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

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Tissue-non-specific alkaline phosphatase (TNSALP) is an ecto-enzyme anchored to the plasma membrane via glycosylphosphatidylinositol (GPI). A TNSALP mutant with an Asn^{153} → Asp (N153D) substitution was reported in a foetus diagnosed with perinatal hypophosphatasia (Mornet, Taillandier, Peyramaure, Kaper, Muller, Brenner, Bussiere, Freisinger, Godard, Merrer et al. (1998) Eur. J. Hum. Genet. **6**, 308–314). When expressed ectopically in COS-1 cells, the wild-type TNSALP formed active non-covalently associated dimers, whereas TNSALP (N153D) formed aberrant disulphide-bonded high-molecular-mass aggregates devoid of enzyme activity. Cell-surface biotinylation and digestion with phosphatidylinositol-specific phospholipase C showed that TNSALP (N153D) failed to reach the cell surface. Instead, double immunofluorescence demonstrated that TNSALP (N153D) partially co-localized with a *cis*-Golgi marker (GM-130) at the steady-state. Upon treatment with brefeldin A, TNSALP (N153D) was still co-localized with GM-130, further supporting the finding that this mutant is localized in the *cis*-Golgi. Consistent with morphological results, pulse–chase experiments showed that newly synthesized TNSALP (N153D) remained endo-β-*N*-acetylglucosaminidase H-sensitive throughout the chase. Eventually, after a prolonged chase time, the mutant was found to be partly degraded in a proteasome-dependent manner. Since the mutant TNSALP was significantly labelled with [^{3}H]ethanolamine, a component of GPI, comparable with the wild-type enzyme, it is unlikely that the abortive synthesis of the mutant is due to a defect in GPI-attachment. Interestingly, when asparagine was replaced by glutamine at position 153 (N153D), TNSALP (N153Q) was indistinguishable from the wild-type enzyme in terms of its molecular properties, suggesting the possible importance of amino acids with a polar amide group at position 153. Taken together, these findings indicate that replacing asparagine with aspartic acid at position 153 causes misfolding and incorrect assembly of TNSALP, which results in its retention at the *cis*-Golgi *en* route to the cell surface, followed by a delayed degradation, presumably as part of a quality-control process. We postulate that the molecular basis of the perinatal hypophosphatasia associated with TNSALP (N153D) is due to the absence of mature TNSALP at the cell surface.

Key words: aggregation, hypomineralization, intracellular transport, missense mutation, proteasomes.

INTRODUCTION

Hypophosphatasia is a genetic disease characterized by reduced levels of tissue-non-specific alkaline phosphatase activity (TNSALP) in serum and tissues, and bone and tooth defects [1–3]. Clinical symptoms of hypophosphatasia are highly variable, from stillbirth in uterus, with profound skeletal hypomineralization, to pathologic fractures first presenting in adulthood. Six clinical types are recognized: perinatal, infantile, childhood, adult, odonto and pseudophosphatasia. Perinatal and infantile hypophosphatasia are severe and inherited in an autosomal-recessive manner, whereas other clinically mild forms are likely to be transmitted as an autosomal recessive or dominant trait [4,5]. Inorganic pyrophosphate, phosphoethanolamine and pyridoxal 5′-phosphate are reported to be elevated in serum and urine of patients with hypophosphatasia, thus implying that three phospho-compounds are natural substrates for TNSALP [2,3]. These compounds are also elevated in serum and urine of TNSALP-deficient mice [6–8]. However, it remains unknown how a metabolic disorder of the putative substrates culminates in hypomineralization of hard tissues *in vivo*.

More than 65 different mutations in the TNSALP gene have been reported in patients with hypophosphatasia, most of which are missense mutations [9]. To date, however, only a few studies have related the molecular defects of TNSALP mutants to cell physiology and clinical phenotypes. By expressing TNSALP mutants in cells and following their biosynthesis, we have previously reported [10–12] that several alleles cause defective protein folding and aberrant aggregation of TNSALP mutants, which resulted in degradation in a proteasome-dependent manner. Misfolded and incorrectly assembled proteins are generally recognized and degraded by the endoplasmic reticulum (ER) quality-control system. The ER quality-control system is crucial for securing the fidelity of gene expression at the post-translational level and permits only correctly folded and completely assembled proteins to proceed to the Golgi and final

Abbreviations used: BFA, brefeldin A; DMEM, Dulbecco's modified Eagle's medium; Endo H, endo-β-*N*-acetylglucosaminidase H; LLnL, *N*-acetyl-L-leucinyl-L-leucinyl-L-norleucinal; MG-132, benzyloxycarbonyl-L-leucinyl-L-leucinyl-L-leucinal; PI-PLC, phosphatidylinositol-specific phospholipase C; TNSALP, tissue-non-specific alkaline phosphate; ER, endoplasmic reticulum; GPI, glycosylphosphatidylinositol; GRP78, glucose-regulated protein; MEM, minimal essential medium; ECL*, enhanced chemiluminescence (Amersham Biosciences).

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destinations. Thus the degradation of TNSALP mutants in the ER fits this pattern.

Recently, a TNSALP (N153D) mutation, in which asparagine at position 153 is replaced by aspartic acid, was found in the homozygous state in a patient affected by perinatal hypophosphatasia [13]. This residue is proposed to be localized in a loop, close to the active-site positions His^{154} and Thr^{156}, by a computer-assisted modelling of human TNSALP [14]. So far, effects of this particular mutation on the TNSALP molecule have not been examined in detail. In the present study we have expressed TNSALP (N153D) in COS-1 cells and have defined abnormalities of this mutant. It is of interest that unlike other TNSALP mutants previously reported [10–12], TNSALP (N153D) was localized at the *cis*-Golgi at the steady-state and then underwent degradation within the cell.

MATERIALS AND METHODS

Materials

Express $^{35}S^{35}S$ protein-labelling mix (> 1000 Ci/mmol) was obtained from Dupont–New England Nuclear (Boston, MA, U.S.A.), and [1-^{3}H]ethanolamine hydrochlorine (12.0 Ci/mmol), ^{14}C-methylated proteins, enhanced chemiluminescence (ECL®) Western blotting detection reagent and Protein A–Sepharose CL-4B were from Amersham Biosciences (Arlington Heights, IL, U.S.A.); pALTER-MAX Vector and Altered sites II mammalian mutagenesis system were from Promega (Madison, WI, U.S.A.). Lipofectamine Plus Reagent from Gibco–BRL (Gaithersburg, MD, U.S.A.), immobilized streptavidin was from Pierce (Rockford, IL, U.S.A.); *N*-acetyl-L-leucinyl-L-leucinyl-L-norleucinal (LLnL) and saponin (Quillaja Bark) were from Sigma Chemical Co. (St Louis, MO, U.S.A.); Alexa488-conjugated goat anti-(mouse IgG) was from Molecular Probes, Inc. (Eugene, OR, U.S.A.); rhodamine-conjugated goat anti-(rabbit IgG) was from Cappel Laboratories (Malvern, P.A., U.S.A.); various DNA-modifying enzymes and restriction endonucleases from New England Biolabs, Inc. (Beverly, MA, U.S.A.) and Takara Shuzo (Kyoto, Japan); leupeptin and benzyloxycarbonyl-L-leucinyl-L-leucinyl-L-leucinal (MG-132) were from Protein Research Foundation (Osaka, Japan); phosphatidylinositol-specific phospholipase C (PI-PLC) was from Funakoshi Co. (Tokyo, Japan); bovine liver catalase and brefeldin A (BFA) were from Wako Pure Chemical Co. (Tokyo, Japan); sulphosuccinimidyl *N*-(D-biotinyl)-6-aminohexanoate was from Dojindo Laboratories (Kumamoto, Japan); endo-β-*N*-acetylglucosaminidase H (*Streptomyces griseus*; Endo H) was from Seikaguku Kogyo (Tokyo, Japan); anti-GM130 monoclonal antibody was from Transduction Laboratories (Lexington, KY, U.S.A.); anti-Bip/78 kDa glucose-regulated protein (GRD78; Bip/GRP78) monoclonal antibody was from StressGen (Victoria, Canada). Antiserum against recombinant human TNSALP was raised in rabbits as described previously [15]. COS-1 cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10 % foetal-bovine serum [10]. Saos-2 cells were cultured in α-minimum essential medium (MEM) supplemented with 10 % foetal-bovine serum [15]. MG-132 and LLnL were dissolved in DMSO (50 mM stock solution) and stored at −20 °C. BFA was dissolved in methanol (5 mg/ml stock solution) and kept at −20 °C.

Plasmids and transfection

Construction of pSG5-TNSALP encoding a full-length wild type human TNSALP was as described previously [10]. The plasmid was digested with *Eco*RI and the purified fragment was subcloned into a unique *Eco*RI site of pALTER-MAX Vector. Point mutations were introduced at desired sites using an Altered sites II mammalian mutagenesis system, essentially as described in the manufacturer's protocol. Oligonucleotides used were: 5′-GGGTGGCATGGTCGACTCTCGTGGT-3′ for TNSALP (N153D) and 5′-GGGGGTGGCATGCTGCACTCTCGTG-3′ for TNSALP (N153Q). The plasmid encoding TNSALP (R54C), in which arginine at position 54 of TNSALP is replaced with cysteine, was constructed as described previously [12]. Mutations were verified by restriction enzyme digestion, and nucleotide sequences of mutation sites were determined further by the dideoxynucleotide chain-termination method. 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 [12], and the transfected cells were incubated for 24 h in a 5 % CO_2/95 % air incubator before use.

Metabolic labelling and immunoprecipitation

For pulse–chase experiments, cells were preincubated for 0.5–1 h in the methionine/cysteine-free MEM and labelled with 50–100 μCi of [^{35}S]methionine/cysteine for 0.5 h in fresh methionine/cysteine-free MEM. After a pulse period, cells were washed and chased in MEM as described previously [10]. Cells were metabolically labelled with [^{3}H]ethanolamine as described previously [10]. The medium was removed, and 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]. The lysates were incubated for 20 min at 37 °C to extract TNSALP. The lysates and media were subjected to immunoisolation as described previously [10,12]. The immune complexes/Protein A beads were either used for digestion with Endo H or boiled directly in Laemmli's sample buffer [16] in the absence or presence of 1 % (v/v) 2-mercaptoethanol, and then analysed by SDS/PAGE [9 % (w/v) gels]. Gels were fixed and processed for fluorography as described previously [10].

Miscellaneous procedures

Immunofluorescence and cytohistochemical staining for alkaline phosphatase were performed as described previously [12]. For co-localization experiments, cells expressing TNSALP (N153D) were initially incubated with primary mouse and rabbit antibodies and then with Alexa488-labelled anti-mouse and rhodamine-labelled anti-rabbit secondary antibodies. Electric transfer of proteins and subsequent procedures were as described previously [15], and proteins on membranes were detected with ECL®Western blotting detection reagents. PI-PLC digestion of transfected cells using PI-PLC and digestion of [^{35}S]TNSALP with Endo H were carried out as described previously [10–12]. Sucrose-density-gradient centrifugation, cell-surface biotinylation using sulphosuccinimidylbiotin and protein and alkaline phosphatase assays were performed as described previously [12].

RESULTS

Alkaline phosphatase activity of the TNSALP (N153D) mutant

Replacement of asparagine at position 153 of TNSALP with aspartic acid has been reported in the TNSALP gene of a patient diagnosed with perinatal hypophosphatasia, who was homozygous for this mutant allele [13]. To investigate if this amino acid substitution affects catalytic activity of the enzyme, COS-1 cells were transiently transfected with a cDNA construct encoding TNSALP (N153D), and cell homogenates were assayed quan-

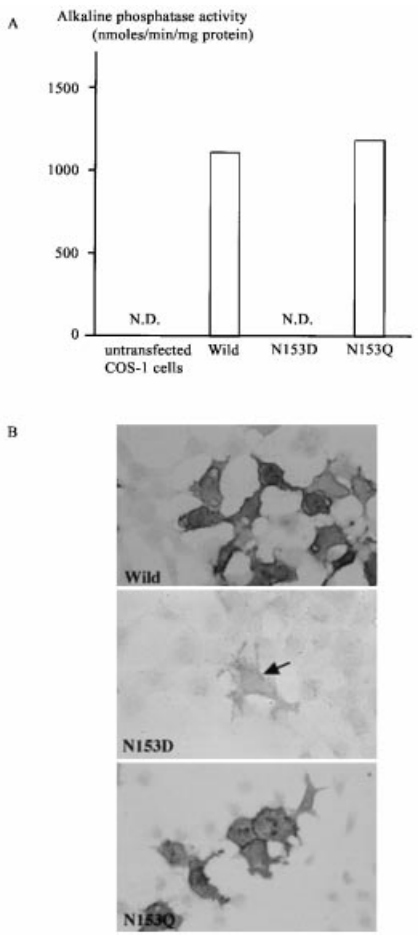

Figure 1 Alkaline phosphatase activity of the transfected COS-1 cells

COS-1 cells were transfected with the cDNA encoding the wild-type TNSALP (Wild), TNSALP (N153D) or TNSALP (N153Q). (**A**) Cells were homogenized and assayed for alkaline phosphatase activity. Values are means of two experiments. (**B**) Cells were stained for alkaline phosphatase for 10 min at 25°C and counterstained further with Methyl Green. Weakly staining cells were detected very occasionally in the cells expressing TNSALP (N153D) (arrow). N.D., not detectable.

titatively for alkaline phosphate activity (Figure 1A). In contrast with the cells expressing the wild-type enzyme, the cells expressing TNSALP (N153D) failed to exhibit measurable alkaline phosphatase activity. Since TNSALP (N153D) was expressed in amounts comparable with the wild-type enzyme in the transfected cells, as described below (Figure 3B), this indicates that TNSALP (N153D) expressed in the COS-1 cells is impaired in its ability to hydrolyse *p*-nitrophenyl phosphate. Cytohistochemical staining confirmed this results (Figure 1B), although very occasionally we detected weak alkaline phosphatase activity in cells expressing TNSALP (N153D). Thus we can not exclude the possibility that even this mutant undergoes a productive folding and assembly, albeit far less efficiently than the wild-type enzyme.

Intracellular transport of TNSALP (N153D)

To examine possible effects of this missense mutation on the biosynthesis and intracellular transport of TNSALP, cells expressing wild-type TNSALP or TNSALP (N153D) were pulse-labelled with [^{35}S]methionine/cysteine and chased for various time intervals. At the indicated times, the cells were lysed, the

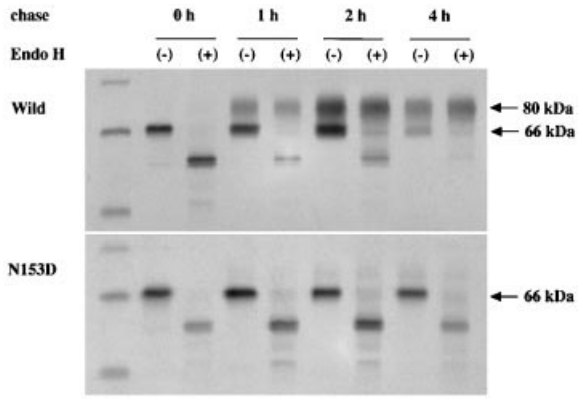

Figure 2 Acquisition of Endo-H resistance

Cells transfected with the cDNA encoding the wild-type TNSALP (Wild) or TNSALP (N153D) were pulse-labelled with [^{35}S]methionine/cysteine for 0.5 h and chased for 1 h, 2 h or 4 h. Cell lysates were subjected to immunoisolation and the immune complexes were incubated in the absence (−) or presence (+) of Endo H. Each sample was analysed by SDS/PAGE/fluorography. The furthermost lane on the left shows ^{14}C-methylated protein markers: 97.4, 66 and 46 kDa, from the top of the gel.

TNSALP molecules were immunoprecipitated and then analysed by SDS/PAGE, followed by fluorography. At least three of five consensus sites for N-glycosylation of the wild-type TNSALP have attached oligosaccharides when it is expressed in COS-1 cells [10]. The wild-type enzyme was synthesized as a 66-kDa Endo H-sensitive form and became an 80-kDa H-resistant terminally glycosylated mature form (Figure 2). The difference in molecular mass between the 66-kDa and 80-kDa form is ascribed solely to the processing of N-linked oligosaccharide chains on TNSALP as reported previously [12]. In marked contrast, newly synthesized TNSALP (N153D) remained Endo H-sensitive throughout the chase time, suggesting that this mutant failed to reach the medial-Golgi, where N-linked oligosaccharide chains on glycoproteins are trimmed to acquire Endo H-resistance.

We previously reported that TNSALP (R54C) with an $Arg^{54} \rightarrow Cys$ substitution, and TNSALP (G317D) with an $Gly^{317} \rightarrow Asp$ substitution, which were found in hypophosphatasia patients, formed disulphide-linked high-molecular-mass aggregates in the transfected cells [11,12]. To investigate if this was also the case with TNSALP (N153D), the mutant protein was immunoprecipitated from transfected cells and analysed by SDS/PAGE under non-reducing condition. As shown in Figure 3(A), a large proportion of newly synthesized TNSALP (N153D) was detected at the top of the resolving gel. Since no other protein band other than the 66-kDa form was found under reducing conditions, it is likely that TNSALP (N153D) formed homo-oligomers covalently linked by disulphide bond(s) in the transfected cells. A small amount of aggregate was also found in cells expressing the wild-type enzyme, probably due to over expression of the enzyme in the transient-expression system. Next, the transfected cells were homogenized and dissolved directly in SDS with or without a reducing agent and then analysed by immunoblotting (Figure 3B). About two-thirds of the 66-kDa form of TNSALP (N153D) existed as a high-molecular-mass aggregate in transfected cells, this was in contrast with Saos-2 cells (human osteosarcoma cell), which express high levels of TNSALP [17], and cells expressing wild-type TNSALP. These results strongly indicate that TNSALP (N153D) tends to assume aberrant folding structures, resulting in the formation of multiple interchain disulphide-linkages in transfected cells.

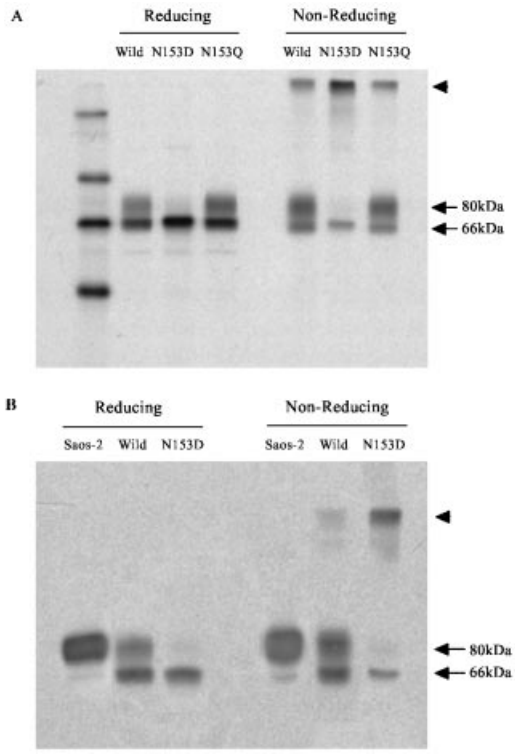

Figure 3 Aggregate formation of TNSALP (N153D)

(**A**) COS-1 cells were transfected with the cDNA encoding the wild-type TNSALP (Wild), TNSALP (N153D) or TNSALP (N153Q). Cells were labelled with [^{35}S]methionine/cysteine for 3 h. Cell lysates were subjected to immunoisolation and the immune complexes were analysed by SDS/PAGE under reducing or non-reducing conditions, followed by fluorography. Leftmost lane: ^{14}C-methylated protein markers: from the top of the gel, 200, 97.4, 66 and 46 kDa. An arrowhead indicates the top of the resolving gel. (**B**) Saos-2 cells and COS-1 cells expressing the wild-type TNSALP or TNSALP (N153D) were homogenized. Samples of the cell homogenates (7.5 μg) were analysed by SDS/PAGE under reducing or non-reducing conditions, followed by immunoblotting. Protein bands were detected using ECL®. An arrowhead indicates the top of the resolving gel.

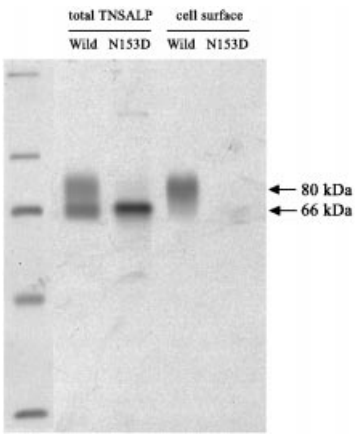

Figure 4 Cell-surface biotinylation

Cells transfected with the cDNA encoding the wild-type TNSALP (Wild) or TNSALP (N153D) were labelled with [^{35}S]methionine/cysteine for 6 h. The cells were further incubated with biotin succinimidylester on ice. Cell lysates were subjected to immunoisolation. The immune complexes were divided into two equal parts: one part was directly analysed by SDS/PAGE, whereas the other was boiled and incubated further with streptavidin beads before analysis. Leftmost lane: the same ^{14}C-methylated protein markers as shown in Figure 3(A).

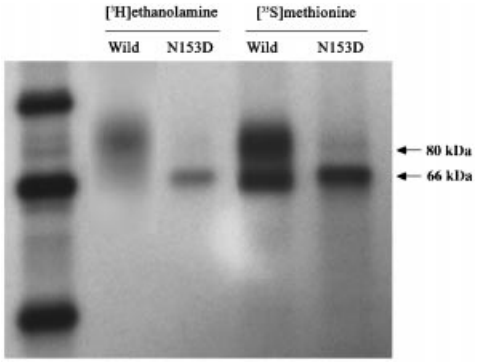

Figure 5 Labelling of TNSALP (N153D) with [^{3}H]ethanolamine

Cells transfected with the cDNA encoding the wild-type TNSALP (Wild) or TNSALP (N153D) were labelled with 50 μCi of [^{35}S]methionine/cysteine or 300 μCi of [^{3}H]ethanolamine for 6 h. Cell lysates were subjected to immunoisolation and analysed by SDS/PAGE/fluorography. The samples were run on the same gel. Leftmost lane: the same ^{14}C-methylated protein markers as shown in Figure 2(A).

Cell-surface appearance of TNSALP (N153D)

Cell-surface appearance of TNSALP (N153D) was investigated as shown in Figure 4. Metabolically labelled transfected cells were incubated with a membrane-impermeant biotinylation reagent, followed by immunoprecipitation with anti-TNSALP. Biotin-labelled cell surface TNSALP molecules were separated further from intracellular TNSALP using a streptavidin resin. Only the 80-kDa form was labelled with biotin in cells expressing the wild-type, whereas virtually no biotinylated TNSALP (N153D) was detected, indicating that the 66-kDa form was not able to gain access to the cell surface. In support of this, immunofluorescence observations showed that, in contrast with the wild-type, TNSALP (N153D) was not detected on the cell surface and accumulated within the cell (results not shown).

TNSALP is an ectoenzyme anchored to the cell membrane via glycosylphosphatidylinositol (GPI) [1]. GPI-anchored proteins are synthesized as a precursor with a C-terminal-peptide extension. Following its synthesis and translocation into the ER lumen, precursor molecules are rapidly cleaved and simultaneously attached with GPI to become embedded on the inner leaflet of the ER [18,19]. Failure in GPI-attachment often leads to the accumulation of precursor forms in the ER [20–22], presumably because the peptide extensions are thought to act as a transmembrane ER retrieval signal [23]. Metabolic labelling of the transfected cells with [^{3}H]ethanolamine, a component of GPI, showed that both the wild-type enzyme and TNSALP (N153D) were labelled strongly with [^{3}H]ethanolamine (Figure 5). Thus it is unlikely that the defect in GPI-anchoring causes the diminished cell-surface expression of TNSALP (N153D).

Localization of TNSALP (N153D)

In permeabilized cells, TNSALP (N153D) exhibited strong perinuclear staining in the cytosol resembling the Golgi apparatus (Figure 6A). However, there is increasing evidence that misfolded and incorrectly assembled proteins in the ER are dislocated into the cytosol and targeted for degradation by proteasomes and/or form an inclusion body (aggresome) at juxtanuclear locations [24,25], which resembles Golgi staining under an immuno-

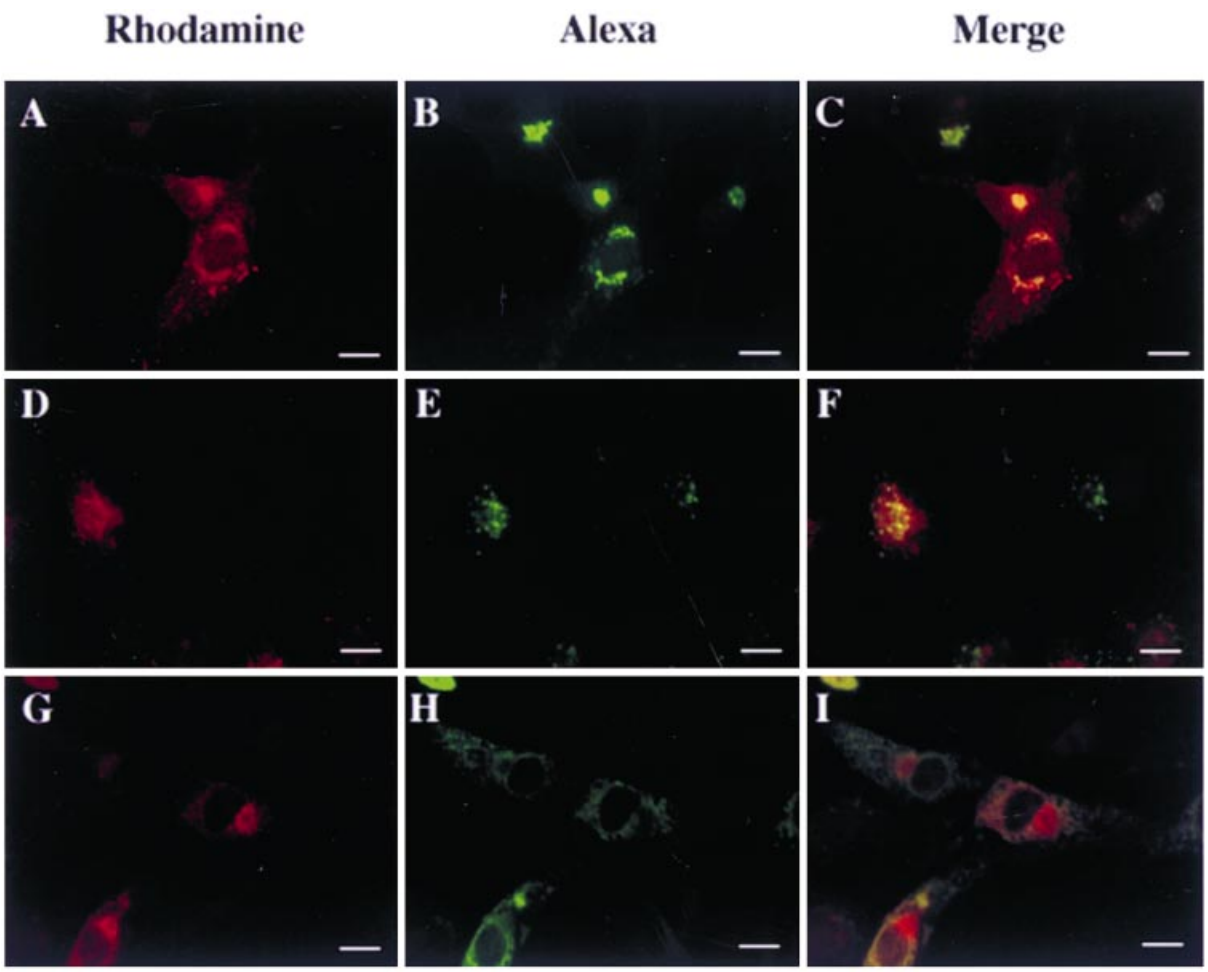

Figure 6 Co-localization of TNSALP (N153D) with a *cis*-Golgi marker

Cells transfected with the cDNA encoding TNSALP (N153D) were fixed and labelled with either anti-GM130 or anti-Bip/GRP78 monoclonal antibodies in combination with anti-TNSALP serum, followed by Alexa488-conjugated anti-(mouse IgG) and rhodamine-conjugated anti-(rabbit IgG). For treatment with BFA, the cells were incubated in the presence of 5 μg/ml BFA for 1 h before fixation (**D**–**F**). Rhodamine-labelled TNSALP (**A**, **D**, **G**), Alexa488-labelled GM-130 (**B**, **E**) and Alexa488-labelled Bip/GRP78 (**H**) are shown. Superimposed images are shown (Merge: **C**, **F**, **I**). Scale bars, 30 μm.

fluorescence microscope. To determine if the concentrated perinuclear fluorescent pattern of TNSALP (N153D) indeed reflects its localization to the Golgi, cells expressing TNSALP (N153D) were double labelled with a monoclonal antibody against a Golgi (GM130) or ER (Bip/GRP78) marker protein in combination with a polyclonal antibody against TNSALP. TNSALP (N153D) extensively co-localized with GM-130 (Figure 6C), a *cis*-Golgi matrix protein of 130 kDa [26], but not with Bip/GRP78 (Figure 6I), an ER-resident chaperon. The localization of this mutant protein was further substantiated with the aid of BFA. BFA is reported to redistribute most Golgi enzymes to the ER, but not proteins in the *cis*-Golgi network. When cells expressing TNSALP (N153D) were incubated with BFA, the Golgi stacks were distrupted and the *cis*-Golgi marker GM-130 displayed dispersed, punctuated structures, as described previously [26]. Again, TNSALP (N153D) significantly co-localized with GM-130. In agreement with the double immunofluorescence findings, immunoperoxidase electron microscopy showed that TNSALP (N153D) was found to accumulate in the Golgi apparatus (results not shown). When expressed in CHO cells, TNSALP (N153D) also co-localized with GM-130, although the co-localization was less prominent in CHO cells than in COS-1 cells (results not shown).

Degradation of TNSALP (N153D)

As reported previously [11,12], TNSALP (G317D) and TNSALP (R54C) were rapidly degraded in the transfected COS-1 cells. Furthermore, the degradation of these mutants was sensitive to lactacystin, a specific inhibitor of proteasomes, implying that these abnormal molecules are degraded as part of the ER quality-control system. Interestingly, TNSALP (N153D) was found to be more stable than TNSALP (R54C) even after a 6-h chase period (Figure 7A). In conjunction with morphological data, this strongly suggests that TNSALP (N153D) might somehow escape initial detection by the ER quality-control system and gain access to the *cis*-Golgi along the secretory pathway. After a prolonged chase time, most of newly synthesized TNSALP (N153D) disappeared from the cell (Figure 7B). Since the mutant was not released into the medium (results not shown), the decrease in the intensity of the mutant can be accounted for by its degradation within the cell. The degradation of TNSALP

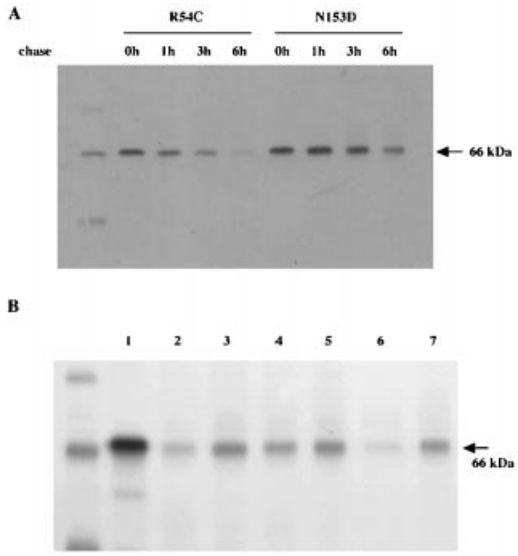

Figure 7 Degradation of TNSALP (N153D)

(**A**) COS-1 cells transfected with the cDNA for TNSALP (R54C) or TNSALP (N153D) were pulse-labelled with [^{35}S]methionine/cysteine for 0.5 h and chased for 0 h, 1 h, 3 h or 6 h. Cell lysates were subjected to immunoisolation and the immune complexes were analysed by SDS/PAGE. Leftmost lane: the same ^{14}C-methylated protein markers as shown in Figure 3(A). (**B**) COS-1 cells expressing TNSALP (N153D) were pulse-labelled with [^{35}S]methionine/cysteine for 0.5 h (lane 1) and chased for 12 h in the absence (lane 2) or presence of 50 μM MG132 (lane 3), 50 μM LLnL (lane 4), 100 μM LLnL (lane 5), 20 μM leupeptin (lane 6) and a combination of 50 μM MG132 and 20 μM leupeptin (lane 7). Protease inhibitors were added at the start of the chase. Cell lysates were subjected to immunoisolation and the immune complexes were analysed by SDS/PAGE/fluorography. Leftmost lane: the same ^{14}C-methylated protein markers as shown in Figure 2(A).

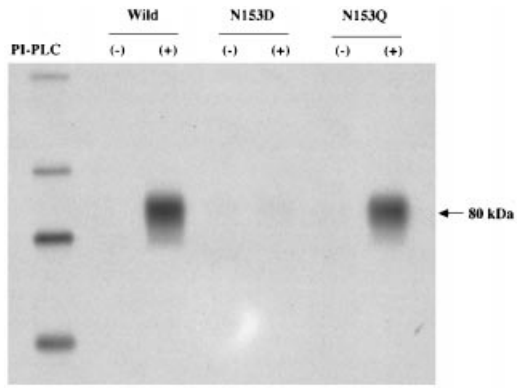

Figure 8 Digestion with PI-PLC

Cells transfected with the cDNA for the wild-type TNSALP (Wild), TNSALP (N153D) or TNSALP (N153Q) were labelled with [^{35}S]methionine/cysteine for 4 h and chased for 1 h. The cells were washed and further incubated in the absence (−) or presence (+) of PI-PLC in a CO_2 incubator at 37 °C for 1 h. Media were harvested, subjected to immunoisolation and analysed by SDS/PAGE/fluorography. Leftmost lane: the same ^{14}C-methylated protein markers as shown in Figure 3(A).

(N153D) was found to be significantly blocked with the proteasome inhibitors, MG132 and LLnL, but not with leupeptin, an inhibitor of lysosomal cysteine proteases, suggesting involvement of proteasomes in the degradation of TNSALP (N153D).

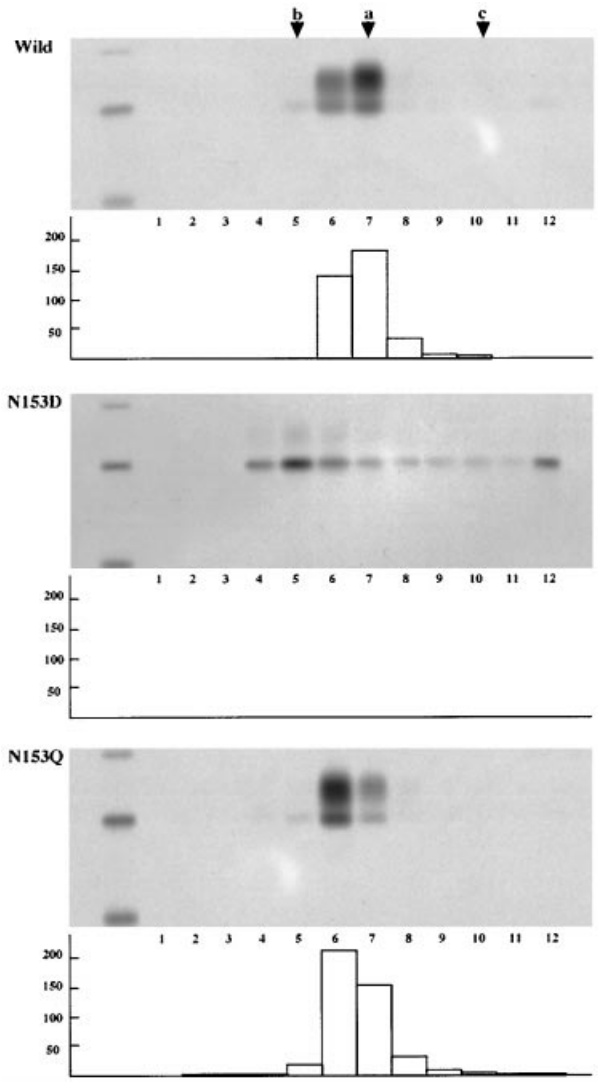

Figure 9 Sucrose-density-gradient centrifugation

COS-1 cells transfected with the cDNA encoding the wild-type TNSALP (Wild), TNSALP (N153D) or TNSALP (N153Q) were pulse-labelled with [^{35}S]methionine/cysteine for 0.5 h and chased for 2 h. Cell lysates were prepared and loaded on to a 5%–35% (w/w) sucrose gradient and centrifuged. Twelve fractions of 0.4 ml were collected from the top (fraction 1) and used for enzyme assay (lower panel of gel/histogram pairing: ordinate, alkaline phosphate activity expressed as units/ml) and immunoprecipitation (upper panel). Left lane: the same ^{14}C-methylated protein markers as shown in Figure 2(A). BSA (b; 68 kDa), alcohol dehydrogenase (a; 141 kDa) and catalase (c; 250 kDa) were loaded on to a separate gradient as molecular-mass markers.

Comparison of TNSALP (N153Q) and the wild-type enzyme

In addition to *Escherichia coli* alkaline phosphatase, three other human tissue-specific isoenzymes also have a glutamine residue corresponding to position 153 of TNSALP [27–30]. Thus we reasoned that asparagine can be replaced with glutamine at position 153 of TNSALP. To test this, we created a cDNA construct encoding TNSALP (N153Q), a mutant that has not been described in humans, and transfected it into COS-1 cells. Similar to the wild-type enzyme, the 80-kDa mature form of TNSALP (N153Q) was released into the medium upon digestion with PI-PLC (Figure 8), which cleaves the GPI-anchor, demonstrating that both the wild-type and TNSALP (N153Q) are

anchored to the surface of transfected cells via GPI. Consistent with the cell-surface biotinylation results (Figure 4), TNSALP (N153D) was not found in the media after the digestion with PI-PLC. TNSALP (N153Q) exhibited a catalytic activity comparable with the wild-type enzyme both quantitatively and qualitatively, as judged by alkaline phosphate assays (Figure 1). K_m values determined by the Lineweaver–Burk method for the wild-type and TNSALP (N153Q) were 1.05×10^{-4} M and 1.18×10^{-4} M respectively, indicating that the replacement of asparagine with glutamine at position 153 does not significantly change the substrate affinity of TNSALP. As reported previously [12], the wild-type enzyme is likely to form a dimer on the basis of analysis by sedimentation on a sucrose gradient. Figure 9 shows that TNSALP (N153Q) was detected at the same positions (fractions 6 and 7) as the wild-type on a sucrose-density-gradient. In marked contrast, TNSALP (N153D) formed heterogeneous aggregates with a considerable proportion of it being recovered at the bottom of the gradient (fraction 12). Using the criteria tested here, we demonstrate that TNSALP (N153Q) is very similar to the wild-type enzyme.

DISCUSSION

Mutation of the TNSALP gene is believed to be a cause of hypophosphatasia. Missense mutations represent the bulk of TNSALP gene mutations [9]. To gain an insight into the molecular basis underlying the clinical phenotype of hypophosphatasia, it is necessary to elucidate the structure and function of protein products encoded by the affected genes. This may also provide an understanding of the physiological role of TNSALP. In order to define molecular defects of TNSALP (N153D), which is associated with perinatal hypophosphatasia, COS-1 cells were transfected with the cDNA encoding this mutant and expressed TNSALP molecules were examined biochemically and morphologically. In contrast with the observation in cells expressing the wild-type enzyme, cells expressing TNSALP (N153D) had negligible enzyme activity, as determined by alkaline phosphatase assay (Figure 1). This explains why the foetus reported by Mornet et al. [13], found to be homozygous for this mutant allele, had extremely low levels of alkaline phosphatase activity. Furthermore, the present study has demonstrated that the lack of catalytic activity of TNSALP (N153D) can be ascribed to improper folding and incorrect assembly of the mutant protein. Analysis by SDS/PAGE under non-reducing conditions revealed that a large proportion of newly synthesized TNSALP (N153D) molecules formed an interchain disulphide-linked high-molecular-mass aggregate within cells (Figure 3A). It is unlikely that the aggregate was an artefact formed during the prolonged immunoprecipitation procedure, since the aggregate was also detected when the cell homogenates were analysed directly by immunoblotting (Figure 3B). In contrast with the dimeric structure of the wild-type enzyme, newly synthesized TNSALP (N153D) consists of heterogeneous aggregates with different molecular masses (Figure 9). It seems unlikely that the aggregation of TNSALP (N153D) results from a failure in modification by GPI, since both the wild-type and mutant were labelled with [^{3}H]ethanolamine, a component of GPI (Figure 5).

When asparagine was replaced with glutamine instead of aspartic acid at position 153 of TNSALP, TNSALP (N153Q) was found to be indistinguishable from the wild-type enzyme on the basis of the following criteria: enzyme activity (Figure 1), K_m value, cell-surface expression (Figure 8) and subunit assembly (Figure 9). Considering that not only *E. coli* alkaline phosphatase, but also three human tissue-specific isoenzymes have glutamine at corresponding positions to 153 of TNSALP [27–30], this finding suggests that the polar amide group on the side chain at position 153 (and corresponding positions) plays a critical role in folding and assembly of alkaline phosphatases. However, more extensive mutagenesis experiments are required to verify this.

The wild-type TNSALP is an ectoenzyme anchored to cell membranes via GPI. Following its modification by GPI and assembly in the ER lumen, the wild-type enzyme moves rapidly and efficiently along the secretory pathway to the cell surface. In contrast, TNSALP (N153D) did not reach the cell surface, as judged by cell surface biotinylation (Figure 4) and digestion with PI-PLC (Figure 8). Instead, TNSALP (N153D) was found to be co-localized with GM-130, a *cis*-Gogi marker protein (Figure 6). Furthermore, the mutant was sensitive to Endo H digestion (Figure 2), therefore, taken together, these observations indicate that TNSALP (N153D) is localized to the *cis*-Golgi at the steady-state.

It is noteworthy that TNSALP (N153D) resembles TNSALP (G317D) and TNSALP (R54C) with respect to their molecular properties, such as disulphide-bonded aggregate formation, lack of enzyme activity and modification by GPI [11,12], although they differ in subcellular localization in transfected cells. TNSALP (G317D) and TNSALP (R54C) are confined largely to the ER in the steady-state and degraded rapidly by the ER quality-control system [11,12]. Since the three mutants were expressed in comparable amounts in the transfected cells, it is unlikely that the *cis*-Golgi localization of TNSALP (N153D) is due simply to over-flow from the ER. What determines the intracellular localization of the different mutants at the steady-state remains unknown at present, although it is conceivable that association with ER-resident chaperones may help retain TNSALP (G317D) and TNSALP (R54C) in the ER and efficiently direct the mutants to the cytosol for degradation. On the other hand, the delayed degradation of TNSALP (N153D) may reflect its *cis*-Golgi localization. After a prolonged chase, TNSALP (N153D) was degraded within the cells. Inhibitors of proteasomes, but not lysosomal hydrolases, significantly, although not completely, block the degradation of TNSALP (N153D) (Figure 7), suggesting that TNSALP (N153D) is recycled back to the ER, translocated into cytosol and eventually degraded by proteasomes. Although we cannot exclude possible involvement of hitherto unknown proteasome inhibitor-sensitive proteases at the *cis*-Golgi, we do not favour this interpretation, because the data are more consistent with the present understanding of Golgi-to-ER retrograde transport. There is increasing evidence showing that Golgi proteins continuously recycle between the Golgi and ER, regardless of authentic or artificial Golgi residents [31–35]. In particular, the *cis*-Golgi may serve as a back-up quality-control system to retain and send back misfolded proteins to the ER, as proposed by Hammond and Helenius [32]. If so, it is unlikely that the recycling of TNSALP (N153D) depends on specific retrieval signals at the C-terminus (KDEL and dilysine motif) or transmembrane domain, considering that TNSALP (N153D) is modified by GPI at the C-terminus of its polypeptide chain. Instead, recycling of the mutant may be mediated by constitutive retrograde bulk-lipid-flow [33].

Unexpectedly, TNSALP knockout mice were found not to develop severe skeletal hypomineralization, symptomatic of perinatal hypophosphatasia, at birth [6–8]. In contrast with null-mutant mice, aberrant TNSALP molecules accumulating within a cell might be responsible for clinical phenotypes of perinatal hypophosphatasia. Several attempts to establish clones of stably expressing TNSALP (N153D) cells have failed to date, which is probably due to its cytotoxity.

We thank Dr Y. Amaya (Niigata University), Dr Y. Ikehara, Dr Y. Misumi (Fukuoka University) and Dr Y. Nishimura (Kyushu University) for their useful suggestions. This work was supported in part by a Grant-in-Aid for Scientific Research from the Ministry of Education, Culture, Sports, Science and Technology of Japan (to K.O.).

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Received 15 June 2001/17 September 2001; accepted 23 November 2001