A Collagen/Gelatin-Binding Decapeptide Derived from Bovine Propolypeptide of von Willebrand Factor†

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ABSTRACT: Propolypeptide of von Willebrand factor (pp-vWF) binds to type I collagen, and we have reported that a binding domain exists in a 21.5/21-kDa fragment originated from the C-terminal portion [Takagi, J., Fujisawa, T., Sekiya, F., & Saito, Y. (1991) *J. Biol. Chem. 266*, 5575–5579]. The collagen-binding property of the 21.5/21-kDa fragment was compared with that of the intact pp-vWF. Although pp-vWF preferentially binds to native type I collagen fibrils, the isolated fragment no longer has this specificity and binds well to collagen of other types in the native and heat-denatured states. In order to determine the critical site that mediates this collagen/gelatin binding, several peptides were synthesized based on the primary structure of the 21.5/21-kDa fragment. Among these, a 25-residue peptide strongly inhibited the binding of the ^{125}I-labeled 21.5/21-kDa fragment to collagen. Using this inhibitory effect as an index, the binding site was defined to the sequence as follows: WREPSFCALS. Furthermore, a decapeptide of this sequence bound to collagen and gelatin, indicating that this sequence is responsible for the binding of the 21.5/21-kDa fragment to collagen/gelatin.

von Willebrand factor (vWF),[1] an adhesive glycoprotein found in plasma, platelets, and endothelial cells, is synthesized as a large precursor polypeptide, and a propolypeptide is cleaved (Wagner & Marder, 1984; Wagner, 1990). The propolypeptide of vWF (pp-vWF) is an about 100-kDa glycoprotein with D-type repeat structure which is also found in the mature vWF (Fay et al., 1986; Verweij et al., 1986). We have studied the collagen-binding properties of this molecule and found that pp-vWF bound very preferentially to type I collagen like mature vWF but with different specificity (Takagi et al., 1989a,b; Usui et al., 1992). In addition, we have determined that at least one collagen-binding site is located in a 21.5/21-kDa fragment generated from bovine pp-vWF by lysyl endopeptidase. The fragment corresponds to the region Phe^{570}–Lys^{682} in pp-vWF. This conclusion was based on two observations: (1) the isolated 21.5/21-kDa fragment inhibited the binding of ^{125}I-pp-vWF to type I collagen (Takagi et al., 1991); (2) the epitope for a monoclonal antibody that strongly inhibited pp-vWF binding to the collagen lay within this fragment (Fujisawa et al., 1991). In this paper, we further investigated the collagen-binding property of the 21.5/21-kDa fragment and found that in contrast to pp-vWF the fragment could bind various types of collagen both in native and in heat-denatured states. The region responsible for this collagen/gelatin binding was determined as a 10-residue portion in the fragment using synthetic peptides.

EXPERIMENTAL PROCEDURES

Collagens and Other Proteins. Native type I collagen fibrils from bovine tendon were kindly provided from Ethicon Inc. (Sommerville, NJ). This collagen was suspended and dialyzed against 10 mM acetic acid and diluted with 10 mM Tris-HCl, pH 7.4, containing 0.15 M NaCl (TBS) to appropriate concentration just before use. Pepsin-solubilized type I–V collagens from bovine tissues (type I from skin, type II from cartilage, and others from placenta) were purchased from KOKEN Co., Ltd. (Tokyo, Japan), and these soluble collagens were reconstituted by dialysis against 20 mM Na_2HPO_4 and diluted with TBS before use. Porcine gelatin was from Sigma Chemicals. Bovine pp-vWF and its 21.5/21-kDa fragment were purified as described previously (Takagi et al., 1991).

Peptide Synthesis. Peptides were synthesized according to the amino acid sequence of the 21.5/21-kDa fragment on a peptide Model 430A synthesizer (Applied Biosystems) using phenylacetamidomethyl resin and *tert*-butyloxycarbonyl-blocked amino acids. The following side-chain-protecting groups were used: His, 2,4-dinitrophenyl; Arg, mesitylenesulfonyl; Trp, formyl; and Cys, acetamidomethyl. In the case of peptides III-1 and III-3, however, the protecting group for cysteine was changed to 4-methoxybenzyl. The peptides were cleaved from the resin, and the side-chain-protecting groups were removed using trifluoromethanesulfonic acid in the presence of 8% (v/v) thioanisole and 4% (v/v) ethanedithiol (Yajima & Fujii, 1981). Crude peptides were extracted with 10% acetic acid and chromatographed on a Sephadex G-25 column (2.5 × 80 cm) equilibrated with 10% acetic acid and further purified by preparative reverse-phase HPLC on an Inertsil PREP-ODS column (20 × 250 mm; GL Sciences, Tokyo, Japan) using a 0.1% trifluoroacetic acid/H_2O and 0.1% trifluoroacetic acid/acetonitrile gradient elution. The purity of the peptides was assessed by analytical HPLC on a C18 column, and the sequences were verified on a gas-phase sequencer (Model 470A, Applied Biosystems).

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[1] Abbreviations: vWF, von Willebrand factor; pp-vWF, propolypeptide of von Willebrand factor; BSA, bovine serum albumin; HPLC, high-performance liquid chromatography; TBS, 10 mM Tris-HCl, pH 7.4, containing 0.15 M NaCl; native collagen fibrils, acid-insoluble fibrillar type I collagen obtained from bovine tendon; reconstituted collagen fibrils, fibrillar collagen reconstituted from soluble collagen preparation obtained from pepsinized bovine tissue.

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Radioiodination of Proteins and Peptide. Bovine pp-vWF and the 21.5/21-kDa fragment were iodinated using Iodobeads as described previously (Fujisawa et al., 1991), and the specific activity of 1–2 μCi/μg of protein was routinely obtained. Radioiodination of a peptide (peptide III-3) was conducted as follows. ^{125}I-Labeled Bolton–Hunter reagent (250 μCi, ICN Radiochemicals Inc.) was allowed to adhere to the inner well of the reaction vial by evaporating benzene with nitrogen gas, and 5 μg of peptide III-3 dissolved in 10 μL of 0.1 M borate buffer, pH 8.5, was immediately added. After incubation for 4 h on ice, 150 μL of TBS containing 0.2 M glycine was added followed by additional incubation for 5 min, mixed with 150 μL of TBS containing 5 mg/mL ovalbumin, and applied to a column of Bio-gel P-2 (200–400) equilibrated with TBS containing 0.05% NaN_3. Fractions containing the labeled peptide were pooled and stored in the presence of 1% bovine serum albumin (BSA) at 4 °C. The specific activity was about 4 μCi/μg of peptide.

Solid-Phase-Binding Assay. The collagen-binding property of pp-vWF, the 21.5/21-kDa fragment, or a peptide was assessed using a solid-phase-binding assay. Each well of a detachable 96-well plate (Breakable Combiplate 8; Labsystems, Helsinki, Finland) was coated with 5 μg of collagen or gelatin in 50 μL of TBS by overnight incubation at 4 °C. The amount of the protein adsorbed to the surface of the wells were determined using a bicinchoninic acid protein assay kit (Pierce Chemical Co., Rockford, IL) containing 5 mM NaOH and 0.05% SDS, and it was confirmed that about 30–40% of the protein (1.5–2 μg) was adsorbed to the wells regardless of the difference in collagen preparation. The nonspecific protein-binding sites were blocked with 1% BSA in TBS for more than 1 h at room temperature. ^{125}I-Labeled proteins or a peptide in 1% BSA–TBS was added in duplicate and incubated for 90 min at room temperature together with peptides or other competing ligands to be tested. After being washed 3 times with cold TBS, the plate was divided into separate wells, and the radioactivity of bound ligand was counted on a γ-counter. The value obtained with wells that had not been coated with collagen was subtracted as it represented nonspecific binding.

RESULTS

In a previous paper (Takagi et al., 1991), we showed that the 21.5/21-kDa fragment inhibited the binding of pp-vWF to collagen in a dose-dependent manner and speculated that this inhibition was a result of competitive binding of the fragment to the collagen molecule. As we have not shown direct binding of the fragment to collagen in previous work, purified 21.5/21-kDa fragment was labeled with ^{125}I, and binding to collagen was evaluated. The fragment actually bound to native type I collagen fibrils coated on microtiter wells in a saturable and specific manner (Figure 1). The binding was dependent on the concentration of collagen used in the coating step, increased with increasing amounts of fragment added using a fixed concentration of the collagen, and reached a plateau, indicating saturation of the binding site(s) in the coated collagen molecule. Furthermore, the apparent binding was completely reduced by the addition of excess unlabeled fragment, which is to be shown later in Figure 4.

The binding specificity of intact pp-vWF and the 21.5/21-kDa fragment was compared using the same technique. The microtiter wells were coated with the same concentration of various collagens, and ^{125}I-labeled pp-vWF or its fragment was added. The collagen preparations used in this assay

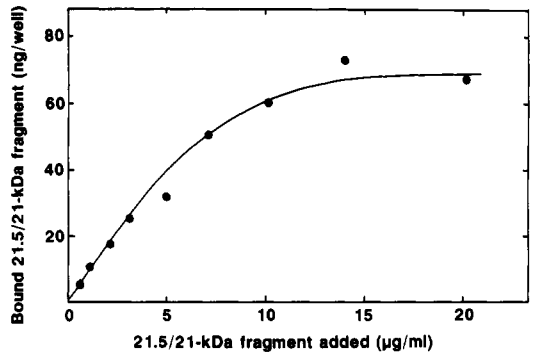

FIGURE 1: Saturable binding of the 21.5/21-kDa fragment to type I collagen immobilized on a microtiter well. Wells were coated with native type I collagen fibrils as described under Experimental Procedures, and increasing amounts of the 21.5/21-kDa fragment were added in a total volume of 40 μL. The total amounts of 21.5/21-kDa fragment bound were calculated from the specific activity.

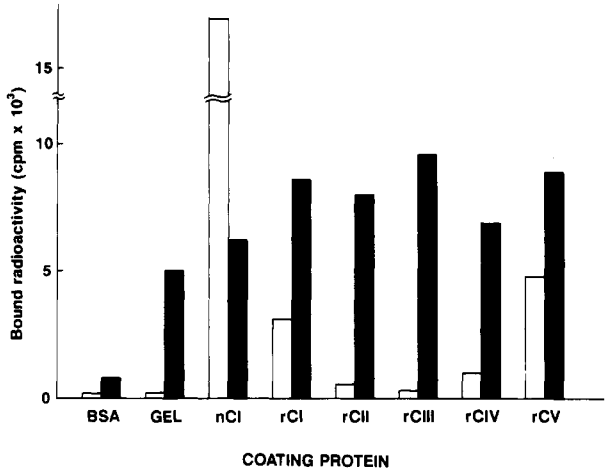

FIGURE 2: Difference between pp-vWF and the 21.5/21-kDa fragment in collagen-binding specificity. Wells were coated with various collagen preparations using the same concentration (100 μg/mL), and ^{125}I-pp-vWF (white bars) or the ^{125}I-21.5/21-kDa fragment (black bars) was added at about 80 000 cpm/well. The coated proteins were BSA, gelatin (GEL), native type I collagen fibrils (nCI), and reconstituted type I–V collagen fibrils (rCI–V) as described under Experimental Procedures.

included native type I collagen fibrils, porcine gelatin, and reconstituted type I–V collagen fibrils prepared from pepsin-treated soluble collagens. The purity of all these collagens was checked by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) under reducing condition. The native type I collagen fibrils, obtained from bovine tendon, were free from any soluble collagen because it did not enter even the stacking gel upon SDS–PAGE in the presence of urea and the band corresponding to α1(I) and α2(I) chains is observed only after extensive treatment with pepsin (Usui et al., 1992). This preparation retained normal biological activity such as platelet aggregating activity. In contrast, the porcine gelatin was freely soluble in buffer of neutral pH and composed of α, β, and γ chains of type I collagen but totally devoid of the ability of platelet aggregation or fibril formation. Reconstituted type I–V collagen fibrils were also analyzed by SDS–PAGE and confirmed that they consisted of more than 95% pure α, β, or γ chains of each type. These collagens were reconstituted into fibers by the method described under Experimental Procedures and were coated on wells at the same concentration (100 μg/mL), and it was confirmed that almost the same amount was absorbed to the wells in spite of the variation in collagen preparation. As shown in Figure 2, intact pp-vWF and the fragment showed significant difference

Table I: Synthetic Peptides Derived from the Sequence of the 21.5/21-kDa Fragment

peptide	sequence	position[a]	MW
I	FEACHSAVSPLPYLRNCRYDV	570–590	2583.2
II	ACSDGRDCLCDAVANYAAACAR	592–613	2503.9
III	RGVHVGWREPSFCALSCPHGQVYQQ	614–638	2986.4
IV	GTPCNLTSCSLSYPDEECNES	640–660	2462.8
V	LEGCFCPPGLFLDENGSCHPK	662–682	2477.1
III-N	ARRGVHVGWREPSFCALS	612–629	2099.7
III-C	ALSCPHGQVYQQCGTP	627–642	1831.2

[a] The numbers indicate the position of the first and the last amino acid of the designated peptide, numbered from the N-terminal of the pp-vWF assuming the location of the 21.5/21-kDa fragment as Phe^{570}–Lys^{682}.

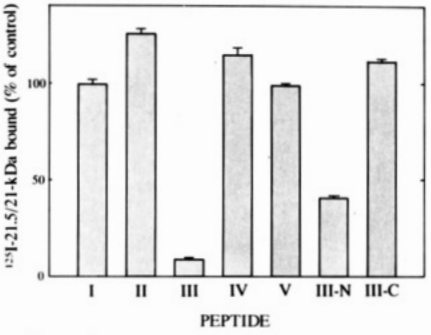

FIGURE 3: Effect of synthetic peptides on binding of the 21.5/21-kDa fragment to collagen. Binding of the ^{125}I-21.5/21-kDa fragment to native collagen fibrils was evaluated in the presence of peptides at 300 μg/mL. Relative binding compared to the value obtained in the absence of any peptide (mean ± SEM) is shown. The amino acid sequences of peptides are found in Table I.

in binding specificity. Intact pp-vWF selectively bound to the native type I collagen fibrils but did not to gelatin at all, and it bound a little to the reconstituted type I and V collagen fibrils among those tested. This result is consistent with our result that pp-vWF recognizes the pepsin-sensitive structure of type I collagen (Usui et al., 1992). In contrast, the 21.5/21-kDa fragment bound fairly equally to these collagens tested and even to gelatin. When the native collagen fibrils were denatured by heat treatment of 100 °C for 5 min, the binding of intact pp-vWF was diminished while that of the 21.5/21-kDa fragment was almost unchanged (data not shown). This result is consistent with the experiment conducted with the commercially available gelatin preparation, indicating this fragment recognizes heat-denatured collagen as well as native fibrillar collagen, in contrast to native pp-vWF. Apparently, the collagen-binding site(s) in the 21.5/21-kDa fragment has (have) less tight specificity, probably due to the change of conformation of surrounding residues.

We then decided to determine the critical site(s) responsible for the binding of the 21.5/21-kDa fragment to type I collagen/gelatin. Five synthetic peptides (peptides I–V) were prepared according to the amino acid sequence of the 21.5/21-kDa fragment of bovine pp-vWF (Table I). Though this region in the intact molecule contains many disulfide bridges, the isolated fragment that has collagen/gelatin-binding activity is reduced and alkylated. We, therefore, synthesized peptides with (acetamidomethyl)cysteine (Acm-cysteine) instead of cysteine. Residues that had been assumed to be glycosylated asparagine and sulfated tyrosine (Asn^{644}, Asn^{658}, Asn^{676}, and Tyr^{652}) in bovine pp-vWF (Takagi et al., 1991) were replaced by Asn and Tyr, respectively, in these peptides. Among the synthetic peptides tested, only peptide III had an inhibitory effect on the binding of the 21.5/21-kDa fragment to collagen (Figure 3). When added at 300 μg/mL, peptide III showed more than 90% inhibition, while four other peptides had no or even a slightly potentiating effect on the binding. It became apparent that only peptide III interacted with the collagen. It was also shown that only peptide III bound to the gelatin among those peptides. When the mixture of the five peptides was applied to gelatin–Sepharose, only peptide III was retarded (data not shown). Taking all these data together, we assumed that peptide III (25 amino acid residues) represented the collagen/gelatin-binding domain of the 21.5/21-kDa fragment (113 amino acid residues). It was found, however, that this peptide cannot inhibit the binding of intact pp-vWF to native collagen fibrils even when added at high concentration (1 mg/mL). It may be possible that the affinity of this peptide to the collagen is not high enough to compete with intact pp-vWF, which has an affinity more than 20 times greater than that of the 21.5/21-kDa fragment. It is also possible that intact pp-vWF can utilize another collagen-binding site(s) that is (are) different from the sequence corresponding to peptide III. Two more peptides containing either N- or C-terminal portions of peptide III were then synthesized to further clarify the binding domain of peptide III (Table I). Peptides corresponding to the N-terminal (peptide III-N) but not the C-terminal (peptide III-C) portion of peptide III sequence had an inhibitory effect on the binding of the 21.5/21-kDa fragment to collagen (Figure 3), and it became evident that the essential sequence was located in the N-terminal half of peptide III. Figure 4 shows the dose-dependent inhibition of peptide III, peptide III-N, and unlabeled 21.5/21-kDa fragment on the binding of the 21.5/21-kDa fragment to collagen. Along with the shortening of polypeptide, the inhibition curve shifted to the right, suggesting a decrease of the affinity for collagen.

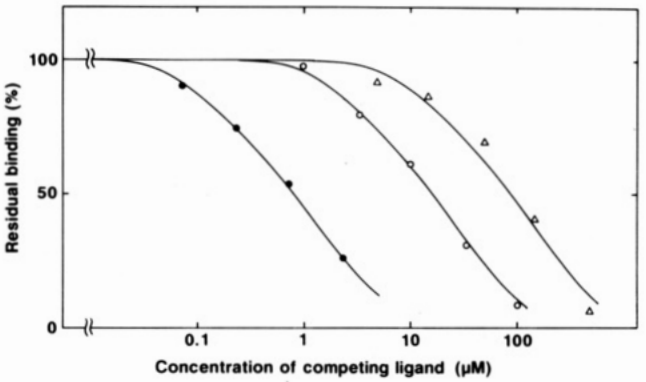

FIGURE 4: Dose-dependent inhibition of the binding of the 21.5/21-kDa fragment to collagen by peptides and the fragment. Increasing amounts of unlabeled 21.5/21-kDa fragment (●), peptide III (○), and peptide III-N (△) were added, and the binding of the 21.5/21-kDa fragment to native type I collagen fibrils was evaluated. The values shown represent residual binding determined as described in the legend to Figure 3.

In order to further define the critical sequence within peptide III-N for collagen/gelatin binding, three shorter peptides with overlapping sequence were synthesized (Table II). Among them, only a decapeptide (peptide III-3) which has the sequence WREPSFCALS was active in the inhibition assay. The IC_{50} value of this peptide was about 130 μg/mL, while other overlapping peptides, CARRGVHVG and RGVHVGWREP, had no effect on the binding of the 21.5/21-kDa fragment to collagen when added up to 3 mg/mL. Shorter peptides (such as WREPSF or SFCALS) were examined using the same assay, but we could not detect the inhibition (data not shown). We then investigated the interaction between peptide III-3 and collagen. Since peptide III-3 does not contain tyrosine residues, the peptide was labeled with ^{125}I-labeled Bolton–Hunter reagent on its N-

Table II: Determination of the Inhibitory Activity of the Synthetic Peptide Derived from the Peptide III Sequence on Binding of the 21.5/21-kDa Fragment to Type I Collagen

peptide	sequence	IC_{50} (μg/mL)[a]
III	RGVHVGWREPSFCALSCPHGQVYQQ	38.7 ± 13.7
III-N	ARGVHVGWREPSFCALS	149.7 ± 62.2
III-1	CARGVHVG[b]	>3000
III-2	RGVHVGWREP	>3000
III-3	WREPSFCALS[b]	132.5 ± 42.5

[a] The IC_{50} values were determined by the same method as that utilized in Figure 4, and are expressed as mean ± SEM of three independent experiments. [b] The cysteine residues in these peptides are deprotected and have free SH groups.

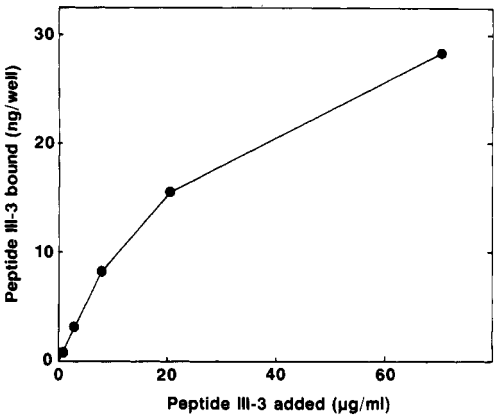

FIGURE 5: Binding of peptide III-3 to type I collagen. Peptide III-3 was iodinated by ^{125}I-labeled Bolton–Hunter reagent, and the binding to native type I collagen fibrils immobilized on a microtiter well was determined as described under Experimental Procedures. The amount of bound peptide was calculated from its specific activity.

terminal or on the cysteine residue. As clearly depicted in Figure 5, peptide III-3 actually bound to the collagen, and the binding was reduced by the addition of excess unlabeled peptide (data not shown). The binding specificity was almost same as that of the 21.5/21-kDa fragment; it bound to all types of collagen tested and gelatin.

DISCUSSION

In this paper, we investigated the collagen-binding properties of the 21.5/21-kDa fragment obtained from bovine pp-vWF and found a significant difference between the fragment and intact pp-vWF in collagen recognition specificity. As to the binding to type I collagen, pp-vWF requires native and fibrillar conformation of the collagen and prefers a cross-linked insoluble state, and the binding is greatly diminished by pepsin treatment of the collagen (Usui et al., 1992). Unlike intact pp-vWF, the 21.5/21-kDa fragment binds well to gelatin and reconstituted various types of collagen fibrils from pepsin-treated collagens. Apparently, the 21.5/21-kDa fragment acquired the ability to interact with gelatin, a feature that had not been shared by the intact molecule. It might be more appropriate to state that the fragment lost the ability to distinguish between the native type I collagen fibrils and gelatin or reconstituted collagen fibrils from pepsin-treated collagens. It is not so unusual that isolated fragments are conformationally different from the corresponding regions within the original proteins and this change leads to significant alteration in the biological functions. It is also possible that amino acid residues surrounding the essential site within the native molecule may confer a more rigid environment and give higher specificity.

Though the 21.5/21-kDa fragment contains several carbohydrate chains (Takagi et al., 1991), it seems unlikely that

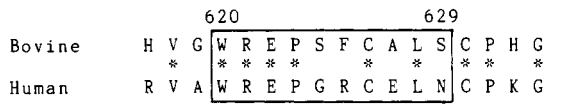

FIGURE 6: Comparison of the amino acid sequence of bovine and human pp-vWF in a region corresponding to peptide III-3. The bovine sequence was determined by direct amino acid sequencing of the 21.5/21-kDa fragment (Takagi et al., 1991), and the human sequence was predicted from the cDNA sequence (Verweij et al., 1986). Amino acids are denoted by single-letter codes, and the conserved amino acids are indicated by asterisks. The sequence corresponding to peptide III-3 is boxed.

these carbohydrate chains are essential for the collagen/gelatin-binding activity because synthetic peptides without sugar chain can bind to the collagen. It is still possible, of course, that the carbohydrate moiety has some roles in collagen binding by, for example, modulating the recognition specificity or constructing a desired conformation of this portion. The fact that peptide III-3 almost completely inhibits the binding of the 21.5/21-kDa fragment to collagen suggests that the linear sequence of this peptide is responsible for the binding. In contrast to the effective inhibition of the binding of the 21.5/21-kDa fragment to collagen, peptide III-3 failed to inhibit intact pp-vWF from binding to collagen. The reason for this failure is not clear. It may be due to the inability of the small peptide to cover the binding site on the collagen molecule or, more simply, due to the decreased affinity compared with the 21.5/21-kDa fragment.

The binding of the peptide to collagen/gelatin was confirmed by the direct-binding assay. The binding was specific and saturable because the addition of an excess amount of unlabeled peptide reduced the apparent binding. All peptides we used in this study were synthesized according to the sequence of bovine pp-vWF. Several residues in peptide III-3 sequence are not conserved in human sequence (Figure 6). If this region is responsible for the collagen/gelatin-binding activity of pp-vWF in physiological condition, the conserved amino acid should be essential. Moreover, it may be possible that a homologous sequence is shared by other collagen/gelatin-binding proteins. We compared the sequence of peptide III-3 with the protein sequence recorded in the National Biomedical Research Foundation (NBRF) Protein Data Bank (release 29) but could not find a significantly homologous sequence in the known collagen-binding proteins such as fibronectin (Ingham et al., 1989), collagen-binding heat shock protein HSP47 (Hirayoshi et al., 1991), or α_2 subunit of integrin VLA-2 (Takada & Hemler, 1989). At the amino acid level, several proteins are found to contain regions homologous to this decapeptide though all these proteins are unlikely to have collagen-binding activity. Peptides III-2 and III-3 have overlapping sequence, WREP, but they have very different affinity (Table II). This may suggest that this sequence is not essential. However, peptide III-3 devoid of this sequence, that is to say peptide SFCALS, did not have affinity to the collagen. It is clear that a combination of some of the left four residues and some of the right six residues is necessary for binding. Determination of the distinctive motif in peptide III-3 sufficient to express the activity is required to clarify this point.

We believe that this is the first paper to report a synthetic peptide that has affinity to collagen/gelatin. There are a growing number of investigations dealing with peptide analogues that mimic the biological activity of the original protein. The well-known examples of such investigations are those dealing with synthetic peptides that have cell adhesion activity (Yamada, 1991). These recognition sequences of adhesion

receptors are derived from fibronectin (Pierschbacher & Ruoslahti, 1984; Haugen et al., 1990; Komoriya et al., 1991), laminin (Graf et al., 1987; Kanemoto et al., 1990), fibrinogen (Smith et al., 1990), von Willebrand factor (Ruoslahti & Pierschbacher, 1987), and collagen types I (Staatz et al., 1991) and IV (Chelberg et al., 1990). It is particularly interesting to know whether the DGEA sequence, identified as the primary recognition sequence in type I collagen by VLA-2, has any effect on the collagen binding of peptide III-3. What kind of structure in the collagen molecule is specifically recognized by the peptide is not clear. The fact that both the 21.5/21-kDa fragment and peptide III-3 recognize most of all types of collagen, and even the gelatin, may suggest that they recognize the general "Gly-X-Y" repeat structure. Experiments with an artificial collagen mimetic, such as $(Gly\text{-}Pro\text{-}Pro)_n$ polymer, would be necessary for such consideration. Moreover, it is possible to expect that the peptide may modulate some cellular events such as platelet aggregation or the cell attachment process utilizing its collagen/gelatin-binding activity. Elucidation of these in vitro activities of the peptide awaits further study.

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