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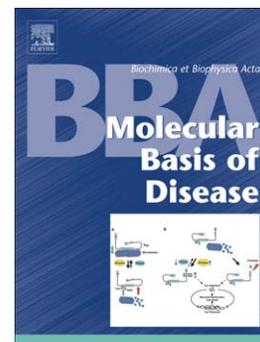
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Molecular characterization of tissue-nonspecific alkaline phosphatase with an Ala to Thr substitution at position 116 associated with dominantly inherited hypophosphatasia

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Abbreviations: DMEM, Dulbecco's modified minimum essential medium; ER, endoplasmic reticulum; GPI, glycosylphosphatidylinositol; PI-PLC, phosphatidylinositol-specific phospholipase C; TNSALP, tissue-nonspecific alkaline phosphatase; TNSALP (A116T), TNSALP with an alanine to threonine substitution at position 116; TNSALP (D306V), TNSALP with an aspartate to valine substitution at position 306; TNSALP (W), the wild-type TNSALP

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ABSTRACT

Mutations in the tissue-nonspecific alkaline phosphatase (TNSALP) gene are

responsible for hypophosphatasia, an inborn error of bone and teeth metabolism associated with reduced levels of serum alkaline phosphatase activity. A missense mutation (c.346G>A) of TNSALP gene, which converts Ala to Thr at position 116 (according to standardized nomenclature), was reported in dominantly transmitted hypophosphatasia patients (A.S. Lia-Baldini et al. Hum Genet. 109 (2001) 99-108). To investigate molecular phenotype of TNSALP (A116T), we expressed it in the COS-1 cells or Tet-On CHO K1 cells. TNSALP (A116T) displayed not only negligible alkaline phosphatase activity, but also a weak dominant negative effect when co-expressed with the wild type enzyme. In contrast to TNSALP (W, wild type), which was present mostly as a non-covalently assembled homo-dimeric form, TNSALP (A116T) was found to exist as a monomer and heterogeneously associated aggregates covalently linked via disulfide bonds. Interestingly, both the monomer and aggregate forms of TNSALP (A116T) gained access to the cell surface and were anchored to the cell membrane via glycosylphosphatidylinositol (GPI). Co-expression of secretory forms of TNSALP (W) and TNSALP (A116T), which are engineered to replace the C-terminal GPI-anchor with a tag sequence (*his*-tag or *flag*-tag), resulted in the release of heteromeric complexes consisting of TNSALP (W)-*his* and TNSALP (A116T)-*flag*.

Taken together, these findings strongly suggest that TNSALP (A116T) fails to fold properly and forms disulfide-bonded aggregates, though it is indeed capable of interacting with the wild type and reaching the cell surface, therefore explaining its dominant transmission.

1. Introduction

Hypophosphatasia is a genetic disease caused by various mutations in the

tissue-nonspecific alkaline phosphatase (TNSALP) gene [1-4]. To date, a total of 224 mutations have been reported worldwide as of Oct. 2010 (http://www.sesep.uvsq.fr/03_hypo_mutations.php). Hypophosphatasia is characterized by reduced levels of serum alkaline phosphatase activity and defective bone and teeth mineralization. One of two natural substrates which relate to clinical manifestation of hypophosphatasia, inorganic pyrophosphate is thought to play a crucial role as a negative regulator at the site of mineralization. That is: TNSALP enhances the mineralization by hydrolyzing pyrophosphate to phosphate and therefore decline in the activity due to mutations in the TNSALP gene leads to various degrees of hypomineralization [2,3]. Symptom of hypophosphatasia varies widely and in general its severity is inversely related to serum alkaline phosphatase levels of the patients. Clinically, hypophosphatasia is classified into five major categories depending on the age at diagnosis: perinatal, infantile, childhood, adult and odonto forms. Recently, the perinatal form is further divided into lethal and benign types [4]. Severe hypophosphatasia (perinatal and infantile forms) is transmitted in an autosomal recessive way, while mild hypophosphatasia (childhood, adult and odonto forms) is transmitted in a recessive or dominant way.

Severe hypophosphatasia patients are homozygote or compound heterozygotes with severe mutations, while mild form results from compound heterozygosity for severe and moderate mutations or single heterozygosity for severe mutations [5]. Mutations in TNSALP gene not only reduce the enzyme activity of TNSALP mutant proteins to a various degree, but also residual activities often exhibit different enzymatic properties from TNSALP (W) [6], probably providing additional variability in clinical symptoms. Apart from their direct effects on the catalytic function, TNSALP mutant proteins display a different magnitude of trafficking defects. We, along with other groups, have reported that defective trafficking of TNSALP mutant protein contributes a molecular mechanism of hypophosphatasia [7-12]. In one extreme case where T is deleted at 1559 of TNSALP cDNA, this frame-shift TNSALP with an additional 80-amino acid extension at the C-terminus failed to be modified by glycosylphosphatidylinositol (GPI) and resultantly is released into the medium [13,14].

There is an increasing number of mild hypophosphatasia with a dominant negative effect [5,15-17]. These dominant-type TNSALP mutants suppress the enzyme activity of TNSALP (W) to a various degree when they are co-expressed in a cell [5,15-17]. Thus this inhibition of the enzyme activity implies that TNSALP mutant proteins with a dominant effect and TNSALP (W) are able to interact with each other and form heteromeric enzyme complexes. However, molecular characterization of dominantly inherited TNSALP mutations has not been studied in detail so far. In this report, we

examined the molecular defect of TNSALP (A116T, according to standardized nomenclature, where the ATG initiator codon is numbered as 1). Extensive study on familial pedigree and its laboratory data unequivocally show that TNSALP (A116T) is dominantly transmitted [17,18]. Also, the co-expression data showed that TNSALP (A116T) inhibits TNSALP (W) albeit to a lesser degree compared to other dominant negative mutants such as TNSALP (D378V) and TNSALP (G63V) [17]. Surprisingly, our data indicate that the replacement of alanine with threonine at position 116 of TNSALP (A116T) renders TNSALP incompetent to form a dimeric structure and resultant monomeric polypeptides associate with each other to become disulfide-bonded high molecular mass aggregates. Moreover, we have also indicated that TNSALP (W) is trapped in the aggregate of TNSALP (A116T) when they are co-expressed.

2. Materials and methods

2.1. Materials

Express³⁵S³⁵S protein labeling mix (>1000 Ci/mmol) was obtained from Dupont-New England Nuclear (Boston, MA.), and¹⁴C-methylated proteins and enhanced chemiluminescence (ECL[®]) western blotting detection reagent, peroxidase-conjugated donkey anti-(rabbit IgG) and Protein A-Sepharose CL-4B from Amersham Pharmacia Biotech (Arlington Heights, IL, USA); pALTER[®]-MAX, Altered sites[®] II mammalian mutagenesis system from Promega (Madison, WI, U.S.A.); G418 and pansorbin from Calbiochem (La Jolla CA, U.S.A.); Lipofectamine Plus Reagent from Invitrogen (Carlsbad, CA, U.S.A.); phosphatidylinositol-specific phospholipase C (PI-PLC) from BIOMOL International, L.P. (Plymouth Meeting, PA, U.S.A.); aprotinin, baker's yeast alcohol dehydrogenase, bovine serum albumin, doxycycline, and ANTI-FLAG[®]M2 Agarose from Sigma Chemical Co. (St. Louis, MO.U.S.A.); Ni-NTA (nickel- nitrilotriacetic acid) resin and plasmid Midi-kit from Qiagen (Hilden, Germany); pTRE2 and BD[®] CHO-K1 Tet-On cell line and Tet system approved Fetal bovine serum from Clontech (Palo Alto, CA, U.S.A.); sulphosuccinimidyl N-(D-biotinyl)-6-aminohexane from Dojindo Laboratories (Kumamoto , Japan); antipain, chymostatin, elastatinal, leupeptin and pepstatin A from Protein Research Foundation (Osaka, Japan); bovine liver catalase and hygromycin from Wako Pure Chemicals (Tokyo, Japan), Antiserum against recombinant human TNSALP was raised in rabbits as described previously [19].

2.2. Plasmids and transfection

The pALTER-MAX[®] encoding TNSALP (W) was constructed as described previously [9]. Mutations were introduced at specific sites using Altered sites[®] II mammalian mutagenesis system essentially according to the manufacturer's protocol [10]. Oligonucleotides used are: TNSALP (A116T), 5'- CCCACACAGGTACGTAGTG-GCGGTGCC -3'; TNSALP (W) *his*-tag, 5'-GCAGCAAGGCTGCCTGCCTAGT-GATGGTGATGGTGATGGCTGGCAGGAGCACA-3'; TNSALP (W or A116T) *flag*-tag, 5'-GCAGCAAGGCTGCCTGCCTACTTATCGTCGTCATCCTTGTAATCGCTGGCAGGAGCACA-3'. The DNA sequence of the mutation sites was verified by DNA sequence analysis. The cDNA encoding TNSALP (W) or TNSALP (A116T) was further subcloned into pTRE2 to establish stable cell lines. Transfection and screening of stable cell lines were performed essentially according to the manufacturer's protocol. Tet-On cells, which successfully produced TNSALP (W) or the mutant TNSALP in the presence of doxycycline, but not in its absence, were identified using immunofluorescence. Established Tet-On cells were cultured and passaged in the absence of doxycycline until they were used for experiments. For immunoblotting or

immunofluorescence studies, the cells were cultured in the presence of 1 µg/ml doxycycline for 24 h before used. Alternatively, cells were cultured 0.2 µg/ml of doxycycline for 14 h before biosynthetic experiments. For transient expression, COS-1 cells [(1.0 -1.3) x 10⁵ cells/ 35-mm dish] were transfected with 0.8 µg of each plasmid using Lipofectamine Plus according to the manufacturer's protocol as described previously [10] and the transfected cells were incubated for 24 h in 5 % CO₂/95 % air incubator before use. COS-1 cells were cultured in Dulbecco's modified Eagle's minimum essential medium (DMEM) supplemented with 10% fetal bovine serum [10].

2.3. Metabolic labeling and immunoprecipitation

For pulse-chase experiments, cells were preincubated for 0.5 - 1 h in the methionine/cysteine-free DMEM and labeled with 50 - 100 µCi of [³⁵S] methionine/cysteine for 0.5 h in the fresh methionine/cysteine-free MEM. After a pulse period, cells were washed and chased in the DMEM as described previously [10]. After metabolic labeling, the medium was removed, and the cells were lysed in 0.5 ml of lysis buffer [1% (w/v) Triton X-100/0.5% (w/v) sodium deoxycholate/ 0.05% (w/v) SDS in PBS]. A protease inhibitors cocktail (antipain, aprotinin, chymostatin, elastatinal, leupeptin, pepstatin A) was added to cell lysates and media (10 µg each/ml). The lysates were incubated for 20 min at 37°C to extract TNSALP. The lysates and media were subjected to immunoisolation as described previously [10]. The immune complexes/ Protein A beads were boiled in the absence or presence of 1% (v/v) 2-mercaptoethanol, and then analyzed by SDS/PAGE [9% (w/v) gels], followed by fluorography [7]. For sequential purification of soluble forms of heteromeric TNSALPs, the medium was adjusted to 20 mM imidazole and incubated with Ni-chelate resin in the presence of 20 mM imidazole. After washing the resin with PBS containing 20 mM imidazole, TNSALP-*his* was released with PBS containing 200mM imidazole. This fraction was diluted 3-fold with PBS and mixed with anti-*flag* antibody beads. After washing with PBS containing 500 mM NaCl and then PBS, TNSALP-*flag* was released by boiling in the presence of SDS.

2.4. Miscellaneous procedures

Immunofluorescence for alkaline phosphatase was performed as described previously [9,10]. Electric transfer of proteins and subsequent procedures were described as before [19]. Proteins on membranes were detected with ECL[®] western blotting detection reagents. Cell surface biotinylation and PI-PLC digestion were carried out as described previously [8,10]. Sucrose-density-gradient centrifugation was performed as described previously [8].

Protein and alkaline phosphatase assays were performed as described previously [7,10]. 1 unit of alkaline phosphatase activity is defined as nmoles of *p*-nitrophenylphosphate hydrolyzed per min at 37°C.

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3. Results

3.1. Transient expression of TNSALP (A116T) in COS-1 cells

In order to define the molecular property of TNSALP (A116T), the wild type

enzyme and this mutant protein were expressed in COS-1 cells ectopically. Contrast to TNSALP (W), TNSALP (A116T) exhibited only negligible alkaline phosphatase activity as shown in Fig. 1A. In agreement with the previous report [17], when co-expressed with the wild enzyme, the activity of TNSALP (W) was slightly suppressed by TNSALP (A116T), indicative of a dominant negative effect of the latter (Fig. 1B). We have previously shown that the wild type enzyme is synthesized as a 66 kDa form with high mannose-type oligosaccharides and is converted to a 80 kDa mature form with complex type oligosaccharides as it migrates from the endoplasmic reticulum (ER) to the Golgi apparatus and finally is localized on the cell surface as a GPI-anchored protein [7,20]. Besides, these two molecular species are thought to exist as a non-covalently assembled homodimer at least in transfected COS-1 cells [8].

Transfected cells were continuously labeled with [³⁵S] methionine/ cysteine and then TNSALP was immunoprecipitated with an antibody against TNSALP, followed by SDS-PAGE under a reduced or non-reducing condition/ fluorography. Both the 66 kDa and 80 kDa forms were apparent in the cells expressing TNSALP (W) (Fig. 1, lane 1). Similarly, the 80 kDa mature form was also found in the cells expressing TNSALP (A116T) but not in the cells expressing TNSALP (D306V) (Fig. 1, lanes 2 and 3). The aspartic acid at position 306 is closely involved in the coordination of Ca²⁺ [21] and TNSALP (D306V) is retained in the endoplasmic reticulum (ER), followed by ubiquitination and degradation in proteasomes [10]. Under a non-reducing condition, a small amount of aggregates was detected even in the cell expressing TNSALP (W) (Fig. 1, lane 4), probably due to shortage of GPI precursors in transiently transfected cells [14]. On the other hand, larger amounts of the aggregate were found in the cells expressing TNSALP (A116T) or TNSALP (D306V) (Fig. 1, lanes 5 and 6).

3.2. Expression of TNSALP (A116T) in the Tet-On conditional expression system

To determine if the aggregation of TNSALP (A116T) is due to an excessive amount of this mutant generated in transiently transfected cells, we attempted to establish Tet-On CHO K1 cells expressing TNSALP (A116T) in response to doxycycline. Immunofluorescence pattern of Tet-On cells expressing TNSALP (A116T) was indistinguishable from that of the cells expressing TNSALP (W) (Fig 2A). Both TNSALP (W) and the TNSALP mutant were observed on the cell surface only in the presence, but not in the absence of the antibiotic (data not shown). In accordance with the result of transiently transfected cells, TNSALP (A116T) lacked the enzyme activity as shown in Fig. 2B. Next, the steady state expression level of TNSALP (A116T) was assessed by

immunblotting. No protein band was observed in the absence of doxycycline. Only the 80 kDa band was detected in the cells expressing TNSALP (W) irrelevant to a reducing or non-reducing conditions (Fig. 3A, lanes 2 and 6). In contrast, in addition to the 80 kDa band a large amount of disulfide-bonded aggregate appeared in the established cell expressing the mutant protein (Fig 3A. lanes, 4 and 8). The presence of the 66 kDa form in the cells expressing TNSALP (A116T) but not TNSALP (W) raises a possibility that some part of TNSALP (A116T) exits from the ER only at a reduced rate (Fig. 3A, lanes 4 and 8). To further examine the aggregation state of TNSALP (W) and TNSALP (A116T), we subjected each cell lysate to sucrose-density-gradient centrifugation and then the fractions were analyzed by SDS-PAGE under a non-reducing condition as shown in Fig. 3B. Most of TNSALP (W) was recovered in fractions 7 and 8, where alcohol dehydrogenase (141 kDa) also appeared, demonstrating that TNSALP (W) exists as a homo-dimeric enzyme in Tet-On CHO. As SDS breaks this homo-dimeric structure even in the absence of a reducing reagent, the dimer was not detected on the gel. Markedly, a considerable fraction of TNSALP (A116T) appeared with bovine serum albumin (68kDa) (Fig. 3B, fractions 5 and 6), strongly indicating that TNSALP (A116T) is incapable of assuming a correct tertiary structure and it tends to form disulfide-bonded aggregates with heterogeneous sizes, instead of a dimer.

3.3. Biosynthetic study of TNSALP (A116T) in the established cell line

To examine the biosynthesis of TNSALP (A116T) in detail, pulse-chase studies were carried out. The mutant protein is synthesized as the 66 kDa form (Fig 4, lane 1), however, the disulfide-bonded aggregate started to appear during a 30-min pulse labeling time (Fig 4, lanes 1 and 5) and was present throughout the chase periods (Fig 4, lanes 6-8). This indicates that part of the newly synthesized 66 kDa form of TNSALP (A116T) aggregates in the ER before it moves to the Golgi. At a 30 min chase period, the 80 kDa form was clearly detected under a reducing condition, while this mature form was less clear under a non-reducing condition (Fig 3, lanes 2 and 6), indicating that a considerable amount of the 80 kDa form is in an aggregate state in the established cell. Also, we noticed that newly synthesized TNSALP (A116T) was decreased in its total intensity during the experiment. Longer chase experiments suggest that TNSALP (A116T) is less stable than the wild type enzyme (Fig. 4B).

3.4. Cell surface appearance of disulfide-bonded aggregates of TNSALP (A116T)

The presence of the 80 kDa mature form in the aggregate raises a possibility that the high-molecular-mass aggregate of TNSALP (A116T) synthesized in the ER moves to the Golgi, acquires terminal sugar residues during passage across the Golgi stack and finally is conveyed to the cell surface. Next, we examined the molecular species of TNSALP (A116T) exposed on the cell surface. Biosynthetic studies in combination with cell surface biotinylation (Fig. 5A) or digestion with PI-PLC (Fig. 5 B) were performed. Not only the 80 kDa mature form, but also the aggregate on and near the top of the gel were reactive with a non-permeable biotinylation reagent. Furthermore, PI-PLC, which cleaves the GPI-anchor portion from the TNSALP molecule, rendered the aggregate as well as the 80 kDa form released into the medium, indicating that both molecular species reside on the cell surface via the GPI-anchor

3.5. TNSALP (W) and TNSALP (A116T) interact with each other

Dominantly transmitted TNSALP mutant protein including TNSALP (A116T) are known to suppress the catalytic activity of TNSALP (W) to various degrees when they are expressed together (Fig. 1A)[15-17]. However, direct association between TNSALP (W) and a dominant negative TNSALP mutant protein has not been demonstrated to date. To confirm if TNSALP (W) and TNSALP (A116T) interact with each other, we co-expressed secretory versions of TNSALP (W) and TNSALP (A116T), which are modified by either a C-terminal *his* or *flag* tags (Fig 6). TNSALP-*his* and TNSALP-*flag*, which possess each tag sequence instead of a C-terminal prosequence serving as a GPI-anchor signal sequence, are secreted into the medium as a dimeric enzyme [6,19]. The levels of TNSALP in the media were more or less similar whether TNSALP (W)-*his* was co-expressed with TNSALP (W)-*flag* or TNSALP (A116T)-*flag* as assessed by direct immunoprecipitation using anti-TNSALP (Fig. 6, lanes 1 and 2). However, the aggregate was observed only in the culture medium of the cells expressing TNSALP (W)-*his* and TNSALP (A116T)-*flag* (Fig. 6, lane 2), implying that the GPI-anchor portion of TNSALP (A116T) is not responsible for the formation of the aggregation. After being eluted with imidazole from the Ni-NTA agarose, the aggregate was no more evident (Fig. 5, lane 4). This is probably because TNSALP-*flag*/ TNSALP-*flag* is not absorbed to the resin among the three possible combinations [TNSALP (W)-*his*/ TNSALP (W)-*his*, TNSALP (W)-*his*/ TNSALP (A116T)-*flag*, TNSALP (A116T) -*flag* / TNSALP-*flag*], resulting in the lower ratio of TNSALP (A116T)-*flag* to TNSALP (W)-*his* in the eluate. By a subsequent immunoabsorption using the anti-*flag* antibody, both the 80 kDa form and the aggregate were captured (Fig. 6, lane 6), indicating that TNSALP (W) is able to interact with the 80 kDa form non-covalently and the aggregate form of TNSALP (A116T) covalently, respectively.

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4. Discussion

Based on 3D structure, TNSALP missense mutations associated with hypophosphatasia are classified as 1) active site or active site vicinity, 2) active site valley,

3) homo-dimer interface, 4) crown domain, 5) calcium site or calcium site vicinity and 6) others [21]. So far mutations with a dominant effect are restricted to three regions: active site, homo-dimer and the crown domain [5]. A116T is assigned to active site or active site vicinity, as serine at position 110 is an active center of TNSALP [21]. The patients heterozygous for TNSALP (A116T) displayed enamel hypoplasia and premature loss of fully rooted primary anterior teeth [18], corresponding to adult or odontohypophosphatasia. Familial pedigree analysis demonstrated that TNSALP (A116T) is dominantly transmitted [17,18]. Milder phenotypes are probably attributed to its weak dominant negative effect on the wild-type enzyme, as shown by co-expression study (Fig 1A) [17]. Initially we thought that TNSALP (W) and TNSALP (A116T) monomers form a simple non-covalently assembled hetero-dimer when they are co-expressed, however, their interaction turned out to be more complex.

To gain insight into the molecular mechanism of mild hypophosphatasia caused by this particular dominant negative mutation, we expressed TNSALP (A116T) as well as TNSALP (W) in COS-1 cells and Tet-On CHO K1 cells. Cell surface appearance of TNSALP (A116T) is indistinguishable from that of the wild enzyme as evaluated by immunofluorescence (Fig. 2). However, whether expressed in COS-1 cells transiently or Tet-On conditional expression system, TNSALP (A116T) does not exhibit measurable alkaline phosphatase activity. Besides, we found that TNSALP (A116T) tends to generate the disulfide-bonded high-molecular-mass aggregate in contrast to a non-covalently assembled dimer structure of TNSALP (W) (Fig3. A, B). The aggregate form of TNSALP (A116T) appeared in the cell within a 30-min pulse period, indicating that this aggregate is produced in the ER (Fig. 4). Surprisingly, the disulfide-bonded aggregate as well as the 66 kDa monomeric form of TNSALP (A116T) are transport-competent and appear on the cell surface as judged by cell surface biotinylation and PI-PLC digestion (Fig. 5).

To determine if TNSALP (A116T) is capable of interacting with TNSALP (W), we took an advantage that TNSALP (W) can be secreted as a homo-dimeric protein if its C-terminal prosequence serving as a GPI anchor signal is appropriately replaced with *his* or *flag*-tag [6,19]. Co-expression of TNSALP (W)-*his* and TNSALP (A116T)-*flag* in COS-1 cells allowed us to demonstrate that the wild and the mutant TNSALP interact with each other and are secreted into the medium (Fig. 6). Although the sequential affinity procedure is not quantitative, our finding strongly suggests that TNSALP (W) and TNSALP (A116T) form heterogeneous complexes and are anchored to the cell membrane via GPI in patients carrying this particular dominantly transmitted missense mutation.

Mornet's group has reported that dominant negative effects of TNSALP mutant proteins on the wild type enzyme vary from one mutation to another [5,17]. TNSALP

(D378V) and TNSALP (G63V) inhibit the enzyme activity far more strongly than TNSALP (A116T) does when co-expressed with TNSALP (W) [17]. On the other hand, TNSALP (G249V) exerts its dominant effect without suppressing the wild-type enzyme activity. TNSALP (G249V) likely sequesters the wild-type enzyme at the Golgi, thus blocking its cell surface appearance when co-expressed with the wild type [22]. In this study our results demonstrated that the substitution of alanine with threonine at position 116 of TNSALP abrogates its tertiary structure. TNSALP (A116T) exists as a monomeric polypeptide and its randomly cross-linked aggregates via disulfide bonds. Considering that its dominant negative effect is weak [17], it is likely that only a small portion of newly synthesized TNSALP (W) may be associated with TNSALP (A116T) and entrapped in the aggregate when co-expressed, while the rest of the TNSALP (W) is able to assemble into the native homo-dimeric structure with a full enzyme activity. This result, along with others, leads to a speculation that molecular mechanisms whereby mild hypophosphatasia exerts its dominant negative effects are highly variable.

Contrast to mild one with phenotypes of such as premature loss of deciduous teeth and pseudo-fracture, severe hypophosphatasia develops rickets or osteomalacia and sometimes hypercalcemia and vitamin B₆-dependent seizures [2-4]. The causative mutations for severe forms are transmitted in a recessive trait and also found to affect the TNSALP molecule to varying degrees, resulting in a complete loss or marked reduction of the catalytic function. Residual activities of these TNSALP mutants even exhibit different catalytic properties compared with the wild-type enzyme [6]. In addition, some TNSALP mutants showed folding and trafficking defects [7-12, 20, 23]. These broad effects of the mutations on the TNSALP molecule contribute the remarkably variable expressivity of this inborn error of metabolism. Obviously, more works will be necessary to be done until we will get a whole picture of molecular basis of hypophosphatasia.

There is no established medical treatment for hypophosphatasia. However, several attempts have recently been made to aim at developing the cure for this rare disease [24-26]. Especially, an enzyme replacement therapy using a soluble human recombinant TNSALP with a bone-targeted deca-aspartate motif is promising [24] and clinical trials are underway in Canada and U.S.A..

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ACCEPTED MANUSCRIPT

Legend to Figures

Fig. 1. Transient expression of TNSALP mutant proteins in COS-1 cells. (A) COS-1 cells were co-transfected with plasmids encoding TNSALP (W) or TNSALP (A116T) in various ratios (1: 0, 0.9: 0.1, 0.5: 0.5, 0.1: 0.9, 0: 1, X axis). The transfected cells were

homogenized and assayed for alkaline phosphatase activity (expressed in % wild enzyme, Y axis). The dotted line expects variation of the enzyme activity in the recessive model. The values are the means of two independent experiments. (B) The cells expressing TNSALP (W) (lanes 1 and 4), TNSALP (A116T) (lanes 2 and 5) or TNSALP (D306V) (lanes 3 and 6) were steadily labeled with [³⁵S] methionine/ cysteine for 3 h and lysed for immunoadsorption using anti-TNSALP antibody. Each immunoprecipitate was analyzed by SDS-PAGE under a reducing (lanes 1-3) or non-reducing condition (lanes 4-6), followed by fluorography. An arrowhead indicates the top of the resolving gel, while a double arrowhead indicates the top of the stacking gel. Left lane, ¹⁴C-methylated protein markers of 200, 97.5, 66, 46 kDa, from the top of the gel.

Fig. 2. Expression of TNSALP (W) and TNSALP (A116T) in established Tet-On cells. (A) Established cells harboring a pTRE2 encoding TNSALP (W) or TNSALP (A116T) were cultured for 24 h in the presence of doxycycline (1 µg/ml) and processed for immunofluorescence using anti-TNSALP serum. (B) Like in (A), the cells were induced to express TNSALP (W) or TNSALP (A116T). The cell homogenates were assayed for alkaline phosphatase activity. The values are the means of two independent experiments. The specific activity was expressed in unit mg⁻¹ protein. ND denotes not detected.

Fig.3. Molecular size of TNSALP (W) and TNSALP (A116T).

(A) The cells harboring pTRE2 encoding TNSALP (W) or TNSALP (A116T) were cultured for 24 h in the absence (lanes 1,3,5,7) or presence (lanes 2,4,6,8) of doxycycline (1 µg/ml) and then the cell homogenates (5 µg each) were analyzed by immunoblotting. An arrowhead indicates the top of the resolving gel. (B) The established cells were induced to express TNSALP (W) or TNSALP (A116T) like in (A). Each cell lysate was layered on the top of a linear sucrose gradient consisting of 5 % (w/w) sucrose and 35% (w/w) sucrose and centrifuged at 4°C for 18 h at 163,000g. Each 400 µl of fraction was collected from the top of the gradient (total 12 fractions). Each 20 µl of the fractions was subjected to SDS-PAGE under a non-reducing condition, followed by immunoblotting. Size marker: b (bovine serum albumin, 68 kDa, a (alcohol dehydrogenase, 141 kDa) and c (catalase, 250 kDa).

Fig. 4. Biosynthetic study of TNSALP (A116T). (A) The cells harboring pTRE2 encoding TNSALP (A116T) were cultured for 14 h in the presence of 0.2 µg/ml of doxycycline before experiments. The cells were pulse-labeled with [³⁵S] methionine/ [³⁵S]cysteine for 30 min (lanes 1, 5), and chased for 30 min (lanes 2 and 6), 1 h (lanes 3

and 7) and 2 h (lanes 4 and 8) as described in Materials and Methods. The immunoprecipitates were divided into two equal portions, followed by SDS-PAGE under reducing (lanes 1-4) or non-reducing condition (lanes 5-8) / fluorography. Left lane, the same ^{14}C -methylated protein markers as in Fig 1. (B) The cells harboring pTRE2 encoding TNSALP (W) or TNSALP (A116T) were cultured like in A. The cells were pulse-labeled for 30 min (lane 1 and 5) and chased for 2 h (lane 6), 4 h (lanes 2 and 7), 6 h (lanes 3 and 8) and 8 h (lanes 4 and 8). The immunoprecipitates were analyzed by SDS-PAGE under a reducing condition / fluorography. Left lane, the same ^{14}C -methylated protein markers as in Fig 1.

Fig. 5. Cell surface appearance of TNSALP (A116T). The cells harboring pTRE2 encoding TNSALP (A116T) were cultured for 14 h in the presence of 0.2 $\mu\text{g}/\text{ml}$ of doxycycline and subjected to cell surface biotinylation or PI-PLC digestion. (A) After being steadily labeled with [^{35}S] methionine/ cysteine for 3 h, the cells were washed and incubated with biotin succinimidylester on ice. The cell lysate was immunoprecipitated using anti -TNSALP antibody. The immune-complexes were divided into two equal parts: One part is directly analyzed by SDS-PAGE under a non-reducing condition/ fluorography as total TNSALP, while the other was boiled, diluted and further incubated with streptavidin beads before analysis (cell surface). Left lane, the same ^{14}C -methylated protein markers as in Fig. 1. An arrowhead indicates the top of the resolving gel. (B) The cells were steadily labeled like in (A) and further incubated in the absence (-) or presence (+) of PI-PLC in the DMEM. The cells and media were subjected to immunoprecipitation and then analyzed by SDS-PAGE under a non-reducing condition / fluorography. Left lane, the same ^{14}C -methylated protein markers as in Fig 1. An arrowhead indicates the top of the resolving gel.

Fig. 6. Interaction between TNSALP (W) and TNSALP (A116T). COS-1 cells were transfected with cDNAs coding TNSALP (W)-*his* and TNSALP (W)-*flag* (lanes 1, 3 and 5) or TNSALP (W)-*his* and TNSALP (A116T)-*flag* (lanes 2, 4 and 6). The cells were labeled with [^{35}S] methionine/ cysteine for 8 h. Each culture medium was collected and divided into three equal portions and was adsorbed with anti-TNSALP antibody (lanes 1 and 2), Ni-chelate beads (lanes 3 and 4) or Ni-chelate beads and subsequently with anti-*flag* antibody (lanes 5 and 6). Left lane, the same ^{14}C -methylated protein markers as in Fig 1. An arrowhead indicates the top of the resolving gel.

Figure 1 Ishida et al. Molecular characterization of tissue-nonspecific---

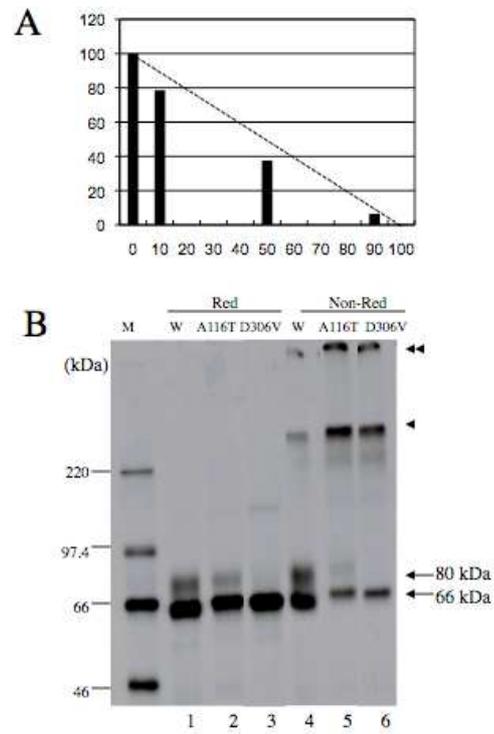


Figure 2 Ishida et al. Molecular characterization of tissue-nonspecific---

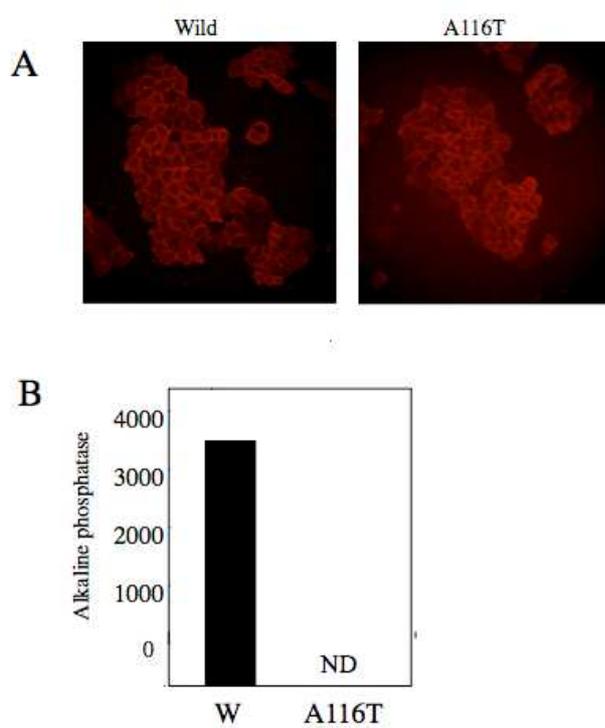
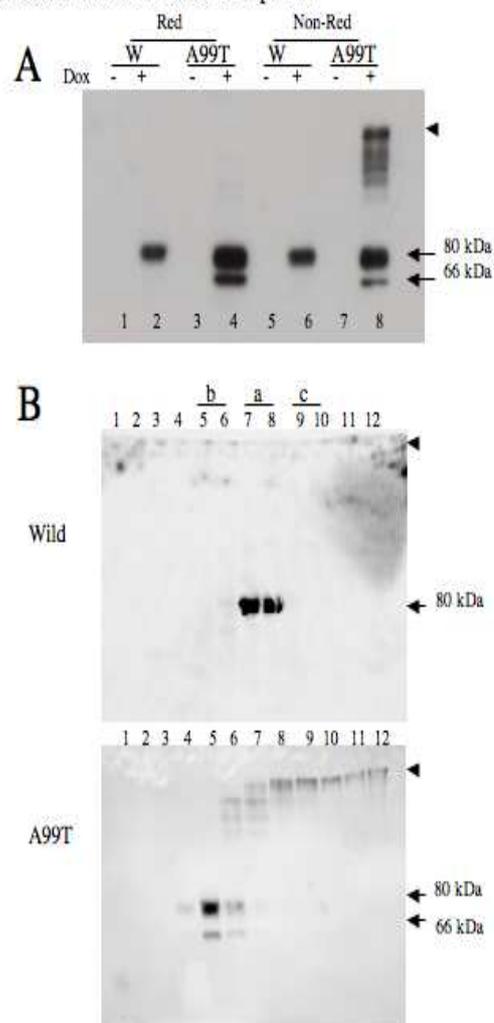
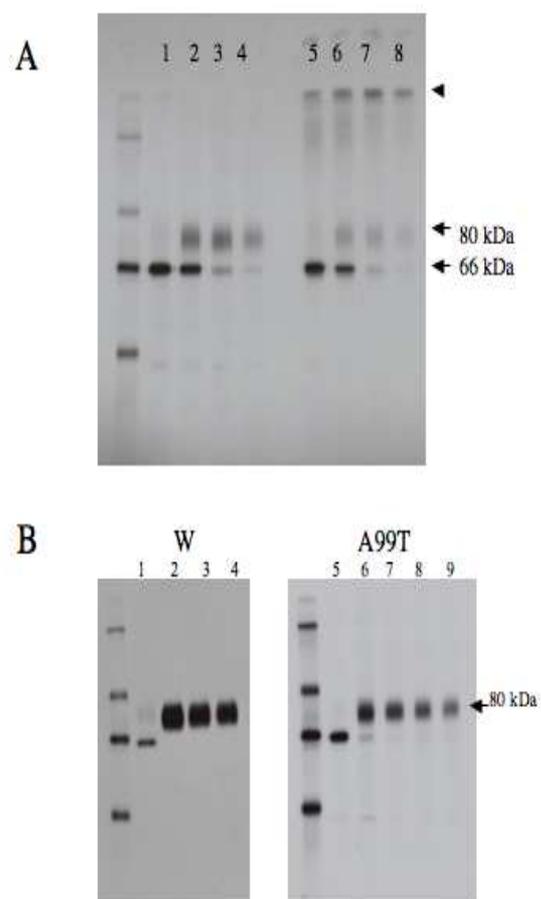


Figure 3 Ishida et al. Molecular characterization of tissue-nonspecific---



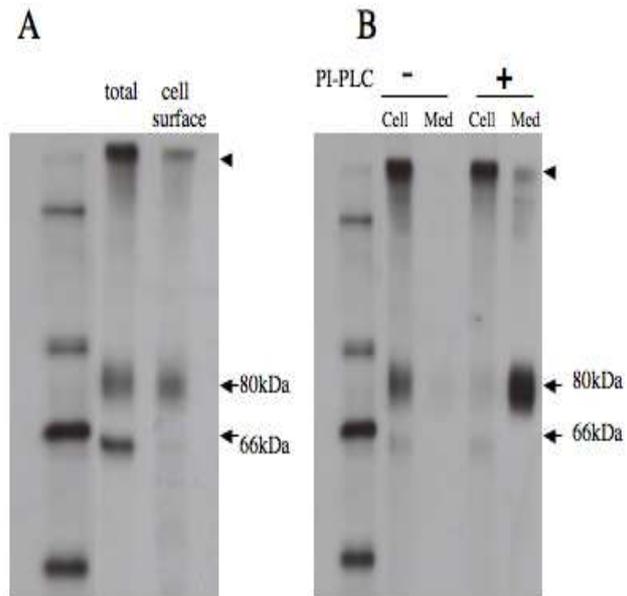
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Figure 4 Ishida et al. Molecular characterization of tissue-nonspecific---



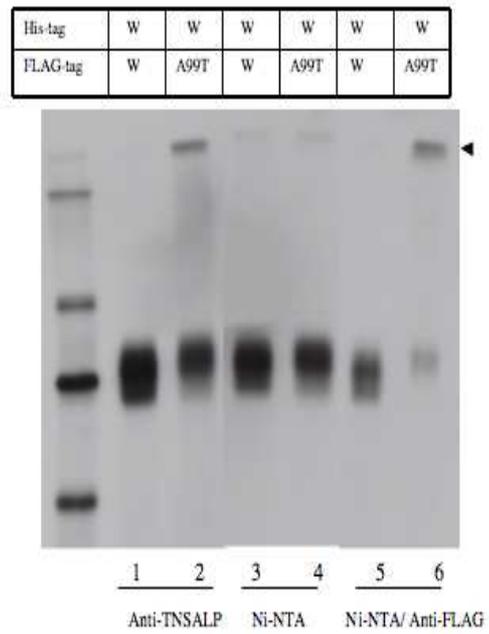
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Figure 5 Ishida et al. Molecular characterization of tissue-nonspecific---



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Figure 6 Ishida et al. Molecular characterization of tissue-nonspecific---



A

Research highlights (Y. Ishida et al.)

- TNSALP (W) monomer assembles into non-covalently associated homo-dimer.
- TNSALP (A99T) fails to form non-covalent homo-dimer.
- TNSALP (A99T) forms disulfide-bonded aggregates.
- The aggregate of TNSALP (A99T) traps TNSALP (A99T), suggestive of dominant negative inhibition.