to the immobilized β peptide.** Coated γ-globulin was conjugated with the β peptide by the bifunctional linker SPDP. Then biotinylated fibrinogen or fibronectin was added at the desired concentrations. The binding assays were carried out as described in the experimental procedures. Other procedures were the same as described for Fig. 2. The time for color development for this assay was 12 min.

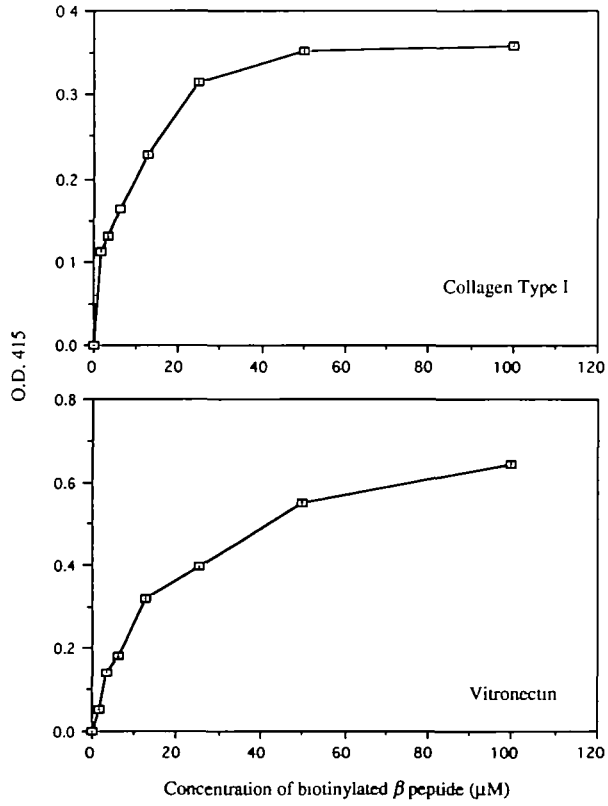

Fig. 4. **Binding of the biotinylated β peptide to collagen Type I and vitronectin.** The microtiter plates were coated with collagen Type I (500 μg/ml) and vitronectin (30 μg/ml). Then the procedure described for Fig. 2 was carried out.

shown in Fig. 2. Similar binding assays of the β peptide to collagen Type I and vitronectin were carried out, and similar results were obtained, as shown in Fig. 4. Analysis of the data obtained confirmed that the β peptides bound independently to the matrix-adhesive proteins.

The Inhibitory Effect of RGD-Containing Peptides and Calcium Ions on the Binding between the β Peptide and Adhesive Protein—In the binding assay, the β peptide simply acted as part of the binding domain of the β subunits of integrins. RGD-containing proteins or polypeptides were able to bind to this fragment. However, there may have been some effect from the interaction of the β peptide in the presence of two RGD-containing polypeptides; one in liquid, the other, in the immobilized phase. Taking this into account, we co-incubated the β peptide and GRGDS in the same system. The results, shown in Figs. 5, 6, and 7, indicate that the binding rate of the biotinylated β peptide to immobilized fibrinogen or fibronectin and collagen Type I or vitronectin can be reduced to some degree in this system. In the presence of 0.5 or 0.1 mM GRGDS, the inhibitory rates for 30 μM biotinylated β peptide binding to coated fibronectin were 19.5 and 16.4%, respectively; for its contacting to coated fibrinogen, the values were 24 and 20%, respectively. In the presence of 1 or 0.5 mM GRGDS, the inhibitory rates for biotinylated β peptide binding to collagen Type I were 16.7 and 14.3%, respectively; to coated vitronectin, the values were 23.4 and 19.4%, respectively. This indicates that the GRGDS peptide was able to contact with the β peptide and temporarily abrogate β peptide binding to coated fibronectin during the incubation. In the presence of different concentrations of EDTA, the binding rate of the biotinylated β peptide to fibrinogen was reduced to 50-60% of maximal binding. Under the same conditions, the values for binding to fibronectin were reduced to 17-30% of maximal binding. This experiment also showed that the SDGRG peptide had no significant effect on the binding of the biotinylated β peptide to fibrinogen, fibronectin, collagen Type I and vitronectin. The same experiments were also carried out with the β peptide as the immobilized phase. The biotinylated fibronectin or fibrinogen was coincubated with the unlabeled GRGDS peptide. Because the interaction between short oligopeptides is much less stable than that between proteins (*37*), no significant inhibition was observed (not shown).

The Inhibitory Effect of the β Peptide on Cell Adhesion to Immobilized Fibronectin—Cell adhesion is characterized by a multi-step process involving the special recognition of

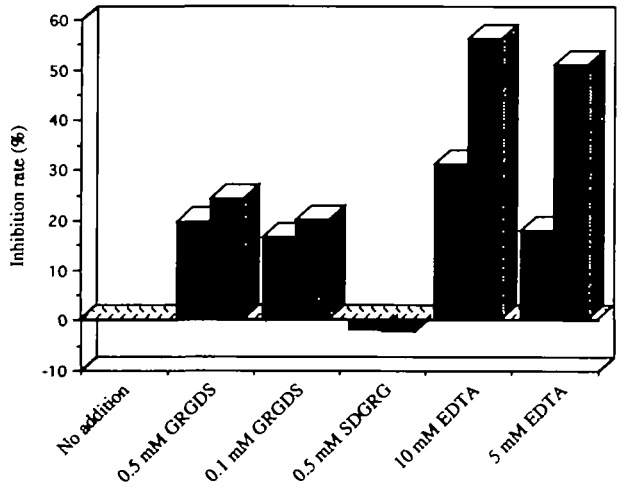

Fig. 5. **Inhibitory effects of GRGDS and EDTA on the binding of the biotinylated β peptide to immobilized fibrinogen and fibronectin.** The wells were coated with fibrinogen (1 mg/ml) (▨) or fibronectin (10 μg/ml) (■). The β peptide was added to the test wells at a final concentration of 30 μM and coincubated with the indicated concentration of GRGDS or EDTA. The data were expressed as the inhibition rate (%) of binding.

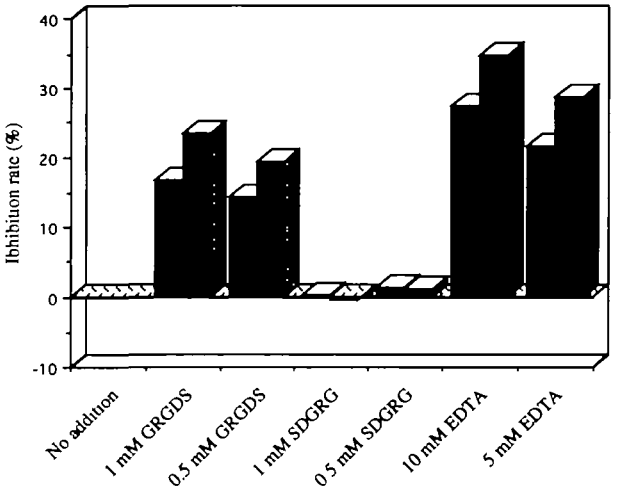

Fig. 6. **Inhibitory effects of GRGDS and EDTA on the binding of the biotinylated β peptide to collagen Type I and vitronectin.** Wells were coated with collagen Type I (500 μg/ml) or vitronectin (30 μg/ml). The procedure was similar to that described for Fig. 5.

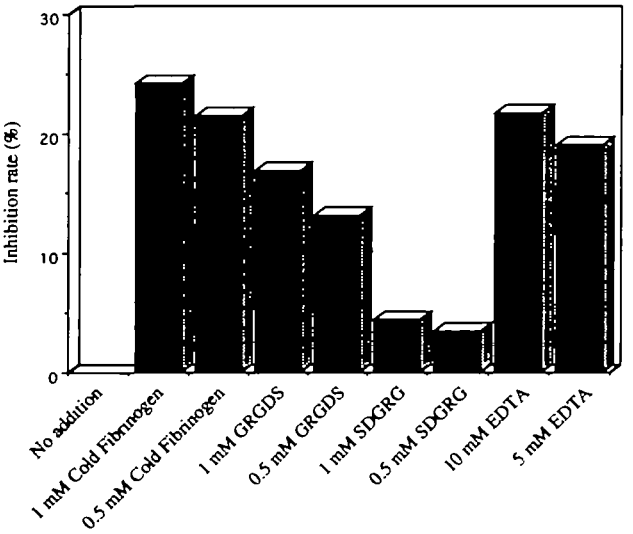

Fig. 7. **Inhibitory effects of GRGDS and EDTA on the binding of biotinylated fibrinogen to the immobilized β peptide.** Wells were first coated with γ-globulin, then SPDP and the β peptide were added at a concentration of 20 μg/ml. After blocking of the non-specific binding sites with buffer B+35 mg/ml BSA, biotinylated fibrinogen at a final concentration of 0.5 nM was loaded into each well and coincubated with cold fibrinogen, GRGDS, SDGRG, and EDTA. The data were expressed as the inhibition rate of binding. All results were significantly different from the control, except for coincubation with SDGRG. Similar results were obtained from the same assays on the binding of biotinylated fibronectin to coated β peptides.

extracellular molecules by the cell-surface receptors, integrins, and leading to focal contact and the rearrangement of the cytoskeletal structure inside the cell. To investigate the integrin and its derived polypeptide-ligand recognition, cells were allowed to adhere to coated fibronectin containing an RGD tripeptide sequence in the presence of the β peptide (coincubation), or bound cells were challenged with the β peptide (pre-incubation).

Fibroblast L929 and MC3T3-E1 osteoblastic cell lines were chosen for this adhesion assay. Because the L929 cell is a typical fibroblast cell line including many kinds of integrins on the cell surface, it can serve as a good model cell system for this kind of assay. Our main interest in integrin work is in the role of bone protein-integrin binding in osteoblast differentiation. For our preliminary work, we chose an MC3T3-E1 cell, one of the most typical osteoblastic cell lines. The optimum conditions for fibroblast adhesion to fibronectin were as follows: the concentration of fibronectin for coating was 10 μg/ml (1 μg per well), and the cell number was 5×10^4 for each well (not shown). Cells were added into each coated well and coincubated with the β peptide at the concentrations indicated in Fig. 8. The maximum concentration of the β peptide was 100 μM.

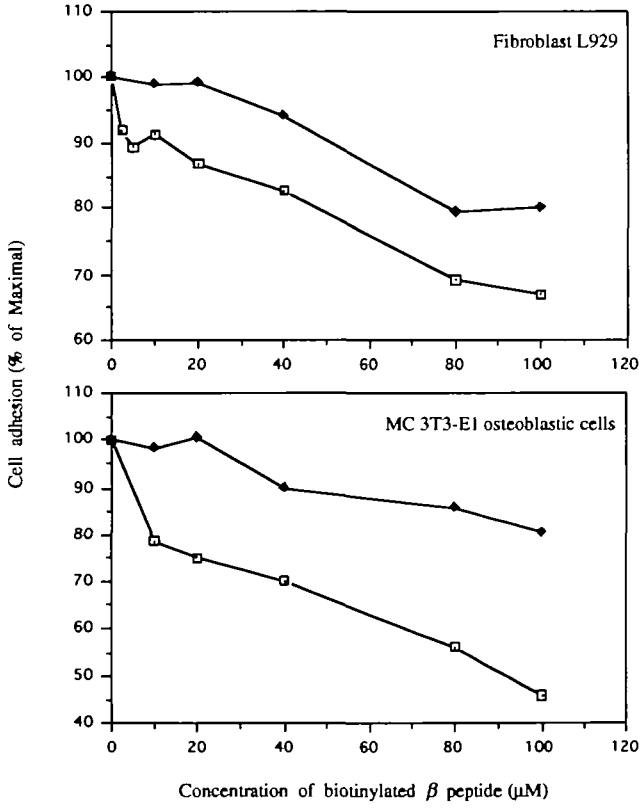

Fig. 8. **Inhibition of attachment of fibroblast L929 and MC 3T3-E1 osteoblastic cells to coated fibronectin by the β peptide.** Plates were coated with 10 μg/ml of fibronectin and the non-specific binding sites were saturated with adhesion buffer for 2 h at 30°C. In the coincubation assays 5×10^4 cells per well of fibroblast L929 or 2.5×10^4 cells of MC 3T3-E1 osteoblastic cells, were then added and incubated with the desired concentration of the β peptide (—□—). For preincubation, the cells were allowed to adhere to coated fibronectin, then challenged with the β peptide (—◆—). The data obtained in the adhesion assays in the absence of the β peptide indicated maximal binding, and results were expressed as the percentage of maximal binding.

Inhibition of the attachment for fibroblast L929 binding to fibronectin was observed in a dosage-dependent manner. With 100 μM β peptide, the inhibition rate reached 34%. The same concentration of fibronectin was used in the adhesion assays for MC3T3-E1 cells, and the cell number for each well was 2.5×10^4. In the presence of 100 μM β peptide, the inhibitory rate was 54.1%. This was significantly different from the same assay in the absence of the β peptide, which acts for maximal binding, and from the assay on the well coated with the non-adhesive protein BSA (no adhesion).

A pre-incubation assay was also carried out. First, the cells were added into fibronectin-coated wells and incubated for 1 h at 37°C. After washing the wells, the indicated concentration of the β peptide was added to each well, followed by two washings with buffer B, and the bound cells were measured as described in the experimental procedure. The main purpose of this experiment was to determine the competition between the cell-surface integrin-binding site and the β peptide. The data shown in Fig. 8 indicate that there was no inhibitory effect on cell adherence if the concentration of the β peptide was lower than 20 μM. On the other hand, when the concentration of the β peptide was higher than 20 μM, inhibition was apparent in the adhesion of both fibroblast L929 and MC 3T3-E1 osteoblastic cells. With 100 μM β peptide, the inhibition rate was 19.4% for fibroblast cells and 20% for MC3T3-E1 cells. Comparison of this data with that from the coincubation experiment suggests that the bound cells may have been partially removed from the adhesion regions within fibronectin and replaced by the β peptide. Another reason for this inhibitory effect might be that the peptide itself bound to cell-surface integrins (*42*), but this possibility needs further study.

Comparison of the Effect of the GRGDS Peptide and the

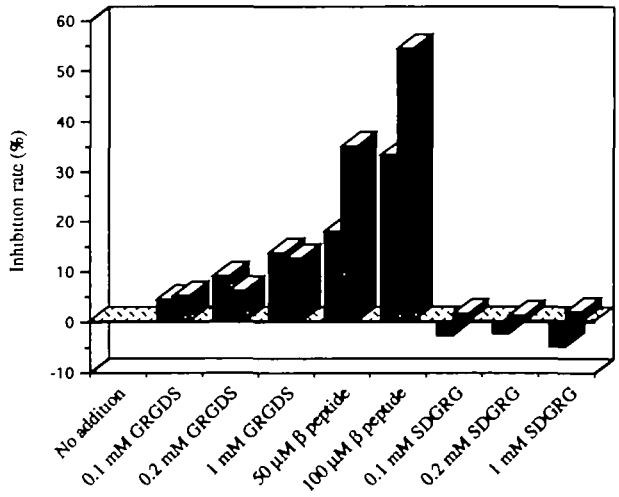

Fig. 9. **Comparison of the effect of the GRGDS peptide and the β peptide on attachment of fibroblast L929 and MC3T3-E1 osteoblastic cells to fibronectin.** Plates were coated with 10 μg/ml of fibronectin. Then 5×10^4 cells per well of fibroblast L929 (■) or MC3T3-E1 osteoblastic cells (▨) were added and coincubated with a designated concentration of the GRGDS or SDGRG peptide. The bound cells were determined according to the stated experimental procedure. All the data obtained were expressed as the inhibition rate (%) of cell adhesion.

β Peptide on Cell Adhesion—Cell adhesion to matrix proteins is now known to be mediated by cell-surface glycoprotein receptors or integrins. A GRGDS peptide containing an RGD tripeptide sequence can block cell attachment by binding to a cell-surface receptor. On the other hand, the β peptide contacts and binds to matrix-adhesive proteins and occupies their binding domains. Both of these effects lead to abrogation or partial blocking of cell adhesion. For comparison, cells were added to fibronectin-coated wells and coincubated with GRGDS, SDGRG, and the β peptide. The data in Fig. 9 indicate that the inhibition rate of the β peptide at the μM level of concentration was much higher than that of GRGDS at the mM level, and that there was no inhibition on treatment with another peptide, SDGRG. These results may suggest that blocking the adhesive region of fibronectin with the β peptide may have been more efficient than abrogating cell adhesion by contacting the cell surface with the short peptide, GRGDS. The results also showed a difference between the inhibitory effect on the two cell lines used in this experiment.

DISCUSSION

The main aspects of the highly conserved sequence corresponding to integrin-ligand binding and cell-matrix protein interaction within β subunits have recently been reviewed (*2-5*) as follows: (1) two putative sites in β integrins, especially in β_3, have been identified as the regions from amino acid residue 109-123 and 211-222 respectively, and the former is known to be a common ligand-contacting site; (2) high affinity ligand recognition requires both α and β subunits and may involve multiple ligand-contact sites; (3) the interaction between an integrin and ligand protein or polypeptide requires the participation of divalent cations and conformational changes leading to the formation of a transient ternary complex. The aim of this study was to identify the functions of a synthetic polypeptide derived from the highly conserved amino acid sequence (113-125) of the β subunit of integrins involved independently in ligand binding, and to determine the polypeptide's effect on cell attachment to immobilized fibronectin.

Considerable progress has been made in defining integrin-recognition sites in ligands and counterreceptors. The first binding site to be defined was the Arg-Gly-Asp (RGD) sequence present in fibronectin, fibrinogen and other adhesive proteins. Several integrins containing $\alpha_5\beta_1$, $\alpha_{IIb}\beta_3$, and most $\alpha_v\beta$ can recognize this tripeptide. With regard to other recognition sites within fibrinogen, $\alpha_{IIb}\beta_3$ recognizes the sequence KQAGDV, and $\alpha_x\beta_2$ binds to GPRP (*40*). Except for the RGD sequence in fibronectin, additional $\alpha_4\beta_1$ binds EILDV in an alternative-spliced segment of fibronectin.

Rapid and sensitive solid-phase binding assays using the β peptide and adhesive proteins labeled with biotin, followed by enzyme-linked immunodetection, are useful tools to investigate binding ability. Our results for β peptide binding ability (shown in Figs. 2, 3, and 4) also support the findings referred to above. Both the biotinylated β peptide bound to immobilized fibrinogen or fibronectin, and the biotinylated adhesive proteins bound to the coated β peptide suggest that this was direct and independent binding, since the recognition site of the β integrin subunit was determined by a cross-linking experiment with labeled RGD containing oligopeptide. In the competition assay, the GRGDS peptide partially inhibited the biotinylated β peptide from binding to coated adhesive proteins. It was hypothesized that the GRGDS peptide bound to the β peptide and blocked its binding to adhesive proteins. Because the interaction between peptides is less stable, more reversible, and has lower affinity than that between proteins (*37*), this could explain why the inhibition rate obtained was not as high as for blocking the binding of ligand proteins to integrins. In the binding experiments, when the system contained EDTA to remove divalent cations, especially calcium ions, inhibitory effects were also observed to a high degree. The data obtained indicate that this binding needed divalent cations. A possible model has been proposed for this kind of binding (*41*), in which the divalent cations attach to and occupy the binding sites within integrins, followed by a conformational change of integrins and initiation of recognition and binding through the RGD tripeptide sequence replacement of bound divalent cations.

Cell adhesion to immobilized adhesive proteins initially includes cell-matrix protein recognition and involves multistep processes, including clustering of integrin receptors, transmembrane signal transduction, and rearrangement of the cytoskeletal structure inside the cells. The data in Fig. 8 demonstrate that the β peptide was partially able to abrogate the attachment of fibroblast L929 and MC3T3-E1 osteoblastic cells, on which many integrins were expected to be induced and expressed on the cell surface during the period of cell attachment. Our research has proven, first of all, that the β peptide can efficiently bind to fibrinogen and fibronectin. Further, the research on the GP_{III} integrin also indicates that the peptide derived from GP_{III}, with the amino acid sequence RNRDA, can bind to the platelet $GP_{IIb\text{-}III_a}$ and inhibit binding of the ligands (*42*). Accordingly, it is possible that the inhibitory effects of the β peptide on cell adhesion might be of two different types: occupation of the adhesive site in fibronectin, and direct binding to cell-surface integrins. However, this possibility needs further investigation. The present study also shows that the β peptides were able to replace the bound cells under preincubation conditions. This may suggest that the β peptides can compete with cell surface integrins or are more efficient in occupying the adhesive sites within fibronectin. Further investigation is needed of the recognition sites of fibronectin and fibrinogen for the stable binding of the β peptides, and of details of the mechanism for the inhibitory effects of β peptides during cell attachment.

Our main interest in integrin work is to study the role of binding between integrin and bone proteins such as osteopontin. We have already obtained results showing that the β peptide efficiently bound to osteopontin, and an experiment involving photo cross-linking of osteopontin with the SASD-linked β peptide is now in progress. In this experiment, the β peptide partially inhibited photo cross-linking between osteopontin and SASD-linked β peptide, and the RGD sequence was proved to be in the binding region of the osteopontin. These results also support the results presented in this paper, and are to be published elsewhere (Y. Feng *et al.*, in preparation).

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