

Transglutaminase-catalyzed Cross-linking of Osteopontin Is Inhibited by Osteocalcin*

(Received for publication, April 15, 1997, and in revised form, June 9, 1997)

Mari T. Kaartinen‡, Arja Pirhonen, Annikka Linnala-Kankkunen, and Pekka H. Mäenpää

From the Department of Biochemistry and Biotechnology, University of Kuopio, FIN-70211 Kuopio, Finland

Osteocalcin, the most abundant noncollagenous protein of bone matrix, has been demonstrated to inhibit bone growth by gene knockout experiments (Ducy, P., Desbois, C., Boyce, B., Pinero, G., Story, B., Dunstan, C., Smith, E., Bonadio, J., Goldstein, S., Gundberg, C., Bradley, A., and Karsenty, G. (1996) *Nature* 382, 448–452). Its specific functional mechanism in bone metabolism is, however, largely unknown. In this study, we provide evidence that osteocalcin has an inhibitory effect on tissue transglutaminase activity, as measured by cross-linking of osteopontin, another bone matrix protein. Using a set of synthetic peptides, we found that the inhibitory activity resided within the first 13 N-terminal amino acid residues of osteocalcin. An N-terminal peptide also inhibited cross-linking of another tissue transglutaminase substrate, β -casein. The inhibitory peptide was shown to have affinity for the substrates of transglutaminase rather than for the enzyme. Since the N terminus of osteocalcin exhibits homology to the substrate recognition site sequences of two transglutaminases, we conclude that the inhibitory effect is most likely due to competition with the enzyme for the transglutaminase-binding region of the substrates, osteopontin and β -casein, which prevents access of the enzyme to them to perform its function. The interference of osteocalcin with osteopontin cross-linking gives osteocalcin a new potential function as the first protein inhibitor of tissue transglutaminase. This suggests a specific role and a plausible mechanism for it as a modulator of maturation, stabilization, and calcification of bone matrix.

The organic matrix of mineralized tissues is composed of several noncollagenous proteins, whose functions in bone, dentine, and cartilage development and remodeling are not presently fully understood. In addition to their possible contribution to the structural integrity of hard tissues, the biochemical and medical evidence suggests their involvement in the regulation of bone turnover. The control of bone remodeling involves numerous extracellular matrix events, including various protein-protein interactions, which eventually lead to the arrangement of proteins into larger complexes, which finally form the strong supramolecular architecture of bone matrix.

One of the most abundant noncollagenous proteins of adult

bone is osteocalcin (OCN),¹ which is a small osteoblast-specific calcium-binding protein of 46–50 amino acid residues, containing three vitamin K-dependent Gla residues (1, 2). The abundance of OCN in the mineralized matrix and its well conserved amino acid sequence emphasize the importance of OCN in bone, but its functions have still remained unclear. Data accumulated thus far indicate that OCN acts as a negative regulator of bone turnover and a suppressor of mineralization (3–6). OCN deficiency, resulting from interference with the vitamin K-dependent Gla synthesis, causes poor accumulation of OCN in bone and results in excessive calcification and resistance to bone resorption (4, 5). The gene knockout experiments by Ducy *et al.* (6) have demonstrated that mice lacking OCN develop bones with increased mass and strength. This implies the importance of OCN in promoting bone resorption and inhibiting mineralization.

OCN is known to interact with osteopontin (OPN) *in vitro* (7). OPN is a prominent highly acidic phosphoglycoprotein of mineralized bone with multiple potential roles (8, 9). OPN was originally isolated from the mineralized matrix of bone (10), but it is now known to be expressed and secreted also by other tissues (8). In addition, it is found in physiological fluids such as milk and urine (11, 12, 25). In bone, OPN is expressed at an early stage of bone formation (13, 14); laid into unmineralized matrix prior to calcification; and localized at matrix-matrix, matrix-mineral, and matrix-cell interfaces and between collagen fibrils of fully matured hard tissue (15). Since OPN is present in most normal tissues undergoing mineralization and is also known to accumulate at the site of healing calcified tissue interfaces (16), it may be involved in the regulation of bone mineralization. OPN is a substrate of tissue transglutaminase (TG) (17–19).

Tissue transglutaminase (EC 2.3.2.13) belongs to a family of widely distributed calcium-dependent enzymes, which catalyze the formation of γ -glutamyl- ϵ -lysyl cross-links, inducing the formation of high molecular mass complexes of proteins (20, 21). The covalent bonds formed are stable and resistant to proteolysis, and where present, they increase the durability and integrity of the tissue (21). TG is produced in mineralizing cartilage and bone (22, 23), and it is thought to participate in matrix cross-linking before the tissue undergoes calcification (21). Therefore, it might be involved in the initiation and regulation of the mineralization processes (22).

In view of the observation that OCN is known to interact with OPN, we investigated whether OCN has an effect on TG-catalyzed cross-linking of OPN. In this paper, we provide evidence that OCN reduces the formation of TG-catalyzed high molecular mass complexes of OPN most likely by competing

* This work was supported by the Academy of Finland and the Protein Structure and Function Graduate School of the Finnish Ministry of Education. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

‡ To whom correspondence should be addressed: Dept. of Biochemistry and Biotechnology, University of Kuopio, P. O. Box 1627, FIN-70211 Kuopio, Finland. Tel.: 358-17-163068; Fax: 358-17-2811510; E-mail: Mari.Kaartinen@uku.fi.

¹ The abbreviations used are: OCN, osteocalcin; OPN, osteopontin; TG, transglutaminase; HATU, *O*-(7-azabenzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate; Fmoc, *N*-(9-fluorenyl)methoxycarbonyl; HPLC, high performance liquid chromatography; PAGE, polyacrylamide gel electrophoresis.

with the enzyme for the binding site of the protein substrate by a sequence homologous to tissue transglutaminase.

EXPERIMENTAL PROCEDURES

Materials—OPN antibody and β -casein were generous gifts from Dr. E. Sørensen (University of Aarhus, Aarhus, Denmark). Alkaline phosphatase-conjugated anti-rabbit IgG, alkaline phosphatase-conjugated anti-biotin IgG, guinea pig tissue TG, pepsin, 5-bromo-4-chloro-3-indolyl phosphate/nitro blue tetrazolium and FAST *p*-nitrophenyl phosphate alkaline phosphatase substrate tablets, and biotinamidocaproate *N*-hydroxysuccinimide ester were purchased from Sigma. Endoproteinase Asp-N and the blocking reagent from the digoxigenin luminescent detection kit for nucleic acids were from Boehringer Mannheim GmbH (Mannheim, Germany). HATU coupling reagent was from PerSeptive Biosystems, Inc. (Framingham, MA), and Fmoc-(*tert*-butyl ester)₂-Gla amino acid derivative was from Bachem Feinchemikalien AG (Bubendorf, Switzerland). Polyvinylidene difluoride Immobilon P membranes were from Millipore Corp. (Bedford, MA), and polyvinyl chloride microtiter plates were from Costar (Cambridge, MA). Instaview Universal stain was purchased from BDH Laboratory Supplies (Poole, United Kingdom).

Instrumentation—Chromatography was performed with a Pharmacia Biotech GradiFrac chromatography system, peptide syntheses with an Applied Biosystems Model 431 synthesizer (Biocenter, University of Oulu, Oulu, Finland), and HPLC purifications with a Hewlett-Packard 1050 HPLC system. Protein and peptide sequencing was performed with Applied Biosystems Model 477A sequencer, amino acid analysis with an LKB 4151 Alpha Plus amino acid analyzer, and mass spectrometry with a Kratos Compact MALDI III mass spectrometer (Biocenter, University of Oulu).

Protein Purification—Bovine OCN was isolated from bovine bone powder using a previously described method (24) and further purified by reverse-phase HPLC on a Vydac C₁₈ semipreparative column using 0.1% trifluoroacetic acid (buffer A) and 70% acetonitrile in 0.1% trifluoroacetic acid (buffer B) in a 40-min linear gradient from 5 to 100% buffer B at a flow rate of 2 ml/min. Bovine OPN was purified from nonfat dry milk according to a previously described method (25), replacing trichloroacetic acid precipitation with 50–80% ammonium sulfate fractionation. Homogeneity of both proteins was confirmed by amino acid analysis, N-terminal sequencing, and SDS-PAGE.

Synthesis and Purification of Osteocalcin-derived Peptides—Three human OCN peptides covering amino acids 1–25, 26–41, and 42–49 were synthesized by solid-phase peptide synthesis using 0.1-mmol scale Fmoc chemistry and HATU coupling. Gla residues were coupled to peptide 1–25 during synthesis as a Fmoc-(*tert*-butyl ester)₂-Gla amino acid derivative. The cysteine residues of OCN were replaced with methionines to avoid intermolecular dimerization during synthesis, purification, and usage. Human OCN peptides 1–25, 26–41, and 42–49 were used without further purification and named Peptides 1, 2, and 3, respectively. Peptide 4 was obtained from the HPLC purification as a breakdown product of intact bovine OCN. It was verified by sequence analysis to contain the first 14 amino acid residues of the N terminus of OCN.

Preparation of Peptides 1a and 1b from Peptide 1 by Asp-N Digestion—Peptides 1a and 1b were simultaneously obtained from Peptide 1 by endoproteinase Asp-N cleavage. Two mg of Peptide 1 was dissolved in 50 mM sodium phosphate buffer, pH 8.0. Endoproteinase Asp-N was dissolved according to the instructions of the manufacturer and added to the peptide solution to give an enzyme/substrate ratio of 1:1000 (w/w). The reaction was terminated with 0.1 M EDTA after a 10-h incubation at 37 °C. Peptides were purified by reverse-phase HPLC using a Vydac C₁₈ semipreparative column and a buffer system of buffers A and B in a linear 45-min gradient from 0 to 100% buffer B at a flow rate of 2 ml/min. Purity of peptides was analyzed with a matrix-assisted laser desorption/ionization time-of-flight mass spectrometer and by N-terminal sequencing.

Preparation of Peptides 1c and 1d from Peptide 1 by Pepsin Digestion—Peptides 1c and 1d were prepared by digesting Peptide 1 with pepsin in 0.01 M HCl, pH 2.0, using an enzyme/substrate ratio of 1:100 (w/w) and incubating the mixture overnight at 37 °C. The reaction was stopped by the addition of 0.1 M NaOH. The peptides were purified and characterized by N-terminal sequencing as Peptides 1c and 1d.

Cross-linking of Osteopontin by Tissue Transglutaminase in the Presence and Absence of Osteocalcin and Its Peptides—Cross-linking experiments were conducted in a total volume of 50 μ l of 50 mM Tris-HCl, pH 8.0, containing 2.5 mM CaCl₂ and 1 mM dithiothreitol. Reaction mixtures contained 1.2 μ g of OPN, and the enzyme/substrate ratio was 1:5

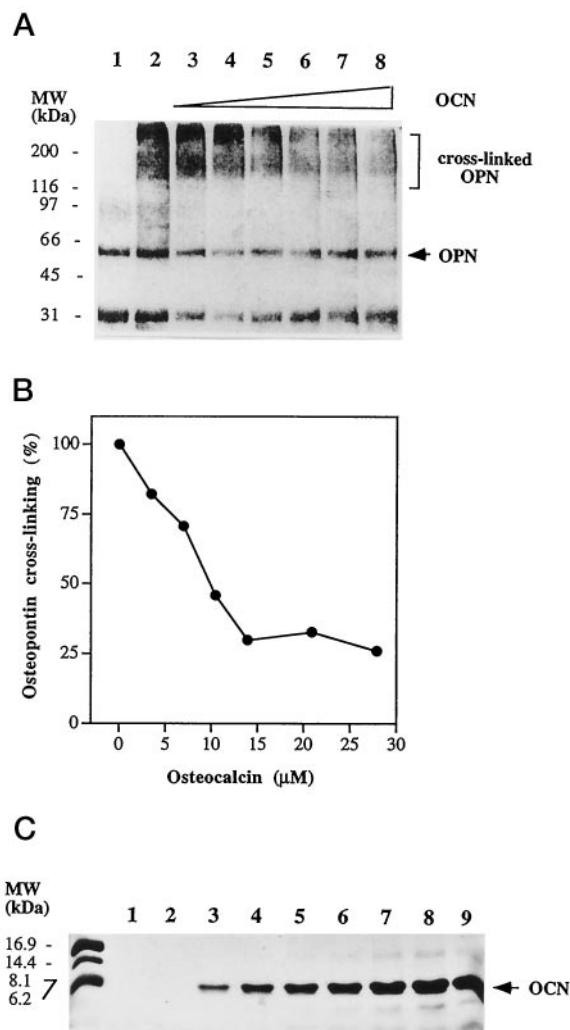


FIG. 1. Inhibitory effect of osteocalcin on tissue transglutaminase-catalyzed cross-linking of osteopontin analyzed by Western blotting. A, bovine OPN was incubated for 2 h at 37 °C with tissue TG in the presence of increasing amounts of OCN. Samples were subjected to 8.5% SDS-PAGE followed by electrophoretic transfer to polyvinylidene difluoride membranes and staining with polyclonal antibody against bovine OPN. Lane 1, OPN; lane 2, cross-linked high molecular mass OPN; lanes 3–8, cross-linked OPN in the presence of 3.5, 7.0, 10.5, 14.0, 21.0, and 28.0 μ M bovine OCN, respectively. Molecular mass standards are indicated on the left. B, filters were scanned, and the intensities of the bands representing the high molecular mass complexes of OPN were measured using the NIH Image 1.55 noFPU program. Relative cross-linking of OPN was plotted against increasing concentrations of OCN. C, OPN cross-linking reactions performed as described above were run on 18% SDS-polyacrylamide gels and stained with Instaview Universal. The lanes are the same as described for A, except for the negative control containing 21 μ M OCN in lane 9.

(w/w). Reactions were carried out for 2 h at 37 °C. OPN was preincubated for 10 min at 37 °C with increasing amounts of OCN or the peptides before adding TG. TG was omitted from control experiments. Reactions were terminated by lyophilization.

Identification of Osteopontin Cross-linking Products by Western Blotting—Reaction products were separated by SDS-PAGE under reducing conditions (26) using 8.5% acrylamide separating gel. Following electrophoresis, the proteins were electroblotted onto polyvinylidene difluoride membranes. The membranes were blocked with 1% blocking reagent (from the digoxigenin luminescent detection kit for nucleic acids) in 0.1 M maleic acid buffer; washed with 0.1% Tween 20 in 50 mM Tris-buffered saline, pH 7.4; and then incubated with rabbit anti-bovine OPN IgG diluted in the washing buffer. After washing, the membranes were subjected to alkaline phosphatase-conjugated anti-rabbit IgG. The proteins were detected with 5-bromo-4-chloro-3-indolyl phosphate/nitro blue tetrazolium alkaline phosphatase substrate following the instructions of the manufacturer.

Quantification of the Degree of Osteopontin Cross-linking—Western blot filters were scanned with the DeskScan IIp/3p program, and the intensities of the bands representing high molecular mass complexes of OPN were measured using the NIH Image 1.55 noFPU program.

β -Casein Cross-linking with Tissue Transglutaminase and Identification and Quantification of Complexes Formed in the Presence and Absence of Osteocalcin Peptides—The reaction conditions for β -casein cross-linking were as described above for OPN cross-linking, except that the reaction buffer was 50 mM Tris-HCl, pH 8.5. The enzyme/substrate ratio was 1:20 (w/w). Each reaction mixture contained 20 μ g of β -casein and, when present, increasing amounts of the OCN peptides. Reaction products were separated by SDS-PAGE using 10% acrylamide separating gel. Proteins were detected by Instaview Universal staining and destained with 5% acetic acid. Gels were scanned, and the intensities of the cross-linked β -casein bands were measured as described above for OPN.

Biotinylation of Peptide 1—To avoid possible disturbance in binding caused by biotin substitutes in the middle of the peptide, a modified OCN Peptide 1 was synthesized, containing extra Lys residues at both ends of the peptide. Biotinylation was performed for 18 h at ambient temperature in 0.1 M phosphate buffer with dimethyl sulfoxide-dissolved biotinamidocaproate *N*-hydroxysuccinimide ester. The biotinylated peptide was immediately purified by reverse-phase HPLC using a Vydac C₁₈ semipreparative column with a 45-min linear acetonitrile gradient as described above and characterized by mass spectrometry. The molecular mass of Peptide 1 was found to have increased by a mass corresponding to two biotin molecules.

Enzyme-linked Immunosorbent Binding Assay—Polyvinyl chloride microtiter wells were coated with 1 μ g of TG, OPN, or β -casein diluted in 0.1 M borate buffer. After a 4-h incubation at ambient temperature, the wells were washed with 0.05% Tween 20 in 50 mM Tris-buffered saline, pH 8.0, and blocked for 30 min with 2% bovine serum albumin in the washing buffer. The wells were washed as described above, and the bound proteins were allowed to interact for 1 h with 10 μ g of biotinylated Peptide 1 in 0.1 M borate buffer, followed by washing. The bound peptide was detected with alkaline phosphatase-conjugated anti-biotin IgG and visualized by FAST *p*-nitrophenyl phosphate alkaline phosphatase substrate. The absorbance values were measured at 405 nm.

RESULTS

Effect of Bovine Osteocalcin on Transglutaminase-catalyzed Cross-linking of Osteopontin

—To test whether OCN had an

TABLE I
Peptide concentrations required for 50% inhibition of transglutaminase-catalyzed cross-linking of osteopontin

The other peptides showed no inhibition when tested at up to a molar ratio of 1:80 (OPN/peptide).

| Peptide | IC ₅₀ for OPN cross-linking μ M | OPN/OCN (or peptide) molar ratio for IC ₅₀ |
|------------|---|---|
| Bovine OCN | 9.9 | 1:18 |
| Peptide 1 | 12.9 | 1:22 |
| Peptide 1a | 14.6 | 1:26 |
| Peptide 4 | 38.4 | 1:63 |

effect on TG-mediated OPN polymerization, we cross-linked bovine OPN with tissue TG in the presence and absence of bovine OCN. Due to poor stainability of OPN with common polyacrylamide gel stains, Western blotting was used to visualize the high molecular mass OPN complexes. The complexes differed in multimer size, ranging from 120 kDa to >250 kDa (Fig. 1A). A 30-kDa band with an N terminus identical to that of OPN was also detected by Western blotting. It possibly represents an OPN breakdown fragment or a dephosphorylated form of the protein. The addition of OCN to the cross-linking reaction mixture produced a concentration-dependent inhibition of OPN complex formation (Fig. 1B), with an IC₅₀ value of 9.9 μ M. An 18-fold molar excess of OCN to OPN was required for 50% inhibition (Table I). Excess CaCl₂ (2.5 mM) was added to reaction mixtures to rule out the possibility that OCN would inhibit the cross-linking of OPN by chelating Ca²⁺ ions essential for TG activity. The possibility of a pseudoinhibitory activity resulting from OCN competition with OPN as a TG substrate was ruled out since the TG treatment did not result in shifting of the OCN bands as analyzed by 18% SDS-polyacrylamide gel electrophoresis (Fig. 1C).

Localization of the Inhibitory Region in Osteocalcin—To identify the amino acid sequence involved in the inhibitory activity of OCN, partial peptides covering the whole protein were synthesized. The peptides were designed to represent the known structural features of human OCN (1) (Table II). Results of cross-linking experiments using these peptides, shown in Fig. 2, indicated that Peptide 1, consisting of amino acids 1–25, was the most potent inhibitor of OPN cross-linking, with an IC₅₀ value of 12.9 μ M. Cross-linking experiments performed in the presence of its cleavage products, Peptides 1a and 1b, indicated that only Peptide 1a was capable of reducing OPN cross-linking. The inhibitory potency was, however, slightly decreased as compared with intact Peptide 1 (Table I). Peptide 1b did not appreciably inhibit OPN cross-linking since 93% of the OPN cross-links were formed when tested at up to a 43 μ M peptide concentration. This indicated that the elements required for the inhibition were located within the first 13 N-terminal amino acid residues and that the Gla residues were not essential for inhibition. The entire Peptide 1 was, however, needed to achieve the maximum inhibitory effect. Thus, residues 14–25 may to some extent contribute to its binding affinity. Peptides 1c and 1d did not cause inhibition of TG activity. This implies that the minimum elements needed for the inhibition were within the first 13 N-terminal amino acid residues. No inhibition was observed with either Peptide 2 or 3 since, in both cases, >90% of the OPN complexes were still formed when tested at 43 and 67 μ M, respectively. The maximum peptide concentrations tested corresponded to molar ratios of 1:2000

TABLE II
Osteocalcin peptides used in the structural localization of the inhibitory activity

Peptides 1, 2, and 3 were synthesized with an Applied Biosystems Model 431 peptide synthesizer. Peptides 1a–1d were obtained from Peptide 1. Peptides 1a and 1b were prepared by Asp-N cleavage, and Peptides 1c and 1d by pepsin digestion. Peptide 4 was obtained as a breakdown product of intact bovine OCN. The amino acid sequences and structural features are as reported by Hauschka *et al.* (1, 2).

| Osteocalcin peptide | Sequence | Sequence position | Structural feature |
|---------------------------|--|-------------------|--|
| Human | | | |
| Peptide 1 ^{a,b} | YLYQWLGA ^a VPYPDPLXPRRXVMXL | 1–25 | β -Sheet, β -turn, Gla helix |
| Peptide 1a | YLYQWLGA ^a VPYP | 1–13 | β -Sheet, β -turn |
| Peptide 1b ^{a,b} | DPLXPRRXVMXL | 14–25 | Gla helix |
| Peptide 1c | YLYQ | 1–4 | β -Sheet |
| Peptide 1d | WLGA ^a VPYPDP | 5–15 | β -Turn |
| Peptide 2 ^b | NPDMDELADHIGFQEA | 26–41 | β -Turn, Asp-Glu helix |
| Peptide 3 | YRRFYGPV | 42–49 | β -Sheet |
| Bovine | | | |
| Peptide 4 | YLDHWLGA ^a PAPYPD | 1–14 | β -Sheet |

^a X = γ -carboxyglutamic acid.

^b Cys²³ and Cys²⁹ residues were replaced by Met.

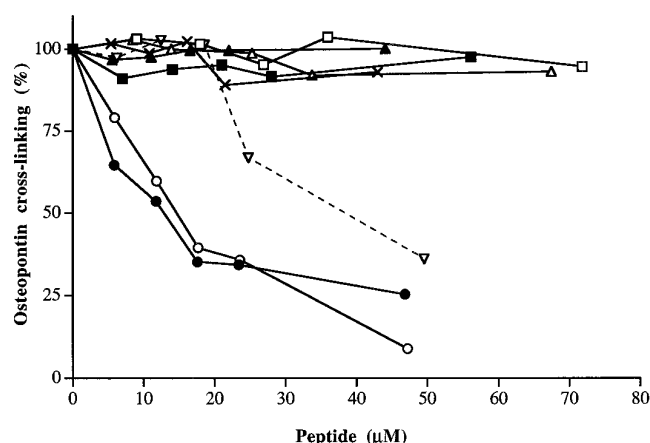


FIG. 2. Localization of the osteocalcin amino acid sequence responsible for the inhibitory effect on osteopontin cross-linking. OPN cross-linking reactions were performed in the presence of increasing amounts of synthetic human OCN peptides as described under "Experimental Procedures." Reaction products were separated electrophoretically and subjected to Western analysis. Western blots were scanned, and the intensities of the bands representing complex forms of OPN were analyzed using the NIH Image 1.55 noFPU program. The relative cross-linking of OPN was plotted against peptide concentration. ●, Peptide 1; ○, Peptide 1a; ×, Peptide 1b; ■, Peptide 1c; □, Peptide 1d; ▲, Peptide 2; △, Peptide 3; ▽, Peptide 4.

(TG/peptide) and 1:80 (OPN/peptide).

The possibility that the peptides could have acted as pseudosubstrates and competed with OPN in the TG reaction, therefore producing inhibition, was ruled out by two observations: Peptides 1c and 2, which both contain a glutamine residue, did not show inhibition, whereas Peptide 4 inhibited the cross-linking without having a glutamine residue in its sequence. At lower concentrations, bovine Peptide 4 was less potent than the corresponding region of human OCN in inhibiting OPN cross-linking. At higher concentrations, however, the inhibitory effect of the bovine peptide approached that of the human peptide. In summary, the inhibitory effect of OCN on OPN cross-linking appears to be caused by the N-terminal region of OCN.

Effect of Osteocalcin Peptides on the Cross-linking of β -Casein—TG treatment of β -casein, a 30-kDa protein of milk (27, 28), resulted in cross-linking, which appeared to produce dimeric β -casein according to molecular mass. When β -casein was pre-incubated with Peptide 1 before adding TG, an inhibitory effect, similar to that observed in the OPN reaction, was achieved. As demonstrated in Fig. 3, Peptide 1 inhibited the cross-linking of β -casein in a concentration-dependent manner (Fig. 3, A and B). Peptides 2 and 3 were not inhibitory. A 50% inhibition of β -casein cross-linking was achieved with a molar ratio of 1:20 (substrate/inhibitor), which was close to the ratio observed in the OPN reaction.

Affinity of Peptide 1 for Osteopontin, β -Casein, and Transglutaminase—An enzyme-linked immunosorbent binding assay was used to determine whether the TG inhibition was mediated by the binding of Peptide 1 to the substrate or to the enzyme. The results shown in Fig. 4 indicated that Peptide 1 bound to both substrates, OPN and β -casein, whereas binding to TG was negligible.

DISCUSSION

Previous studies have indicated that OPN may participate in the assembly of bone matrix as a substrate for tissue TG (18, 21). As OCN has been identified as a negative regulator of bone mineralization (6) and is capable of *in vitro* interaction with OPN (7), we studied whether OCN had an effect on TG activity with OPN as a substrate. Our results demonstrate that OCN is

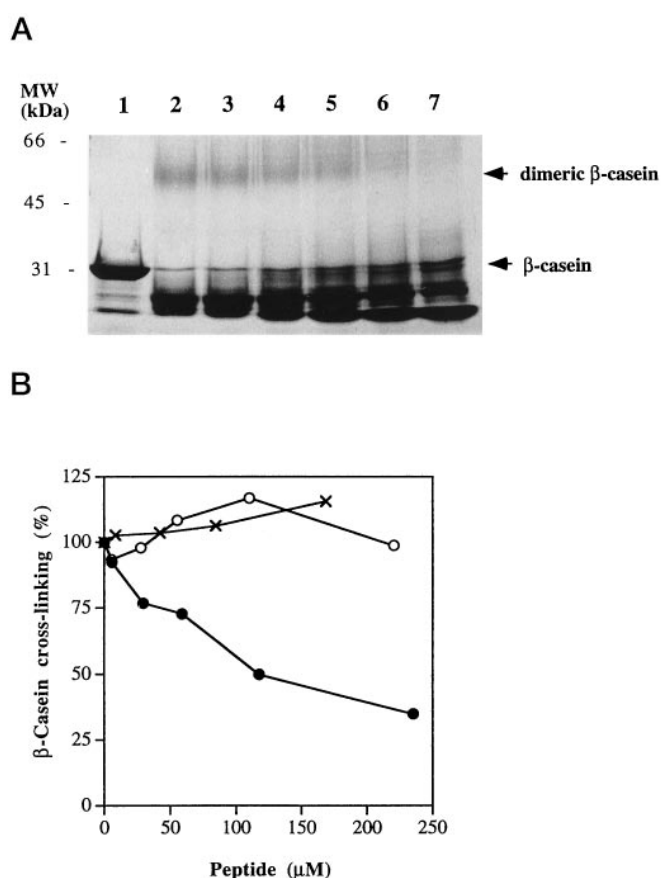


FIG. 3. Effect of osteocalcin peptides on the cross-linking of β -casein. β -Casein was cross-linked by TG in the presence of Peptides 1, 2, and 3 as described under "Experimental Procedures" and analyzed on 10% SDS-polyacrylamide gels. A, shown are the products from the cross-linking reaction in the presence of Peptide 1. Lane 1, β -casein; lane 2, cross-linked β -casein; lanes 3–7, cross-linked β -casein in the presence of 6, 29, 59, 118, and 235 μ M Peptide 1, respectively. Molecular mass standards are shown on the left. B, the intensities of the cross-linked β -casein bands obtained in the presence of Peptide 1 (●), 2 (○), or 3 (×) were measured using the NIH Image 1.55 noFPU program. The relative cross-linking of β -casein was plotted against peptide concentrations.

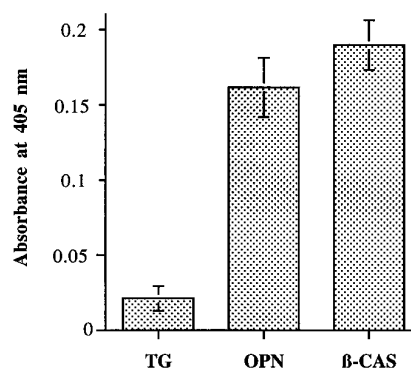


FIG. 4. Affinity of biotinylated Peptide 1 for transglutaminase, osteopontin, and β -casein. TG, OPN, and β -casein (β -CAS), at 20 μ g/ml concentrations in 0.1 M borate buffer, pH 8.8, were applied to microtiter wells and allowed to incubate for 4 h at ambient temperature. After washing and blocking the wells with 2% bovine serum albumin, the immobilized proteins were allowed to interact with biotinylated Peptide 1. Protein-bound Peptide 1 was detected as described under "Experimental Procedures" using an alkaline phosphatase-conjugated biotin antibody detection system. Error bars represent the S.E. of values from four independent determinations.

capable of inhibiting TG-mediated cross-linking of OPN. Experiments with a set of human OCN-derived synthetic peptides revealed that the first 13 N-terminal amino acid residues in-

TABLE III
Amino acid sequence comparison of the human and bovine inhibitory osteocalcin peptides with tissue transglutaminase and factor XIII substrate recognition and active sites

The amino acid sequences of human OCN are as reported by Hauschka (1) and those of factor XIII by Ichinose *et al.* (1986). The sequence of guinea pig liver TG is as reported by Ikura *et al.* (1988). Homologous or similar elements are designated by boldface letters. Sequences marked with asterisks represent well conserved OCN amino acid residues. Dashes were added to obtain maximum homology.

| Peptide | Sequence |
|--|---|
| Human OCN Tyr ¹ -Val ¹⁰ | YLYQWL G APV |
| Guinea pig liver TG Tyr ²⁷³ -Ala ²⁸³ | YGQ C WVFAAVA ^a |
| Human OCN Tyr ¹ -X ²⁵ | *** YLYQWL G APV PYPDPLXPRRXVMXL ^b |
| Factor XIII A-chain Asn ⁷² -Asp ⁹⁷ | NKLIVRRGQSFYV-QIDFSR-PY---DPRRD |
| Bovine OCN Tyr ¹ -Pro ¹³ | *** YLDHWLGAPVPYP |
| Factor XIII A-chain Asp ¹⁹⁰ -Asn ²⁰⁷ | DDAV YLD NEKEREEYVLN |

^a Sequence is 100% homologous to that of factor XIIIa.

^b X, γ -carboxyglutamic acid.

clude the elements required for this inhibitory effect. The cross-linking of a non-bone TG substrate, β -casein, was inhibited by an N-terminal OCN peptide as well. The binding assay further demonstrated an interaction of the biotinylated inhibitory peptide with OPN and β -casein, showing only minor interaction with TG. This indicates that OCN interferes with TG activity by interacting with its substrates.

The inhibitory N-terminal region of OCN possesses elements homologous to transglutaminase regions, reported to be involved in the substrate recognition of tissue TG and factor XIIIa by Achyuthan *et al.* (29). Factor XIIIa is a transglutaminase of the blood coagulation cascade (21). The substrate recognition sites were localized by Achyuthan *et al.* by testing the effects of peptides derived from factor XIIIa on factor XIIIa and tissue TG activities. Two peptides outside the active-site pocket were capable of inhibiting the cross-linking of three different substrates of the enzymes, suggesting that these regions are involved in the substrate recognition. These factor XIIIa peptides, Asn⁷²-Asp⁹⁷ and Asp¹⁹⁰-Phe²³⁰, exhibit 70 and 61% homology, respectively, to similar regions of liver tissue TG (30). Sequence comparison (Table III) indicated that OCN has elements similar and homologous to both factor XIIIa peptides and that the homologous OCN residues are well conserved. OCN is also homologous to the region around the active-site cysteine of TG and factor XIIIa. The similarity of the N terminus of OCN to the three TG regions may imply that OCN binds to multiple sites in the protein substrates. The 1:20 substrate/inhibitor ratio required to produce a 50% decrease in the cross-linking of both OPN and β -casein also lends support to this idea. In light of these observations, we suggest that the inhibitory effect of OCN results from its mimicking the TG sequences that contribute to its substrate binding activity and therefore also to its enzymatic activity. This could result in a reversible binding of OCN to as yet unknown enzyme contact sites of OPN and β -casein, therefore preventing the access of the enzyme to its substrates.

TG is expressed in mineralizing cartilage and bone and is active in tissues undergoing calcification (22, 23). Aeschlimann *et al.* (23) demonstrated this by staining developing long bones and maturing tracheal cartilage with an isopeptide-specific antibody, which revealed the existence of TG-catalyzed cross-links in the mineralization front. Only osteopontin and osteonectin (31) from bone phosphoproteins have been identified as its *in vitro* substrates. They are both ultrastructurally localized in areas undergoing calcification (23, 33). Large OPN and osteonectin complexes have also been shown to exist in bone *in vivo* (18, 23). The role of these complexes is not clear, but as it is thought that the initiation of hydroxyapatite crystallization requires protein aggregates rather than single molecular

monomers (32), it is possible that the TG activity in the mineralization front could provide such protein clusters for hydroxyapatite nucleation seeds and a cross-linked protein scaffold for matrix assembly (21, 34). In other words, the cross-linking products of TG, such as the OPN complexes, could be involved in the initiation of calcification and contribute to the stability and strength of the organic matrix of bone. Our observation that an established inhibitor of mineralization, OCN, inhibits TG-mediated cross-linking may imply *per se* that TG activity is directed to enhance calcification.

Studies demonstrating that low levels or the total absence of OCN in bone causes extensive calcification and bone overgrowth indicate that OCN acts as a negative regulator of bone turnover (5, 6). *In vitro* experiments point to a dual role for OCN since it is thought to directly participate in the inhibition of calcification (3) and in the promotion of bone resorption (4). In light of the present findings, we propose that one of the roles of OCN could be in the interference with TG activity. Based on previously published data, this kind of inhibitory effect could not only restrict the superfluous strengthening of the organic matrix of bone, but could simultaneously result in suppression of mineralization and excessive calcification by interfering with the formation of protein aggregates, which possibly act as initiators of calcification. The suppressive action directed toward TG activity could therefore have *in vivo* importance for the maintenance of flexibility of hard tissue.

To our knowledge, there have been no previous reports on protein inhibitors of tissue TG activity. This paper therefore describes the first protein regulator of tissue TG activity, suggesting a novel potential function for OCN in this process. The fact that OCN is expressed only in calcifying tissues, but that both OPN and TG are present in, for example, atherosclerotic plaques (35, 36), elicits an intriguing possibility that there could be OCN-like control proteins in other tissues for the extracellular regulation of TG activity and possibly for prevention of pathological calcification. This view is supported by the recent finding that mice lacking OCN-related matrix Gla protein show extensive calcification of arteries (37).

Acknowledgments—We thank Dr. E. Sørensen for providing β -casein and the polyclonal antibody for osteopontin and for helpful discussions. We also thank E.-L. Stefanius for help in peptide synthesis and P. Keinänen for excellent technical assistance.

REFERENCES

1. Hauschka, P. V., Lian, J. B., Cole, D. E. C., and Gundberg, C. M. (1989) *Physiol. Rev.* **69**, 990–1047
2. Hauschka, P. V., and Carr, S. A. (1982) *Biochemistry* **21**, 2538–2547
3. Romberg, R. W., Werness, P. G., Riggs, B. L., and Mann, K. G. (1986) *Biochemistry* **25**, 1176–1180
4. Lian, J. B., Tassinari, M., and Glowacki, J. (1984) *J. Clin. Invest.* **73**, 1223–1226

5. Price, P. A., Williamson, M. K., Haba, T., Dell, R. B., and Jee, W. S. S. (1982) *Proc. Natl. Acad. Sci. U. S. A.* **79**, 7734–7738
6. Ducy, P., Desbois, C., Boyce, B., Pinero, G., Story, B., Dunstan, C., Smith, E., Bonadio, J., Goldstein, S., Gundberg, C., Bradley, A., and Karsenty, G. (1996) *Nature* **382**, 448–452
7. Ritter, N. M., Farach-Carson, M. C., and Butler, W. T. (1992) *J. Bone Miner. Res.* **7**, 877–885
8. Butler, W. T. (1989) *Connect. Tissue Res.* **23**, 123–136
9. Denhardt, D. T., and Guo, X. (1993) *FASEB J.* **7**, 1475–1482
10. Franzén, A., and Heinegård, D. (1985) *Biochem. J.* **232**, 715–724
11. Senger, D. R., Peruzzi, C. A., Papadopoulos, A., and Tenen, D. G. (1989) *Biochim. Biophys. Acta* **996**, 43–48
12. Shiraga, H., Min, W., VanDusen, W. J., Clayman, M. D., Miner, D., Terrell, C. H., Sherbotie, J. R., Foreman, J. W., Przysiecki, C., Neilson, E. G., and Hoyer, J. R. (1992) *Proc. Natl. Acad. Sci. U. S. A.* **89**, 426–430
13. Mark, M. P., Prince, C. W., Oosawa, T., Gay, S., Bronckers, A. L. J. J., and Butler, W. T. (1987) *J. Histochem. Cytochem.* **35**, 707–715
14. Mark, M. P., Butler, W. T., Prince, C. W., Finkelman, R. D., and Ruch, J.-V. (1988) *Differentiation* **37**, 123–136
15. McKee, M. D., and Nanci, A. (1996) *Microsc. Res. Tech.* **33**, 141–164
16. McKee, M. D., and Nanci, A. (1996) *Anat. Rec.* **245**, 394–409
17. Prince, C. W., Dickie, D., and Krundieck, C. L. (1991) *Biochem. Biophys. Res. Commun.* **177**, 1205–1210
18. Sørensen, E. S., Rasmussen, L. K., Møller, L., Jensen, P. H., Hørup, P., and Petersen, T. E. (1994) *Biochem. J.* **304**, 13–16
19. Beninati, S., Senger, D. R., Cordella-Miele, E., Mukherjee, A. B., Chackalaparampil, I., Shanmugam, V., Singh, K., and Mukherjee, B. B. (1994) *J. Biochem. (Tokyo)* **115**, 675–682
20. Greenberg, C. S., Birkbichler, P. J., and Rice, R. H. (1991) *FASEB J.* **5**, 3071–3077
21. Aeschlimann, D., and Paulsson, M. (1994) *Thromb. Haemostasis* **71**, 402–415
22. Aeschlimann, D., Wetterwald, A., Fleisch, H., and Paulsson, M. (1993) *J. Cell Biol.* **120**, 1461–1470
23. Aeschlimann, D., Kaupp, O., and Paulsson, M. (1995) *J. Cell Biol.* **129**, 881–892
24. Colombo, G., Fanti, P., Yao, C., and Malluche, H. H. (1993) *J. Bone Miner. Res.* **8**, 733–743
25. Sørensen, E. S., and Petersen, T. E. (1993) *J. Dairy Res.* **60**, 189–197
26. Laemmli, U. K. (1970) *Nature* **227**, 680–685
27. Ikura, K., Kometani, T., Yoshikawa, M., Sasaki, R., and Chiba, H. (1980) *Agric. Biol. Chem.* **44**, 1567–1573
28. Christensen, B. M., Sørensen, E. S., Højrup, P., Petersen, T. E., and Rasmussen, L. K. (1996) *J. Agric. Food Chem.* **44**, 1943–1947
29. Achyuthan, K. E., Slaughter, T. F., Santiago, M. A., Enghild, J. J., and Greenberg, C. S. (1993) *J. Biol. Chem.* **268**, 21284–21292
30. Ikura, K., Nasu, T., Yokota, H., Tsuchiya, T., Sasaki, R., and Chiba, H. (1988) *Biochemistry* **27**, 2898–2905
31. Hohenadl, C., Mann, K., Mayer, U., Timpl, R., Paulsson, M., and Aeschlimann, D. (1995) *J. Biol. Chem.* **270**, 23415–23420
32. Glimcher, M. J. (1989) *Anat. Rec.* **224**, 139–153
33. Hultenby, K., Reinholt, F. P., Oldberg, Å., and Heinegård, D. (1991) *Matrix* **11**, 206–213
34. Boskey, A. L. (1992) *Clin. Orthop. Relat. Res.* **281**, 244–274
35. Giachelli, C. M., Bae, H., Almeida, M., Denhardt, D. T., Alpers, C. E., and Schwartz, S. M. (1993) *J. Clin. Invest.* **92**, 1686–1696
36. Wiebe, R. I., Tarr, A. H., and Bowness, J. M. (1991) *Biochem. Cell Biol.* **69**, 821–827
37. Luo, G., Ducy, P., McKee, M. D., Pinero, G. J., Loyer, E., Behringer, R. R., and Karsenty, G. (1997) *Nature* **386**, 78–81
38. Ichinose, A., McMullen, B. A., Fujikawa, K., and Davie, E. W. (1986) *Biochemistry* **25**, 4633–4638