Post-translational processing of chicken bone phosphoproteins

Identification of the bone phosphoproteins of embryonic tibia

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In order to understand the mechanism of the post-translational processing of bone phosphoproteins in embryonic bone, periosteal bone strips isolated from 12-day-embryonic-chick tibiae were cultured and the bone proteins labelled with $\text{Na}_2\text{H}^{32}\text{PO}_4$. Of the total radiolabelled proteins recovered from the medium and bone extracts in the absence of SDS ('medium', 'EDTA extract' and 'EDTA/guanidinium chloride extract'), nearly 80% of the radioactivity was found in the EDTA extract. The three major radiolabelled phosphoproteins in the EDTA extract of apparent M_r 68 000, 63 000 and 58 000 reacted with polyclonal as well as monoclonal antibodies raised against '32-kDa' and '150-kDa' bone phosphoproteins which were derived from 14-week-old chicken. Therefore these phosphorylated embryonic proteins are identified as chicken bone phosphoproteins. Judging from their common *N*-terminal sequences, differences in the patterns obtained by labelling them with several radioisotopes, and slightly different amino acid compositions, these components seem to have been derived from the same original protein by sequential proteolytic cleavage and other processing such as glycosylation and phosphorylation.

INTRODUCTION

The phosphoproteins of avian [1,2] and mammalian bone {sialoprotein I (osteopontin) and II [3–5]} all belong to a class of non-collagenous glycosylated sialoproteins [6]. Because of their high capacity for Ca^{2+} binding [7], their high affinity for collagen [8], their ability to form ternary complexes [9] and their reported secretion site in the vicinity of the mineralization front [10–12], it has been postulated that they may be directly involved in the nucleation and growth of the hydroxyapatite crystals [6,13]. There are as many as 16 bone phosphoprotein components in the diaphyses of 14-week-old chicken metatarsals as described in the previous studies from this laboratory [2,14]. It was suggested that extracellular proteolytic cleavage is largely responsible for the heterogeneity, although at least two genetically independent classes of bone phosphoproteins have been identified in chick [1,2]. Other mechanisms of the age-dependent heterogeneity of phosphoproteins have also been proposed [15].

To understand the mechanisms of post-translational processing of bone phosphoproteins (BPPs) in relation to the heterogeneity, we studied the BPPs from the strips of periosteal bone isolated from 12-day-embryonic-chick tibiotarsus. The BPPs were extracted either directly from dissected bone strips or after isotope incorporation in tissue culture. We expected only a limited extent of extracellular degradation of these proteins in culture medium of neutral pH, since our previous studies have shown that this tissue does not secrete detectable amounts of neutral proteinases in culture [16].

MATERIALS AND METHODS

Materials

DEAE membrane filters (NA 45) were purchased from Schleicher and Schuell (Keene, NH, U.S.A.), and Zeta-Probe membrane filters from Bio-Rad Laboratories (Richmond, CA, U.S.A.). Nylon Plus TM-NYP4-1515 membrane filters were a

gift from Hoefer Scientific Instruments (San Francisco, CA, U.S.A.). Affinity-purified goat anti-[mouse IgG (H+L)]-horseradish peroxidase (HRP) conjugate, HRP substrate (4-chloro-1-naphthol), electrophoresis purity reagents for SDS/PAGE were all purchased from Bio-Rad. Goat anti-[mouse IgA, IgG and IgM (H+L)]-HRP conjugate was purchased from Cappel/Cooper Biochemical (Malvern, PA, U.S.A.). Specially purified SDS (BDH product 44215) was purchased from Gallard Schlesinger Chemical Manufacturing Corp. (Carle Place, NY, U.S.A.). $\text{Na}_2\text{H}^{32}\text{PO}_4$ (1000 mCi/mmol) and D-[1- ^{14}C]glucosamine hydrochloride (50 mCi/mmol) were purchased from ICN Radiochemicals (Irvine, CA, U.S.A.) and L-[2,3- ^3H]arginine (53 Ci/mmol), L-(3,4,5- ^3H (n))leucine (144 Ci/mmol) and L-[4,5- ^3H (n)]lysine (97 Ci/mmol) from NEN du Pont (Boston, MA, U.S.A.). Potato acid phosphatase (P0157) was purchased from Sigma Chemical Co. (Chicago, IL, U.S.A.), and mixed glycosidases from the gastropod *Turbo cornutus* purchased from Miles Laboratories (Naperville, IL, U.S.A.), was used after removal of proteinases by gelatin-Sepharose (Pharmacia, Uppsala, Sweden) chromatography. All other chemicals were the best grade available. Charged poly(vinylidene difluoride) membrane, Immobilon-N, was kindly supplied by Dr. Nancy Legendre, Millipore Corporation, Bedford, MA, U.S.A.

Dissection and culture

Chick tibiae obtained from 12-day embryos were dissected into zones as described previously [16]. Periosteal bone strips were incubated in F-12 medium or minimal essential medium (MEM; GIBCO, Grand Island, NY, U.S.A.) in the presence of antibiotics (GIBCO), with or without 10% (v/v) fetal-calf serum (FCS) (Hyclone Laboratories, Logan, UT, U.S.A.).

Isotope incorporation and fractionation of matrix proteins

Metabolic radiolabelling was carried out for 24 h unless described otherwise. In all, 14 periosteal strips were incubated with 1 mCi of $\text{Na}_2\text{H}^{32}\text{PO}_4$ in F-12 medium (4 ml each) in the

Abbreviations used: HRP, horseradish peroxidase; FCS, fetal-calf serum; PMSF, phenylmethanesulphonyl fluoride; *p*-MB, *p*-hydroxymercuribenzoate; BPPs, bone phosphoproteins; H+L, heavy and light chains; (D)MEM, (Dulbecco's) minimal essential medium; TBS, Tris-buffered saline (for composition and pH, see the text); Caps, 3-(cyclohexylamino)propane-1-sulphonic acid.

presence of antibiotics with or without 10% FCS for 24 h. The final specific radioactivity of $^{32}\text{PO}_4$ was 0.2 mCi/ μmol . After the incubation, the bone matrix was extracted from the washed bone strips, first with 10 ml of 0.5 M-EDTA buffered with 25 mM-Tris/HCl, pH 7.8, containing 2 mM-phenylmethanesulphonyl fluoride (PMSF), 1 mM-*p*-hydroxymercuribenzoate (*p*-MB) and 20 mM-NaF (phosphoprotein phosphatase inhibitor). After being extracted for 24 h at 4 °C on a shaker, residual bone matrix was extracted for another 24 h with 4 M-guanidinium chloride containing 0.5 M-EDTA and proteinase inhibitors, pH 6.5 [17]. These extracts were dialysed against two changes of the dialysis buffer containing 25 mM-Tris/HCl, pH 7.8, 5 mM-EDTA, 1 mM-NaF, 0.5 mM-PMSF and 0.1 mM-*p*-MB. The extracts were freeze-dried, dissolved to 1 ml of water, and redialysed against 1:20 concentration of the above dialysis buffer for 16 h. Samples were freeze-dried again, kept at -80 °C and dissolved in 100 μl of water before use. Residual materials in the bones were solubilized by boiling in Laemmli's SDS sample buffer [18], which was prepared without Bromophenol Blue, for 5 min. ^{32}P radioactivity was quantified by Čerenkov counting in 10 ml of water. Portions of each fraction were used for protein assay with protein assay dye (Bio-Rad) and also subjected to SDS/PAGE to analyse radioactive proteins. In some instances, radiolabelled proteins were extracted with 0.25 M-HCl containing pepstatin A and leupeptin (50 $\mu\text{g}/\text{ml}$ each) before the EDTA extraction. In that case HCl extracts were dialysed against 1 mM-HCl instead of Tris/HCl.

For labelling with ^3H -labelled amino acids, 500 μCi of each radioactive amino acid was added to 10 ml of the medium, which contains only 25% of that particular amino acid as compared with that of the regular DMEM medium. MEM Select-Amine Kit (GIBCO) was used to prepare the media. The specific radioactivity of each amino acid was approx. 600 mCi/mmol. In all, 28 bone strips were incubated for 14 h. In each case of [^{14}C]glucosamine, 125 μCi was added to 10 ml of DMEM containing 1000 mg of glucose/l. Each isotope was tested in two experiments which gave essentially the same results.

Immuno-adsorption

Immuno-complexes were adsorbed to Protein A-Sepharose as follows. A 100 μl portion of serum was mixed with 20 μl of Protein A-Sepharose, which was equilibrated with 20 mM-Tris/HCl, pH 7.4, containing 150 mM-NaCl (TBS). After 1.5 h incubation at 4 °C on a shaker, Sepharose beads were washed with three changes of 1 ml of TBS. A 200 μl portion of TBS solution containing 1 mg of protein/ml of HCl or EDTA extract was incubated with the washed beads at 4 °C on a shaker. After the 1.5 h incubation, the Sepharose beads were spun down, and the pellet was washed with five changes of 1 ml of TBS. Immunoprecipitated proteins were solubilized either directly or after incubation with acid phosphatase by boiling for 2 min in SDS sample buffer and revealed by autoradiography after SDS/PAGE.

SDS/PAGE

Linear 5–15%-(w/v)-polyacrylamide gradient gels (17 cm $\times$ 11 cm) were used for electroblotting to recover samples in the Laemmli buffer systems [18]. For analytical purposes, 8- or 10%-polyacrylamide Minigels (8 cm $\times$ 6 cm) were used. The gels were stained with either Coomassie Brilliant Blue R-250 or Stains-All.

Although it is reported that a 15% gel is better than a gradient gel for the estimation of M_r of these phosphoproteins [3], we used gradient gels throughout our studies for two reasons: first, we cannot reveal high- M_r proteins in a 15% gel and, secondly, a 15% gel is not suitable for the electroelution which

is essential to the immunochemical analysis. Therefore we describe the apparent M_r of phosphoproteins on the basis of their migration in a gradient gel (e.g. M_r , 68000). The reported molecular mass of a BPP is expressed, for example as '32-kDa BPP'.

Electroblotting

On completion of electrophoresis, gels were washed for 30 min in Tris/HCl, pH 7.8, containing 0.1% SDS at room temperature. The buffer used for blotting was 50 mM-Tris/HCl, pH 7.8, containing 0.2% Nonidet P40 and 5 mM-EDTA, which was also used to equilibrate NA45 DEAE membranes for 15 min. By using an electroblotting unit from Hoefer Scientific Instruments (San Francisco, CA, U.S.A.), proteins were transferred either at 0.6 A for 4 h or at 1.0 A for 2 h at 7–10 °C. Prestained standard proteins (Bethesda Research Laboratories, Bethesda, MD, U.S.A.) were used as references. Molecular sizes of prestained standard proteins, which are different from their original sizes, were determined by comparing them with those of standard proteins from Bio-Rad. Electroblotting with nitrocellulose and charged nylon membranes was performed under the conditions described by Towbin *et al.* [19].

N-Terminal sequencing of chicken bone phosphoprotein components on charged PVDF membrane

BPP extracts were electrophoresed in SDS/8%-polyacrylamide gel in the presence of 10 mM-thioglycolic acid and washed in two changes of blotting buffer {10 mM-Caps [3-(cyclohexylamino)propane-1-sulphonic acid] buffer, pH 10.0, containing 0.2 mM-6-aminoheptanoic acid}. The extracts were then transferred to charged PVDF membranes, which were wetted with methanol and equilibrated with the blotting buffer for 15–30 min. After 30 min of transfer at 7 °C and at 0.3 A, which completes the transfer of prestained standard proteins on to the membrane, the blot was washed in three changes of water for 5 min each and air-dried. BPPs were detected by ^{32}P autoradiography and/or by staining the blot with 0.2% Indian ink. Corresponding bands measuring 1–2 mm $\times$ 4 mm were cut out and sequenced by the reported method for PVDF membrane (Immobilon-P) [20]. Automated Edman degradation of proteins was performed on an Applied Biosystems 470A protein sequencer with a model 120A h.p.l.c. system for the analysis of amino acid phenylthiohydantoin derivatives.

Identification of phosphoamino acids

The phosphoamino acids in the blotted BPP components were analysed by a modification of the method of Hunter & Sefton [21]. ^{32}P -labelled bands were located on the NA45 blot by autoradiography, cut out, then washed in 0.1 M-sodium acetate, pH 6.0, and recovered from the membrane with formamide/high-salt buffer [55% (v/v) formamide/1.8 M-sodium acetate/2 mM-EDTA/0.2% SDS, pH 7.4]. After extensive dialyses against 0.1% CHAPS (Calbiochem) and water, samples were freeze-dried with BSA for hydrolysis. The above elution buffer was prepared according to the manufacturer's application notes. The acid hydrolysis was carried out for 2, 4, 6 and 8 h at 105 °C in 850 μl of 4 M-HCl with 0.3 mg of BSA/ml. The samples were then dried, redissolved in water and dried again to remove residual HCl. The residues were mixed with 3 μl of water containing (1 mg/ml each of) phosphoserine, phosphothreonine and phosphotyrosine and applied on to wet cellulose thin-layer plates (20 cm $\times$ 25 cm). Electrophoresis was performed on a flat-bed electrophoresis apparatus (Multiphor II; LKB Produkter A.B., Bromma, Sweden) at 750 V for 1.5 h at 15 °C in a pyridine/acetic acid/water (1:10:89, by vol.) buffer, containing Xylene Cyanol tracking dye. Radioactive phosphoamino acids

were detected by autoradiography and the standard phospho-amino acids with ninhydrin. They were measured by scraping off each ninhydrin-positive spot, followed by counting radioactivity in Hydrofluor and by densitometry of autoradiographs using an UltroScan XL densitometer (LKB Produkter).

Microanalysis of amino acid composition of BPP components

BPP components were recovered from NA45 transfers as described in the above subsection, hydrolysed in constant-boiling HCl and then converted into amino acid phenylthiohydantoin derivatives, which were analysed by using an Applied Biosystem 420A Derivatizer and a 130A Separation System.

Immunological detection of transferred proteins

NA45 transfers were soaked in the blocking solution in 50 mM-Tris/HCl, pH 7.5, containing 3% BSA (Sigma A7906), 10% horse serum (GIBCO) and 0.2% (we now use 0.1%) Tween 20 (EIA purity; Bio-Rad) for 30 min to saturate non-specific protein-binding sites. The transfers were incubated overnight at 4 °C in the primary-antibody solution (Protein A–Sepharose-purified monoclonal IgG at 4 µg/ml or ascites at 1:500 dilution in the above blocking solution). The following morning, the sheets were washed in 50 mM-Tris/HCl, pH 7.5, containing 0.2% Tween 20 (five changes, for 5 min each on a shaker), and incubated with the secondary antibody for 1.5–2 h at room temperature. Appropriate goat anti-(mouse IgG)–HRP conjugate was used at 1:1000 dilution in the above blocking solution. For the colour reaction, the blots were washed in the above Tris/Tween solution and soaked in a solution of 4-chloro-1-naphthol at 0.5 mg/ml [22]. When the blue colour appeared, the blots were washed and dried. Autoradiograms were produced using Kodak X-Omat AR film.

RESULTS

$^{32}\text{PO}_4$ incorporation into bone matrix proteins

The results of three experiments showed that $^{32}\text{PO}_4$ incorporation into the macromolecular components of the matrix (EDTA and EDTA/guanidinium chloride extract) and medium of 14 periosteal bone strips during a 24 h period was $(211 \pm 38) \times 10^3$ d.p.m. in the presence of 10% FCS and $(130 \pm 20) \times 10^3$ d.p.m. in the absence of 10% FCS. In the presence of FCS, $69 \pm 14\%$ of the incorporated $^{32}\text{PO}_4$ radioactivity (312 ± 10 µg) was recovered from EDTA extract, whereas $16 \pm 2\%$ (109 ± 28 µg) and $15 \pm 1\%$ were recovered from the EDTA/guanidinium chloride extract and medium. In the absence of FCS, $80 \pm 14\%$ (316 ± 29 µg), $10 \pm 1\%$ (87 ± 22 µg) and $10 \pm 1\%$ (95 ± 22 µg) were recovered from each fraction respectively (values in parentheses represent the amount of protein recovered in each fraction). Further extraction of the bone strips by boiling them in 1% SDS yielded the matrix components containing $(439 \pm 26) \times 10^3$ d.p.m. (480 ± 22 µg) in the presence of FCS and $(220 \pm 22) \times 10^3$ d.p.m. (452 ± 44 µg) in the absence of 10% FCS. Phosphate incorporation was least affected in the EDTA extract by removing FCS (28% less), the other fractions showing greater decreases (50–63%). In the presence of 10% FCS, $^{32}\text{PO}_4$ incorporation into the matrix proteins of the periosteal bone strips was monitored during the 44 h incubation in F-12 medium (Fig. 1). Under the conditions described in the Materials and methods section, the incorporation was linear up to approx. 30 h in all three fractions. Labelled materials in the EDTA extract were analysed by SDS/5–15% gradient PAGE (Fig. 1, inset). There was essentially no difference in the pattern among the samples of different incubation times. All the components increase with time. In the EDTA extract, incor-

Fig. 1. Time course of [^{32}P]phosphate incorporation into the bone matrix proteins

Periosteal bone strips dissected from 12-day-chick embryos were labelled with $\text{Na}_2\text{H}^{32}\text{PO}_4$ during 44 h of incubation in F-12 medium in the presence of 10% FCS. [^{32}P]Phosphate incorporation into the three sequential extracts [EDTA (○), guanidinium chloride/EDTA (△) and SDS (□)] were measured under the conditions described in the Materials and methods section. In the inset, samples containing approx. 5000–10000 d.p.m. of $^{32}\text{PO}_4$ were drawn from several time points (1–30 h as indicated) and analysed by SDS/5–15%-gradient PAGE.

Fig. 2. Identification of BPPs in the EDTA extract of embryonic tibia periosteal bones

$^{32}\text{PO}_4$ -labelled proteins in the EDTA extract were examined. Each blot of approx. 30 μg of sample was treated with 60k4D7 (a), /32kIE2 (b) or 150kIE4 (c) antibody, and immunostained blots (marked 'Ab') and their autoradiographs (marked ' ^{32}P ') are shown. Two arrows indicate the position of prestained standard proteins: BSA (upper arrow; M_r 72000) and ovalbumin (lower arrow, M_r 50000). Prestained standard proteins, which were run next to the EDTA extract on the left in (b) and on the right in (a) and (c) included myosin heavy chain (M_r 214000), phosphorylase *b* (110000), BSA, ovalbumin, carbonic anhydrase (24000), β -lactalbumin (18000) and lysozyme (15000).

poration was mostly into the proteins of apparent M_r 68000, 63000, 58000 and 54000. But, depending on the experiment, a protein of M_r 54000 may or may not be seen. In the EDTA/guanidinium chloride extract, only lower- M_r ^{32}P -labelled proteins ($M_r < 35000$) were recovered (results not shown). The 'medium' fraction exhibited some incorporation into proteins similar to the M_r -68000 and -63000 ones (results not shown). Patterns of radioactive proteins were essentially the same in the presence or absence of FCS. In order to avoid contamination by serum proteins, we therefore prepared bone phosphoproteins for analytical purposes in the absence of FCS.

Immunological detection of embryonic BPPs

To identify $^{32}\text{PO}_4$ -labelled proteins as BPPs, monoclonal antibodies (32kIE2, 150kIE4 and 60k4D7) which were raised against 14-week-old chicken '32-kDa', '150-kDa' and '60-kDa' BPPs [1] were used to immunostain a Western blot on NA45 membrane as described in the Materials and methods section. The transfer of the EDTA extract of the $^{32}\text{PO}_4$ -labelled bones (approx. 30 μg of protein) showed three [$^{32}\text{PO}_4$]phosphoprotein bands of M_r 68000, 63000 and 58000, which were stained by using 32kIE2 and 150kIE4 antibodies (Fig. 2b and 2c). In addition, two apparently non-phosphorylated protein bands of approx. M_r 180000 and 160000 were also immunostained, but the phosphoproteins of the low M_r (< 40000) were not. Staining of these three bands (M_r 68–58000) was intensified when the extracts were treated with mixed glycosidase (results not shown). Immunostaining of the embryonic BPPs with 150kIE4 antibody was similar to that with 32kIE2 antibody, except that high- M_r proteins of undetectable radioactivity also reacted with this antibody (Fig. 2b). Monoclonal antibody 60k4D7, which binds to another type of less-phosphorylated BPP found in 14-week-old chicken [1,2], was also used to immunostain the EDTA extract (Fig. 2a). Although there is a protein of M_r 50000 which binds to the antibody, it seems that the protein does not have a detectable amount of phosphorylated amino acid residues.

Fig. 3. Comparative labelling pattern of BPP components by [^3H]arginine, [^3H]leucine, [^3H]lysine, [^{14}C]glucosamine and $^{32}\text{PO}_4$

Bone proteins were labelled under the conditions described in the Materials and methods section. In expts. 2 and 3, $\text{Na}_2\text{H}^{32}\text{PO}_4$ was added to the medium used for ^{14}C and ^3H labelling. Before the usual EDTA extraction, washed bone strips were treated with 0.25 M-HCl (25 ml/40 bones) in the presence of 50 μg of pepstatin A/ml for 24 h at 4 $^\circ\text{C}$. Residual proteins were extracted with 0.5 M-EDTA. Combined results from three duplicate experiments (panels 1–3) are shown. A, L and K on top represent arginine, leucine and lysine respectively; ^{32}P and ^{14}C refer to $^{32}\text{PO}_4$ and [^{14}C]glucosamine labelling respectively; H and E represent HCl and EDTA extracts respectively. The three arrowheads ($\blacktriangleleft$) indicate the positions of the three major BPP components. Arrows ($\blacktriangleleft$) indicate the positions of the same prestained standard proteins as those described in Fig. 2.

Nitrocellulose is capable of binding only 2.5 μg of these substances/ cm^2 , which is less than 10% of the materials bound to NA45 and is also inferior in retention of bound material during incubation with antibodies. Another problem is that proteins of different charges, hydrophobicity and M_r transfer and then go through the membrane at different speeds. Charged nylon membranes such as Zeta-Probe or Nylon Plus were comparable with NA45 in binding capacity and retention, but do not have the advantage of sample recovery. Although the combination of biotinylated secondary antibodies, HRP-conjugated avidin and the substrate was tried, staining did not improve because of the higher background.

Comparative labelling pattern of BPP components by [^3H]arginine, [^3H]leucine, [^3H]lysine, [^{14}C]glucosamine and $^{32}\text{PO}_4$

To characterize the BPP components, bone proteins were labelled with three different radioactive amino acids and a radioactive hexosamine; their SDS/PAGE patterns were then compared with that of $^{32}\text{PO}_4$ (Fig. 3). Bone matrix proteins were sequentially extracted with 0.25 M-HCl and 0.5 M-EDTA to compare the differences in extraction. The acid-extraction step [2] was added here, since it had become apparent that there was a qualitative difference in the extracts as shown in Fig. 3. The M_r -58000 component was not completely extracted by HCl under the conditions employed, whereas the other two components were completely removed from the bone by HCl. The results also show that the components are labelled to different extents by the labelled precursors; the smallest component of M_r 58000 was more efficiently labelled by leucine than by lysine or arginine, and showed negligible glucosamine incorporation. The extent of labelling among the components showed the highest contrast in a leucine-labelled sample.

Immunoprecipitation of radiolabelled BPP

Antiserum against '32-kDa' BPP [1] specifically precipitated M_r -68000, -63000 and -58000 proteins from the HCl extract (cf. Figs. 4 and 3). It is obvious that M_r -58000 component is not significantly glycosylated. The right-hand panel in Fig. 4 shows

Fig. 4. Identification of radiolabelled BPPs

Lanes A–C, immunoprecipitation of $^{32}\text{PO}_4^-$ and $[^{14}\text{C}]$ glucosamine-labelled BPPs. Radioactive proteins in HCl extract similar to those described in Fig. 3 were immunoadsorbed to Protein A–Sepharose by using antiserum against '32-kDa' BPP [1]. Lanes A and A' represent $^{32}\text{PO}_4^-$ -labelled BPPs, and lanes B and C represent ^{14}C -labelled BPPs. Lanes A, A' and C were precipitated with antiserum and lane B with preimmune serum. Lane A' contains five times as much sample as in lane A. Lanes D–E, immunoprecipitation of $[^3\text{H}]$ leucine- and $[^3\text{H}]$ lysine-labelled BPPs. The same procedure as in the left-hand panel was used for lane D ($[^3\text{H}]$ leucine) and lane E ($[^3\text{H}]$ lysine). Lane F shows immunoadsorbed $[^3\text{H}]$ lysine-labelled BPPs after acid phosphatase treatment.

Fig. 5. Identification of $[^{32}\text{P}]$ phosphoserine and $[^{32}\text{P}]$ phosphothreonine

The radiolabelled proteins were recovered from transfers, hydrolysed and electrophoresed for the identification under the conditions described in the Materials and methods section. The time course of the hydrolysis of the two major bone $^{32}\text{PO}_4^-$ -labelled phosphoproteins was plotted every 2 h during an 8 h period. ●, Free $^{32}\text{PO}_4^-$; ■, $[^{32}\text{P}]$ phosphoserine; ▲, $[^{32}\text{P}]$ phosphothreonine.

that the M_r values for the immunoadsorbed BPPs decrease after incubation with acid phosphatase. However, their relative mobility does not seem to change, suggesting that the difference is not only due to the extent of phosphorylation.

Identification of $[^{32}\text{P}]$ phosphoserine and $[^{32}\text{P}]$ phosphothreonine

Fig. 5 shows the time course of hydrolysis of two smaller $[^{32}\text{P}]$ phosphoprotein components of M_r 63000 and 58000, which were eluted from the NA45 blot. In these proteins phosphoserine residues are generated very quickly and are unstable. On the other hand, phosphothreonine residues are generated more slowly and the half-life seems to be much longer. Almost 70 % of

the total radioactivity was recovered as free $^{32}\text{PO}_4^-$ after 8 h of hydrolysis. In both proteins, the estimated ratio of phosphoserine to phosphothreonine is approx. 3:1, which is in agreement with the reported ratio in chicken BPPs [2,23]. Phosphotyrosine was not detected in either protein.

Amino acid composition of the major BPPs

Eluted phosphoproteins were also subjected to amino acid analysis. The results are summarized in Table 1. Compositions of the three components differ slightly from each other. The ratios of lysine/leucine and arginine/leucine are 16 % lower in the BPP component of M_r 58000 compared with the component of M_r 68000.

N-Terminal sequence of the major BPPs

To overcome limited availability of samples, amino acid sequencing was attempted by using a charged PVDF membrane, Immobilon-N. For the same reason that we use DEAE membrane rather than nitrocellulose, Immobilon-N turned out to be a better support than PVDF membrane, Immobilon-P, for the binding of phosphoproteins. Approx. 1 μg (~ 20 pmol) of $[^{32}\text{P}]$ BPP was bound on the 1 mm $\times$ 4 mm area of the membrane, which was excised and subjected to analysis. The N-terminal sequence obtained:

Ala?-Pro-Val-Ser-Lys

is common in all three components at the level of recovery between 8.6 and 0.8 pmol and is different from the phospho/sialoproteins of mammalian bone [5,24–26]. (In proteins prepared from diaphyses of 14-week-old-chicken metatarsals or from osteoblast cultures derived from embryonic-chick calvaria, there are phosphoproteins of similar sizes ($M_r \sim 55000$ – 68000) [34] which bind to the monoclonal antibodies used in the present study. The N-terminal amino acid sequence of the equivalent bone phosphoproteins from chickens of different ages were found to be identical at least from the second to the fifth amino acid:

Xaa-Pro-Val-Ser-Lys

(Y. Gotoh, L. Gerstenfeld, Y. Mikuni-Takagaki & M. J. Glimcher, unpublished work.)

Unphosphorylated protein of $\sim M_r$ 50000, which reacted with 4D7 antibody [1] to less phosphorylated adult BPP [2], was also subjected to sequence analysis. The N-terminal sequence obtained:

Ala-Pro-Ala-Ala-Pro-Pro-Pro-Leu-Gly-Xaa-Asp-Asp-Pro-Glu

is identical with that of the adult BPP reported previously [2].

DISCUSSION

In mineralized tissues there is a group of proteins which are highly phosphorylated. These phosphoproteins of bone, cementum, calcified cartilage and calcified tendon are phosphorylated via their serine and threonine residues [27–30], whereas only serine is phosphorylated in dentine [31,32] and enamel [33]. In chicken bone, the ratio of phosphoserine to phosphothreonine is approx. 3:1 [2,23]. Rat and bovine sialoprotein I (osteopontin) and sialoprotein II, which have Arg-Gly-Asp ('RGD') cell-binding sequences, were also found to contain both phosphoserine and phosphothreonine [3,4,27]. The reported amino acid and sugar compositions of chicken bone phosphoprotein components [2] are similar to those of the above rat and bovine sialoproteins. The data strongly suggest that all of these bone matrix proteins represent the same class of glycosylated phosphoproteins (or phosphorylated sialoproteins).

It was reported from this laboratory that the 14-week-old-chicken BPPs can contain as many as 16 diverse components [2], a far higher number than that of the BPP components we found in the present study of embryonic bone. In the embryonic bone we found only three major ^{32}P -labelled proteins, all of which belong to only one of the immunochemically distinct two groups of phosphoproteins found in 14-week-old-chicken bone [1,2]. The results of isotope incorporation, amino acid analysis and enzyme treatment suggest that proteolytic processing is at least in part involved in the production of the three components. Neither the result shown in Fig. 1 in a 30 h incubation nor the immunostaining pattern of the bone extract in Fig. 2 indicates that there is any cumulative increase of any particular component among these three major BPP components. Also, the presence of proteinase inhibitors such as leupeptin, chymostatin, antipain and pepstatin at 100 $\mu\text{g}/\text{ml}$ in culture media did not affect the pattern of radioactive components (results not shown). Therefore this heterogeneity seems to derive from the intracellular processing. The fact that two different extractants (0.25 M-HCl in the presence of pepstatin and leupeptin, and 0.5 M-EDTA at neutral pH in the presence of *p*-MB and PMSF) resulted in a similar proportion of BPP components suggests that the diversity is not derived from the proteolytic cleavages during the extraction. However, whether the cleavage actually occurs intracellularly before and/or during the phosphorylation or, possibly, at a later stage, has yet to be determined. In the bone matrix which was metabolically radiolabelled with isotopes, sequential extraction with 0.25 M-HCl and 0.5 M-EDTA showed that only the lowest- M_r component failed to be completely solubilized with HCl (Fig. 3). Although all three components share the same *N*-terminal sequence, the M_r -58000 component is less (or not) glycosylated, shows lower arginine/leucine and lysine/leucine ratios (Fig. 3) and has a lower concentration of basic amino acid residues (Table 1). This component may be more firmly bound to the mineralized matrix than others because of these chemical differences. Assuming that these BPP components originate from the same translation product, it is conceivable that the loss of peptides rich in basic amino acid residues contributes to the heterogeneity. It should be noted that casein kinase II binds to basic peptides such as polylysine and is activated significantly (see the following paper [35]). It was shown that most of the lysine and arginine residues are clustered in the *C*-terminal half of the osteopontin molecules [24]. If the abovementioned variation derives only from glycosylation and phosphorylation, without the involvement of proteolytic cleavage, the ratio of [^3H]lysine (or [^3H]arginine) to [^3H]leucine should be consistent among the components. Moreover, the treatment with phosphatase and/or glycosidase would have resulted in a conversion of certain components into others. However, this does not seem to be the case. We have shown that the high- M_r embryonic-bone proteins of M_r 160000 and 180000, which do not have detectable amounts of $^{32}\text{PO}_4$, share epitopes with the major chicken BPPs (Fig. 2), suggesting the possibility that they have certain common amino acid sequences. In 14-week-old-chicken bone, there is an equivalent component of a similar size namely '150-kDa' BPP, which was found to contain only a small amount of phosphoserine [1,2]. We may speculate that these high- M_r proteins may be related to the low- M_r chicken BPPs and that certain serine and threonine residues that are extensively phosphorylated in the low- M_r BPPs may be sterically hindered from the protein kinase in the high- M_r proteins. Possibly only the molecules proteolytically processed to certain sizes may be fully phosphorylated and function as components of the mineralizing matrix. Further degradation of these components may occur in a more mature bone matrix, contributing to the diversity of BPPs which was observed in 14-week-old chicken [2,15]. There is also another

group of less phosphorylated proteins in a 14-week-old chicken bone [2] which have a unique *N*-terminal sequence:

Ala-Pro-Ala-Ala-Pro-Pro-Leu-Gly-Ser-Asp-Asp-Pro-Glu

A protein of $M_r \sim 50000$ was identified in the embryonic-bone matrix by immunostaining with 60k4D7 monoclonal antibody to this group of proteins (Fig. 2a). The embryonic protein has the same *N*-terminal sequence, but is not phosphorylated as far as we can detect by ^{32}P radioactivity. No ^{32}P -labelled proteins were immunochemically identified as BPP components in this group (Fig. 2).

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