Purification and partial identification of bone-inducing protein from a murine osteosarcoma

Yasuhiko ITO*

Institute for Protein Research, Osaka University, Suita, Osaka 565, Japan

A highly purified fraction with bone-inducing potential was obtained from a murine osteosarcoma using initial fractionation, gel filtration on Sephacryl S-200, and cation-exchange, hydroxyapatite and reverse-phase h.p.l.c. Protein sequencing of the sample derived from this active fraction revealed the *N*-terminal amino acid sequence to be Ala-Ala-Leu-Arg-Pro-Leu-Val-Lys-Pro, which is identical to the *N*-terminal sequence of mouse, human and rat ribosomal protein L32, except for an additional methionine residue at the *N*-terminus of the three latter proteins. Amino acid analysis of the sample also showed its similarity to L32. Two lines of evidence using an antibody specific for the above peptide strongly suggest that this L32-related protein from a murine osteosarcoma has the potential to induce ectopic bone formation. First, the protein specifically detected by the antibody co-distributes with the bone-inducing active fraction. Secondly, a highly purified sample obtained using the antibody was shown to induce the formation of bone with active haematopoiesis.

INTRODUCTION

Under pathological conditions, bone has the potential to develop in almost any organ of the body [1,2]. Also, ectopic bone formation can be induced experimentally by transplantation of various biological materials into certain connective tissues. These include the living epithelial tissues of the renal pelvis, ureter, urinary bladder [1], and gall bladder [3], which induce bone via intramembranous bone formation; devitalized bone, fracture callus, cartilage [4], dentin [5], osteosarcoma [6], and even skeletal, cardiac and visceral muscles [4] induce bone via endochondral bone formation. Of special interest is the case where living epithelial cell lines such as FL and WISH cells (both of which originate from normal amnion tissue), Hep-2, HeLa and KB cells (neoplastic tissues), and CLV-X and CLV-4 cells (transformed by vaccinia virus) become invested with bone when grafted into the connective tissue of cortisol-treated mice [7–9]. Furthermore, these events seem to proceed via endochondral bone formation.

Since Urist demonstrated that the implantation of demineralized bone matrix elicits ectopic bone formation [10], much attention concerning the purification of inducing factor(s) [originally designated as bone morphogenetic protein (BMP) by Urist] has been focused on this experimental model, presumably due to the ease of handling and an adequate supply of the starting material. Moreover, Sampath & Reddi [11] have developed an effective assay method by combining the residue of 4 M-guanidine hydrochloride (Gdn-HCl)-extracted bone matrix with the active fractions as a reconstituted form of the assay sample *in vivo*.

Evidence has accumulated indicating that BMPs are members of the transforming growth factor- β (TGF- β) superfamily [12–18]. In addition, studies involving the purification and characterization of inducing factor(s) from a murine osteosarcoma, which produces 15–75 times greater bone-inducing activity than normal mouse calvaria [19], have also been performed [20–23]. So far, a crude bone-inducing fraction has been prepared by gel filtration on Sephacryl S-200 from the defatted osteosarcoma tissue [21].

In this paper, I describe a detailed procedure for obtaining a highly purified fraction with bone-inducing potential from a murine osteosarcoma, and indicate that the identified protein, (which is closely related to ribosomal protein L32) is likely to represent the inducing factor. This type of inducing factor has been named bone-inducing protein (BIP) in order to distinguish it from TGF- β -type BMPs.

EXPERIMENTAL

Materials

A murine osteosarcoma derived from Thelma Dunn osteosarcoma was a gift from Dr. K. Takaoka, University of Osaka Medical School [21].

Purification procedures

Purification was performed at 4 °C, unless otherwise stated, for steps (i) to (v). In a different purification procedure, step (iv) was replaced by the procedure using hydroxyapatite h.p.l.c. (step vi).

(i) Initial fractionation. The tumour tissue (36 g) was defatted as described in [21], and this defatted material (2.9 g) was solubilized in 4 M-Gdn-HCl/10 mM-sodium phosphate buffer (pH 6.8)/0.5 mM-phenylmethanesulphonyl fluoride (PMSF) (buffer A), and the extract was precipitated with cold acetone (–20 °C). The acetone precipitate (between 50 and 80 %) was collected and homogenized with 0.9 and 2 M-Gdn-HCl successively in 10 mM-sodium citrate buffer (pH 3.0)/0.5 mM-PMSF. The two extracts thus obtained were combined and dialysed successively against 10 mM-sodium phosphate buffer (pH 6.8)/0.05 mM-PMSF and deionized water. Materials precipitated upon dialysis were collected by centrifugation (10000 g, 30 min) and lyophilized (the amount of the lyophilizate was 156 mg).

(ii) Gel filtration. A 153 mg portion of the above lyophilizate was dissolved in 5 ml of buffer A and subjected to gel filtration on Sephacryl S-200 (Pharmacia) (3.0 cm $\times$ 95 cm) in the same buffer, and arbitrary fractions were dialysed successively against

Abbreviations used: BIP, bone-inducing protein; BMP, bone morphogenetic protein; Gdn-HCl, guanidine hydrochloride; PMSF, phenylmethanesulphonyl fluoride; RP, reverse phase; TFA, trifluoroacetic acid; TGF- β , transforming growth factor- β .

* Present and correspondence address: Osaka Bioscience Institute, 6-2-4 Furuedai, Suita, Osaka 565, Japan.

0.01 M-HCl and deionized water and lyophilized. An aliquot of each of the lyophilizates was used for the *in vivo* assay. One of the active fractions (47 mg) was dissolved in 2.5 ml of buffer A and re-chromatographed on Sephacryl S-200 (column 2.1 cm × 74 cm) in the same developing buffer. Another active fraction was used for the next chromatography.

(iii) **Cation-exchange h.p.l.c.** One-third of each lyophilizate (26/3 mg) was dissolved in 2 ml of 0.2 M-Gdn-HCl/10 mM-sodium phosphate buffer (pH 6.8)/0.5 mM-PMSF and chromatographed on SynChropak CM-300 (column 0.41 cm × 25 cm; SynChro Inc.) with a linear gradient of 0 to 2.5 M-Gdn-HCl over 51 min in 10 mM-sodium phosphate buffer (pH 6.8)/0.5 mM-PMSF at a flow rate of 1 ml/min at room temperature. The active fractions from each chromatograph were combined and lyophilized as described above.

(iv) **Reverse-phase (RP) h.p.l.c.** Three-quarters of the above lyophilizate (2.1 mg) was chromatographed on a C₄ RP column (YMC Packed Column; 0.46 cm × 25 cm, 5 µm; Yamamura Chemical) with linear gradients of 0–50 and 50–80% (v/v) acetonitrile/propan-2-ol (2:1, v/v) (solvent B) over 60 and 10 min, respectively in 0.1% (v/v) trifluoroacetic acid (TFA) at a flow rate of 1 ml/min at room temperature.

(v) **RP h.p.l.c. (re-chromatography).** Half of the active fraction obtained in step (iv) (approx. 9 µg) was re-chromatographed on the same column with linear gradients of 0–20, 20–50, and 50–90% (v/v) acetonitrile/propan-2-ol (2:1, v/v) over 10, 60 and 20 min respectively in 0.1% TFA at the same flow rate and temperature as (iv).

(vi) **Hydroxyapatite h.p.l.c.** An active fraction (2.4 mg) obtained from cation-exchange h.p.l.c. (step iii) was dissolved in 2 ml of 0.5 M-Gdn-HCl/83 mM-potassium phosphate buffer, pH 6.8, and applied to hydroxyapatite h.p.l.c. (HCA Column; Mitsui Toatsu Chemicals; 0.76 cm × 10 cm). The column was washed with the above buffer and developed with a co-linear gradient of 0.5–3.0 M-Gdn-HCl and 0.083–0.5 M-potassium phosphate buffer, pH 6.8 (K-P_i) over 50 min at a flow rate of 1 ml/min at room temperature. An active fraction was lyophilized as described above, dissolved in 2 ml of 0.1% TFA and chromatographed on an RP h.p.l.c. column as described in step (v).

In vivo assay

The assay for bone-inducing activity was performed *in vivo* throughout this study. Three equal aliquots were subjected to the assay from each fraction. The lyophilized sample to be assayed was first dissolved in 0.6 ml of 0.01 M-HCl and then combined with 0.5 ml of bovine skin collagen (3.0 mg/ml, pH 3.0; Cellmatrix Type LA; Nitta Gelatin Ltd.), prepared by the procedure described in [24]. After thorough mixing, the collagen/sample mixture was made insoluble by the addition of 0.11 ml of 0.1 M-sodium phosphate buffer, pH 7.4, pelleted by centrifugation (approx. 5000 g, 20 min) and lyophilized. The whole lyophilizate was implanted into the dorsal muscles of allogeneous ddY mice: 19 days later, the implants were recovered from the hosts and bone formation was examined as described elsewhere [21] by soft X-ray photography and histology. The bone-inducing potential of each fraction was judged by histological examination. The criteria used were that at least one of the recovered implants (out of three) was replaced by (a) slight, (b) moderate or (c) intense bone formation (see Figs. 2a–2d of [6]), representing (a) bone containing osteoblasts and basophilic and metachromatic intercellular material, (b) woven bone with haematopoiesis, or (c) bone containing ossicles with active haematopoiesis. These criteria were originally described by Amitani *et al.* [6].

SDS/PAGE and protein measurement

SDS/PAGE was performed according to the method of Laemmli [25] on a 15% (w/v) separating gel. Protein samples were electrophoresed either with or without reduction by 1% (v/v) 2-mercaptoethanol. Protein was measured by the method of Lowry *et al.* [26] using BSA as a standard.

Amino acid analysis and protein sequencing

Amino acid composition was determined with a Hitachi L-8500S or -835S automatic amino analyser. Hydrolysis of the purified protein was performed in an evacuated tube with 5.7 M-HCl containing 0.2% (v/v) phenol at 110 °C for 24 h. The N-terminal amino acid sequence of the purified protein was analysed with a 470A or 477A gas-phase protein sequencer (Applied Biosystems).

Immunoabsorption

(i) **Preparation of antibody.** Antibody was raised in rabbit (New Zealand White, male) against the nonapeptide Ala-Ala-Leu-Arg-Pro-Leu-Val-Lys-Pro (synthesized by the solid-phase method and derivatized with *S*-acetylmercaptosuccinic anhydride at amino groups), which was conjugated to ovalbumin using *N*-(*m*-maleimidobenzoyloxy)succinimide [27]. The IgG fraction was prepared from the serum and was further affinity-purified using the nonapeptide. The anti-nonapeptide IgG was conjugated to CNBr-activated Sepharose 4B (Pharmacia) (4 mg of protein per ml of the resin).

(ii) **Purification of BIP by using anti-nonapeptide IgG.** An active fraction (30 mg), obtained by repeated gel filtrations, was dissolved in 12 ml of 0.1 M-NaCl/20 mM-sodium phosphate buffer (pH 7.2)/0.5 mM-PMSF (buffer C) containing 62 mg of SDS, and then mixed with anti-nonapeptide IgG-conjugated Sepharose 4B (6.3 ml bed volume) previously equilibrated with buffer C containing 0.1% (w/v) SDS [overall SDS concentration was 0.4% (w/v)]. The suspension was kept shaking at room temperature for 2 days and was then packed into a glass column. After three recyclings of the aqueous phase, the column was washed with buffer C containing 0.01% (w/v) SDS, followed by washing with buffer C alone to remove SDS. The adsorbed proteins were eluted with 5 M-Gdn-HCl/10 mM-sodium citrate buffer, pH 2.5, and immediately chromatographed by C₄ RP h.p.l.c. as described above.

Western hybridization

Protein samples obtained after various purification steps were electrophoresed on SDS/PAGE, and Western hybridization was performed according to the manufacturer's guidelines [28]. After transfer, nitrocellulose filters were incubated with 10–50-fold diluted serum or approx. 100 µg of anti-nonapeptide IgG/ml, and subsequently with approx. 200 µg of peroxidase-conjugated goat anti-rabbit immunoglobulin/ml (Cappel). The filter was developed using 4-chloro-1-naphthol (0.7 mg/ml) and 0.03% (w/v) H₂O₂.

RESULTS

Purification of BIP

Before carrying out the various chromatographic steps, initial fractionation of the tumour tissue was performed. This is summarized in Table 1. From 36 g of the osteosarcoma, 156 mg of a 2 M-Gdn-HCl-soluble/water-insoluble fraction was obtained, a 0.9 mg sample of which induced ectopic bone formation. In contrast, more than 2.1 mg of the 2 M-Gdn-HCl-soluble/water-soluble fraction was unable to induce bone

formation. Here, the strong tendency of the active material to aggregate in the solution during the initial fractionation steps

Table 1. Initial fractionation of BIP

A 36 g sample of a murine osteosarcoma was used for the purification of BIP. The detailed procedure for obtaining each fraction is described in the text. The weight of each fraction and the weight used for the *in vivo* assay were measured directly. The incidence is the (number of implants replaced by a bone)/(number of implants recovered).

Fraction	Weight (mg)	Weight used for <i>in vivo</i> assay (mg)	Incidence
Defatted powder	2900	9.4	2/2
Acetone precipitate			
0–50 %	1400*	10.3	0/3
50–80 %	1100*	3.1	3/3
Soluble at 80 %	160*	10.0	0/3
2 M-Gdn-HCl extraction			
Residue	833	5.9	2/3
Extract			
Insoluble after dialysis against water	156	0.9	3/3
Soluble after dialysis against water	172	2.1	0/2

* Calculated values from another set of experiments.

could be seen. This characteristic was also observed in purification using bovine bone as a starting material [29].

In the first gel filtration on Sephacryl S-200 (Fig. 1a), bone-inducing activity was detected in proteins covering a relatively wide range of molecular mass (approx. 20–40 kDa). This breadth of activity was also observed after re-chromatography on Sephacryl S-200, and corresponded to a large histone peak spreading over about 20–40 kDa (Fig. 1b). Histone proteins have a molecular mass of 10–20 kDa/molecule, but it is known that they behave anomalously in gel filtration, exhibiting molecular masses higher by factors of 2–6 than their actual ones [30].

In the next step, SynChropak CM-300 cation-exchange h.p.l.c. (Fig. 1c), bone-inducing activity was detected in the slowest-eluting fraction. This fraction was then applied to C₄ RP h.p.l.c. (Fig. 1d), from which approx. 3 µg of a single active fraction induced slight bone formation (Fig. 2). However, this fraction failed to induce the intense bone formation which had been observed with all of the active fractions obtained in previous steps.

After re-chromatography on the same column, the above active sample was eluted as two overlapping peaks (Fig. 3a, designated Y1 and Y2). N-Terminal amino acid sequencing revealed nine residues in both Y1 and Y2, despite the fact that it was not possible to identify all amino acids (Table 2). This ambiguity was resolved by the sequencing of another portion of sample obtained by the same purification procedure as described above, except that the first RP h.p.l.c. was replaced by hydroxyapatite h.p.l.c. (Fig. 3b). The active fraction from this

Fig. 1. Fig. 1. Purification of BIP

(a) Sephacryl S-200 gel filtration of the active material (153 mg) from the last step of the initial fractionation (2 M-Gdn-HCl-soluble/water-insoluble fraction). The elution positions of BSA (66.2 kDa) and soybean trypsin inhibitor (21.5 kDa) are indicated. Closed and open rectangular bars indicate fractions with and without bone-inducing activity respectively. The fraction marked with an asterisk (tube nos. 86–96; intense bone formation induced by 480 µg) was subjected to re-chromatography. (b) Sephacryl S-200 gel filtration (re-chromatography) of the active fraction obtained in the previous step (47 mg). The fraction marked with an asterisk (tube nos. 55–62) induced intense bone formation (330 µg). (c) Cation-exchange h.p.l.c. on SynChropak CM-300 of the active fraction obtained in the previous step (8.8 mg). Intense bone formation was induced by 231 µg of the active fraction (fraction 5). (d) C₄ RP h.p.l.c. on YMC Packed Column of the active fraction obtained in the previous step (2.1 mg). An approx. 3 µg sample of the active fraction induced slight bone formation. This fraction was subjected to rechromatography on the same column as shown in Fig. 3(a).

Fig. 2. Bone induced by the sample purified from RP h.p.l.c.

Histological appearance (haematoxylin and eosin stain) of a cross-section of a bone which replaced an implant of bovine skin collagen/sample (approx. 3 μ g) from C₄ RP h.p.l.c. magnifications: upper, $\times 22.1$; lower, $\times 55.3$.

Table 2. *N*-Terminal amino acid sequence of purified samples

Peaks Y1 and Y2 from RP h.p.l.c. (Fig. 3a) were subjected to *N*-terminal sequencing, whereas about 64% of the collected Y4 peak (Fig. 3c) was used for sequencing.

Cycle no.	Y1	Y2	Y4
1	?	Ala	Ala
2	?	Ala	Ala
3	?	Leu	Leu
4	?	?	Arg
5	Pro	Pro	Pro
6	Leu	Leu	Leu
7	Val	Val	Val
8	Lys	Lys	Lys
9	Pro	Pro	Pro
10	?	?	?

chromatography was subjected to RP h.p.l.c. (Fig. 3c), and two relatively sharp overlapping protein peaks (corresponding to Y1 or Y2 with respect to the retention time) were obtained (designated Y3 and Y4 respectively). The sample Y4 was used for confirmation of *N*-terminal sequencing, and the sequence was determined completely to the ninth residue (Table 2). The amino acid residues determined at each cycle were the same for all three samples. The sharpness of these protein peaks compared with those of the active fraction in the earlier RP h.p.l.c. (Fig. 1d) might be due to the effective enrichment of the active protein after hydroxyapatite h.p.l.c., implying that Y3 and/or Y4 is the active substance.

A National Biomedical Research Foundation Database search

Fig. 3. Preparation of samples for amino acid sequencing

(a) RP h.p.l.c. (re-chromatography) of the active sample (approx. 9 μ g) obtained in Fig. 1(d). Two overlapping peaks designated Y1 and Y2 were collected separately and subjected to *N*-terminal amino acid sequencing (Table 2). (b) Hydroxyapatite h.p.l.c. on HCA column of the active fraction (2.4 mg) obtained by cation-exchange h.p.l.c. The entire active fraction was subjected to the next chromatography. An *in vivo* assay of each fraction was initially performed using fractions obtained by the prior chromatography of 1 mg of the same material under the same conditions. (c) RP h.p.l.c. of the active fraction obtained in (b). Two overlapping peaks designated Y3 and Y4, corresponding to Y1 and Y2 respectively in (a), were collected separately and subjected to *N*-terminal amino acid sequencing (Y4 only) and preliminary amino acid analysis (both Y3 and Y4).

revealed that the above *N*-terminal sequence completely matches the nine *N*-terminal amino acid residues of mouse, human and rat ribosomal protein L32, except for an additional methionine residue at the *N*-termini of the three latter sequences [31–33]. Moreover, preliminary amino acid analyses of Y3 and Y4 indicated a close similarity in amino acid composition to L32.

These results, together with the failure to induce intense bone formation by the sample from which the above sequence was determined, raised the possibility that the synergistic action of some other substance is required for intense bone formation, or that some other unanalysed substance is the real active entity. On the other hand, it is possible that the above L32-related protein is solely responsible for the bone-inducing activity, but that the amount implanted into mice was not sufficient to induce an intense response. Using the latter as a working hypothesis, an

Fig. 4. Co-distribution of bone-inducing activity and L32-related protein in three types of fractionation

(a) Gdn-HCl (2 M)-soluble/water-insoluble or -soluble fractionation. Active 2 M-Gdn-HCl-soluble material was fractionated into active water-insoluble (lanes 1) and inactive water-soluble (lanes 3) fractions (see Table 1). Both active and inactive material (1 mg of each) together with 1 mg of an active fraction obtained from Sephacryl S-200 gel filtration (lanes 2) were electrophoresed and Western blotted. The filter on the left was hybridized with 10-fold-diluted anti-nonapeptide serum and the filter on the right with control anti-ovalbumin serum (10-fold dilution). The specific 17–18 kDa band is seen only on the left filter in the lanes 1 and 2. Note the more intense band in lane 2 than in lane 1. (b) Cation-exchange h.p.l.c. As shown in Fig. 1(c), bone-inducing activity is eluted after the longest retention time in cation-exchange h.p.l.c. In this experiment, another sample of active material (8.9 mg) was chromatographed under the same conditions, and the same fractionation was performed as in Fig. 1(c). One-quarter of each fraction was Western-hybridized with 50 fold-diluted anti-nonapeptide serum (Antiserum), purified anti-nonapeptide IgG (about 200 μ g/ml) (Anti-IgG), or 25-fold-diluted anti-ovalbumin serum (Contl.). Fraction numbers correspond to those in Fig. 1(c). (c) RP h.p.l.c. About 6 mg of crude active fraction was chromatographed on RP h.p.l.c. under the conditions described in 'Purification procedure' step (v), and an arbitrary fractionation was performed. A total of 29 and 13% of each fraction was used for *in vivo* assay and Western blotting respectively. Hybridization was performed using purified anti-nonapeptide IgG (200 μ g/ml). Bands of approx. 55 kDa (corresponding to IgG heavy chain) and approx. 35 kDa (presumably its degraded product) seen on the Western blot are the result of contamination of rabbit IgG to the material applied to the RP h.p.l.c. and are of no significance to the interpretation of the results. (d) Histological appearance (haematoxylin and eosin stain) of a cross-section of the bone induced by fractions 3 (left) and 4 (right) of (c) (magnification $\times 22.1$). Lines on the right side of Western hybridizations indicate molecular masses from top to bottom: 75, 50, 39, 27 and 17 kDa. SDS/PAGE was performed under reducing conditions.

antibody against the above amino acid sequence was raised and used in the two following experiments.

Co-distribution of bone-inducing activity and of the protein specifically detected by antibody in three types of fractionation

In order to determine whether the L32-related protein and the bone-inducing activity always occurred together in the purification procedure, the three types of fractionations described above were performed again in essentially the same way and the fractionated materials were Western-hybridized to the antibody against the determined nonapeptide.

(i) **2 M-Gdn-HCl-soluble/water-insoluble or -soluble fractionation.** In Western hybridization of these two fractions, anti-nonapeptide serum clearly distinguished the presence and the absence of L32-related protein. As shown in Fig. 4(a), a protein band of 17–18 kDa could be detected in the lane containing

active material, while no corresponding band could be seen in the lane containing inactive material.

(ii) **SynChropak CM-300 cation-exchange h.p.l.c.** A protein band of 17–18 kDa that specifically interacted with antiserum or anti-nonapeptide IgG was observed only in the lane in which an active fraction of this h.p.l.c. was electrophoresed (Fig. 4b).

(iii) **RP h.p.l.c.** Again, a protein band of 17–18 kDa was specifically detected by anti-nonapeptide IgG only in the lanes in which active fractions of this h.p.l.c. were electrophoresed (Fig. 4c). Moreover, a good correlation between the intensity of bone formation and that of the stained band in Western hybridization was demonstrated. Thus, fraction 4 produced a dark band in Western hybridization and intense bone formation in the *in vivo* assay, while fraction 3 produced a faint band and moderate bone formation (Figs. 4c and 4d). Fractions not containing this 17–18 kDa protein failed to induce any bone formation. These

Fig. 5. Purification of BIP using immunoaffinity chromatography

(a) Immunoaffinity chromatography. The column containing anti-nonapeptide IgG/Sepharose 4B incubated with active material (30 mg) was washed with buffer C/0.01% (w/v) SDS (tube nos. 4–21) and subsequently with buffer C only (tube nos. 22–43). The adsorbed proteins were eluted with 5 M-Gdn-HCl/10 mM-sodium citrate buffer, pH 2.5 (tube nos. 44–49; see the Experimental section). (b) RP h.p.l.c. of adsorbed proteins in (a). Half the amount of active material from tube nos. 44–49 in (a) was subjected to C_4 RP h.p.l.c. Protein peaks designated as A1 and A2 were pooled.

Fig. 6. SDS/PAGE of protein peaks A1 and A2

Aliquots of A1 and A2 (5.4 and 4.1% respectively) from Fig. 5(b) were analysed in a 15% (w/v)-separating gel under non-reducing conditions.

results (i–iii) indicate that the purification procedure described earlier indeed enriched an L32-related protein as well as the bone-inducing activity.

Purification of BIP by using anti-nonapeptide IgG

A crude active material was incubated with the anti-nonapeptide IgG coupled to CNBr-activated Sepharose 4B, and adsorbed proteins were further purified by RP h.p.l.c. (Figs. 5a and 5b) as described in the Experimental section. Two protein peaks (designated A1 and A2) were obtained from the RP

Table 3. Amino acid compositions of affinity-purified samples

Peaks A1 and A2 from Fig. 5(b) (17 and 24% respectively) were hydrolysed with 5.7 M-HCl containing 0.2% (v/v) phenol at 110 °C for 24 h. The hydrolysates were analysed using an automatic amino acid analyser. Because of degradation during HCl hydrolysis, the amount of Cys and Trp could not be determined in this experiment. The data for L32 are presented in [31–33]. The *N*-terminal Met is omitted.

Amino acid	A1 (mol %)	A2 (mol %)	Means		L32 (residues/mol)
			(mol %)	(Relative)*	
Lys	11.9	11.0	11.5	15.7	18
His	3.0	3.1	3.1	4.2	4
Arg	13.5	13.0	13.3	18.1	17
Asx	9.1	8.8	9.0	12.3	13
Thr	3.5	3.6	3.6	4.9	3
Ser	5.1	5.0	5.1	7.0	7
Glx	8.7	9.1	8.9	12.1	10
Pro	5.0	4.9	5.0	6.8	6
Gly	5.4	6.1	5.8	7.9	5
Ala	6.5	6.7	6.6	9.0	9
Val	7.1	6.9	7.0	9.5	10
Met	1.6†	1.8	1.7	2.3	3
Ile	5.6	5.4	5.5	7.5	9
Leu	8.1	8.2	8.2	11.2	10
Tyr	2.6	3.2	2.9	4.0	3
Phe	3.2	3.3	3.3	4.5	4
Cys	–‡	+§	+	+	2
Trp	–	–	–	–	1

* Relative to Ala = 9.0.

† Summed value of 0.8% methionine and 0.8% methionine sulphoxide.

‡ Not determined.

§ A small amount of cystine residue was detected.

h.p.l.c., which corresponded to Y1 and Y2 in Fig. 3a, respectively, and gave the same single band of molecular mass 18.7 kDa by SDS/PAGE (Fig. 6). The *N*-terminal amino acid sequence was determined to the fifteenth residue for both A1 and A2. Again, the determined residues (Ala-Ala-Leu-Arg-Pro-Leu-Val-Lys-Pro-Lys-Ile-Val-Lys-Lys-Arg) completely matched those of ribosomal protein L32, except for the additional methionine in the *N*-terminus of the latter [31–33]. No other amino acid residue was detected in any cycle of Edman degradation, indicating that the sample was highly purified. Furthermore, amino acid analysis revealed that A1 and A2 are virtually identical and that they are closely related to L32 (Table 3). Oxidation of methionine residue(s) was presumably the cause of this trivial modification between Y1 and Y2, Y3 and Y4, and A1 and A2.

A mixture of A1 and A2 (13 µg, estimated by amino acid analysis; this amount was achieved by adding another portion of A1 and A2) was implanted into mice and recovered 19 days later. As shown in Fig. 7, the implant was replaced by bone having typical ossicles with active haematopoiesis. This result strongly suggests that a single protein (rather than many indispensable components in the osteosarcoma) is solely responsible for the induction of intense bone formation as is obtained with implantation of crude active material, and that the most probable candidate for BIP is the above L32-related protein.

DISCUSSION

Since it was demonstrated that a tissue lyophilizate of mouse Telmar Dunn osteosarcoma possesses the potential to induce ectopic bone formation [6], attempts have been made to purify

Fig. 7. Bone induction by the sample purified using anti-nonapeptide IgG

Histological appearance (haematoxylin and eosin stain) of a cross-section of a bone which replaced an implant of bovine skin collagen/sample (13 μ g) from C₄ RP h.p.l.c. (Fig. 5b). Magnifications: upper, $\times 22.1$; lower, $\times 55.3$.

the protein responsible for this phenomenon. Thus far a partially purified fraction has been obtained [21].

In the present study, the preparation of a highly purified active sample was achieved using various chromatographic methods together with initial fractionation. *N*-Terminal amino acid sequencing of the sample revealed that the protein has the same nine *N*-terminal amino acid residues as ribosomal protein L32, and a preliminary analysis of amino acid composition also showed close similarity between the two proteins. The use of an antibody specific for the nine-amino-acid peptide proved that the bone-inducing activity and the presence of the identified protein in various fractionations corresponded completely, and that the identified protein is indeed directly responsible for ectopic bone formation. The bone-inducing potential of a murine osteosarcoma has been shown to be lost upon reduction of the active material [22], in good agreement with the presence of cystine residue(s) in L32-related protein (Table 3). Thus the data presented here strongly suggest that the L32-related protein represents the BIP from a murine osteosarcoma.

Several proteins possessing bone-inducing potential derived from bovine bone matrix, and of human origin, have been reported and identified as members of the TGF- β superfamily [12–18]. It is unlikely that the L32-related protein and a TGF- β -type protein were co-purified during the purification procedure in the present study, since their molecular masses (18.7 kDa according to SDS/PAGE and approx. 30 kDa [12] respectively) and especially their pI values [approx. 12 (predicted from the value of L32) and 8.8 [12] respectively] are too different for the two types of protein to behave in the same manner in the various fractionations. This assumption is confirmed by the fact that one of the TGF- β -type proteins (BMP-3) from bovine bone is eluted

at almost the same retention time as histone H2B on RP h.p.l.c. (Dr. Y. Kuboki, personal communication), whereas the L32-related protein is eluted much earlier than histone H2B (results not shown). It remains to be elucidated whether the above protein is L32 itself, a closely related protein arising from post-translational modification for commitment to a bone-inducing role, or from a distinct gene product. Of course, molecular cloning and the demonstration of induction of intense bone formation by a recombinant protein, together with a thorough structural analysis of the purified protein, are necessary to prove that the above protein in fact represents the BIP. However, the biochemical and immunochemical studies described above may provide sufficiently convincing evidence to guarantee the future success of such studies.

It was demonstrated previously that the bone formation elicited by the implantation of active material from a murine osteosarcoma proceeds via cartilage formation by day 7. Ossicle formation takes place by day 14 [34], although this time schedule can be accelerated or delayed depending on the amount of active substance implanted. Moreover, during the present study, a histological picture of a recovered implant was obtained showing that the cartilage formed was just about to be replaced by young bone (results not shown). Thus it is most likely that the intense bone formation elicited by 13 μ g of the highly purified sample proceeded via cartilage formation and subsequent absorption. Thus both L32-type BIP and TGF- β -type BMPs [35] are capable of inducing endochondral bone formation at an ectopic site, despite differences in their molecular structures. A clue for resolving this curiosity resides in an awareness of the different carriers used in the *in vivo* assays.

According to Reddi [36], the extracellular matrix plays a critical role in influencing the behaviour of mesenchymal cells in the developmental cascade of ectopic endochondral bone formation. This assumption led to the demonstration that the original potential of demineralized bone matrix for ectopic bone formation is restored by the reconstruction of its Gdn-HCl-extracted residue and the active fraction comprising proteins smaller than 50 kDa from the Gdn-HCl extract [11]. Since then, this bone matrix has been widely used as the reconstitution material for *in vivo* assays in BIP purification attempts, including those resulting in the identification of TGF- β proteins as the active substance. This may not have been possible in the absence of a reconstitution material containing Gdn-HCl-extracted bone matrix residue. This speculation is supported by the work of Sampath *et al.* [17], who showed that TGF- β -type proteins implanted in the presence of other carrier materials, such as the porous hydroxyapatite disc coated with bovine skin collagen developed by Takaoka *et al.* [24], could not achieve the same degree of bone formation as those implanted with bone matrix residue.

In contrast, ectopic bone formation has been demonstrated to occur in the absence of bone matrix after fractionating the active sample to include proteins smaller than 50 kDa [21], or in the presence of the carrier material developed by Takaoka *et al.* [24], when the starting material for purification was a murine osteosarcoma. Furthermore, in the present study, also using an osteosarcoma as a starting material, the highly purified active protein could induce intense bone formation in the presence of a carrier consisting only of bovine skin collagen (in this study, porous hydroxyapatite was not added as a carrier, in contrast to [24]; thus it did not contain bone matrix components). In other experiments, Urist *et al.* [29] have indicated that a fraction free from bone matrix proteins possesses bone-inducing potential, and composites of bone gelatin, collagen or matrix decreased rather than increased the yield of new bone. Under the assay conditions that excluded bone matrix, these workers

demonstrated that the pI values of the active fractions from bovine bone were 4.9 and 5.3, and that, in addition, an 18.5 kDa gel slice from SDS/PAGE had chondrogenetic activity in tissue culture assay *in vitro* [29,37]. These values are substantially different from those of TGF- β -type proteins which were also purified from bovine bone. How can we explain these discrepant results? If in fact the extracellular matrix exerts a synergistic effect with BIP on the anchorage-dependent behaviour of mesenchymal cells in ectopic bone formation, there would be some differences at the molecular and cellular level between the endochondral bone formation observed in the present study and other experiments in which other carriers were used. From this point of view, it is not surprising that, although the protein isolated as BIP in the present study was structurally different from the ones already identified as TGF- β -type proteins, the effects of the two proteins *in vivo* appear to be the same.

Thus the suggestion that the L32-related protein elicits ectopic bone formation implies that the factors responsible for ectopic bone formation under various biological conditions [1–9] are not represented solely by TGF- β -type proteins. Instead, the bone formation induced by implantation of TGF- β -type proteins may be one example of artificially elicited bone development, which may reflect, for example, fracture healing or bone remodelling in the adult body, where previously formed bone matrix is available. According to this view, it is necessary to determine what is represented by the artificial bone formation induced by implantation of the L32-related protein under normal physiological conditions, and the nature of proteins capable of inducing other ectopic bone formation.

I thank Professor F. Sakiyama, Dr. S. Tsunasawa and Dr. M. Hirado at the Institute for Protein Research, Osaka University, Dr. K. Takaoka at Osaka University Medical School, and M. Puri at Osaka Bioscience Institute for valuable discussions.

REFERENCES

- Huggins, C. B. (1931) *Arch. Surg.* **22**, 377–408
- Urist, M. R. & McLean, F. C. (1952) *J. Bone Jt. Surg. Am. Vol.* **34-A**, 443–476
- Huggins, C. B. & Sammet, J. F. (1933) *J. Exp. Med.* **48**, 393–403
- Bridges, J. B. (1959) *Int. Rev. Cytol.* **8**, 253–278
- Urist, M. R., Dowell, T. A., Hay, P. H. & Strates, B. S. (1968) *Clin. Orthop. Relat. Res.* **59**, 59–96
- Amitani, K., Nakata, Y. & Stevens, J. (1974) *Calcif. Tissue Res.* **16**, 305–313
- Anderson, H. C. & Coulter, P. R. (1967) *J. Cell Biol.* **33**, 165–177
- Wlodarski, K. (1969) *Exp. Cell Res.* **57**, 446–448
- Wlodarski, K., Hinek, A. & Ostrowski, K. (1970) *Calcif. Tissue Res.* **5**, 70–79
- Urist, M. R. (1965) *Science* **150**, 893–899
- Sampath, T. K. & Reddi, A. H. (1981) *Proc. Natl. Acad. Sci. U.S.A.* **78**, 7599–7603
- Wang, E. A., Rosen, V., Cordes, P., Hewick, R. M., Kriz, M. J., Luxenberg, D. P., Sibley, B. S. & Wozney, J. M. (1988) *Proc. Natl. Acad. Sci. U.S.A.* **85**, 9484–9488
- Wozney, J. M., Rosen, V., Celeste, A. J., Mitscock, L. M., Whitters, M. J., Kriz, R. W., Hewick, R. M. & Wang, E. A. (1988) *Science* **242**, 1528–1534
- Wang, E. A., Rosen, V., D'Alessandro, J. S., Bauduy, M., Cordes, P., Harada, T., Israel, D. I., Hewick, R. M., Kerns, K. M., LaPan, P., Luxenberg, D. P., McQuaid, D., Moutsatsos, I. K., Nove, J. & Wozney, J. M. (1990) *Proc. Natl. Acad. Sci. U.S.A.* **87**, 2220–2224
- Celeste, A. J., Iannazzi, J. A., Taylor, R. C., Hewick, R. M., Rosen, V., Wang, E. A. & Wozney, J. M. (1990) *Proc. Natl. Acad. Sci. U.S.A.* **87**, 9843–9847
- Luyten, F. P., Cunningham, N. S., Ma, S., Muthukumar, N., Hammonds, R. G., Nevins, W. B., Wood, W. I. & Reddi, A. H. (1989) *J. Biol. Chem.* **264**, 13377–13380
- Sampath, T. K., Coughlin, J. E., Whetstone, R. M., Banach, D., Corbett, C., Ridge, R. J., Ozkaynak, E., Oppermann, H. & Rueger, D. C. (1990) *J. Biol. Chem.* **265**, 13198–13205
- Ozkaynak, E., Rueger, D. C., Drier, E. A., Corbett, C., Ridge, R. J., Sampath, T. K. & Oppermann, H. (1990) *EMBO J.* **9**, 2085–2093
- Heiple, K. G., Herndon, C. H., Chase, S. W. & Wattleworth, A. (1968) *J. Bone Jt. Surg. Am. Vol.* **50-A**, 311–325
- Takaoka, K., Ono, K., Amitani, K., Kishimoto, R. & Nakata, Y. (1980) *Clin. Orthop. Relat. Res.* **148**, 274–280
- Takaoka, K., Yoshikawa, H., Shimizu, N., Ono, K., Amitani, K., Nakata, Y. & Sakamoto, Y. (1981) *Biomed. Res.* **2**, 466–471
- Yoshikawa, H., Takaoka, K., Shimizu, N., Ono, K., Nakata, Y. & Amitani, K. (1982) *Clin. Orthop. Relat. Res.* **163**, 248–253
- Yoshikawa, H., Takaoka, K., Shimizu, N. & Ono, K. (1984) *Clin. Orthop. Relat. Res.* **182**, 231–235
- Takaoka, K., Nakahara, H., Yoshikawa, H., Masuhara, K., Tsuda, T. & Ono, K. (1988) *Clin. Orthop. Relat. Res.* **234**, 250–254
- Laemmli, U. K. (1970) *Nature (London)* **227**, 680–685
- Lowry, O. H., Rosebrough, N. J., Farr, A. L. & Randall, R. J. (1951) *J. Biol. Chem.* **193**, 265–275
- Kitagawa, T., Ohotani, W., Maeno, Y., Fujiwara, K. & Kimura, Y. (1985) *J. Assoc. Off. Anal. Chem.* **68**, 661–664
- Bio-Rad Laboratories (1988) in *Instruction Manual for Immuno-Blot Assay Kit*
- Urist, M. R., Chang, J. J., Lietze, A., Huo, Y. K., Brownell, A. G. & DeLange, R. J. (1987) *Methods Enzymol.* **146**, 294–312
- Phillips, D. M. P. & Clarke, M. (1970) *J. Chromatogr.* **46**, 320–323
- Dudov, K. P. & Perry, R. P. (1984) *Cell* **37**, 457–468
- Young, J. A. T. & Trowsdale, J. (1985) *Nucleic Acids Res.* **13**, 8883–8891
- Rajchel, A., Chan, Y. & Wool, I. G. (1988) *Nucleic Acids Res.* **16**, 2347
- Nakahara, H., Takaoka, K., Koezuka, M., Sugamoto, K., Tsuda, T. & Ono, K. (1989) *Clin. Orthop. Relat. Res.* **239**, 299–305
- Reddi, A. H. (1981) *Collagen Relat. Res.* **1**, 209–226
- Reddi, A. H. (1984) in *Extracellular Matrix Biochemistry* (Piez, K. A. & Reddi, A. H., eds.), pp. 375–412, Elsevier, New York
- Urist, M. R., Huo, Y. K., Brownell, A. G., Hohl, W. M., Buyske, J., Lietze, A., Tempst, P., Hunkapiller, M. & DeLange, R. J. (1984) *Proc. Natl. Acad. Sci. U.S.A.* **81**, 371–375

Received 24 September 1991/23 December 1991; accepted 17 January 1992