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Isoforms of Bone Alkaline Phosphatase: Characterization and Origin in Human Trabecular and Cortical Bone

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ABSTRACT

Alkaline phosphatase (ALP) is a glycoprotein and functions as an ectoenzyme attached to the cell membrane by a hydrophobic glycosyl-phosphatidylinositol (GPI) anchor. Three bone ALP (BALP) isoforms in human serum were separated and quantitated by high-performance liquid chromatography. B/I, a minor fraction, is composed on average of bone (70%) and intestinal (30%) ALP, and two major isoforms, B1 and B2. Treatment with GPI-specific phospholipase C (GPI-PLC) did not influence the activities or retention times for B1 and B2, indicating that the biochemical differences between B1 and B2 are likely to be due to different glycosylation patterns. The B/I fraction in serum, on average 4% of total ALP, was found to be composed of B1 and B2 isoforms, each with an intact hydrophobic GPI cell membrane anchor. We investigated the origin of these three BALP isoforms and osteocalcin in human femora from five healthy individuals (four males), mean age 51 years, obtained from a tissue bank. Bone was sampled from three sites: cortical bone, trabecular bone from the diaphysis, and trabecular bone from the greater trochanter. Trabecular bone, from both sites, had higher BALP activities compared with cortical bone. Conversely, the osteocalcin content of cortical bone was more than 3-fold greater than that of trabecular bone. Cortical bone had approximately 2-fold higher activity of B1 compared with B2, whereas trabecular bone had ~2-fold higher activity of B2 compared with B1. We observed a previously undescribed BALP isoform (B1x) in all bone samples. B1x was also observed in sera from some patients (60%) with severe renal insufficiency and on chronic dialysis therapy (*n* = 20). The isoforms of BALP may provide information relating to bone metabolism within specific bone compartments. (J Bone Miner Res 1999;14:1926–1933)

INTRODUCTION

ALKALINE PHOSPHATASE (ALP; EC 3.1.3.1) is the most frequently used biochemical marker of osteoblastic bone formation. In humans, four gene loci encode for the ALP isoenzymes: "tissue nonspecific," placental, germ cell, and the intestinal locus.[1] ALP from the "tissue nonspecific" locus is expressed in many tissues including bone and liver, which in healthy adults constitute ~95% of the total ALP activity found in serum with a ratio of ~1:1.[2,3] Because bone ALP (BALP) and liver ALP (LALP) are encoded by the same gene locus, they are referred to as isoforms of the same isoenzyme. Different carbohydrate side chains or maybe remaining fragments of the in situ cell membrane glycosyl-phosphatidylinositol (GPI) anchor, or both, yield "tissue-specific" structures in the ALP isoforms from this gene locus.[4–6] Currently, no consensus exists regarding these possible structural differences between the BALP isoforms. Explanations incorporating differences in both glycosylation and remaining GPI anchor fragments have been cited, often independently of each other.[7,8] In

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this study, we have used a previously described weak anion-exchange high-performance liquid chromatography (HPLC) method which can resolve six different ALP isoforms in serum from healthy adults: three BALP (B/I, B1, and B2), and three LALP (L1, L2, and L3) isoforms.[9,10] Selective differences between these BALP isoforms have previously been described during the pubertal growth spurt,[11] and in disease states such as growth hormone deficiency,[2] hypophosphatasia, hypophosphatemic vitamin D–resistant rickets, Paget's disease, stress fractures,[9,12] and metastatic bone disease.[13]

The human skeleton is a heterogeneous organ with a wide morphologic, functional, and metabolic variety. Cortical bone mainly fulfills mechanical and protective functions while trabecular bone, in addition to mechanical support, provides metabolic functions including hematopoiesis and calcium homeostasis.[14] Cortical bone constitutes ~75% of the skeletal volume and mass, but only ~25% of the surface area.[15] Bone turnover, which is higher in trabecular compared with cortical bone, refers to volume replacement and depends on both the surface-defined activation frequency and on the surface-to-volume ratio. The difference in turnover rates between trabecular and cortical bone depends much on the difference in surface-to-volume ratio, which is about four times higher in typical trabecular bone than in cortical bone.[16] Despite these known differences, reports of biochemical markers of bone turnover in relation to skeletal heterogeneity are few. Bone markers are thus far not able to distinguish between metabolic events in trabecular and cortical bone compartments,[17–19] although differences in composition have been reported.[20]

This study was designed to characterize and investigate the origin within human bone of the three BALP isoforms previously reported to be found in serum samples of healthy children and adults.[2,11] In addition, we also present data on the osteocalcin content of the various bone fractions, and of BALP isoforms in patients with severe renal insufficiency undergoing dialysis therapy. The results are evaluated and discussed in relation to recent findings of selective differences between the BALP isoforms in other disorders of bone and mineral metabolism.[2,9,12,13,21]

MATERIALS AND METHODS

Bone tissues and serum samples

Human bone tissues were obtained from a tissue bank and comprised femora from five healthy individuals, four males and one female, mean age 51 years (range 20–66 years). All bone samples were harvested within 24 h after death and stored at –70°C. None of the tissue donors had a medical history of any bone pathology or metabolic disorder, nor had they received drugs known to affect bone metabolism. The control group for serum BALP was composed of 123 healthy adult blood donors, 45 males and 78 females, mean age 39 years (range 21–65 years). The study group with severe renal insufficiency and on chronic dialysis therapy was composed of 20 adults, 9 males and 11 females, mean age 63 years (range 33–87 years). This study was conducted in accordance with the Declaration of Helsinki and approved by the Ethical Committee of Linköping University Hospital, Sweden.

To examine a possible GPI-specific phospholipase C (GPI-PLC; EC 3.1.4.10) resistance due to inositol-palmitoylation[22] of BALP, we investigated a serum sample from a 72-year-old female diabetic patient with slightly increased total ALP, 5.7 μkat/l (upper reference limit 4.6 μkat/l). This serum sample was chosen because agarose gel electrophoresis with Triton X-100 had indicated an ALP pattern similar to that obtained with tissue homogenates,[23] that is, most likely a sample with ALP attached to an intact GPI cell membrane anchor. A limited sample volume excluded a more thorough characterization of this particular sample. Six months after we received this sample, a renal tumor was located in this patients' right kidney, in addition to some liver metastasis, and the patient died shortly thereafter.

To rule out a possible kidney ALP fraction[24,25] or intestinal ALP type from human kidney,[26] a serum sample from a 65-year-old bilaterally nephrectomized patient was analyzed.

Preparation of bone samples

Femora were cleaned of adhering soft tissues and sawn into sections. Bone marrow was removed by water jetting. Diaphyses were split lengthwise and trabecular bone was scraped from the endosteal surface, while strips of cortical bone were removed from the periosteal surface. Cubes of trabecular bone were cut from the greater trochanter with a band saw. Bone was sampled from three sites within each femora: cortical bone from the diaphysis, and two samples of trabecular bone taken from the endosteal surface of the diaphysis and the greater trochanter regions of the proximal femora. The bone samples were initially washed twice for 30 minutes with cold trichloromethylene in an ultrasonic bath, before being cut into small pieces and powdered under liquid nitrogen using an impaction freezer mill (Spex 6700; Spex Industries Inc., Edison, NJ, U.S.A.). Bone powders were washed in cold acetone for 15 minutes, centrifuged, and the sediments lyophilized.

Biochemical determinations

The BALP isoforms, B/I, B1, and B2, were determined by a previously described HPLC method.[9,10] The B/I peak is a minor fraction in serum composed on average of BALP (70%) and intestinal ALP (30%).[9] A weak anion-exchange column, SynChropak AP300 (250 × 4.6 mm internal diameter) (Micra Scientific, Inc., Lafayette, IN, U.S.A.) was used in place of the previously referred SynChropak AX300. The SynChropak AP300 is a modified SynChropak AX300, optimized for BALP isoform analysis.[2] For the electrophoretic separation of BALP and LALP isoforms, agarose gel with Triton X-100 was used as supporting media as reported elsewhere.[23] Serum total ALP activity was measured on a Hitachi 917 analyzer (Boehringer Mannheim GmbH, Mannheim, Germany) at

37°C.[27] The relationship between the enzyme activity units kat and U is 1/60, i.e., 1.0 μkat/l corresponds to 60 U/l. All homogenized bone and serum samples were filtered through low protein-binding Cameo 25 A AcetatePlus filters, pore size 0.45 μm (Micron Separations, Inc., Westborough, MA, U.S.A.).

Prior to HPLC analysis of BALP isoforms, 230 μl of serum was treated with 230 μl of 25 U/ml GPI-PLC (EC 3.1.4.10, from *Bacillus cereus*; Sigma Chemical Co., St. Louis, MO, U.S.A.), at pH 7.4, 37°C, for 2 h. The powdered bone samples (20 mg dry weight) were treated with 230 μl of isotonic saline and 230 μl of 25 U/ml GPI-PLC, at pH 7.4, 37°C, for 2 h.

To verify that the observed peaks were of BALP origin, several different approaches were used, including heat inactivation at 56°C for 15 minutes,[9,28] inhibition by 10 mmol/l L-phenylalanine (Sigma),[9,29] desialylation by neuraminidase (EC 3.2.1.18, from *Clostridium perfringens*; Sigma), incubated at 37°C for 30 minutes with a final concentration of 400 U/l,[30] and treatment with a monoclonal antibody against human intestinal ALP, 1.5 mg/l (Monosan MIG-I15; Sanbio bv, 5400 AM Uden, The Netherlands), incubated overnight at room temperature with a final concentration of 1.0 mg/l.[31]

Osteocalcin was extracted from the powdered bone samples (50 mg dry weight) with 500 μl of 0.5 M ammonium EDTA at pH 7.4 containing protease inhibitors,[32] for 2.5 h at 4°C on a rotary mixer. After this time, centrifugation at 12,000*g*, 4°C, for 20 minutes, yielded a supernatant which was carefully removed by aspiration. Osteocalcin immunoreactivity in dilutions of the EDTA bone powder extracts was determined by a competitive radioimmunoassay procedure using a polyclonal antiserum raised in a rabbit against purified bovine osteocalcin.[32]

Statistical analysis

All calculations were performed with the StatView 4.5 program (Abacus Concepts, Inc., Berkeley, CA, U.S.A.). The Mann–Whitney test was used to test for differences between the groups of healthy individuals and patients with severe renal insufficiency, between different blood groups for healthy individuals, and between the different skeletal sites. A difference was considered statistically significant at $p < 0.05$.

RESULTS

BALP and LALP isoforms in serum from healthy adults

We separated and quantitated six ALP isoforms in each investigated serum sample: three BALP (B/I, B1, and B2) and three LALP (L1, L2, and L3) isoforms. The BALP and LALP isoform reference intervals by 2.5% and 97.5% for healthy adults ($n = 123$) were: B/I, 0.04–0.17 μkat/l; B1, 0.19–0.60 μkat/l; B2, 0.34–1.63 μkat/l; B1/B2 ratio, 0.23–0.72; L1, 0.20–0.83 μkat/l; L2, 0.36–0.86 μkat/l; and L3, 0.10–0.34 μkat/l. In healthy individuals, the three BALP isoforms, B/I, B1, and B2, account for ~4, 16, and 37%, respectively, of the total serum ALP activity. We also observed that healthy individuals with blood group O and B ($n = 37$) had a significantly ($p < 0.01$) higher B/I activity in comparison with blood group A ($n = 79$), mean 0.11 μkat/l and 0.07 μkat/l, respectively, due to a larger proportion of the intestinal ALP component.

Effect of GPI-PLC on the BALP isoforms in serum

To investigate a possible GPI-PLC resistance of BALP due to inositol-palmitoylation, we analyzed a serum sample with a possible intact GPI cell membrane anchor, total ALP: 5.7 μkat/l. Agarose gel electrophoresis with Triton X-100 showed ALP activity near the application point as previously reported for tissue homogenates.[23] Analysis by HPLC resulted in one large tailing peak with a retention time of 16.38 minutes. A smooth baseline appeared in front of this peak, and no other peak with ALP activity was detectable. However, treatment with GPI-PLC exposed both B1 and B2, in addition to intestinal ALP of the B/I fraction, each with retention times as for healthy adults demonstrating cleavage of the GPI anchor. Accordingly, it is likely that no palmitoylated inositol is linked to the GPI anchor of BALP.

Treatment of serum with GPI-PLC did not influence the activities or retention times for B1 and B2, the two major BALP isoforms. However, the minor B/I peak decreased, and only a small fraction of B/I activity remained after the GPI-PLC treatment. This remaining fraction was resistant to heat inactivation at 56°C, but was not visible after treatment with a monoclonal antibody against human intestinal ALP, indicating an origin of intestinal ALP in this peak. In addition, B1 and B2 increased equally with the lost activity of the B/I component, indicating that the BALP fraction of B/I in serum was composed of the B1 and B2 isoforms with a ratio of 1:1, each with an intact hydrophobic GPI cell membrane anchor.

Effect of GPI-PLC on the BALP isoforms in bone samples

To retain the same terminology for description of the eluted BALP peaks from bone samples, as for serum samples, the name of the B/I peak has not been changed despite the fact that no intestinal ALP exists in the bone sample fractions.

Bone samples not treated with GPI-PLC displayed a B/I peak, representing the anchor-intact BALP (Fig. 1A); however, treatment with GPI-PLC eliminated this peak, indicating cleavage of the GPI membrane anchor (Figs. 1B–1D).

To investigate the possibility of innate GPI-PLC or GPI-PLD activity in serum, bone and serum samples of known BALP isoform activities were mixed and incubated under the same conditions as for the GPI-PLC treatment. No differences in either the activities or retention times of the B/I fraction were found, indicating little or no GPI-PLC or GPI-PLD activity in serum.

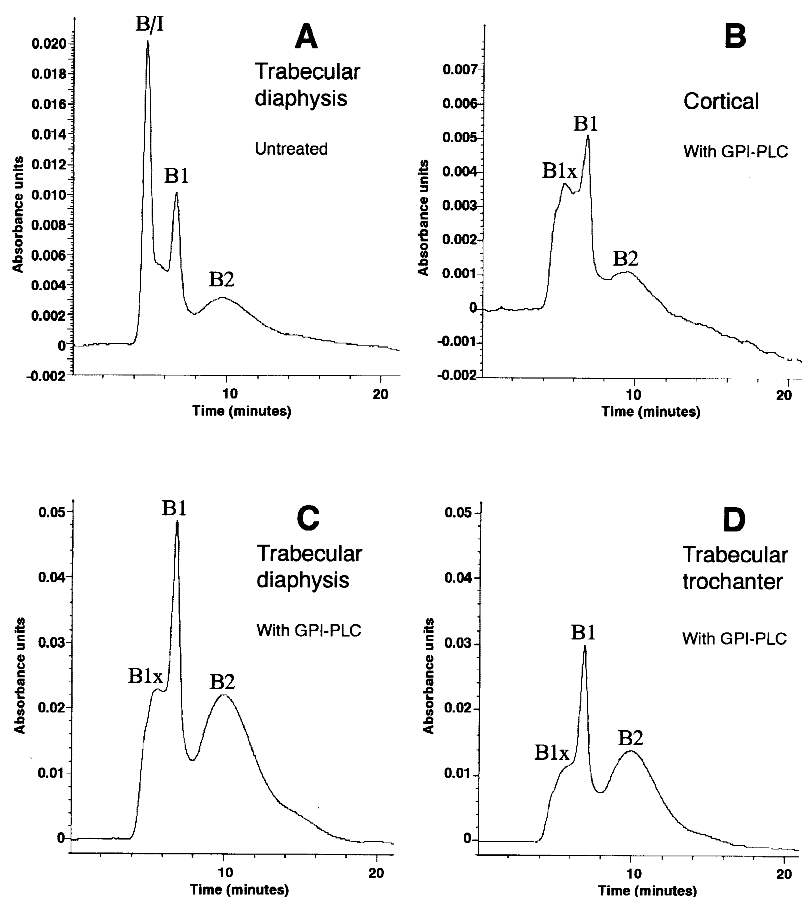

FIG. 1. BALP profiles of bone samples from a female, 48 years of age. The bone sample shown in (A) was untreated. The bone samples shown in (B–D) were treated with GPI-PLC. (A) Trabecular diaphysis bone (untreated), with total ALP 90.5 μkat/l/g of dried bone. Peaks, retention times, and activities, in order of elution, are: B/I, 4.83 minutes, 37.0 μkat/l/g dried bone; B1, 6.80 minutes, 20.5 μkat/l/g dried bone; B2, 9.91 minutes, 33.0 μkat/l/g dried bone. (B) Cortical bone (with GPI-PLC), with total ALP 28.5 μkat/l/g dried bone. Peaks, retention times, and activities, in order of elution, are: B1x, 5.35 minutes, 10.0 μkat/l/g dried bone; B1, 6.85 minutes, 12.5 μkat/l/g dried bone; B2, 10.08 minutes, 6.0 μkat/l/g dried bone. (C) Trabecular diaphysis bone (with GPI-PLC), with total ALP 394.0 μkat/l/g dried bone. Peaks, retention times, and activities, in order of elution, are: B1x, 5.40 minutes, 81.0 μkat/l/g dried bone; B1, 6.87 minutes, 94.5 μkat/l/g dried bone; B2, 10.00 minutes, 218.5 μkat/l/g dried bone. (D) Trabecular trochanter bone (with GPI-PLC), with total ALP 216.5 μkat/l/g dried bone. Peaks, retention times, and activities, in order of elution, are: B1x, 5.39 minutes, 39.0 μkat/l/g dried bone; B1, 6.90 minutes, 58.0 μkat/l/g dried bone; B2, 9.97 minutes, 119.5 μkat/l/g dried bone.

Origin of BALP isoforms and osteocalcin in human trabecular and cortical bone

Trabecular bone from both diaphyseal and trochanteric sites had higher BALP activities per gram of dried bone compared with cortical bone (Table 1, Figs. 1B–1D). Conversely, the osteocalcin content of cortical bone was more than 3-fold greater than that of trabecular bone (Table 1). Cortical bone had ~2-fold higher activity of B1 compared with B2, whereas trabecular bone had ~2-fold higher activity of B2 compared with B1 (Table 1, Figs. 1B–1D). Significant differences were found between cortical bone and both trabecular sites for each BALP isoform (Table 1). No significant differences were found between trabecular bone fractions from the diaphyseal and trochanteric sites.

Identification of a new BALP isoform (B1x) found in bone samples and in serum from patients with severe renal insufficiency

In all bone samples treated with GPI-PLC, an additional soluble BALP isoform (B1x) with a retention time of ~5.4 minutes was detected, eluting prior to the B1 peak (Figs. 1B–1D). The B1x activity was particularly high in trabecular bone from the diaphyseal region, and was of the same magnitude as B1 in all bone samples (Table 1).

B1x was not resolved in serum from healthy individuals but was identified in serum samples from 12 (60%) of the patients with severe renal insufficiency (n = 20) (Fig. 2). Higher activities of total ALP and of the BALP isoforms were found in these patients compared with healthy adults (Table 2). Heat inactivation of B1x yielded a mean remaining activity of 18%, a value similar to that previously reported for B1 and B2, 14% and 5%, respectively.[9] Inhibition of B1x by L-phenylalanine yielded a mean remaining activity of 65%, a value similar to that previously reported for B1 and B2, 68% and 62%, respectively.[9] Desialylation by neuraminidase altered the retention times for the BALP isoforms B1 and B2, and resulted in a large peak covering the fractions B/I and B1x. However, B1x was not visible after the combined procedure of heat inactivation and thereafter neuraminidase treatment, indicating an ALP fraction of bone origin. Moreover, B1x was not affected by incubation with the monoclonal antibody against human intestinal ALP. The B/I fraction in serum samples was the only peak with an altered retention time after incubation with this monoclonal antibody. B1x was also found in serum from a bilaterally nephrectomized patient. Thus, B1x is not of kidney origin,[24,25] nor is it an intestinal ALP type of human kidney.[26]

Table 1. BALP Isoforms and Osteocalcin in Cortical and Trabecular Bone

Biochemical markers	*Cortical*	*Trabecular diaphysis*	*Trabecular trochanter*
Total BALP	14.4 ± 2.1 (100%)	152 ± 29* (100%)	79 ± 17† (100%)
B1x	6.0 ± 1.2 (42%)	33 ± 9* (22%)	15 ± 5 (19%)
B1	5.5 ± 0.6 (38%)	42 ± 9* (28%)	23 ± 5† (29%)
B2	2.9 ± 0.4 (20%)	77 ± 11‡ (51%)	41 ± 7§ (52%)
B1/B2 ratio	1.72 ± 0.21	0.65 ± 0.06‡	0.72 ± 0.10†
Osteocalcin	72 ± 11	13 ± 4‡	19 ± 7§

BALP, bone alkaline phosphatase; B1x, additional bone 1 alkaline phosphatase isoform at retention time ~5.4 minutes; B1, bone 1 alkaline phosphatase; B2, bone 2 alkaline phosphatase; B1/B2 ratio, bone 1 alkaline phosphatase/bone 2 alkaline phosphatase ratio. The activities for BALP and the isoforms, are expressed in microkat per liter per gram of dried bone and osteocalcin in microgram per gram of dried bone. Values are given as mean ± SEM.

* $p < 0.05$, Mann–Whitney test between cortical and trabecular diaphysis.

† $p < 0.05$, Mann–Whitney test between cortical and trabecular trochanter.

‡ $p < 0.01$, Mann–Whitney test between cortical and trabecular diaphysis.

§ $p < 0.01$, Mann–Whitney test between cortical and trabecular trochanter.

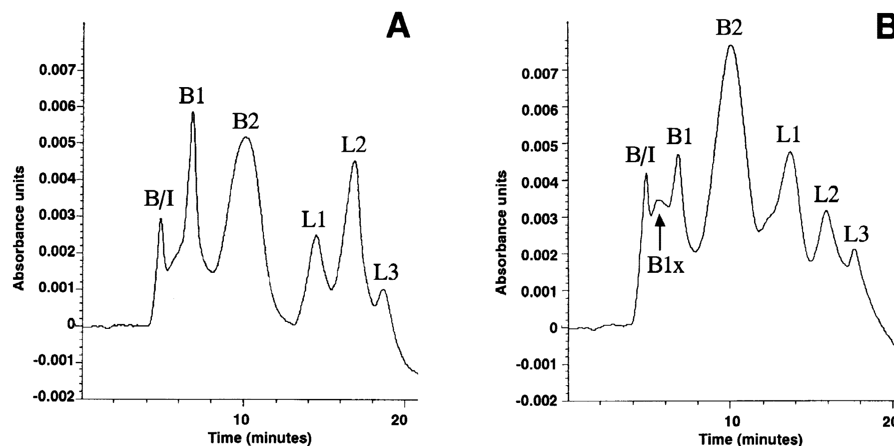

FIG. 2. (A) A serum ALP isoform profile from a healthy female, 53 years of age, with total ALP 2.8 μkat/l. Peaks, retention times, and activities, in order of elution, are: B/I, 4.85 minutes, 0.11 μkat/l; B1, 6.82 minutes, 0.51 μkat/l; B2, 10.07 minutes, 0.97 μkat/l; L1, 14.45 minutes, 0.42 μkat/l; L2, 16.85 minutes, 0.64 μkat/l; L3, 18.56 minutes, 0.15 μkat/l. (B) A serum ALP isoform profile from a female patient with severe renal insufficiency and on chronic dialysis therapy, 70 years of age, with total ALP 3.6 μkat/l. Peaks, retention times, and activities, in order of elution, are: B/I, 4.88 minutes, 0.16 μkat/l; B1x, 5.40 minutes, 0.19 μkat/l; B1, 6.85 minutes, 0.45 μkat/l; B2, 10.02 minutes, 1.25 μkat/l; L1, 13.92 minutes, 0.79 μkat/l; L2, 16.06 minutes, 0.49 μkat/l; L3, 17.72 minutes, 0.27 μkat/l.

DISCUSSION

The results of the present study demonstrate that the biochemical differences between the two major BALP isoforms, B1 and B2, found in serum from healthy adults are most likely to be due to different glycosylation patterns. We also explored the alternative explanation, that is, the differences of B1 and B2 may be due to the presence of an attached GPI anchor, by examining the retention characteristics of these isoforms after GPI-PLC treatment. Positive results, such as those obtained using GPI-PLC for the removal of the hydrophobic GPI membrane anchor are powerful criteria of the presence of the GPI anchor. However, negative results can be ambiguous as some GPI anchors are inherently resistant to GPI-PLC due to inositol-palmitoylation.[22] To investigate a possible GPI-PLC resistance of BALP, we analyzed a serum sample with an intact GPI cell membrane anchor attached to BALP as shown by agarose gel electrophoresis and HPLC. Treatment with GPI-PLC exposed both B1 and B2, each with retention times as for healthy adults, demonstrating cleavage of the GPI anchor. Thus, it is likely that no palmitoylated inositol is linked to the GPI anchor of BALP and that no GPI anchor fragments are presented on B1 and B2. In further support of our data, Wallace et al.[33] using isoelectric focusing, have reported, in some serum samples, two bands of bone origin (band 5a and 5b) which were resistant to GPI-PLC. Our experimental data confirm previous reports that little or no GPI-PLC activity exists in serum, and also probably no GPI-PLD activity despite its reported abundance in the circulation.[6,34–37]

To study if the BALP isoforms found in serum are the

TABLE 2. TOTAL ALP AND BALP ISOFORMS FOR PATIENTS WITH SEVERE RENAL INSUFFICIENCY COMPARED WITH HEALTHY ADULTS

	Healthy adults	*Renal patients*	p *value**
Total ALP (μkat/l)	2.5 ± 0.05	4.2 ± 0.51	<.0001
B/I (μkat/l)	0.09 ± 0.003	0.17 ± 0.04	<.05
B1x (μkat/l)	Not resolved	0.10 ± 0.02†	
B1 (μkat/l)	0.39 ± 0.01	0.42 ± 0.05	NS
B2 (μkat/l)	0.94 ± 0.03	1.73 ± 0.32	<.005
B1/B2 ratio	0.44 ± 0.01	0.30 ± 0.03	<.0001

BALP, bone alkaline phosphatase; B/I, bone/intestinal alkaline phosphatase; B1x, additional bone 1 alkaline phosphatase isoform at retention time ~5.4 minutes; B1, bone 1 alkaline phosphatase; B2, bone 2 alkaline phosphatase; B1/B2 ratio, bone 1 alkaline phosphatase/bone 2 alkaline phosphatase ratio; NS, not significant. Healthy adults, $n = 123$. Patients with severe renal insufficiency, $n = 20$. Values are given as mean ± SEM.

* Mann–Whitney test between healthy adults and patients with severe renal insufficiency.

† Patients with severe renal insufficiency expressing B1x, $n = 12$.

result of different glycosylation patterns, or remaining GPI cell membrane anchor fragments, it is crucial to use serum as a source of BALP and not bone cell cultures such as SaOS-2. Although specific BALP isoforms such as B1 and B2 were not investigated, Anh et al.[38] have recently reported that BALP is released from human osteoblasts primarily in the anchor-intact hydrophobic form and appears in serum as the anchorless hydrophilic form. However, we also observed hydrophobic ALP, that is, the B/I component, in bone samples not treated with GPI-PLC. The minor B/I fraction, which represented 4% of total serum ALP, was found to be composed of B1 and B2, each with an intact hydrophobic GPI cell membrane anchor. This finding corresponds with Anh et al.[38] who reported that 98 ± 3% of all BALP activity was in a hydrophilic form (and ~2% in the hydrophobic form) in serum from patients with Paget's disease.

Thus far, no consensus has been reported with regard to the structural differences between the ALP isoforms found in serum. Explanations, incorporating both glycosylation differences and remaining GPI anchor fragments have been cited, often independently of each other.[7,8] We suggest that the difference between the BALP isoforms B1 and B2 is due to different glycosylation patterns as a result of post-translational modification. However, differences between the LALP isoforms appear to be more complex and possibly due to a combination of remaining cell fragments with a GPI anchor,[6,33] and different glycosylation patterns[33] undetected by techniques such as electrophoresis. In essence, it is noteworthy that divergent BALP results, obtained with different methods for determination of BALP, should be interpreted with caution since different isoforms of BALP exist.[2,21,39,40]

Our results demonstrate that cortical bone has ~2-fold higher activity of B1 compared with B2, whereas that trabecular bone had ~2-fold higher activity of B2 compared with B1. Our finding of a higher B2 activity, in comparison with B1, in serum from healthy adults probably reflects the higher turnover rate of trabecular than cortical bone. Recent clinical studies have revealed increased activities of B1 and B2 during childhood when compared with adults, especially during the adolescent growth spurt. Moreover, we have previously shown that the B1/B2 ratio in adolescent girls reaches higher levels than in boys at Tanner stage IV–V, due to a more rapid decline of the B2 isoform compared with B1 after puberty.[11] There is a reported gender and age variability in the mineralization of bone at trabecular and cortical sites at the forearm. Substantial mineralization of cortical bone occurs about 1–2 years later than at trabecular sites.[41] Trabecular bone is made by endochondral ossification and thus a product of longitudinal growth, whereas cortical bone is made by periosteal apposition and thus a product of circumferential growth.[42] The increased incidence of upper extremity fractures, particularly in the lower forearm, is coincident with the adolescent growth spurt. This may be due to an increase in cortical porosity as a consequence of the increased intracortical bone turnover, which supplies some of the calcium and phosphate needed by the growing ends of the long bones.[42] Taken together, it is reasonable to imply that the B1 isoform is more related to cortical bone and that B2 is more related to trabecular bone metabolism.

Furthermore, patients with prostate cancer and skeletal metastases have B2 activities which correspond to 75% of the total ALP activity, significantly higher than the value of 35% found in healthy men.[13] These increased B2 levels could be explained by the fact that secondary bone tumors often develop in vertebral bones.[43] Individual vertebra have a greater proportion of trabecular than cortical bone, and it is possible that increased B2 activities could be related to tumor invasion of the trabecular bone compartment.

As presented, we found significant differences between B1 and B2; however, neither of these isoforms were totally specific for cortical or trabecular bone. Therefore, taken together with previously reported clinical data, we propose that B1 and B2 may reflect different stages in osteoblast differentiation during osteogenesis, where one isoform is presented before and the other during the extracellular matrix maturation phase.[2,13,44] The BALP isoforms may indicate further osteoblast differentiation, for example when osteoblasts are entrapped in bone matrix becoming osteocytes, or remain on the surface as bone lining cells, or undergo apoptosis.[45]

We have used the term B1x to describe the newly characterized BALP isoform in patients with severe renal insufficiency, because it most likely coeluates with the peak of B1 and therefore contributes to the activity of B1. There is no complete baseline separation between the B/I and B1 peaks in serum samples. This part of the chromatographic profile is very much dependent on the initial mobile phase composition and cannot be further separated by a change of the gradient profile. B1x has not previously been observed in healthy children,[11] or in disorders including growth hormone deficiency,[2] hypophosphatasia, hypophosphatemic

vitamin D–resistant rickets, Paget's disease, stress fractures,[9,12] and metastatic bone disease.[13] However, the presence of B1x in healthy individuals cannot be excluded since its intermediate retention, and possible lower activity than either of the B/I and B1 peaks, may confound its detection. The high expression of B1x in patients with severe renal insufficiency could be an effect of secondary hyperparathyroidism commonly noted in renal osteodystrophy. Other groups of patients with primary and secondary hyperparathyroidism, as well as more renal patients with unambiguous histomorphometric classifications, will need further investigation before these findings are adequately explained. Schober et al.[46] recently reported differences of mineralized bone loss at different skeletal sites in patients with renal osteodystrophy, which were further classified by histologic criteria as osteitis fibrosa or osteomalacia.

Osteocalcin concentrations were also found to be markedly different, with cortical bone having a higher content of extractable immunoreactive osteocalcin relative to the trabecular fractions. Although our findings in cortical bone are consistent with those of Vanderschueren et al.,[47] we have interpreted these data with caution since the measured concentrations of osteocalcin represent only that immunoreactive protein solubilized from bone using EDTA over a limited period of several hours, and do not necessarily represent the total osteocalcin content of the bone. Exhaustive demineralization protocols involving multiple changes of decalcifying agent over periods of up to 1 week, are required to release mineral-bound osteocalcin. However, due to the lability of human osteocalcin, true total bone osteocalcin measurements are difficult to make. Our data may also relate to the relative extractability of osteocalcin from the cortical and trabecular fractions and may further be influenced by an increased accumulation of osteocalcin within cortical bone, possibly a result of its longer residence time due to its slower turnover rate relative to trabecular bone.

In summary, our data show that the differences between the two major BALP isoforms found in serum, B1 and B2, are likely to be due to different glycosylation patterns of BALP, rather than the presence of GPI cell membrane anchor fragments. We have also found that cortical bone was richer in B1 activity and osteocalcin, whereas trabecular bone was richer in B2. The BALP isoforms, B1 and B2, may provide information relating to bone metabolism within specific bone compartments. In addition, we also identified a previously undescribed soluble BALP isoform, B1x, in serum from some patients (60%) with severe renal insufficiency. B1x was also present in all investigated bone samples. Future investigations including comparative clinical studies using bone histomorphometry and bone mineral density should clarify the clinical significance of the different BALP isoforms described in this study, B/I, B1, B1x, and B2, and their relationships to other markers of bone turnover.

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