RESEARCH COMMUNICATION

Localization of transglutaminase-reactive glutamine residues in bovine osteopontin

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Here we report the identification of two transglutaminase-reactive glutamines (Gln-34 and Gln-36) in bovine osteopontin (OPN). Sequence alignment revealed that these glutamines are conserved in all known OPN sequences, indicating a functional importance of this region of the protein. Furthermore, immunological analysis of bovine bone demonstrated that OPN is present in high-molecular-mass complexes *in vivo*. These findings support the functional aspects of a transglutaminase-catalysed cross-linking of OPN in facilitating cellular attachment and tissue calcification.

INTRODUCTION

Osteopontin (OPN) is an extracellular glycosylated phosphoprotein rich in acidic amino acids (for review see [1]). It contains an RGDS-cell adhesion sequence and mediates cell–matrix and cell–cell interactions, probably via the $\alpha_v\beta_3$ integrin, the vitronectin receptor [2]. OPN was first isolated from the mineralized matrix of bovine bone [3]. Since then it has been characterized in calcified tissues from several species, including man [4], rat [5], mouse [6], pig [7] and chicken [8]. In bone, OPN is synthesized by preosteoblasts, osteoblast and osteocyte, secreted into the osteoid and subsequently incorporated in the bone [9,10].

In addition to bone cells, OPN is present in physiological fluids such as urine [11–13] and milk [14,15]. In urine, OPN stereospecifically regulates the solubility of calcium salts [11], and recently OPN has been identified in the organic matrix of urinary stones [12,13] and atherosclerotic plaques [16–18]. Furthermore, OPN is expressed in activated T-cells and metastazing tumour cells, where roles in the early resistance to bacterial infections [19] and binding of tumour cells at secondary sites [20], respectively, have been hypothesized.

OPN has been reported to associate with fibronectin [21], Type I collagen [22] and osteocalcin [23]. The expression of tissue transglutaminase (TG) in skeletal tissue is strictly regulated at the mineralization front and has been proposed to induce cross-linking of the mineralizing matrix [24]. Moreover, it has been shown that bovine OPN is a substrate for TG [25], and recently OPN has been shown to cross-link to fibronectin in a TG-catalysed reaction [26].

In the present work we have localized the TG-reactive glutamines in OPN and found that these residues are conserved in all known OPN sequences. The significance of OPN as a TG substrate is supported by Western-blot analysis of bovine bone, which reveals the presence of covalently linked OPN in high-molecular-mass complexes *in vivo*. These findings indicate that TG-catalysed cross-linking could be an important mechanism in the functions of OPN.

EXPERIMENTAL

Reagents

Bovine OPN was purified as described previously [15]. Guinea-pig liver TG, thermolysin, 5-bromo-4-chloro-3-indolyl phosphate *p*-toluidine salt and Nitro Blue Tetrazolium were from Sigma, U.S.A. [1,4-^{14}C]Putrescine (109 mCi/mmol) was from Amersham, U.K. Phenylmethanesulphonyl fluoride (PMSF) was from Boehringer Mannheim, Germany. Bovine bone was provided by Dr. Peter Nielsen, National Institute of Animal Science, Research Centre Foulum, Denmark. Vydac C_{18} (10 μm) was from The Separations Group, Hesperia, U.S.A. Polyvinylidene difluoride (PVDF) Problott membrane and reagents used for sequencing were purchased from Applied Biosystems, Foster City, U.S.A. Protein A–Sepharose was from Pharmacia, Sweden. Rabbit anti-(bovine OPN) antiserum was generated with purified bovine OPN at Dako, Denmark. Pig anti-rabbit IgG conjugated to alkaline phosphatase and alkaline-phosphatase-conjugated streptavidin were purchased from Dako. Biotinylated marker proteins for SDS/PAGE were from Bio-Rad Laboratories, U.S.A.

Purification and characterization of [^{14}C]putrescine-labelled peptides

OPN (83 nmol) was labelled with [^{14}C]putrescine (370 nmol) by guinea-pig liver TG in a 1:60 (mol/mol) enzyme:substrate ratio. The reaction was performed in 50 mM Tris/HCl/2.5 mM $CaCl_2$/20 mM dithioerythritol, pH 8.5 (reaction vol. 750 μl), at 37 °C for 18 h. TG was inactivated by 50 mM EDTA and the material was desalted on a PD-10 column (Pharmacia), followed by freeze-drying.

[^{14}C]Putrescine-labelled OPN was digested with thermolysin. The digestion was performed in 250 μl of 0.1 M pyridine/acetate (pH 6.5)/5 mM $CaCl_2$ in a 1:50 (w/w) enzyme:substrate ratio at 55 °C for 18 h. The resulting peptides were separated by reverse-

Abbreviations used: OPN, osteopontin; TG, transglutaminase; PTH, phenylthiohydantoin; PMSF, phenylmethanesulphonyl fluoride; PVDF, polyvinylidene difluoride; PDMS, plasma-desorption mass spectrometry.

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phase h.p.l.c. on a Pharmacia LKB h.p.l.c. system. Incorporation of [^{14}C]putrescine was determined by liquid-scintillation counting of samples from each peak on a Beckman LS 1801 instrument. Amino acid sequence analysis was performed on an Applied Biosystems model 477A sequencer with on-line phenylthiohydantoin (PTH) analysis (Applied Biosystems 120A). Part of the PTH derivative sample left over after on-line injection was used for radioactivity detection as described above.

Plasma-desorption mass spectrometry (PDMS) was carried out with a BioIon 20K plasma-desorption time-of-flight instrument (Applied Biosystems ABI, Sweden). Samples (10–50 pmol) were dissolved in 0.1 % trifluoroacetic acid and applied to nitrocellulose-covered targets, spin-dried and micro-rinsed as described [27]. Spectra were recorded at 15 kV for 10^6 primary fission events.

Western blotting of bovine bone extract

Crushed bovine bone of the longitudinal part of the femur was extracted with 0.1 M EDTA/2 mM PMSF for 24 h at 5 °C. The extract was dialysed against 1 % formic acid and the soluble fraction was freeze-dried. A sample was solubilized in SDS-sample buffer and heated at 95 °C for 15 min before SDS/PAGE using a 10–20 % acrylamide gel, followed by electroblotting to a PVDF membrane. The membrane was rinsed with water, air-dried, blocked with 2 % Tween 20 in 50 mM Tris/0.5 M NaCl, pH 7.4, and washed in 50 mM Tris/0.5 M NaCl (pH 7.4)/0.1 % Tween 20. IgG from rabbit anti-(bovine OPN) serum was purified on a Protein A–Sepharose column and incubated with the membrane in a concentration of 5 μg/ml in 50 mM Tris/0.5 M NaCl (pH 7.4)/0.1 % Tween 20 for 2 h at 20 °C. Finally, the membrane was incubated with alkaline-phosphatase-conjugated pig anti-rabbit IgG (diluted 3000-fold) for 1 h at 20 °C, followed by addition of 50 ml of 0.1 M Tris/0.1 M NaCl/50 mM $MgCl_2$, pH 9.0, containing 100 μl of 5-bromo-4-chloro-3-indolyl phosphate *p*-toluidine salt stock solution (100 mg in 2 ml of dimethylformamide) and 100 μl of Nitro Blue Tetrazolium stock solution (100 mg in 1.33 ml of 70 % dimethylformamide). In the control experiment, the antibodies (5 μg/ml) were preincubated with purified OPN (10 μg/ml) in 50 mM Tris/0.5 M NaCl (pH 7.4)/0.1 % Tween 20 for 1 h at 20 °C, before incubation with the membrane. Biotinylated SDS/PAGE mass-standard proteins were detected by addition of alkaline-phosphatase-conjugated streptavidin.

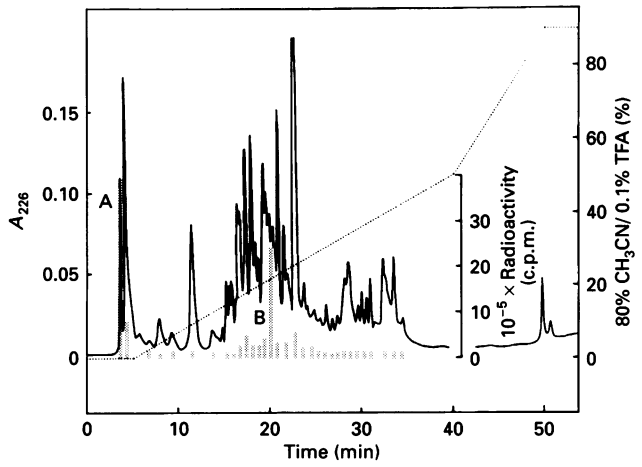

Figure 1 Reverse-phase h.p.l.c. separation of a thermolysin digest of [^{14}C]putrescine-labelled OPN

Peptides were eluted with a stepwise linear gradient of 80 % acetonitrile in 0.1 % trifluoroacetic acid (TFA) (dotted line) from a Vydac C_{18} (10 μm) column (4 mm × 250 mm). The column was operated at 40 °C and the flow rate was 0.85 ml/min. Peptides were detected in the effluent by recording the A_{226} (continuous line) and collected manually. Hatched bars indicate total amount of radioactivity in the respective peaks as determined by liquid-scintillation counting.

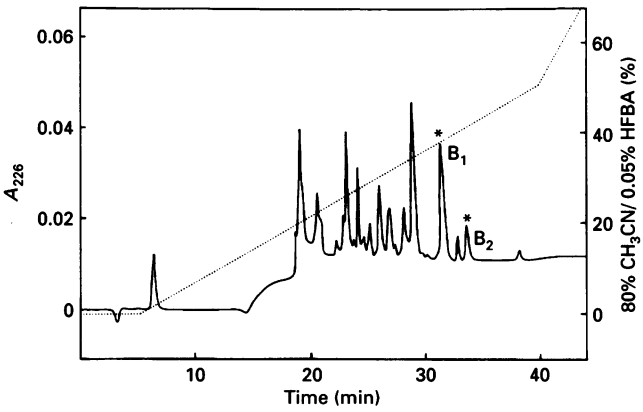

Figure 2 Repurification of peak B

Peak B (Figure 1) was repurified by using the same chromatographic conditions as described for Figure 1, except that 0.05% heptafluorobutyric acid (HFBA) was used as the ion-pairing agent. Radioactive peaks are marked by asterisks.

RESULTS

Identification of transglutaminase acceptor sites in OPN

In order to localize the TG-reactive glutamines, [^{14}C]putrescine-labelled OPN was digested with thermolysin, followed by reverse-phase h.p.l.c. separation. The recovery of [^{14}C]putrescine after the reverse-phase chromatography was calculated as 89 %. Two major radioactively labelled peaks (A and B) were found in the digest (Figure 1). The radioactivity in peak A originated from unbound [^{14}C]putrescine not totally removed by the desalting method used. Repurification of peak B by additional reverse-phase h.p.l.c. resulted in two radioactive peaks, B_1 and B_2 (Figure 2). Sequence analysis of B_1 showed the sequence LKPDPSQKQT, corresponding to residues 28–37 in OPN. In the sequence analysis, yields of PTH-Gln in cycles 7 and 9 were relatively low, indicating putrescine-linked glutamines at these positions. Scintillation counting of the PTH-amino acids showed radioactivity in cycles 7 and 9, thus confirming incorporation of putrescine at Gln-34 and Gln-36 (results not shown). Mass spectrometry of B_1 showed three MH^+ ions, with m/z 1217.5, 1116.2 and 987.9 (Figure 3a), corresponding to the peptides LKPDPSQKQT, LKPDPSQKQ and LKPDPSQK, respectively, all with one putrescine unit attached. Taken together, these results demonstrated that B_1 contained three split variants from the same OPN fragment, each containing a single putrescine moiety at either Gln-34 or Gln-36.

Sequence analysis of B_2 showed the amino acid sequence LKPDPSXKXT, where X denotes that no PTH-amino acids were detected in these cycles. This sequence also aligns with residues 28–37 in OPN. Scintillation counting of the PTH-amino acids showed radioactivity in cycles corresponding to Gln-34 and

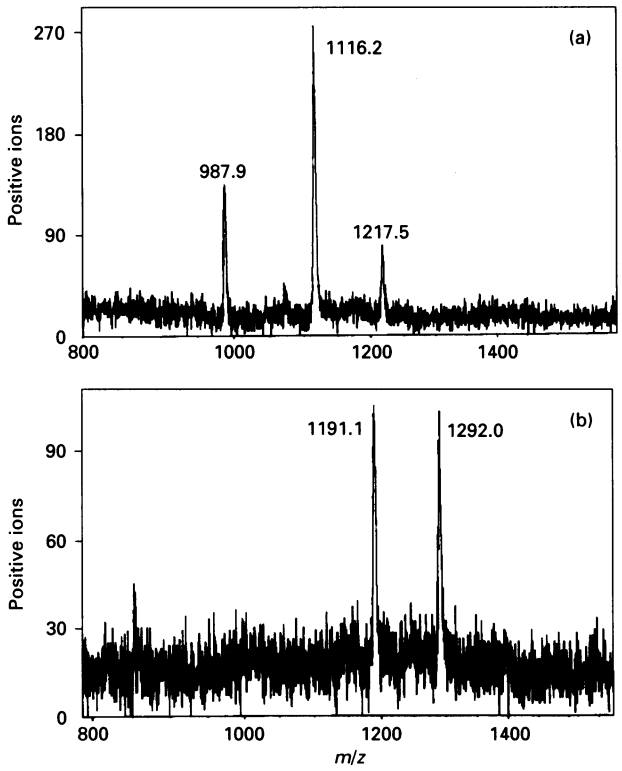

Figure 3 PDMS spectra of B_1 and B_2

(**a**) PDMS spectra of peak B_1 (Figure 2). The MH^+ ions at m/z 987.9, 1116.2 and 1217.5 correspond to residues 28–35, 28–36 and 28–37 of OPN, respectively, each with one putrescine unit attached. (**b**) PDMS spectra of peak B_2 (Figure 2). The MH^+ ions at m/z 1191.1 and 1292.0 correspond to residues 28–36 and 28–37 of OPN, respectively, each with two putrescine units attached.

Gln-36, thereby indicating incorporation of putrescine at these glutamines. Mass spectrometry of B_2 showed two MH^+ ions, with m/z 1292.0 and 1191.1 (Figure 3b), corresponding to the peptides LKPDPSQKQT and LKPDPSQKQ respectively, both with two putrescine units attached. These results demonstrated that B_2 contained the sequenced peptide with two putrescine moieties attached.

Identification of OPN in high-molecular-mass complexes from bovine bone

In order to examine whether OPN is incorporated into high-molecular-mass complexes *in vivo*, we analysed bovine bone. Western blotting of EDTA extracts of bovine bone with OPN antibodies (Figure 4) demonstrates that, apart from OPN itself, high-molecular-mass complexes are recognized by the antibodies (lane 2). In a control experiment, the antibodies were incubated with purified OPN before incubation with the membrane (lane 3). This preincubation effectively blocked all reactivity, showing that the stainings are not a result of non-specific reactivity. The minor band (38 kDa) observed in lane 1 represents a degradation product of OPN which was probably generated during preparation of the SDS/PAGE samples. These results strongly suggest that OPN is covalently cross-linked into high-molecular-mass complexes in bone.

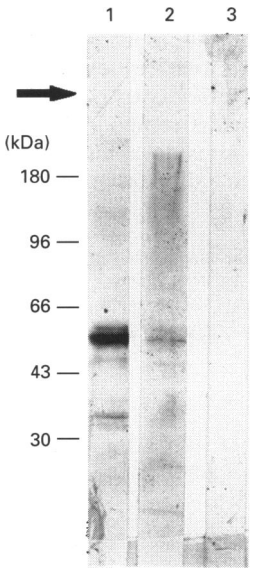

Figure 4 Identification of OPN in high-molecular-mass complexes in bovine bone

Western analysis of bovine bone with OPN antibodies. Lane 1, 5 ng of purified OPN. Lane 2, EDTA extract of bovine bone. Lane 3, control experiment (EDTA extract of bovine bone). Western analysis was performed with antibodies preincubated with purified OPN. The analysis was performed as described in the Experimental section. Molecular-mass markers (kDa) are shown to the left. The arrow indicates the interface between the stacking gel and the resolving gel.

DISCUSSION

A number of reactive glutamine residues have been identified in proteins functioning as TG substrates, but no consensus sequence for this modification has been derived. However, the location of glutamines at the surface of the respective proteins seems to be a common feature [28]. Analysis of the rat OPN sequence places Gln-34 and Gln-36 in a region with no predicted structure linking an α-helix and a β-sheet in the N-terminal part of the protein [29]. Alignment of the TG-reactive region of bovine OPN with the corresponding region in pig [30], man [31], rat [5], mouse [32], rabbit [33] and chicken [8] shows that the glutamines corresponding to Gln-34 and Gln-36 in the bovine sequence are conserved in all known OPN sequences. The conservation of these glutamines in OPN implies that this motif is critical to functional aspects of the protein.

The presence of two adjacent glutamines, where either one can serve as an acceptor in a TG-catalysed amine incorporation, is not uncommon, as has been shown for βA3-crystallin [34], the γ-chain of fibrinogen [35] and plasminogen-activator inhibitor type-2 [36]. In OPN, part of the molecules contains putrescine incorporated at both of the adjacent glutamines Gln-34 and Gln-36 at the same time, and another part of the molecules contains putrescine incorporated at only one of the glutamines.

The proteins to which OPN cross-links *in vivo* have not been identified, but the multiple functions and localizations of OPN leave several possible candidates. The expression of OPN in metastazing tumour cells could suggest that OPN is involved in the binding of tumour cells and subsequent metastasis at secondary sites [20]. Prince et al. [25] hypothesized that the localization of OPN to such sites could be mediated by TG-catalysed cross-linking, thereby providing a cell-adhesion substrate to which the tumour cells could bind and proliferate. In bone, a TG-catalysed formation of complexes between OPN and

other matrix proteins could be involved in the initiation and control of the mineralization processes [24].

In the present report we have identified the TG-reactive region of OPN. Furthermore, we have shown that OPN is incorporated into high-molecular-mass compounds in bovine bone. In conclusion, our results provide a molecular explanation of the mechanism by which OPN becomes covalently anchored in developing matrices.

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