Human tissue non-specific alkaline phosphatases: sugar-moiety-induced enzymic and antigenic modulations and genetic aspects

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To investigate the possible role(s) of glycans in human tissue non-specific alkaline phosphatase (TNAP) activity, the isoenzymes were purified and treated with various exo- and endoglycosidases. Catalytic activity, oligomerization, conformation and immunoreactivity of the modified TNAPs were evaluated. All TNAPs proved to be N-glycosylated, and only the liver isoform (LAP) is not O-glycosylated. Usually, the kidney (KAP) and bone (BAP) isoenzymes are similar and cannot be clearly discriminated. Differences between the immunoreactivity of KAP/BAP and LAP with a BAP antibody were mainly attributed to the N-glycosylated moieties of the TNAPs. In addition, elimination of O-glycosylations moderately affects the TNAP reactivity. Interestingly, N-glycosylation is absolutely essential for TNAP activity, but not for that of the placental or intestinal enzymes. According to the deduced amino acid sequence of TNAP cDNA, Asn-213 is a possible N-glycosylation site, and our present findings suggest that this sugar chain plays a key role in enzyme regulation. With regard to the oligomeric state of alkaline phosphatase (AP) isoforms, the dimer/tetramer equilibrium is dependent on the deglycosylation of glycosyl-phosphatidylinositol(GPI)-free APs, but not GPI-linked APs. This equilibrium does not affect the AP conformation as observed with CD. With regard to TNAPs, no data were available on the gene expression or nature of the 5′-non-translated leader exon of human KAP, as opposed to BAP and LAP genes. cDNA sequencing revealed that cortex/medulla KAP is genetically related to BAP, and medulla KAP to LAP.

INTRODUCTION

Mammalian alkaline phosphatases (AP; EC 3.1.3.1) make up a ubiquitous family of non-specific phosphomonoesterases [1,2]. Human APs are encoded by at least four different genes which express the kidney/bone/liver-type [tissue non-specific (TNAP)], placental-type (PAP), intestinal-type (IAP) and germ-cell-type (or placental variant) enzymes respectively. The gene locus for the TNAPs has been mapped to chromosome 1p34-p36.1 [3], and the loci for PAP, IAP and germ-cell-type AP have all been mapped to chromosome 2q34-q37 [4,5]. The glycosyl-phosphatidylinositol (GPI) moiety of post-translational APs is displaced from the C-terminal hydrophobic sequence [6], and consequently these APs are targeted to the outer leaflet of the cytoplasmic membrane, where the GPI serves as a membrane anchor [7,8]. Very little is known about the glycosylation and physicochemical properties of human APs, principally because of the great diversity of post-translational modifications of the various isoenzymes. The catalytic activity of APs is a more familiar parameter of the enzyme, and the inhibitory effect of different classes of effectors is a common tool used for discriminating between the isoforms. Moreover, mammalian APs are 20–30-fold more active than the corresponding bacterial enzymes. This may be due to the presence of different co-ordination sites for Zn^{2+} and Mg^{2+}, two metal ions necessary for catalytic activity [9]. Alternatively, as mammalian APs are commonly glycosylated, it has not been ruled out that sugar chains play a role in regulating their activity. Significant amounts of APs are found in the serum under both normal and pathological conditions [10–12]. Identification of the different isoforms in serum is a difficult task, but has been facilitated by the study of post-transcriptional modifications. By clarifying further the glycosylation patterns of APs and their role in the immunoreactivity of the enzyme, it will become possible to elucidate the effects of disease on the nature of APs in serum.

In this study, we focused on the glycosylation properties of the human kidney, bone and liver AP isoenzymes. Enzymic stability, immunoreactivity and conformational parameters were investigated after selective deglycosylation of the enzymes. The TNAP gene consists of 12 exons with the coding sequence beginning in the second exon [13]. Recently, an alternative non-coding first exon was identified in the liver message for TNAP [14], which differed from that of the osteoblast-derived cDNA sequence [15]. In addition, Goseki et al. [16] have reported that TNAP in human periodontal ligament cells is translated from mRNA that possesses the same first exon as in osteoblastic cells. To investigate which types of TNAP mRNA are expressed in human kidney, we performed reverse transcriptase (RT)-PCR-based analysis using cDNAs from human kidney cortex and medulla. Further, we partially determined exon 1 of KAP cDNA, and compared it with those of the BAP [13] and LAP [14] genes.

MATERIALS AND METHODS

Purification of APs

KAP, LAP, BAP, PAP and IAP were purified from human organs. The enzymes were prepared as described previously

Abbreviations used: AP, alkaline phosphatase; KAP, kidney AP; BAP, bone AP; LAP, liver AP; TNAP, tissue non-specific AP; bKAP, bovine KAP; GPI, glycosyl-phosphatidylinositol; PAP, placental AP; IAP, intestinal AP; RT, reverse transcriptase; GAPDH, glyceraldehyde phosphate dehydrogenase.

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The partial sequences of human KAP cDNA reported in this paper have been submitted to the GenBank/EMBL Data Bank with accession numbers X95994 (cortex/medulla) and X07598 (medulla).

[17,18]. Briefly, tissues were homogenized in 20 mM Tris-buffered saline, pH 7.5, supplemented with 0.5 mM PMSF. The homogenate was then stirred at 4 °C for 1 h in 50 % butan-1-ol, and, after centrifugation at 15000 ***g*** for 15 min, the aqueous phase obtained was treated with 60 % ice-cold acetone. The protein precipitate was pelleted by centrifugation at 1500 ***g*** for 5 min, redissolved in Tris-buffered saline and dialysed against the same buffer containing 1 mM $MgCl_2$ and 10 μM $ZnCl_2$. The resulting sample was applied to appropriate columns of anti-LAP, anti-PAP and anti-IAP monoclonal antibodies [17,18], coupled to CH-Sepharose 4B and eluted with 0.2 M Na_2CO_3 containing 0.5 M NaCl. The final step was gel filtration on a Sephacryl S-200 column (2 cm × 70 cm; Pharmacia, Uppsala, Sweden). Highly purified bovine KAP (bKAP) was purchased from Sigma Chemical Co. (St. Louis, MO, USA). The specific activities of the final preparations of KAP, BAP, LAP and bKAP were 610 units/mg, 215 units/mg, 750 units/mg and 1650 units/mg respectively. AP activity and protein concentration were determined as described below.

Enzyme assay

AP activity was determined at 37 °C with 5 mM disodium *p*-nitrophenyl phosphate as the substrate in 50 mM Na_2CO_3/$NaHCO_3$ buffer, pH 10.2, supplemented with 1 mM $MgCl_2$. Acid phosphatase activity was measured at 37 °C with 10 mM disodium *p*-nitrophenyl phosphate in 100 mM sodium citrate buffer, pH 5.0, as a modification of the method described by Hudson [19]. One unit of activity was defined as described previously [17]. Protein concentrations were determined by the method of Lowry et al. [20] with BSA as the standard.

Immunological reactivity

Anti-LAP (LAP 1/9) and anti-BAP (BAP 1/9) monoclonal antibodies [21] were obtained from Dr. E. M. Bailyes and Dr. J. P. Luzio (Addenbrooke's Hospital, Cambridge, U.K.). The reactivity of the APs with the antibodies was tested by solid-phase immunoassay. AP samples were blotted on to a nitrocellulose membrane using the Bio-Rad dot-blotting apparatus. After blocking the membrane with Blockase (a casein digestion product; Dainippon Pharmaceutical Co., Osaka. Japan), the membrane was washed with 0.1 % (v/v) Tween 20 in Tris-buffered saline before the addition of antibody at a 1000-fold dilution of the original preparation [22]. Subsequent detection of anti-AP antibody was performed using a Vectastain ABC kit (avidin–biotin system; Vector Laboratories). Tetramethylbenzidine was used as the substrate for the antibody-conjugated peroxidase. Reactivity with antibody was quantified by scanning with a densitometer equipped with a CCD camera.

Gel electrophoresis

The apparent molecular mass of the APs was estimated on a 7.5 % polyacrylamide gel containing 0.1 % SDS. Samples were pretreated with 0.1 % SDS under non-reducing conditions. Protein bands were detected by a silver-staining method (Silver Stain Plus, Bio-Rad, CA, U.S.A.). Specific bands for the phosphatase activities were detected with 5-bromo-4-chloro-3-indolyl phosphate/Nitro Blue Tetrazolium in 0.5 M 2-amino-2-methyl-propane-1,3-diol/HCl buffer, pH 10.2 containing 1 mM $MgCl_2$ and 10 μM $ZnCl_2$ [23] for AP detection and/or in 0.2 M acetate buffer, pH 5.0, for acid phosphatase detection. In these experiments, the separation of GPI-linked and GPI-free APs was carried out as described by Raymond et al. [23]. The unisolated APs (mixed GPI-linked and GPI-free APs) were detected by enzymic staining (Figure 3) and the GPI-linked APs were detected by silver staining (Figure 4).

Treatment with glycosidases

For glycosidase treatment of KAP, LAP, BAP, PAP and IAP, neuraminidase (*Clostridium perfringens*), β-galactosidase (*Streptococcus* 6646K) and N-Glycanase (*Flavobacterium meningosepticum*, EC 3.5.1.52) were purchased from Seikagaku Kogyo Co. (Tokyo, Japan), and O-Glycanase (*Diplococcus pneumoniae*, EC 3.2.1.97) was from Genzyme Co. For glycosidase treatment of bKAP, neuraminidase and O-Glycanase were from Oxford Glycosystems (Oxford, UK). Deglycosylation of about 0.1–0.5 unit/ml (for the enzymic staining) or about 3–7 units/ml (for the silver staining) AP isoenzymes was achieved by exposure to 0.3 unit/ml neuraminidase, 0.3 unit/ml β-galactosidase, 0.3 unit/ml N-Glycanase and 0.1 unit/ml O-Glycanase for 4 h at room temperature. APs were treated with neuraminidase before β-galactosidase, and N- and O-Glycanase digestion.

Treatment of rats with tunicamycin

Tunicamycin from *Streptomyces lysosuperficus* (Funakoshi Pharmaceutical Co., Tokyo, Japan) was injected intraperitoneally into Wistar strain rats weighing about 150 g. After 24 h, the kidney was excised and crushed, and total AP and acid phosphatase activities were determined as described above.

CD

bKAP was investigated by CD. The enzyme solutions were 0.1 mg/ml in 50 mM phosphate buffer, pH 7.0. Spectra were scanned at room temperature with a Jobin-Yvon CD6 polarimeter using 0.5 nm path-length cells. Each spectrum reported is a mean of three scans and has been corrected for buffer or glycosidase contribution. Since the various glycosidases represented 1–2 % of total protein, there was actually little, if any, contribution to the ellipticity.

RNA preparation and PCR

Total RNA from human kidney cortex, medulla and human periodontal ligament cells was isolated by the acid guanidinium thiocyanate/phenol/chloroform method [25]. These polyadenylated RNAs were purified through an oligo(dT)–cellulose column (Type 3; Collaborative Research). Normal human liver polyadenylated RNA was purchased from Clontech.

As a template for PCR, single-stranded cDNA was prepared from 1 μg of RNA using the SuperScript preamplification system (Gibco–BRL). Two sets of specific primers were designed from human TNAP gene sequences. In order to detect LAP, a sense primer AL11 (nucleotide positions 86–106 in exon 1L [14]) and an antisense primer AL12 (nucleotide positions 448–468 in exon 2 [13]) were designed. To detect BAP, we designed other sets of primers, AL12 and AL13. AL13 spans nucleotide positions 44–64 in exon 1B [13]. PCR conditions were as follows: five cycles at 94 °C for 1 min, 50 °C for 1 min and 72 °C for 1 min, and a further 25 cycles at 94 °C for 30 s, 55 °C for 30 s and 72 °C for 30 s, followed by 72 °C for 10 min.

cDNA amplification and sequencing of human KAP

An oligo(dT)-primed cDNA library was prepared by a SuperScript λ system for cDNA synthesis cloning (Gibco–BRL) according to the manufacturer's protocols. Respective cDNA (20 ng) was ligated to a cloning vector, λgt22A (*Not*I–*Sal*I arms),

and assembled into the phage by means of a λ packaging system (Gibco–BRL). We screened approximately 4×10^4 plaques with a human osteoblast-derived TNAP cDNA probe [15], radiolabelled by a random-priming DNA-labelling kit (Boehringer, Mannheim, Germany), by hybridization in 1 M NaCl/1 × Denhardt's solution [26]. The phage DNAs purified from positive clones were digested with *Not*I–*Sal*I, and the isolated inserts were subcloned into pBluescript SK(+) for further analysis.

The nucleotide sequence was determined by the dideoxynucleotide chain-termination method [27] with Sequenase (US Biochemical Co.).

RESULTS

Effect of deglycosylation on enzyme activity

The exoglycosidases tested had little effect on the AP isoenzymes (Table 1). Neuraminidase decreased BAP activity slightly, but further treatment with β-galactosidase enhanced its activity to 140 % of that of the untreated enzyme. However, removal of *N*- or *O*-glycoside chains by endoglycosidases produced marked changes in enzymic activity. N-Glycanase treatment led to complete loss of activity of all TNAPs, but had no effect on human PAP or IAP activity. Thus TNAPs responded similarly to treatment with neuraminidase, β-galactosidase and N-Glycanase. In contrast, O-Glycanase had a different effect on KAP and BAP from that on LAP. As reported previously [28,29], the activity of KAP and BAP, which bear an O-linked sugar chain, was clearly influenced by O-Glycanase treatment. Indeed, the activity of these two enzymes increased to approximately 170 %, compared with controls, whereas the activity of LAP was little changed.

Table 1 Effect of various glycosidases on AP isoenzyme activity

AP activity was measured as described in the Materials and methods section. Results are expressed as a percentage of the control (taken as 100%). n.d., not determined.

Glycosidase treatment	KAP	BAP	LAP	PAP	IAP
Control	100	100	100	100	100
Neuraminidase	94	82	99	107	98
β-Galactosidase	115	140	117	n.d.	n.d.
N-Glycanase	1	0	1	102	84
O-Glycanase	167	169	89	n.d.	n.d.

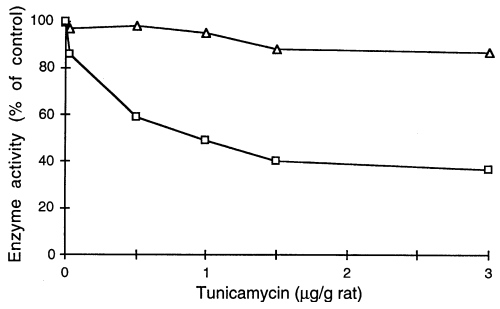

Figure 1 Total acid phosphatase and AP activities in rat kidney after treatment *in vivo* with tunicamycin

Total acid phosphatase (△) and total AP (□) activities in the rat 24 h after administration of various doses of tunicamycin are shown. Phosphatase activities were assayed in whole kidney homogenates.

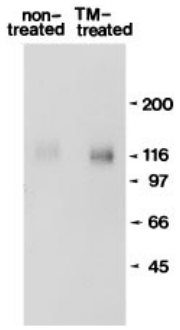

Figure 2 SDS/PAGE under non-reducing conditions of rat renal acid phosphatases with and without tunicamycin (TM) treatment *in vivo*

Electrophoresis and acid phosphatase detection were carried out as described in the Materials and methods section. Numbers on the right indicate molecular size (kDa) markers.

It was of interest to discover whether the loss of TNAP activity on removal of the N-linked sugar chains *in vitro* was reproducible *in vivo*, and tunicamycin, a well-known inhibitor of the first step of biosynthesis of the dolichol-dependent N-glycoside sugar chain [30,31], was used for this purpose. The efficacy of inhibition *in vivo* has already been demonstrated [32], and in our study the inhibitor was administered to laboratory rats 24 h before kidney excision. AP activity was assayed in whole kidney homogenates without butanol extraction, acid phosphatase serving as a control marker enzyme. Acid phosphatase is a highly glycosylated protein, the sugar moieties of which account for about 10 % of its molecular mass [33]. Figure 1 shows a 60 % loss of AP activity at the higher doses of tunicamycin, whereas 90 % of acid phosphatase activity was recovered in the corresponding tissue. In addition, enzymically active bands for the untreated and treated acid phosphatases were found at 120 and 114 kDa respectively (Figure 2).

Effect of deglycosylation on AP molecular mass and its conformation

The quaternary structure of KAP, BAP and LAP was investigated by non-reducing SDS/PAGE. This was possible because of the relatively high resistance of AP activity to SDS. Samples were placed in 0.1 % SDS/2-mercaptoethanol-free buffer without boiling before use. In these preparations, KAP was present as a mixture of GPI-free and GPI-linked proteins, whereas BAP and LAP were not GPI-linked (results not shown). All GPI-free APs formed tetramers after treatment with exo- or endo-glycosidases, as shown by the retardation of migration of the bands (Figure 3). N-Glycanase-treated samples could not be detected enzymically on the gels by their AP activity because N-Glycanase completely inhibited this activity, as shown previously (Table 1). The tetramerization observed with glycosidase-treated GPI-free APs did not occur with glycosidase-treated GPI-linked KAP, in which deglycosylation only provoked a small shift of the band to higher molecular mass. Neuraminidase was added before the other glycosidases. Thus elimination of sialic acids resulted in a slight retardation of migration as the result of the loss of negative charges on the glycosylated APs. Tetramerization overshadowed this small shift effect of GPI-free AP. Nevertheless, the efficacy of the action of the glycosidases was governed by a shift in migration in all cases (Figure 3). When GPI-linked TNAPs were treated with 0.1 % SDS under non-reducing conditions and the protein bands were detected by silver staining, the N- and O-Glycanase-

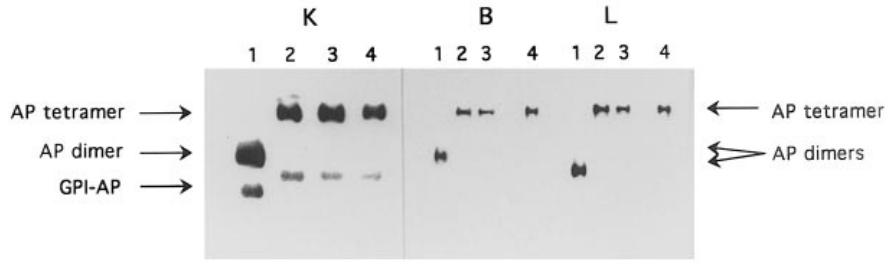

Figure 3 SDS/PAGE of KAP (K), BAP (B) and LAP (L) under non-reducing conditions

Lane 1, control AP; lane 2, neuraminidase-treated AP; lane 3, β-galactosidase-treated AP; lane 4, O-Glycanase-treated AP. Glycosidase treatments and detection of the enzymically active bands were carried out as described in the Materials and methods section.

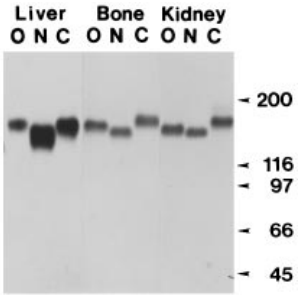

Figure 4 SDS/PAGE of LAP, BAP and KAP enzymes under non-reducing conditions (silver staining)

C, control AP; N, neuraminidase + N-Glycanase-treated AP; O, neuraminidase + O-Glycanase-treated AP. Numbers on the right indicate molecular size (kDa) markers.

treated LAP, BAP and KAP exhibited different molecular masses, as shown in Figure 4. The molecular masses of native LAP, BAP and KAP were found to be 152, 166 and 168 kDa respectively. The molecular masses of N-Glycanase-treated LAP, BAP and KAP were 139, 150 and 150 kDa respectively, and those of O-Glycanase-treated LAP, BAP and KAP were 152, 153 and 152 kDa respectively. These data suggest that the sugar moiety in KAP is the largest in the TNAPs tested, and that there is no O-glycosylated moiety in LAP molecules. We then investigated whether the tetramerization of TNAPs caused by glycosidase treatment was accompanied by secondary-structure changes. CD was used to monitor possible conformational changes in GPI-free bKAP, the only mammalian TNAP available in large quantities at a high grade of purity. The bovine TNAP amino acid sequence deduced from cDNA cloning possesses 91% similarity to the human TNAP sequence and 96% charged amino acid similarity [34,35]. Thus bKAP was a good model for studying the effect of deglycosylation on AP secondary structure. The protein samples were prepared as described in the Materials and methods section. Under these conditions, the glycosidases represented less than 2% of the total amount of protein, thus making a negligible contribution to the CD spectra. There was no significant change in KAP conformation after removal of its N- or O-linked sugar chains. In particular, the molar ellipticities at 208 and 222 nm were unchanged, demonstrating conservation of the α-helix/β-sheet ratio (results not shown). The spectra were typical of an α-helix and β-sheet structure, and closely resembled the spectrum of the bovine IAP isoform [36].

Table 2 Effect of various glycosidases on AP isozyme immunoreactivity with anti-BAP antibodies

AP isoenzymes on nitrocellulose membrane were incubated with anti-BAP antibodies and subsequently detected as described in the Materials and methods section. Results are expressed as a percentage of the control activity (100%).

Glycosidase treatment	KAP	BAP	LAP
Control	100	100	100
Neuraminidase	58	44	38
β-Galactosidase	30	18	163
N-Glycanase	8	6	205
O-Glycanase	62	13	24

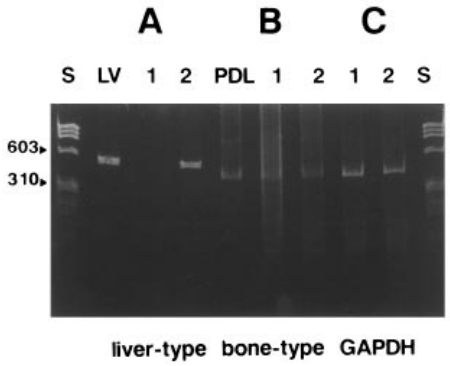

Figure 5 Relative expression of exon 1L for LAP, exon 1B for BAP and GAPDH messages in human renal cortex, renal medulla, liver and periodontal ligament cells

Respective RT-PCR products were detected with exon 1L cDNA (A) for LAP, exon 1B cDNA (B) for BAP and as reference GAPDH cDNA (C) on PAGE (4.25% gel). AL11 and AL12 primers detected exon 1L expression (522 bp), AL12 and AL13 primers detected exon 1B expression (425 bp) and GAPDH primers detected GAPDH expression (450 bp). LV, reference for human liver exon 1L expression [14]; PDL, reference for human periodontal ligament cell exon 1B expression [16]; lane 1, normal cortex from human kidney; lane 2, normal medulla from human kidney; S, molecular mass marker from ϕX174 DNA *Hae*III digest (New England Bio-Labs).

Effect of deglycosylation on the immunoreactivity of APs

KAP, BAP and LAP were tested for their immunoreactivity with anti-BAP and anti-LAP monoclonal antibodies after treatment with various glycosidases (Table 2). Treatment with neur-

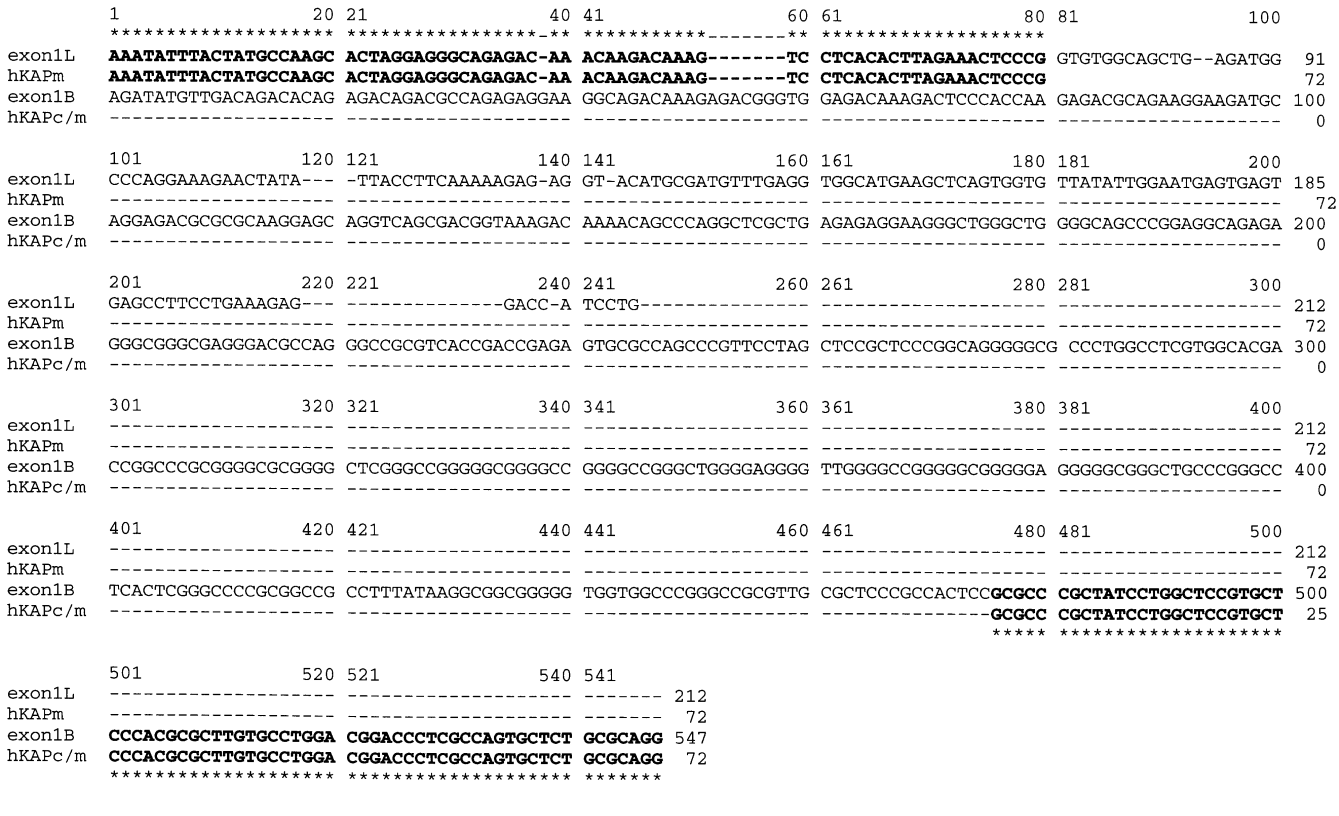

Figure 6 Comparison of 5′-untranslated leader cDNA sequences for human KAP with the complete exon 1L for the LAP-specific gene and part of the exon 1B for the BAP-specific gene

The cDNA sequences of human KAP from medulla (hKAPm, accession number X07598) and cortex/medulla (hKAPc/m, accession number X95994) were identified as described in the Materials and methods section. Sequences that match with exon 1B and/or exon 1L are marked by asterisks. Complementary DNA sequences of exon 1L (D90054) and exon 1B (M24428) were obtained from the data of Matsuura et al. [14] and Weiss et al. [15] respectively and together show 41% sequence similarity.

aminidase or O-Glycanase decreased the ability of all three AP isoforms to be recognized by the anti-BAP antibody. When treated with O-Glycanase, the immunoreactivity of the BAP and LAP enzymes decreased more than that of KAP, demonstrating a difference between BAP/LAP and KAP. This is of interest as it is well known that it is often difficult to differentiate KAP from BAP, but the action of β-galactosidase and N-Glycanase clearly distinguished KAP and BAP from LAP. With these two glycosidases, KAP and BAP lost part (with β-galactosidase) or most (with N-Glycanase) of their reactivity with anti-BAP antibody, whereas LAP reactivity was clearly (with β-galactosidase) or strongly (with N-Glycanase) enhanced. Thus BAP/LAP and KAP could be immunologically differentiated by their O-glycosylation, and BAP/KAP and LAP by their N-glycosylation.

Human KAP mRNA measured by PCR and cDNA sequence

As shown in Figure 5, human liver tissues and periodontal ligament cells appeared to express exon 1L as the LAP gene and exon 1B as the BAP gene respectively. With the same cDNA probes, the 522 bp band of LAP-gene-specific expression was clearly detected in human renal medulla. In contrast, it is interesting to note that the 425 bp band of BAP-gene-specific expression was detected in both human renal cortex and medulla. As a reference, the 450 bp band of the human glyceraldehyde phosphate dehydrogenase (GAPDH)-specific gene was expressed in both renal cortex and medulla. Although it is not known at present why the LAP gene is expressed in the renal medulla, both the renal cortex and medulla in human kidney can express a similar amount of bone and bone-like AP gene.

A cDNA library was constructed from human renal tissues using a λgt22A vector, and screened with TNAP cDNA probes. Two clones of about 2.5 kb were isolated. The 5′-untranslated sequence of KAP cDNA clones are shown in Figure 6, and compared with exons 1B and 1L from human tissues. The identified 5′-untranslated sequence of the leader exon of cDNA for human cortex KAP was identical with exon 1B of BAP cDNA [15], and cDNA for human medulla KAP was similar to part of exon 1B of BAP and/or exon 1L of LAP cDNA [14,15].

DISCUSSION

Although it can be deduced from the primary amino acid sequence [15,35,37,38] that there are five possible N-glycosylation sites (Asn-123, -213, -254, -286, -413) for TNAPs, three possible N-glycosylation sites (Asn-123, -249, -410) for IAP and two possible N-glycosylation sites (Asn-122, -249) for PAP, the role(s) of the sugar chain(s) of human APs is still obscure. The positions in human TNAP (Asn-123, -213, -254, -286, -413) are included in the 91% of amino acids conserved in bovine TNAP [39]. To our knowledge, no data are available on the possible site(s) for Ser/Thr- or O-glycosylation of human TNAPs, IAP or PAP. These two types of glycosylation are currently being investigated by our group.

According to our findings, all human TNAPs bear some N-

linked sugar chains, and BAP and KAP, but not LAP, bear some O-linked sugar chains. Hydrolysis of these sugar chains led to changes in enzyme activity which varied depending on the sugar moiety. There is a clear difference between KAP/BAP and LAP activities after removal of possible O-glycoside sugar chain(s). The presence of certain O-linked sugar chains appeared to down-regulate KAP and BAP activity, but did not significantly affect LAP. These findings are in good agreement with the conclusion of Miura et al. [28], who pointed to O-glycosylations as the main difference between BAP and LAP. Also, we have shown here that LAP does not have any Ser/Thr-linked sugar chains. Most surprising was the effect on all KAP, BAP and LAP activities of the removal of N-glycoside sugar chain(s). Cleavage of the N-linked sugar chains led to complete inactivation of the TNAP activities tested, but there was no effect on the PAP or IAP activities. From the amino acid sequence, we know that PAP can be N-glycosylated on Asn-122 and -249, and IAP on Asn-123, -249, and -410. According to the results of a computer-assisted analysis, the amino acid hydropathy of the regions around Asn-123 and -254 on TNAP was similar to that of the Asn-122 and -249 on PAP or the Asn-123 and -249 on IAP (O. Nosjean, I. Koyama, M. Goseki, B. Roux and T. Komoda, unpublished work). Therefore the similar sites (Asn-123 and -254) on the TNAPs are presumably not involved in the regulation of TNAP activity. In contrast, Asn-213 and -286 on TNAPs can be N-glycosylated, but the corresponding regions of PAP and IAP are not consensus sites for glycosylation. Therefore one or both of these sites must be glycosylated and may play a role in the regulation of TNAP activity. If the tertiary structures of human APs are related to the crystal structure of the *Escherichia coli* enzyme [40,41], then Asn-213 can be located between β-sheet E and the N-terminal-adjacent α-helix. Interestingly, this region is in very close proximity to the active site, which is situated between the α-helix C-terminal to β-sheet B. For a comprehensive spatial representation see ref. [42]. As Asn-286 is located much further from the active centre, Asn-213 is probably N-glycosylated in TNAPs, and could play a key role in regulation of catalytic activity. In addition, we have reported that TNAP and IAP activities are more heat-labile than that of PAP [43]. Furthermore, Asn-413 in TNAPs and Asn-410 in IAP occupy heat-sensitive or collagen-binding sites [43] in AP molecules. Thus Asn-413 in TNAP molecules may also contribute to regulation of activity.

APs are known to be capable of combining together to form tetramers or octamers under certain conditions, especially when anchored to the surface of plasma membranes [44,45], and we have previously shown that GPI-free LAP can be found in a tetrameric form after asialylation [46]. In order to determine whether this oligomerization could partly explain the changes in activity observed after deglycosylation of the GPI-free TNAPs, we estimated the apparent molecular mass of the enzyme-active bands for APs on SDS/PAGE under non-reducing conditions. Tetramer formation was observed after deglycosylation to an equal extent regardless of the nature of the glycosidases used. Thus oligomerization of the deglycosylated TNAPs did not account for the changes in enzyme activity. It is noteworthy that tetramerization of TNAPs in solution was impeded by the presence of a GPI anchor, and only took place in GPI-free TNAPs (see Figures 3 and 4). The action of glycosidases did not provoke any change in secondary structure of the GPI-free APs, as estimated by CD spectrometry. This was not an unexpected finding, as glycosylation occasionally stabilizes secondary structure during the folding of nascent proteins, but usually does not play a key role in maintaining this conformation thereafter. Thus the changes in catalytic activity after glycosidase treatment of the GPI-free TNAPs are probably due to local modulations in tertiary structure, i.e. changes in charge–charge interactions or in steric hindrance.

TNAP reactivity with anti-BAP antibody was also markedly affected by the action of glycosidases. Sialic acid residues were present in KAP, BAP and LAP, and were important for their immunoreactivity with anti-BAP antibody. O-linked sugar chains were also necessary for recognition by the antibodies used here, especially for BAP. These results are in good agreement with the immunological data of Miura et al. [28] on BAP and LAP. In terms of immunoreactivity, we found β-galactosidase and N-Glycanase to be useful for discriminating KAP/BAP from LAP. We therefore concluded that the galactose residues involved in the epitope recognized by BAP 1/9 monoclonal antibody might be derived from one or several N-linked sugar-chain cores. Thus the major immunoreactivity of KAP and BAP was presumably due to the N-glycosylation site. The study undertaken with the anti-LAP antibodies yielded results similar to those described above for the anti-BAP antibodies. Anti-LAP and anti-BAP antibodies cross-react quite well with the TNAPs, as previously observed [28]. Anti-BAP antibody has more limited specificity, as it reacts with a greater amplitude to changes in the antigen.

Meanwhile, transcription of the TNAP gene occurs mainly in the tissue after which the AP isoforms are named, i.e. kidney, bone and liver. The gene is composed of 12 exons, distributed over more than 50 kb [13]. The leader exon is not translated, nor is part of the second exon and most of the 12th exon. The resulting polypeptide structure is believed to be well conserved in the isoforms, as seen from the predicted translation product. However, the primary amino acid structure of mammalian TNAPs has not been determined, and the ubiquitous expression of TNAPs is believed to be regulated by the 5′-untranslated region of the gene. This region has previously been shown to be different in the BAP and LAP isoforms, in which the leader exons have been designated 1B and 1L respectively [13,14]. The two cDNA sequences have low nucleotide homology on computer-assisted sequence alignments (Figure 6). Previous studies on the 5′-untranslated region of bovine KAP cDNA have shown a sequence similarity to human BAP cDNA of 75% [39]. This can be considered good, as it takes the interspecies difference into account. We therefore determined mRNA for exon 1B and 1L by RT-PCR in human renal cortex and medulla. Exon 1B was found to be expressed in both cortex and medulla, and exon 1L was mainly found expressed in medulla, as shown in Figure 5. Thus we sequenced a part of the leader exon of KAP cDNA in human renal cortex and medulla, and concluded that the leader exon in renal cortex is homologous with the exon 1B region in human BAP cDNA, and that renal medulla exhibits two types of exons, homologous with 1B and 1L regions of human BAP/LAP cDNA. The two leader sequences in human KAP cDNAs could be aligned with 100% sequence identity for 72 bases in exons 1B and 1L (Figure 6). We therefore predict that KAP gene expression in renal cortex and BAP gene expression are regulated similarly in the two organs. However the KAP gene in renal medulla expresses both BAP and LAP types. Meanwhile, the leader exons of the TNAPs have no equivalent in the IAP or PAP gene [47,48]. This exon and the adjacent gene promoter located upstream within 610 nucleotides of the major transcription-starting site [13], or silencer, may have been acquired through evolution as an independent genetic unit, allowing multilocalization of TNAP genomes as opposed to tissue-specific IAP or PAP genomes. Finally, the physiological role and transcription regulation of exons 1B and 1L in human kidney, and the exon similarity in renal medulla and liver, are an interesting subject for further study.

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