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Tissue-Nonspecific Alkaline Phosphatase with an Asp^{289}→Val Mutation Fails to Reach the Cell Surface and Undergoes Proteasome-Mediated Degradation

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A missense mutation in the gene of tissue-nonspecific alkaline phosphatase, which replaces aspartic acid at position 289 with valine [TNSALP (D289V)], was reported in a lethal hypophosphatasia patient [Taillandier, A. *et al.* (1999) *Hum. Mut.* 13, 171–172]. To define the molecular defects of TNSALP (D289V), this mutant protein in transiently transfected COS-1 cells was analyzed biochemically and morphologically. TNSALP (D289V) exhibited no alkaline phosphatase activity and mainly formed a disulfide-linked high molecular mass aggregate. Cell-surface biotinylation, digestion with phosphatidylinositol-specific phospholipase C and an immunofluorescence study showed that the mutant protein failed to appear on the cell surface and was accumulated intracellularly. In agreement with this, pulse/chase experiments demonstrated that TNSALP (D289V) remained endo-β-*N*-acetyl- glucosaminidase H-sensitive throughout the chase and was eventually degraded, indicating that the mutant protein is unable to reach the medial-Golgi. Proteasome inhibitors strongly blocked the degradation of TNSALP (D289V), and furthermore the mutant protein was found to be ubiquitinated. Besides, another naturally occurring TNSALP with a Glu^{218}→Gly mutation was also found to be polyubiquitinated and degraded in the proteasome. Since the acidic amino acids at positions 218 and 289 of TNSALP are thought to be directly involved in the Ca^{2+} coordination, these results suggest the critical importance of calcium binding in post-translational folding and assembly of the TNSALP molecule.

Key words: alkaline phosphatase, calcium, hypophosphatasia, proteasome, ubiquitination.

Abbreviations: Bz-Asn-Gly-Thr-NH_2, benzoyl-asparagine-glycine-threonine-amide; DMEM, Dulbecco's modified Eagle's medium; ER, endoplasmic reticulum; ECL®, enhanced chemiluminescence (Amersham Pharmacia Biotech); GPI, glycosylphosphatidylinositol; HA, hemagglutinin of influenza virus; LLnL, *N*-acetyl-L-leucinyl-L-leucinyl-L-norleucinal; LLM, *N*-acetyl-L-leucinyl-L-leucinyl-L-methional; MG-132, benzyloxycarbonyl-L-leucinyl-L-leucinyl-L-leucinal; PI-PLC, phosphatidylinositol-specific phospholipase C; MEM, minimum essential medium; TNSALP, tissue-nonspecific alkaline phosphatase.

Tissue-nonspecific alkaline phosphatase (TNSALP) is an ectoenzyme anchored to the plasma membrane via glycosylphosphatidylinositol (GPI). Its physiological relevance to skeletal mineralization in humans has long been postulated (*1*, *2*), and, in particular, hypophosphatasia, an inborn error of bone metabolism caused by mutations on the TNSALP gene has established the involvement of this enzyme in the formation and maintenance of bone and teeth (*2–5*). Consistent with this, TNSALP-deficient mice have been reported to manifest phenotypes akin to those of patients associated with severe forms of hypophosphatasia (*6–8*). Inorganic pyrophosphate, phosphoethanolamine and pyridoxal-5′-phosphate are known to be elevated in the serum and urine of not only hypophosphatasia patients but also TNSALP knock-out mice, which indicates that these phospho-compounds are natural substrates of TNSALP. Among them, inorganic pyrophosphate has been suggested to control the mineralization process in bone (*9*), although the precise molecular role(s) of TNSALP has remained elusive. Quite recently, it became more evident that TNSALP regulates the formation of hydroxyapatite by hydrolyzing and consequently reducing the local concentration of inorganic pyrophosphate at the site of mineralization, at least in postnatal development (*10*).

Hypophosphatasia is classified into five categories; perinatal, infantile, childhood, adult and odonto hypophosphatasia, depending on the severity and age of onset (*3–5*). Recently, a three-dimensional structural model of human TNSALP was proposed on the basis of that of human placental alkaline phosphatase (*11*, *12*). According to this model, one of the features that differentiate

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alkaline phosphatase of mammals from that of *Escherichia coli* is the acquisition of a calcium binding site (fourth metal binding site) during evolution in addition to two zinc and one magnesium binding sites indispensable for alkaline phosphatase activity. However, the physiological importance of this calcium binding site of TNSALP remains obscure. TNSALP (D289V) has been reported in a patient diagnosed as having perinatal hypophosphatasia, who was homozygous for this disease-causing allele (*13*). A carboxyl group of aspartic acid at position 289 is involved directly in the Ca^{2+} coordination, thus raising the possibility that the substitution of aspartic acid with valine would prevent TNSALP from binding a calcium atom (*11*). However, little is known about the exact effects of this missense mutation on the properties of the TNSALP molecule that lead to the manifestation of severe hypophosphatasia. In this study we attempted to define the molecular defects of TNSALP (D289V), and showed that this mutation causes aggregation and impaired trafficking of TNSALP, culminating in its degradation in the proteasome as part of the ER-quality-control system. Furthermore, we demonstrated that TNSALP (D289V) is heavily ubiquitinated prior to degradation in the proteasome.

MATERIALS AND METHODS

Materials—Express^{35}S^{35}S protein labeling mix (>1,000 Ci/mmol) was obtained from Dupont-New England Nuclear (Boston, MA, USA), ^{14}C-methylated proteins, 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); an Altered Sites® II *in vitro* mutagenesis system, a T_NT®T7 coupled reticulocyte lysate system and canine pancreatic microsomal membranes from Promega (Madison, WI, USA); Lipofectamine Plus Reagent from Gibco-BRL (Gaithersburg, MD, USA); Bz-Asn-Gly-Thr-NH_2 (benzoyl-asparagine-glycine-threonine-amide) from BACHEM AG (Bubendorf, Switzerland); LLnL (*N*-acetyl-L-leucinyl-L-leucinyl-L-norleucinal), LLM (*N*-acetyl-L-leucinyl-L-leucinyl-L-methional), aprotinin and saponin (Quillaja bark) from Sigma Chemical (St. Louis, MO, USA); antipain, chymostatin, elastatinal, leupeptin, pepstatin A and MG-132 (benzyloxycarbonyl-L-leucinyl-L-leucinyl-L-leucinal) from Protein Research Foundation (Osaka); sulphosuccinimidyl N-(D-biotinyl)-6-aminohexane from Dojindo Laboratories (Kumamoto); anti-HA (a YPYDVPDYA epitope from hemagglutinin of influenza virus) antibodies from BAbCO (Richmond, CA, USA); anti-multi ubiquitin monoclonal antibodies from Medical & Biological Laboratories (Nagoya); peroxidase-conjugated goat anti-(mouse IgG) from Molecular Probes (Eugene, OR, USA); rhodamine-conjugated goat anti-(rabbit IgG) from Cappel Laboratories (Malvern, PA, USA); endo-β-*N*-acetylglucosaminidase H (*Streptomyces griseus*; Endo H) from Seikagaku Kogyo (Tokyo); and phosphatidylinositol-specific phospholipase C (PI-PLC) from Funakoshi (Tokyo). Antiserum against recombinant human TNSALP was raised in rabbits as described previously (*18*). COS-1 cells were cultured in Dulbecco's modified Eagle's minimum essential medium (DMEM) supplemented with 10% fetal bovine serum (*14–16*). MG-132, LLnL and LLM were dissolved in DMSO (50 mM stock solution), and stored at –20°C. Bz-Asn-Gly-Thr-NH_2 was dissolved in H_2O (25 mM stock solution) and stored at –20°C.

Plasmids and Transfection—To create a pALTER-MAX vector encoding TNSALP (D289V), site-directed mutation was performed using the Altered Sites®II *in vitro* mutagenesis system essentially according to the manufacturer's protocol as described previously (*17*). The oligonucleotide used was 5′-TCGGAGAGTGACGGTACCGTCACGTTGTT-3′. A plasmid encoding the wild-type TNSALP or TNSALP (E218G) was constructed as described previously (*14*). A plasmid encoding influenza hemagglutinin (HA)-tagged ubiquitin was provided by Dr. Dirk Bohmann (EMBL, Heidelberg, Germany). Cells [$(1.0–1.3) \times 10^5$ cells/35-mm dish] were transfected with 0.8–1 μg of each plasmid using Lipofectamine Plus according to the manufacturer's protocol as described previously (*16*, *17*) and the transfected cells were incubated for 24 h in a 5% CO_2/95% air incubator before use.

In Vitro Transcription/Translation—Transcription-coupled translation was performed using the T_NT®T7 coupled reticulocyte lysate system essentially according to the manufacturer's protocol. Transcription/translation was carried out with [^{35}S]methionine/cysteine at 30°C for 90 min in the absence or presence of canine pancreatic microsomal membranes. Bz-Asn-Gly-Thr-NH_2 was added at the start of the incubation to a final concentration of 0.5 mM.

Metabolic Labeling and Immunoprecipitation—Pulse-chase experiments were performed as described previously (*16*). When cells were incubated in the presence of various proteasome inhibitors, the inhibitors were included throughout the experiment (at the final concentration of 50 μM). Lysis of the cells and the immunoprecipitation procedure were carried out essentially as described previously (*13*). For Endo H digestion, immune-complexes collected on Protein A beads were divided into two equal parts, and then incubated in the absence or presence of Endo H for 16 h at 37°C as described previously (*16*). The immunoprecipitates were analyzed by SDS/PAGE [9% (w/v) gels], followed by fluorography as described previously (*16*).

Miscellaneous Procedures—Cytohistochemical staining for alkaline phosphatase and immunofluorescence observation were performed as described previously (*16*). Electric transfer of proteins and subsequent procedures were performed as before (*16*). Proteins on membranes were detected with the ECL® Western blotting detection reagent. Cell-surface biotinylation was performed as described previously (*14*, *16*). Protein and alkaline phosphatase assays involving *p*-nitrophenylphosphate as the substrate were performed as described previously (*16*).

RESULTS

Alkaline Phosphatase Activity of TNSALP (D289V)—TNSALP (D289V) has been reported in a homozygous patient diagnosed as having perinatal hypophosphatasia (*13*). Previously we demonstrated that several TNSALP missense mutations associated with perinatal hypophosphatasia almost completely abolished alkaline phos-

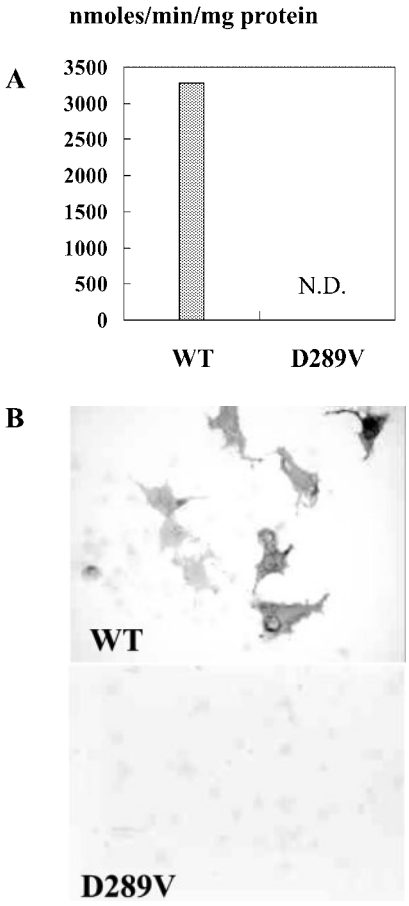

Fig. 1. **Alkaline phosphatase activity of cells expressing TNSALP (D289V).** (A) COS-1 cells expressing the wild-type TNSALP (WT) or TNSALP (D289V) were homogenized and then assayed for alkaline phosphatase activity using *p*-nitrophenylphosphate as the substrate. N.D., not detectable. (B) The transfected cells were stained for alkaline phosphatase and further counterstained with Methyl Green.

phatase activity (*14–17*). To determine if TNSALP (D289V) possesses this enzyme activity, cells ectopically expressing TNSALP (D289V) were assayed for alkaline phosphatase. As shown in Figure 1A, TNSALP (D289V) showed no enzyme activity. In agreement with this, cytohistochemical experiments demonstrated virtually no staining in the cells expressing the mutant protein (Fig. 1B).

In Vitro Translation of TNSALP (D289V)—To determine if this missense mutation affects the overall translation and translocation across the microsomal membrane, *in vitro* translation of TNSALP (D289V) was performed in the absence or presence of microsomes (Fig. 2). A 57-kDa form was a primary translation product of the wild-type enzyme and was converted to a 66-kDa form in the presence of microsomes. The change in the molecular mass is ascribed to *N*-glycosylation, since the 66-kDa band decreased in the presence of an inhibitor of *N*-glycosylation, Bz-Asn-Gly-Thr-NH_2. Previously we reported that TNSALP has at least three *N*-linked oligosaccharide chains at five potential *N*-glycosylation sites when expressed in COS-1 cells (*14*). TNSALP (D289V) was found to be translocated into microsomes to

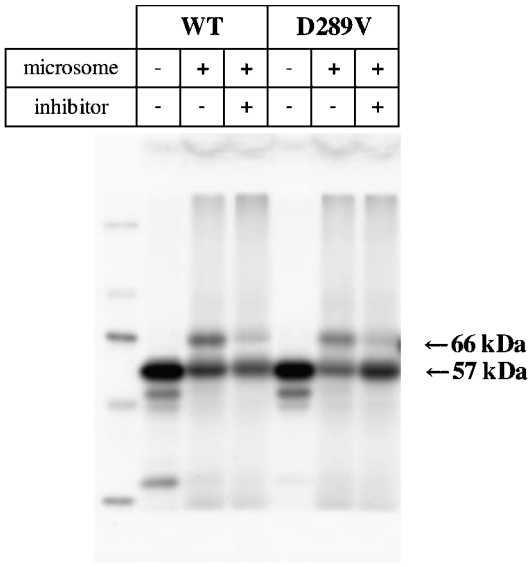

Fig. 2. ***In vitro* translation and translocation.** A plasmid encoding the wild-type TNSALP or TNSALP (D289V) was translated in the absence or presence of canine microsomes with or without an *N*-glycosylation inhibitor (Bz-Asn-Gly-Thr-NH_2). Aliquots of each reaction mixture were analyzed directly by SDS/PAGE/fluorography. Leftmost lane, ^{14}C-methylated protein markers: 200, 97.4, 66, and 46 kDa, from the top of the gel.

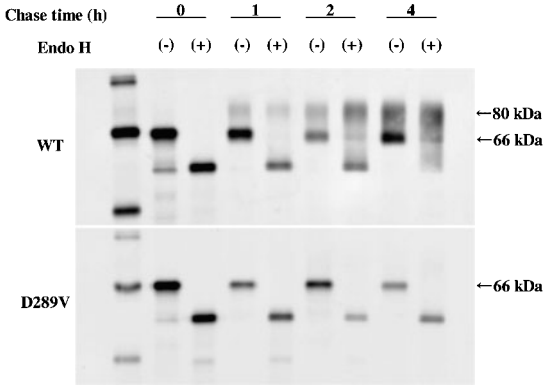

Fig. 3. **Pulse/chase experiments with Endo-H digestion.** Cells expressing the wild-type TNSALP (WT) or TNSALP (D289V) were pulse-labeled with 50 μCi of [^{35}S]methionine/cysteine for 0.5 h, and then chased for 1 h, 2 h, and 4 h. Cell lysates were subjected to immunoprecipitation and the immune complexes were incubated in the absence (–) or presence (+) of Endo H. The samples were analyzed by SDS/PAGE/fluorography. Leftmost lane, ^{14}C-methylated protein markers: 97.4, 66, and 46 kDa, from the top of the gel.

a similar extent to the wild type, as judged from the ratio of the 66-kDa to the 57-kDa form.

Pulse-Chase Experiment on TNSALP (D289V)—The TNSALP (D289V) mutant has a hydrophobic residue substituted for an acidic hydrophilic one, which is needed for Ca^{2+} coordination (*11*). This mutation could easily disrupt the tertiary and quaternary structures of TNSALP. To determine if this replacement at position 289 of TNSALP affects the post-translational folding and subsequent intracellular transport of the mutant protein, pulse-chase experiments were carried out on transfected cells (Fig. 3). Following the pulse, the 66-kDa form was

detected within the cells expressing the wild type. As chase proceeded, it became a 80-kDa Endo H-resistant form. In contrast, the mutant protein remained Endo H-sensitive throughout the experiment. Since *N*-linked oligosaccharides of glycoproteins become Endo H-resistant in the medial-Golgi, this result indicates that in contrast to the wild type, TNSALP (D289V) fails to move beyond the *cis*-Golgi along the secretory pathway.

Cell-Surface Appearance of TNSALP (D289V)—After being properly trimmed and terminally glycosylated through the Golgi apparatus, the wild-type TNSALP finally reaches the plasma membrane and is exposed on the cell surface via a GPI anchor (*14*). To determine if the mutant protein is localized to the cell surface, cell-surface biotinylation was performed. Only the 80-kDa form was strongly stained with a biotinylation reagent in the cells expressing the wild type (Fig. 4A), indicating that the 80-kDa form, but not the 66-kDa one, is expressed on the cell surface. In contrast, no band was detected for the cells expressing the mutant protein, demonstrating that the mutant protein fails to reach the cell surface. In support of this, when the cells were incubated with PI-PLC, which causes TNSALP to be released from the cell surface through hydrolysis between the glycan moiety and phosphatidylinositol of GPI (*19*), the 80-kDa wild type was found in the medium of the cells expressing the wild type, while no band was detected for the medium of those expressing the mutant enzyme (Fig. 4B). Since [^{3}H]ethanolamine, a component of GPI, was found to be incorporated into both the wild-type and mutant TNSALPs, as in the cases of other mutant proteins analyzed so far (*14-17*), it is unlikely that the decreased cell-surface expression is due to a defect of GPI-anchoring of the mutant protein (data not shown).

Consistent with the above results, no visible staining was detected on the surface of the cells expressing the mutant protein on immunofluorescence microscopy, as illustrated in Fig. 5. Instead, TNSALP (D289V) was found to be localized in a reticular structure and/or perinuclear structures resembling the ER and Golgi apparatus, respectively.

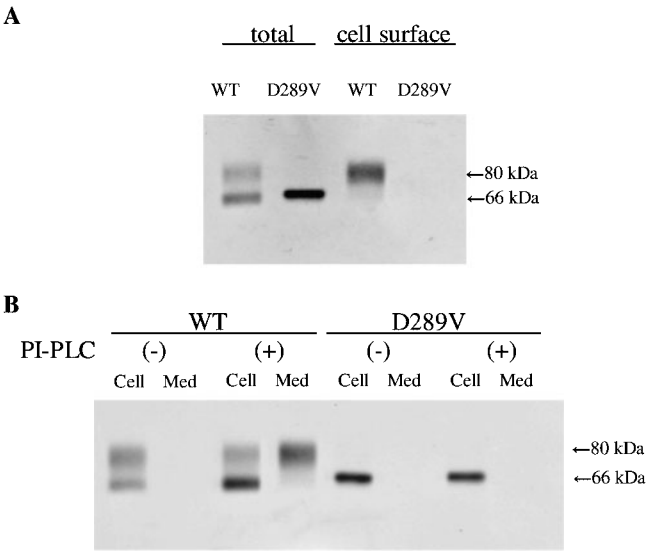

Fig. 4. **Cell surface appearance of TNSALP (D289V).** Cells expressing the wild-type TNSALP (WT) or TNSALP (D289V) were metabolically labeled with [^{35}S]methionine/cysteine for 6 h. (A) The cells were further incubated with biotin succinimidylester on ice. The cells were subjected to immunoprecipitation. The immune complexes were divided into two equal parts: one part was directly analyzed by SDS/PAGE as total TNSALP, while the other was boiled and further incubated with streptavidin beads before analysis (cell surface TNSALP). (B) Cells were incubated in the absence (–) or presence of PI-PLC in a CO_2 incubator for 1 h. The cells and medium were subjected to immunoisolation and then analyzed by SDS/PAGE/fluorography.

Aggregation, Ubiquitination and Degradation of TNSALP (D289V)—As reported previously, several severe alleles, such as TNSALP (R54C), TNSALP (N153D), TNSALP (A162T), and TNSALP (G317D), tend to form aberrant disulfide-bonded high-molecular-mass aggre-

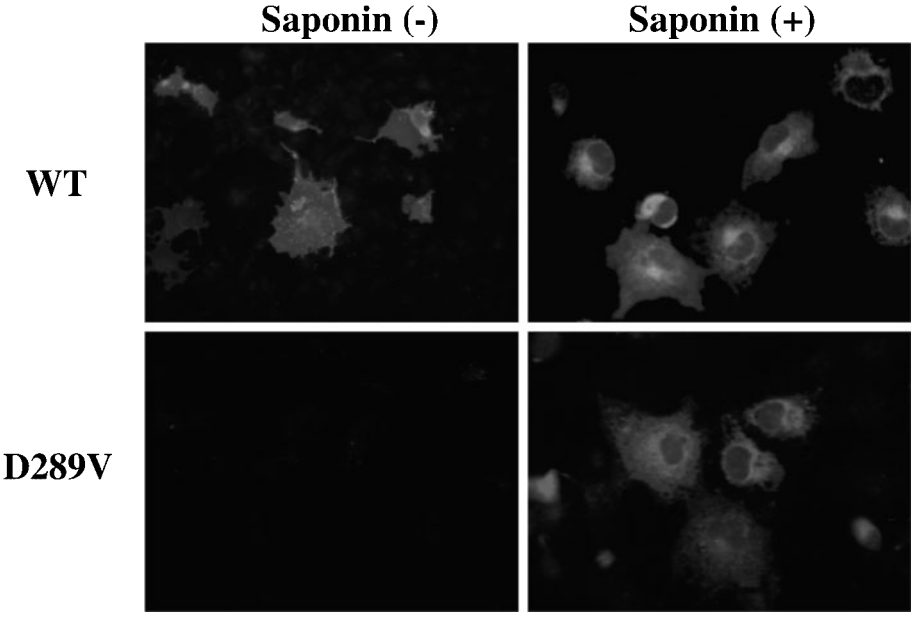

Fig. 5. **Immunofluorescence detection of TNSALP (D289V).** Cells expressing the wild-type TNSALP (WT) or TNSALP (D289V) were fixed and further incubated in the absence or presence of 0.1% (w/v) saponin in phosphate-buffered saline for 20 min on ice, and then stained by means of an indirect immunofluorescence technique.

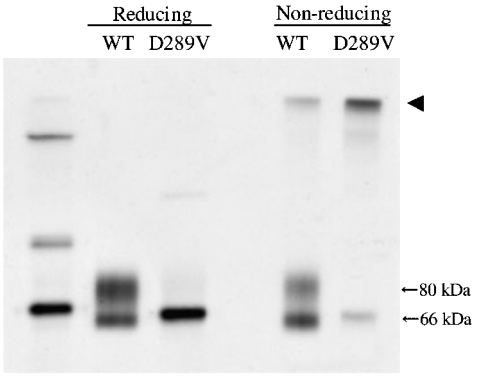

Fig. 6. **SDS/PAGE with or without a reducing agent.** Cells expressing the wild-type TNSALP (WT) or TNSALP (D289V) were metabolically labeled with [^{35}S]methionine/cysteine for 3 h. Cell lysates were subjected to immunoprecipitation and immune complexes were analyzed by SDS/PAGE/fluorography under reducing or non-reducing conditions. Leftmost lane, the same ^{14}C-methylated protein markers as in Fig. 2. The arrowhead indicates the top of the resolving gel.

gates and are subject to degradation in transfected cells (*14–17*). To determine if TNSALP (D289V) undergoes aggregate formation, the mutant protein molecules expressed in transfected cells were immunoprecipitated and analyzed by SDS/PAGE under reducing or non-reducing conditions (Fig. 6). A large fraction of the newly synthesized TNSALP (D289V) was found to form inter-chain high-molecular-mass aggregates on the top of the resolving gel under non-reducing conditions, suggesting improper folding and incorrectly assembly of the mutant protein molecule. On the other hand, the wild-type TNSALP was largely present as a homodimer in transfected COS-1 cells (*16*), and migrated as the 66-kDa or 80-kDa monomer on an SDS-gel, irrespective of the absence or presence of a reducing reagent.

Figure 7 shows that TNSALP (D289V) was significantly degraded at 6 h-chase time, and furthermore this degradation was strongly blocked by proteasome inhibitors (LLnL and MG-132), but not calpain (LLM) or lysosomal protease inhibitors (leupeptin + pepstatin A), which indicates the involvement of the proteasome. Next, we examined if TNSALP (D289V) is ubiquitinated prior to degradation. The plasmid encoding TNSALP (D289V) was transfected into COS-1 cells with or without the

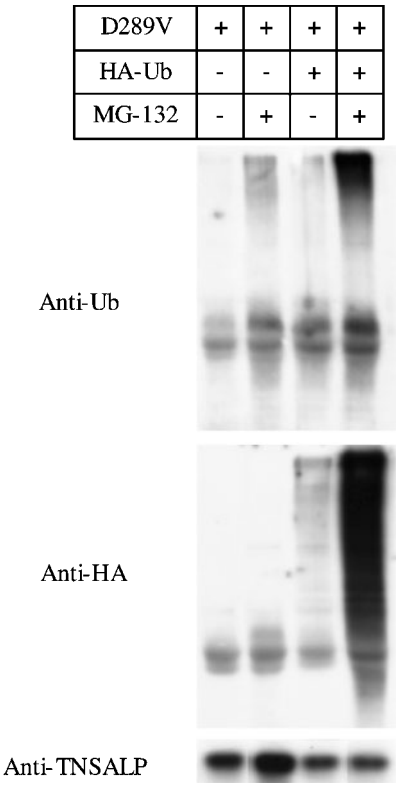

Fig. 8. **Ubiquitination of TNSALP (D289V).** Cells were transfected with a plasmid encoding TNSALP (D289V) only or in combination with a plasmid encoding HA-ubiquitin. After cells had been incubated for 6 h in the absence or presence of 50 μM MG-132, they were homogenized in 50 mM Tris/HCl (pH 7.5). One tenth of each cell homogenate was directly separated by SDS/PAGE and analyzed using anti-TNSALP antibodies. The rest of the homogenate was lysed and subjected to immunoprecipitation with anti-TNSALP, and then the immune complexes were analyzed by immunoblotting using anti-ubiquitin antibodies or anti-HA antibodies.

plasmid encoding ubiquitin tagged with N-terminal HA (hemaggulutinin of influenza virus). The mutant protein molecules were firstly immunoprecipitated with anti-TNSALP and secondly the immunoprecipitate was detected by immunoblotting using anti-ubiquitin antibodies or anti-HA antibodies, as shown in Fig. 8. High-molecular-mass bands corresponding to polyubiquitinated TNSALP (D289V) appeared on the blot of the

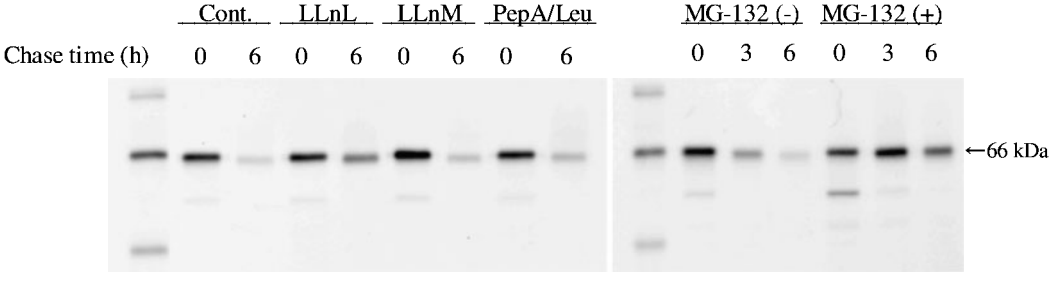

Fig. 7. **Degradation of TNSALP (D289V).** Cells expressing TNSALP (D289V) were pulse-labeled with [^{35}S]methionine/cysteine for 0.5 h, and then chased for 3 and/or 6 h in the absence or presence of 50 μM LLnL, 50 μM LLM, 100 μg/ml of pepstatin A(PepA)/leupeptin (Leu), or 50 μM MG-132. Cell lysates were subjected to immunoprecipitation and the immune complexes were analyzed by SDS/PAGE/fluorography. Leftmost lane, the same ^{14}C-methylated protein markers as in Fig. 3.

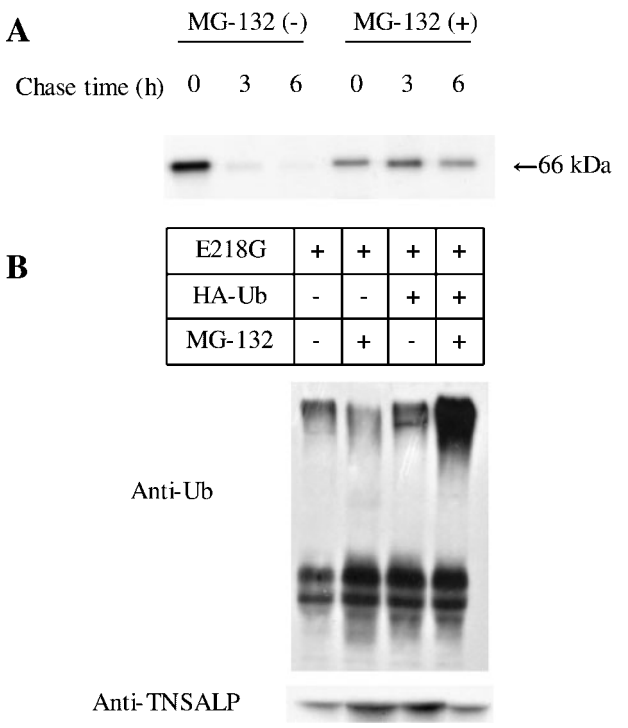

Fig. 9. **Ubiquitination and degradation of TNSALP (E218G).** (A) Cells expressing TNSALP (E218G) were pulse-labeled with [^{35}S]methionine/cysteine for 0.5 h, and then chased for 3 and/or 6 h in the absence or presence of 50 μM MG-132. Cell lysates were subjected to immunoprecipitation and the immune complexes were analyzed by SDS/PAGE/fluorography. Leftmost lane, the same ^{14}C-methylated protein markers as in Fig. 3. (B) Cells were transfected with a plasmid encoding TNSALP (E218G) with or without a plasmid encoding HA-ubiquitin. After the transfected cells had been incubated for 6 h in the absence or presence of 50 μM MG-132, they were homogenized in 50 mM Tris/HCl (pH 7.5). One tenth of each cell homogenate was directly separated by SDS/PAGE and analyzed using anti-TNSALP antibodies. The rest of the homogenate was lysed and subjected to immunoprecipitation with anti-TNSALP, and then the immune complexes were analyzed by immunoblotting using anti-ubiquitin antibodies.

mutant protein TNSALP when its degradation was blocked with MG-132. Furthermore, stronger ubiquitination signals were observed for the cells expressing both the mutant protein TNSALP and HA-ubiquitin. Again, ubiquitination was augmented in the MG-132–treated cells, indicating that the polyubiquitinated mutant protein was accumulated under the conditions where cellular proteasome activity was suppressed. Since the wild-type enzyme was negligibly ubiquitinated (data not shown), it is likely that TNSALP (D289V), but not the wild type, is inspected by the ER quality-control system and ubiquitinated prior to destruction by the 26S proteasome.

Ubiquitination and Degradation of TNSALP (E218G)—TNSALP (E218G) was previously characterized as an artificial mutant protein (*14*), since the original human TNSALP cDNA clone happened to code this particular mutant protein, probably due to an error in the process of reverse transcription (*20*, *21*). This mutant protein mimics TNSALP (D289V), as follows: firstly, the mutant protein exhibited no enzyme activity when expressed in COS-1 cells. Secondly, the mutant protein failed to reach the cell surface and was accumulated in the ER. Thirdly, the mutant protein formed a disulfide-bonded high-molecular-mass aggregate in the transfected cells. Fourthly, the mutant protein never acquired Endo H-resistance and was degraded intracellularly (*14*). However, involvement of the proteasome in the degradation of TNSALP (E218G) remained unexamined. Recently, this mutant protein has been detected as a naturally occurring one in a patient diagnosed as having adult hypophosphatasia with compound heterozygosity (E218G/A382S) (*22*). Together with the knowledge that this mutation also maps to the calcium binding site of TNSALP (*11*), the apparent resemblance of the molecular phenotypes between the two mutant proteins prompted us to examine the degradation of TNSALP (E218G). Figure 9 clearly illustrates that MG-132 inhibited the degradation of TNSALP (E218G), and caused marked accumulation of a ubiquitinated form in the cells coexpressing both the mutant protein and HA-ubiquitin.

DISCUSSION

A computer-assisted three-dimensional structural model of TNSALP, based on the results of an X-ray crystalographic study of placental alkaline phosphatase (which is 74% homologous to TNSALP), allowed us to assign various missense mutations to different functional domains of TNSALP including a putative calcium binding site (*11*). The calcium atom in TNSALP is assumed to be coordinated by four amino acids residues (Glu218, Phe273, Glu274, and Asp289) and a water molecule. Missense mutations at these specific positions in TNSALP could abolish calcium binding. TNSALP (D289V) has been reported in a homozygote patient diagnosed as having perinatal hypophosphatasia (*13*). We have demonstrated for the first time that loss of calcium binding potency has a deleterious effect on biosynthesis of the TNSALP molecule. When expressed in a heterologous mammalian expression system, TNSALP (D289V) exhibited no alkaline phosphatase activity. Since *in vitro* translation/translocation experiments showed that both the wild-type enzyme and mutant protein were core-glycosylated to similar extents in the presence of microsomes, this particular mutation does not seem to affect the overall translation/translocation efficiency across the ER, which suggests post-translational defects of the mutant protein. Pulse-chase experiments confirmed this. In contrast to the wild type, the mutant protein remained Endo H-sensitive throughout the chase, indicating that the mutant protein never reaches the *medial*-Golgi, where glycoproteins undergo oligosaccharide processing and acquire Endo H-resistance. In agreement with this result, the mutant protein never appeared on the cell surface, as evidenced by cell-surface biotinylation, digestion with PI-PLC and immunofluorescence observation. Instead, the mutant protein was mostly localized in the ER, and also immunostaining was low but significantly concentrated in the juxtanuclear region. Considering its sensitivity to Endo H, it is likely that some mutant molecules could reach the *cis*-Golgi. Since TNSALP (D289V), but not the wild type, was found to form a high-molecular-mass

aggregate, improper folding and resultant aggregation of newly synthesized polypeptides through multiple disulfide-bonding is likely to be the cause of the defective trafficking of this mutant protein. It is also likely that these aberrant molecules were scrutinized by the ER-quality-control system and then subjected to degradation. We showed that the degradation of TNSALP (D289V) was insensitive to calpain (LLM) and lysosomal protease inhibitors (leupeptin and pepstatin A), but sensitive to proteasome inhibitors (LLnL, MG-132), indicating involvement of the proteasome. Furthermore, TNSALP (D289V) was found to be polyubiquitinated in MG-132-treated cells, suggesting that TNSALP (D289V) was ubiquitinated prior to degradation in the proteasome.

TNSALP (E218G) is another naturally occurring missense mutant protein as to the calcium binding site. The substitution of glutamic acid at position 218 with glycine results in the loss of carboxylic acid needed for calcium binding in TNSALP. Our previous and present studies demonstrated that the molecular phenotypes of TNSALP (E218G) quite resemble those of TNSALP (D289V): lack of catalytic activity, formation of aggregate, failure of acquisition of Endo H resistance, ubiquitination and degradation. Thus, these two natural mutations occurring at specific amino acid residues involved directly in Ca^{2+} coordination represent severe alleles, therefore strongly arguing that calcium binding is crucial for the proper folding and correct assembly of a newly synthesized TNSALP molecule. Since the patient (E218G/A382S) manifested mild hypophosphatasia (*22*), TNSALP (E218G) is probably transmitted as a recessive trait, as in the case of TNSALP (D289V), and TNSALP (A382S) masks this severe allele (*11*).

It is worth noting that not only TNSALP (D289V) and TNSALP (E218G), but also other severe forms of TNSALP (R54C, N153D, A162T, and G317D), whose mutations are mapped to different domains of the TNSALP molecule such as the active site and homodimer interface, manifested similar defective folding and resultant aggregation (*14–17*). Thus, so far, severe forms of hypophosphatasia can be classified as folding diseases. However, it remains to be determined if the TNSALP mutant proteins form such high-molecular-mass aggregates in the cells of hypophosphatasia patients. Instead of transiently transfected cells, in which a certain expressed protein is supposed to be produced in an excessive amount, we have attempted to establish cells that stably express the TNSALP mutant proteins, but have been unsuccessful so far, probably due to their cytotoxity. Interestingly, Mauro *et al*. recently showed that several mutant proteins including TNSALP (R54C), TNSALP (A162T), and TNSALP (G317D) were secreted as dimeric forms into the medium when expressed as GPI-anchor-free enzymes (*23*), raising the possibility that the GPI-anchor is responsible for the aggregation and entrapment of the mutant proteins in the early stage of the secretory pathway. If calcium-binding defective mutant proteins can also be expressed and purified as soluble enzymes, they will be useful for addressing the biological function of the calcium binding site of TNSALP.

There is increasing evidence that many misfolded proteins and incorrectly assembled ones are retained in the ER or moved from the *cis*-Golgi to the ER as part of the ER quality control system, thus permitting only properly folded and assembled proteins to move to their final destinations (*24–26*). Aberrant proteins are often dislocated or retro-translocated into the cytosol and ubiquitinated before being destroyed in the proteasome (*26*). Several ubiquitin ligases were recently identified as crucial factors for ER-associated degradation (*27–29*). To the best of our knowledge, our results constitute the first evidence that TNSALP mutant proteins can be polyubiquitinated prior to their degradation in the proteasome. However, the cellular mechanism by which how the mutant TNSALP proteins are scrutinized by the ER-quality-control system, ubiquitinated and degraded in the proteasome remain largely unknown. Whether or not high-molecular-mass aggregates of mutant TNSALP proteins become polyubiquitinated is an issue that is currently under investigation. We are also seeking an E3 ubiquitin ligase that contributes to the degradation of TNSALP mutant proteins.

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REFERENCES

1. Robison, J.C. (1923) The possible significance of hexosephosphoric esters in ossification. *Biochem. J.* **17**, 286–293
2. Harris, H. (1989) The human alkaline phosphatases: what we know and what we don't know. *Clin. Chim. Acta.* **186**, 133–150
3. Rathbun, J.C. (1948) Hypophosphatasia: a new developmental anomaly. *Am J. Dis. Child* **75**, 822–831
4. Whyte, M.P. (2001) Hypophosphatasia in *The Metabolic and Molecular Basis of Inherited Disease* (Scriver, C.R., Beaudet, A.L., Sly, W.S., Valle, D., Childs, B., Kinzler, K.W., and Vogelstein, B., eds.) 8th ed., Vol. **4**, pp. 5313–5329, McGraw-Hill, New York, NY
5. Mornet, E. (2000) Hypophosphatasia: The mutations in the tissue-nonspecific alkaline phosphatase gene. *Hum. Mutat.* **15**, 309–315
6. Waymire, K.G., Mahuren, J.D., Jaje, J.M., Guilarte, T.R., Coburn, S.P., and Macgregor, G.R. (1995) Mice lacking tissue non-specific alkaline phosphatase die from seizures due to defective metabolism of vitamin B-6. *Nat. Genet.* **11**, 45–51
7. Narisawa, S., Frohlander, N., and Millán, J.L. (1997) Inactivation of two mouse alkaline phosphatase genes and establishment of a model of infantile hypophosphatasia. *Dev. Dyn.* **208**, 432–446
8. Fedde, K.N., Blair, L., Silverstein, J., Coburn, S.P., Ryan, L.M., Weinstein, R.S., Waymire, K., Narisawa, S., Millán, J.L., Macgregor, G.R., and Whyte, M.P. (1999) Alkaline phosphatase knock-out mice recapitulate the metabolic and skeletal defects of infantile hypophosphatasia. *J. Bone Miner. Res.* **14**, 2015–2026
9. Fleisch, H. and Neuman, W.F. (1961) Mechanisms of calcification: role of collagen, polyphosphates, and phosphatase. *Amer. J. Physiol.* **200**, 94–1300
10. Hessle, L., Johnson, K.A., Anderson, H.C., Narisawa, S., Sali, A., Goding, J.W., Terkeltaub, R., and Millán, J.L. (2002) Tissue-nonspecific alkaline phosphatase and plasma cell membrane glycoprotein-1 are central antagonistic regulators of bone mineralization. *Proc. Natl Acad. Sci. USA* **99**, 9445–9449
11. Mornet, E., Stura, E., Lia-Baldin, A-S., Stigbrand, T., Menez, A., and Le Du, M-H. (2001) Structural evidence for a functional

role of human tissue nonspecific alkaline phosphatase in bone mineralization. *J. Biol. Chem.* **276**, 31171–31178
12. Le Du, M.H., Stigbrand, T., Taussig, M.J., Menez, A., and Stura, E.A. (2001) Crystal structure of alkaline phosphatase from human placenta at 1.8 Å resolution. *J. Biol. Chem.* **276**, 9158–9165
13. Taillandier, A., Zurutuza, L., Muller, F., Simon-Bouy, B., Serre, J.L., Bird, L., Brenner, R., Boute, O., Cousin, J., Gillard, D., Heidemann, P.H., Steinmann, B., Wallot, M., and Mornet, E (1999) Characterization of eleven novel mutations (M45L, R119H, 544delG, G145V, H154Y, C184Y, D289V, 862+5A, 1172delC, R411X, E459K) in the tissue-nonspecific alkaline phosphatase (TNSALP) gene in patients with severe hypophosphatasia. *Hum. Mutat.* **13**, 171–172
14. Shibata, H., Fukushi, M., Igarashi, A., Misumi, Y., Ikehara, Y., Ohashi, Y., and Oda, K. (1998) Defective intracellular transport of tissue-nonspecific alkaline phosphatase with an Ala^{162}→Thr mutation associated with lethal hypophosphatasia. *J. Biochem.* **123**, 968–977
15. Fukushi, M., Amizuka, N., Hoshi, K., Ozawa, H., Kumagai, H., Omura, S., Misumi, Y., Ikehara, Y., and Oda, K. (1998) Intracellular retention and degradation of tissue-nonspecific alkaline phosphatase with a Gly^{317}→Asp substitution associated with lethal hypophosphatasia. *Biochem. Biophys. Res. Commun.* **246**, 613–618
16. Fukushi-Irie, M., Ito, M., Amaya, Y., Amizuka, N., Ozawa, H., Omura, S., Ikehara, Y., and Oda, K. (2000) Possible interference between tissue-non-specific alkaline phosphatase with an Arg^{54}→Cys substitution and a counterpart with an Asp^{277}→Ala substitution found in a compound heterozygote associated with severe hypophosphatasia. *Biochem. J.* **15**, 633–642
17. Ito, M., Amizuka, N., Ozawa, H., and Oda, K. (2002) Retention at the *cis*-Golgi and delayed degradation of tissue-non-specific alkaline phosphatase with an Asn^{153}→Asp substitution, a cause of perinatal hypophosphatasia. *Biochem. J.* **361**, 473–480
18. Oda, K., Amaya, Y., Fukushi-Irie, M., Kinameri, Y., Ohsuye, K., Kubota, I., Fujimura, S., and Kobayashi, J. (1999) A general method for rapid purification of soluble versions of glycosylphosphatidylinositol-anchored proteins expressed in insect cells: An application for human tissue-nonspecific alkaline phosphatase. *J. Biochem.* **126**, 694–699
19. Low, M.G. and Zilversmit, D.B. (1980) Role of phosphatidylinositol in attachment of alkaline phosphatase to membranes. *Biochemistry* **19**, 3913–3918
20. Weiss, J.J., Henthorn, P.S., Lafferty, M.A., Slaughter, C., Raducha, M., and Harris, H. (1986) Isolation and characterization of a cDNA encoding a human liver/bone/kidney-type alkaline phosphatase. *Proc. Natl Acad. Sci. USA* **83**, 7182–7186
21. Weiss, M.J., Ray, K., Henthorn, P.S., Lamb, B., Kadesch, T., and Harris, H. (1988) Structure of the human liver/bone/kidney alkaline phosphatase gene. *J. Biol. Chem.* **263**, 12002–12010
22. Taillandier, A., Lia-Baldini, A.S., Mouchard, M., Robin, B., Muller, F., Simon-Bouy, B., Serre, J.L., Bera-Louville, A., Bonduelli, M., Eckhardt, J., Gaillard, D., Myhre, A.G., Kortge-Jung, S., Larget-Piet, L.I., Malou, E., Sillence, D., Temple, I.K., Voit, G., and Mornet, E (2001) Twelve novel mutations in the tissue-nonspecific alkaline phosphatase gene (ALPL) in patients with various forms of hypophosphatasia. *Hum. Mutat.* **18**, 83–84
23. Mauro, S.D., Manes, T., Hessel, L., Kozlenkov, A., Pizauro J.M., J.M., Hoylaerts, M.F., and Millán, J.L. (2002) Kinetic characterization of hypophosphatasia mutations with physiological substrates. *J. Bone Mineral Res.* **17**, 1383–1391
24. Ellgaard, L., Molinari, M., and Helenius, A. (1999) Setting the standards: quality-control in the secretory pathway. *Science* **286**, 1882–1888
25. Brodsky, J.L. and McCracken, A.A. (1999) ER protein quality control and proteasome-mediated protein degradation. *Semin. Cell Dev. Biol.* **10**, 507–513
26. Tsai, B., Ye, Y., and Rapoport, T.A. (2002) Retro-translocation of proteins from the endoplasmic reticulum into the cytosol. *Nat. Rev. Mol. Cell Biol.* **3**, 246–255
27. Bays, N.W., Gardner, R.G., Seelig, L.P., Joaziero, C.A., and Hampton, R.Y. (2001) Hrd1p/Der3p is a membrane–anchored ubiquitin ligase required for ER-associated degradation. *Nat. Cell Biol.* **3**, 24–29
28. Yoshida, Y., Chiba, T., Tokunaga, F., Kawasaki, H., Iwai, K., Suzuki, T., Ito, Y, Matsuoka, K., Yoshida, M., Tanaka, K., and Tai, T. (2002) E3 ubiquitin ligase that recognizes sugar chains. *Nature* **418**, 438–442
29. Haynes, C.M., Caldwell, S., and Cooper, A.A. (2002) An HRD/DER-independent ER quality control mechanism involves Rsp5p-dependent ubiquitination and ER-Golgi transport. *J. Cell Biol.* **158**, 91–101