# Possible interference between tissue-non-specific alkaline phosphatase with an $Arg^{54} \rightarrow Cys$ substitution and a counterpart with an $Asp^{277} \rightarrow Ala$ substitution found in a compound heterozygote associated with severe hypophosphatasia

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Tissue-non-specific alkaline phosphatase (TNSALP) with an $Arg^{54} \rightarrow Cys$ (R54C) or an $Asp^{277} \rightarrow Ala$ (D277A) substitution was found in a patient with hypophosphatasia [Henthorn, Raducha, Fedde, Lafferty and Whyte (1992) Proc. Natl. Acad. Sci. U.S.A. **89**, 9924–9928]. To examine effects of these missense mutations on properties of TNSALP, the TNSALP mutants were expressed ectopically in COS-1 cells. The wild-type TNSALP was synthesized as a 66-kDa endo-$\beta$-*N*-acetylglucosaminidase H (Endo H)-sensitive form, and processed to an 80-kDa mature form, which is anchored to the plasma membrane via glycosylphosphatidylinositol (GPI). Although the mutant proteins were found to be modified by GPI, digestion with phosphatidylinositol-specific phospholipase C, cell-surface biotinylation and immunofluorescence observation demonstrated that the cell-surface appearance of TNSALP (R54C) and TNSALP (D277A) was either almost totally or partially retarded respectively. The 66-kDa Endo H-sensitive band was the only form, and was rapidly degraded in the cells expressing TNSALP (R54C). In contrast with cells expressing TNSALP (R54C), where alkaline phosphatase activity was negligible, significant enzyme activity was detected and, furthermore, the 80-kDa mature form appeared on the surface of the cells expressing TNSALP (D277A). Analysis by sedimentation on sucrose gradients showed that a considerable fraction of newly synthesized TNSALP (R54C) and TNSALP (D277A) formed large aggregates, indicating improper folding and incorrect oligomerization of the mutant enzymes. When co-expressed with TNSALP (R54C), the level of the 80-kDa mature form of TNSALP (D277A) was decreased dramatically, with a concomitant reduction in enzyme activity in the co-transfected cell. These findings suggest that TNSALP (R54C) interferes with folding and assembly of TNSALP (D277A) *in trans* when expressed in the same cell, thus probably explaining why a compound heterozygote for these mutant alleles developed severe hypophosphatasia.

Key words: autosomal recessive, degradation, glycosylphosphatidylinositol, missense mutation.

## INTRODUCTION

Hypophosphatasia is an inborn error of metabolism characterized by defective mineralization of hard tissues and reduced levels of alkaline phosphatase activity in tissues and serum [1–3]. Mutations in the tissue-non-specific alkaline phosphatase (TNSALP) gene cause hypophosphatasia, pointing towards the involvement of TNSALP in mineralization of bone and teeth, although the precise role that TNSALP plays in mineralization remains elusive. The physiological substrate(s) of TNSALP is/are also still obscure; however, inorganic pyrophosphate, phosphoethanolamine and 5′-pyridoxal phosphate are potential candidates, since levels of these compounds were reported to be increased in both serum and urine of patients with hypophosphatasia. This was supported further by the finding that the levels of these phospho-compounds were elevated in serum and urine of TNSALP-deficient mice [4]. TNSALP-deficient mice did not develop typical skeletal hypomineralization symptomatic of the severe form of hypophosphatasia; however, it seems likely that mouse TNSALP is involved at least in the maintenance of mineralized bone and teeth, and in development of neural tubes [4,5].

The intracellular transport of proteins, which are ferried to their final destinations via vesicular transport, depends on proper folding and correct oligomeric assembly in the endoplasmic reticulum (ER). Many disease-causing mutations are known to impair intracellular transport of proteins by disturbing their folding and assembly [6,7]. Mutated proteins, which fail to acquire transport-competence, are often retained and eventually degraded in the ER [8,9]. Even if misfolded and incorrectly assembled proteins manage to exit from the ER, they are recycled back to the ER from the intermediate compartment and/or the *cis*-Golgi, ensuring that only correctly folded and oligomerized proteins transverse the Golgi stacks and reach the *trans*-Golgi network, where proteins are believed to be sorted for their final destinations.

Abbreviations used: DMEM, Dulbecco's modified Eagle's medium; Endo H, endo-$\beta$-*N*-acetylglucosaminidase H; ER, endoplasmic reticulum; GPI, glycosylphosphatidylinositol; MEM, minimum essential medium; MG132, benzyloxycarbonyl-L-leucyl-L-leucyl-L-leucinal; PI-PLC, phosphatidylinositol-specific phospholipase C; PNGase F, peptide:*N*-glycosidase F; TM, tunicamycin; TNSALP, tissue non-specific alkaline phosphatase; WT, wild-type.

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Recently, there have been an increasing number of reports on the mutation of the TNSALP gene associated with hypophosphatasia [10–19]. Most of the mutations are missense mutations, whereas deletion mutations and mutations in the non-coding region, which might affect splicing and promoter activity, have also been reported. By studying missense mutations associated with hypophosphatasia, it is possible to elucidate how each amino acid change affects the structure and function of TNSALP, leading to an understanding of the mechanism whereby the mutations cause hypophosphatasia. During the course of our studies on biosynthesis of TNSALP mutants to define their molecular defects, we found that TNSALPs with either an $Ala^{162} \rightarrow Thr$ substitution (A162T) or a $Gly^{317} \rightarrow Asp$ substitution (G317D), which were reported in patients who were homozygous for either of these hypophosphatasia alleles, aggregated in an early stage of the secretory pathway and, consequently, the TNSALP mutants largely failed to reach the cell surface, where they are thought to act as a glycosylphosphatidylinositol (GPI)-anchored ectoenzyme [20,21]. In the present study, we examined TNSALP (R54C) and TNSALP (D277A), both of which were found in a patient who developed severe hypophosphatasia. When separately expressed, TNSALP (R54C) was degraded in the cell, whereas TNSALP (D277A) partially appeared as a functional enzyme on the surface of cells expressing this mutant. However, the assembly and subsequent processing of TNSALP (D277A) were found to be markedly impaired, and the resultant alkaline phosphatase activity was reduced in cells co-transfected with cDNAs for TNSALP (R54C) and TNSALP (D277A), thus suggesting that the former exerts *trans*-negative effects on the latter.

## MATERIALS AND METHODS

### Materials

Express $^{35}S^{35}$ protein-labelling mix (> 1000 Ci/mmol) was obtained from Dupont-New England Nuclear (Boston, MA, U.S.A.), and [1-$^{3}$H]ethanolamine hydrochloride (12.0 Ci/mmol), $^{14}$C-methylated proteins, Protein A–Sepharose CL-4B and the Thermo Sequenase fluorescence labelled-primer cycle-sequencing kit were from Amersham Pharmacia Biotech (Arlington Heights, IL, U.S.A.). Lipofectamine Plus Reagent was from Gibco–BRL (Gaithersburg, MD, U.S.A.), immobilized streptavidin was from Pierce (Rockford, IL, U.S.A.); aprotinin, baker's yeast alcohol dehydrogenase, BSA, Fast Blue RR salt, naphthol AS-BI phosphate and saponin (Quillaja Bark) were from Sigma Chemical Co. (St. Louis, MO, U.S.A.); Pansorbin was from Calbiochem–Novabiochem (La Jolla, CA, U.S.A.); rhodamine-conjugated goat anti-(rabbit IgG) was from Cappel Laboratories (Malvern, PA, U.S.A.); Peptide: *N*-glycosidase F (PNGase F), various DNA-modifying enzymes and restriction endonucleases were from New England Biolabs, Inc. (Beverly, MA, U.S.A.) and Takara Shuzo (Kyoto, Japan); antipain, chymostatin, elastatinal, leupeptin, pepstatin A and benzyloxycarbonyl-L-leucyl-L-leucyl-L-leucinal (MG132) were from the Protein Research Foundation (Osaka, Japan); phosphatidylinositol-specific phospholipase C (PI-PLC) was from Funakoshi Co. (Tokyo, Japan); bovine liver catalase and tunicamycin (TM) were from Wako Pure Chemical Co. (Tokyo, Japan); sulphosuccinimidyl-*N*-(D-biotinyl)-6-aminohexanoate was from Dojindo Laboratories (Kumamoto, Japan); and endo-$\beta$-*N*-acetylglucosaminidase H (*Streptomyces griseus*; Endo H) was from Seikaguku Kogyo (Tokyo, Japan). Lactacystin was purified as described previously [22]; antiserum against rat TNSALP or recombinant human TNSALP was raised in rabbits, also as described previously [23,24]. COS-1 cells were cultured in Dulbecco's modified Eagle's minimum essential medium (DMEM) supplemented with 10 % (v/v) fetal-bovine serum [20].

### Plasmids and transfection

Construction of pSG5-TNSALP (Stratagene, San Diego, CA, U.S.A.) encoding a full-length cDNA for wild-type (WT) human TNSALP was as described previously [20]. Point mutations were introduced at desired sites using a Takara Mutan K kit (Takara Shuzo, Kyoto, Japan). Oligonucleotides used were: 5′-CGGC-TGCCTGCATCCTCA-3′ for the C387T (R54C) mutation, and 5′-GCCAGGGGCCATGCAGTA-3′ for A1057C (D277A). Mutations were verified by restriction-enzyme digestion, and nucleotide sequences of mutation sites were determined further by the dideoxynucleotide chain-termination method using a Thermo Sequenase fluorescence-labelled-primer cycle-sequencing kit with 7-deaza-dGTP. pSG5-TNSALP(A162T) encoding TNSALP with an $Ala^{162} \rightarrow Thr$ substitution [10], which was found in a patient who was homozygous for this mutation, was constructed as described previously [20]. For transfection, plasmids were prepared using a plasmid Midi kit (Qiagen, Hilden, Germany). Cells were transfected using Lipofectamine Plus, essentially as described in the manufacturer's protocol. COS-1 cells [$(1.0–1.4) \times 10^5$] were inoculated into 35-mm dishes approx. 24 h before transfection. Unless otherwise stated, 1 $\mu$g of each plasmid was used for a single transfection experiment. After 3 h of incubation with Lipofectamine–plasmid complex, the medium was replaced with 2 ml of DMEM supplemented with 10 % (v/v) fetal-bovine serum, and the cells were incubated further for 24 h before use.

### Metabolic labelling and immunoprecipitation

For pulse–chase experiments, cells were preincubated for 0.5–1 h in methionine-free minimum essential medium (MEM), and labelled with 50–100 $\mu$Ci of [$^{35}$S]methionine for 0.5 h in fresh methionine-free MEM. After a pulse period, cells were washed and chased in MEM as described previously [20,21]. Where indicated, cells were preincubated for 1 h in the presence of 2 $\mu$g/ml of TM, and labelled with [$^{35}$S]methionine while in its presence. For specific labelling of the GPI-anchor moiety of TNSALP, cells were pre-incubated in MEM for 1 h and then labelled with 300 $\mu$Ci of [$^{3}$H]ethanolamine in MEM for 6 h. To prevent degradation of TNSALP mutants, lactacystin (10 $\mu$M) was included throughout the course of the experiment. The medium was removed, and cells were washed with cold PBS and 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], as described previously [20]. A protease inhibitor cocktail (antipain, aprotinin, chymostatin, elastatinal, leupeptin and pepstatin A) was added to cell lysates and media (10 $\mu$g of each/ml). The lysates were incubated for 20 min at 37 °C to extract TNSALP. The lysates and media were centrifuged at 15000 ***g*** for 10 min to remove insoluble materials and incubated with 20–40 $\mu$l of a 10 % suspension of Pansorbin (fixed *Staphylococcus aureus* cells) for 30 min. The precleared supernatants were mixed with 50 $\mu$l of powdered skimmed milk (5 % in PBS) and incubated with 3–4 $\mu$l of anti-TNSALP serum for 3–16 h. The immune complexes were collected on 30–40 $\mu$l of Protein A–Sepharose (50 % suspension in PBS) by shaking for 1 h, washed once with the lysis buffer, and then washed extensively as described before [20]. The immune complexes/Protein A beads were either used for digestion with Endo H or PNGase F or directly boiled in Laemmli's sample buffer [25] in the absence or presence of 2-mercaptoethanol, and then analysed by SDS/PAGE [9 % (w/v) gels]. Gels were fixed and processed for fluorography as described previously [20].

### Sucrose-density-gradient centrifugation

Metabolically labelled cells were lysed as described above and incubated at 37 °C for 20 min. The clarified cell lysates were loaded on to continuous sucrose-density gradients [5 ml, 5–35 % (w/w) sucrose in 10 mM Tris/HCl, pH 7.5, containing 150 mM NaCl and 0.2 % (v/v) Triton X-100] and centrifuged for 18 h at 4 °C in an MLS-50 Rotor (Beckman Coulter, Fullerton, CA, U.S.A.) at 45000 rev./min (163000 ***g***). Twelve fractions of 0.4 ml were collected from the top. Of each fraction, 50 $\mu$l was used for the alkaline phosphatase assay; 300 $\mu$l of each fraction was diluted 3-fold with the lysis buffer and subjected to immunoisolation.

### Enzyme digestions

For PI-PLC digestion, metabolically labelled cells were washed three times with MEM and subsequently incubated with 0.2 unit of PI-PLC in 0.7 ml of MEM for 1 h in a $CO_2$ incubator [20]. Media were harvested and centrifuged at 5000 ***g*** for 5 min to remove detached cells. The resultant supernatants were subjected to immunoisolation as described above. For digestion with Endo H, immune complexes collected on Protein A beads were washed once with distilled water, divided into two equal parts, and then suspended in 50 $\mu$l of 50 mM acetate buffer, pH 5.5, in the presence of the protease inhibitor cocktail. Enzyme digestions were performed in the absence or presence of 0.2 unit/ml Endo H at 37 °C for 16 h. Then, immune complex/Protein A beads were washed once with distilled water and boiled in Laemmli's sample buffer [25]. For PNGase F digestion, immune complexes/Protein A beads were boiled in PBS containing 1 % (w/v) SDS for 3 min, and centrifuged. Then the resultant supernatants were diluted 10-fold with 1 % Nonidet P-40 and incubated in the absence or presence of 1000 units/ml of PNGase F at 37 °C for 16 h, as described previously [20,24].

### Cell-surface biotinylation

Biotinylation was performed essentially as described previously [20]. Metabolically labelled cells were washed three times with 20 mM Hepes buffer, pH 7.3, containing 150 mM NaCl and incubated on ice with 0.5 mg/ml of sulphosuccinimidylbiotin in the same buffer for 15 min. This step was repeated once more. After the biotinylation reagent was removed, the cells were incubated further with the same buffer containing 20 mM glycine on ice for 10 min. Cells were washed with PBS and lysed in 0.5 ml of the lysis buffer containing the protease inhibitor cocktail. The lysates were warmed at 37 °C for 20 min and centrifuged at 15000 ***g*** for 10 min. The resultant supernatants were subjected to immunoisolation as described above. The immune complexes/Protein A–Sepharose were then divided into two equal parts: one was directly boiled in Laemmli's sample buffer, whereas the other was boiled in 50 $\mu$l of PBS containing 1 % SDS and centrifuged. The resultant supernatant was adjusted to final concentrations of 1 % Nonidet P40 and 0.05 % SDS, and incubated further with 40 $\mu$l of streptavidin beads (50 % slurry) at 4 °C overnight. The biotinylated TNSALP/streptavidin beads were washed extensively, and then boiled in Laemmli's sample buffer [25].

### Determinations of protein and alkaline phosphatase activity

Cells were homogenized in 50 mM Tris/HCl, pH 7.5, using a small glass homogenizer with a Teflon pestle. Aliquots were used for the determination of enzyme activity and protein concentration. Alkaline phosphatase was assayed using *p*-nitrophenyl phosphate as a substrate [26] (1 unit of activity is defined as nmol of substrate hydrolysed per min at 37 °C). Protein was assayed using a Bio-Rad protein assay kit (Hercules, CA, U.S.A.) with BSA as a standard.

### Immunofluorescence

COS-1 cells grown on cover slips in a 35-mm dish were transfected with 1.0 $\mu$g of plasmid as described above. After 24 h, the cells were fixed with 4 % (w/v) paraformaldehyde in PBS for 20 min at room temperature. The cells were incubated further with PBS in the absence or presence of 0.1 % (w/v) saponin for 20 min on ice. Then intact cells and permeabilized cells were incubated with PBS containing 5 % skimmed milk in the absence or presence of 0.1 % saponin at 4 °C overnight. The cells were incubated first with anti-TNSALP serum, and then with rhodamine-conjugated goat anti-rabbit IgG. The cover slip was washed with PBS, mounted on a slide glass with 50 % (w/v) glycerol in distilled water, and subjected to microscopic observations.

### Enzyme histochemistry

COS-1 cells expressing TNSALP mutants on cover slips were stained for alkaline phosphatase activity according to the method of Burstone [27]. Cells were fixed with 4 % (w/v) paraformaldehyde in PBS for 30 min on ice and washed three times with 0.1 M Tris/HCl, pH 8.5. Then the cells were incubated with a mixture of 3 mg of naphthol AS-PI as a substrate and 18 mg of Fast Blue RR salt dissolved in 30 ml of 0.1 M Tris/HCl (pH 8.5) at room temperature for 5 min. The cells were counterstained with Methyl Green.

## RESULTS

### Enzyme activities of COS-1 cells expressing TNSALP mutants

COS-1 cells do not express a significant amount of endogenous TNSALP, allowing us to analyse molecular properties of TNSALP mutants that are expressed ectopically in the cells [20]. To examine effects of amino acid substitutions on the catalytic properties of TNSALP, COS-1 cells transiently expressing either the WT or two mutants, TNSALP (R54C) or TNSALP (D277A), were homogenized and assayed for alkaline phosphatase activity. For comparison, TNSALP (A162T) was also expressed in COS-1 cells and assayed for enzyme activity. TNSALP (A162T) was found in a patient who was homozygous for this mutation [10]. As shown in Table 1, TNSALP (WT) showed the highest specific activity among the four TNSALPs. In contrast, the enzyme activity level of cells expressing TNSALP (R54C) was found to be the same as that of untransfected COS-1 cells, suggesting that this mutant is synthesized in a non-active

**Table 1 Enzyme activity of cells expressing TNSALP mutants**

COS-1 cells expressing the WT TNSALP or the TNSALP mutants were homogenized and assayed for alkaline phosphatase activity, as described in the Materials and methods section. Values are averages of two experiments. n.d., not determined.

| Plasmid | Enzyme activity (nmol/min per mg of protein) | $K_m$ ($\times 10^{-4}$ M) |
|---|---|---|
| None | 30 | n.d. |
| TNSALP (WT) | 3080 | 2.3 |
| TNSALP (R54C) | 35 | n.d. |
| TNSALP (D277A) | 1075 | 3.5 |
| TNSALP (A162T) | 749 | 1.3 |

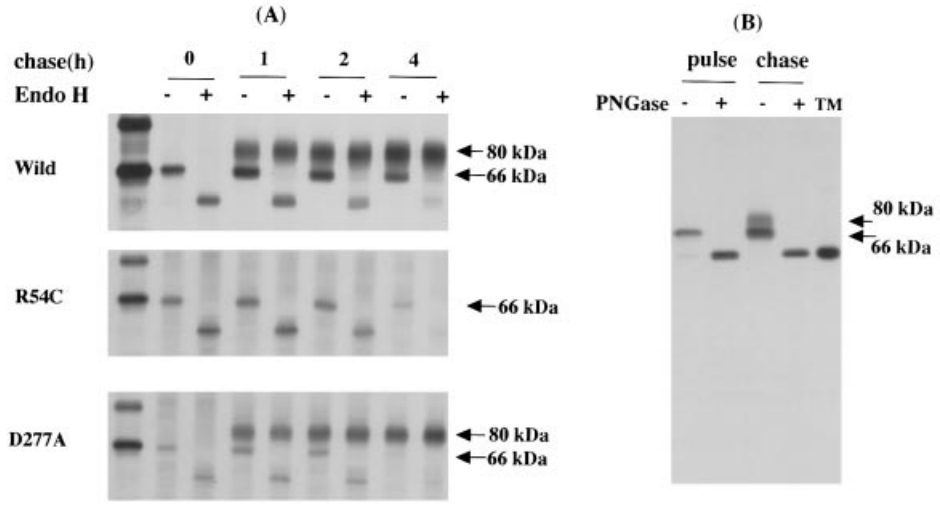


**Figure 1 Acquisition of Endo-H resistance of the TNSALP mutant**

(**A**) Cells transfected with the cDNA encoding TNSALP (WT) (Wild), TNSALP (R54C) or TNSALP (D277A) were pulse-labelled with [$^{35}$S]methionine 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-kDa and 66-kDa, from the top of the gel. (**B**) Cells transfected with the cDNA for TNSALP (WT) were pulsed-labelled with [$^{35}$S]methionine for 0.5 h and chased for 1 h in the absence or presence of TM. Cell lysates were subjected to immunoisolation and the immune complexes were incubated in the absence (−) or presence (+) of PNGase F. Each sample was analysed by SDS/PAGE/fluorography.

form. The specific activity of cells expressing TNSALP (D277A) was roughly one-third of that of cells expressing the WT. We also confirmed that TNSALP (A162T) exhibited a low but significant enzyme activity, as reported previously [20]. $K_m$ values of TNSALP (D277A) and TNSALP (A162T) determined by the Lineweaver–Burk method were similar to that of TNSALP (WT), indicating that replacement of aspartate at position 277 or alanine at position 162 with alanine or threonine respectively does not significantly affect the substrate affinity of TNSALP.

### Intracellular transport of TNSALP mutants

To examine effects of amino acid substitution on biosynthesis of TNSALP, COS-1 cells transiently expressing either TNSALP (WT), TNSALP (R54C) or TNSALP (D277A) were metabolically labelled with [$^{35}$S]methionine for 0.5 h and chased for up to 4 h. TNSALP were immunoprecipitated, further incubated in the absence or presence of Endo H and analysed by SDS/PAGE, followed by fluorography. We reported previously that at least three of five potential N-linked glycosylation sites of the WT human TNSALP are N-glycosylated when it is expressed in COS-1 cells [20]. TNSALP (WT) was synthesized as a 66-kDa Endo H-sensitive form, and became an 80-kDa Endo H-resistant mature form along with chase times (Figure 1A). Upon digestion with PNGase F, which cleaves the linkage between the innermost *N*-acetylglucosamine and asparagine residue of high-mannose, hybrid and complex oligosaccharides from N-linked glycoproteins, the 66-kDa and 80-kDa forms migrated to the same position (Figure 1B), indicating that the size difference was due to modification of N-linked sugar chains. In contrast, TNSALP (R54C) remained Endo H-sensitive, and the 80-kDa mature form was barely detectable throughout the chase time (Figure 1A), pointing towards a block in transport of this mutant from the ER to the Golgi, where N-linked oligosaccharides of glycoproteins become Endo H-resistant. It is evident that TNSALP (D277A) was processed to give rise to the 80-kDa mature form at almost the same rate as was the WT enzyme (Figure 1A). It is noteworthy that the intensity of the 66-kDa form of both TNSALP (R54C) and TNSALP (D277A) declined progressively as the chase time increased, reflecting its degradation within the cell. Lactacystin and MG132, as proteasome inhibitors, strongly inhibited the degradation of the 66-kDa form (results not shown), as reported in the case of TNSALP (G3217D) [21].

### Cell-surface appearance of TNSALP mutants

TNSALP (WT) is anchored to the cell surface via GPI, and is believed to function as an ectoenzyme. To examine whether the TNSALP mutants are expressed on the cell surface via GPI, COS-1 cells expressing either the WT or the mutants proteins were incubated with PI-PLC, which renders membrane-bound GPI-anchored proteins released from the cell surface [28,29]. Upon incubation with PI-PLC, both TNSALP (WT) and TNSALP (D277A) were recovered in the medium, whereas TNSALP (R54C) was not detected in the medium (Figure 2A), indicating that TNSALP (WT) and TNSALP (D277A), but not TNSALP (R54C), are exposed on the cell surface via GPI. We then asked whether the disappearance of TNSALP (R54C) on the cell surface is due to failure in terms of the modification by GPI. Under conditions where GPI attachment is in some way inhibited, precursor proteins of GPI-anchored proteins are known to be retained and subsequently degraded in the ER/pre-Golgi compartment [30–33]. The transfected cells were metabolically labelled with [$^3$H]ethanolamine, a component of GPI, before cell lysis and TNSALP immunoprecipitation, as shown in Figure 2(B). In this experiment, lactacystin was included throughout to inhibit the degradation of the TNSALP mutants. Not only the 80-kDa mature forms of TNSALP (WT) and TNSALP (D277A), which were sensitive to PI-PLC digestion (Figure 2A), but also the 66-kDa form of TNSALP (R54C) were labelled by [$^3$H]ethanolamine, indicating that TNSALP (R54C) is modified by GPI, like TNSALP (WT) and TNSALP (D277A). Another TNSALP mutant with an Ala$^{162}$ → Thr substitution, which showed a lower specific activity relative to TNSALP (D277A)

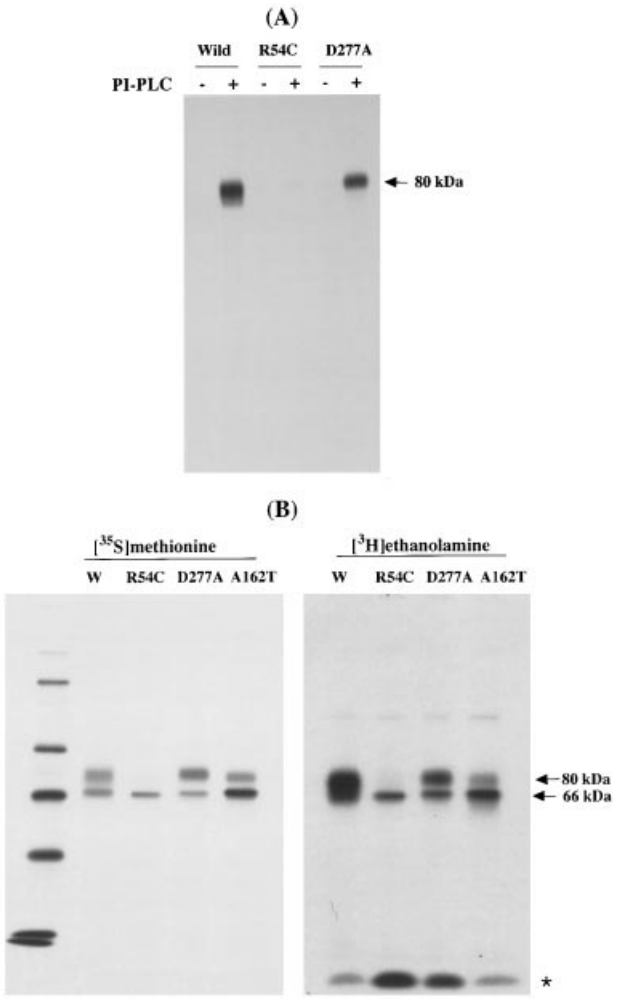


**Figure 2 Modification of TNSALP mutants by GPI**

(**A**) Cells transfected with the cDNA encoding TNSALP (WT) ('Wild'), TNSALP (R54C) or TNSALP (D277A) were labelled with [$^{35}$S]methionine for 3 h. The cells were washed and incubated further in the absence (−) or presence (+) of PI-PLC, as described in the Materials and methods section. Media were subjected to immunoisolation and analysed by SDS/PAGE/fluorography. (**B**) Cells transfected with the cDNA encoding TNSALP (WT) (W), TNSALP (R54C), TNSALP (D277A) or TNSALP (A162T) were labelled with 50 $\mu$Ci of [$^{35}$S]methionine or 300 $\mu$Ci of [$^{3}$H]ethanolamine for 6 h. Lactacystin (10 $\mu$M) was included throughout the experiments to prevent the degradation of the TNSALP mutants. Cell lysates were subjected to immunoisolation and analysed by SDS/PAGE/fluorography. The left lane shows $^{14}$C-methylated protein markers (from the top of the gel, 200, 97.4, 66, 46, 30 and 14.3 kDa). The bands marked by an asterisk presumably represent phosphatidylethanolamine. The samples were run on the same gel; the left half of the gel was exposed for 2 weeks, while the other half was exposed for a month.

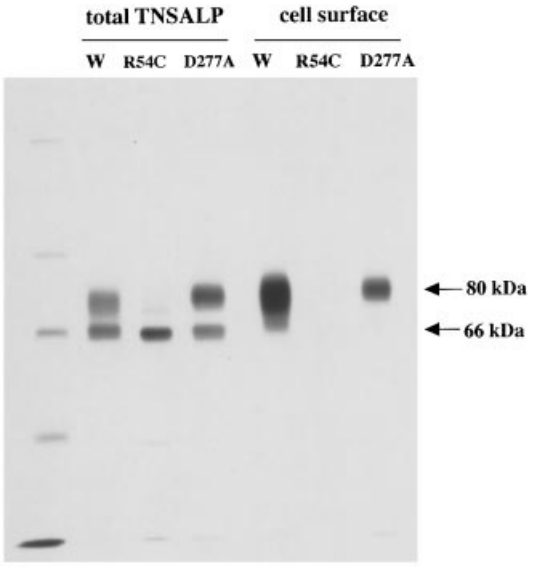


**Figure 3 Cell surface biotinylation**

Cells transfected with the cDNA encoding TNSALP (WT) (W), TNSALP (R54C) or TNSALP (D277A) were labelled with [$^{35}$S]methionine for 6 h. The cells were incubated further with biotin succinimidyl ester on ice, as described in the Materials and methods section. 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. Left lane: the same $^{14}$C-methylated protein markers as shown in Figure 2(B).

(Table 1), was also intensely labelled with [$^{3}$H]ethanolamine in the presence of lactacystin, whereas this mutant was only weakly labelled in its absence [20].

Cell-surface expression of the TNSALP mutants was studied further by labelling transfected cells with biotin (Figure 3). Metabolically labelled cells were first incubated with biotin succinimidyl ester, which reacts with exposed lysine residues on proteins on the cell surface, then cellular TNSALP was immunoprecipitated with anti-TNSALP antibody. Subsequently, biotinylated TNSALP was separated from intracellular TNSALP using a streptavidin resin. In good agreement with the result of PI-PLC digestion (Figure 2A), the 80-kDa form of both TNSALP (WT) and TNSALP (D277A) were labelled with biotin, although TNSALP (D277A) was expressed on the cell surface to a considerably lesser extent than was TNSALP (WT). In contrast, the 66-kDa form of TNSALP (R54C) was not reactive to streptavidin, confirming that TNSALP (R54C) failed to reach the cell surface.

Immunofluorescence observation showed that TNSALP (WT) and TNSALP (D277A) were localized on the surface of transfected cells, while the surface of the cells expressing TNSALP (R54C) were largely devoid of immunofluorescence (Figure 4). When cells were permeabilized, TNSALP (R54C) was found to be accumulated in the intracellular reticular network resembling the ER. Occasionally, we encountered a cell expressing TNSALP (R54C) on the cell surface under a microscope (results not shown). It is probable that the number of cells subjected to the cell-surface staining was too small to be detectable by means of biochemical methods.

### Co-transfection with the cDNA constructs for TNSALP mutants

Purified TNSALPs have been reported to exist as a homodimer [23,34–36], although a tetrameric structure was also proposed for a membrane-bound form of TNSALP [35]. Cells expressing TNSALP mutants were metabolically labelled with [$^{35}$S]-methionine, and TNSALP mutants were immunoprecipitated. The immunoprecipitates were then analysed by SDS/PAGE under reducing or non-reducing conditions (Figure 5A). Irrespective of the absence or presence of a reductant, most TNSALP (WT) migrated as the 66- or 80-kDa monomeric form

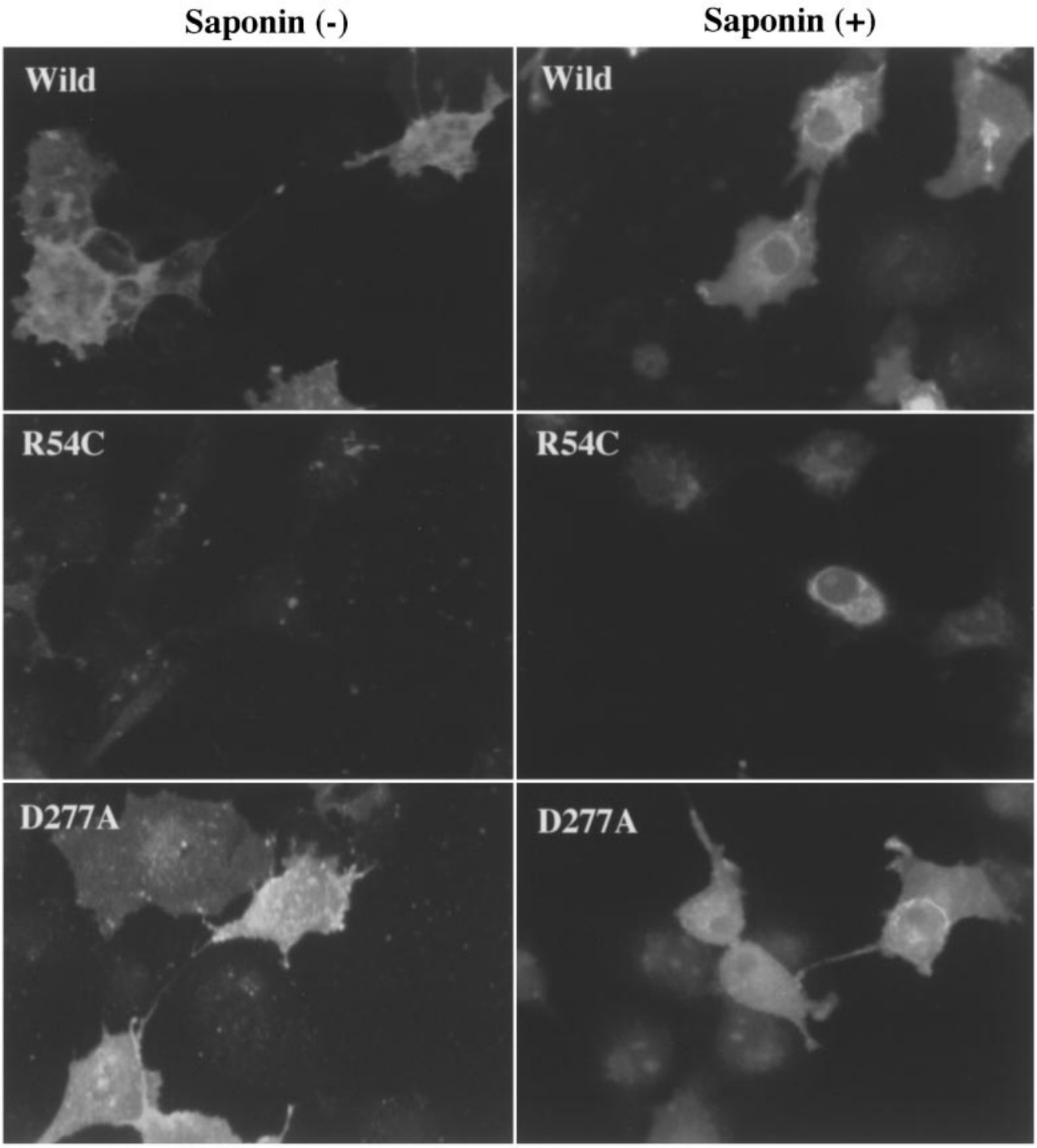


**Figure 4 Immunofluorescence of the cells expressing the TNSALP mutants**

Cells transfected with the cDNA encoding TNSALP (WT) ('Wild'), TNSALP (R54C) or TNSALP (D277A) were fixed, incubated in the absence or presence of saponin and then stained by an indirect immunofluorescence technique, as described in the Materials and methods section.

on an SDS/polyacrylamide gel (Figure 5A), indicating that the oligomeric structure of the WT TNSALP is held non-covalently. A small amount of aggregate on the top of the gel was probably due to overexpression of the enzyme. Compared with the WT, a significantly large amount of aggregate was found in the cells expressing TNSALP (R54C). This was confirmed further by sedimentation-velocity experiments using sucrose-density gradients, as described below in Figure 6. Thus a portion of newly synthesized TNSALP (R54C) formed disulphide-bonded high-molecular-mass aggregates. Although TNSALP (D277A) is able to fold and assembles properly, as shown by its enzyme activity (Table 1) and the appearance of the 80-kDa mature form on the cell surface of the transfected cells (Figures 1–3), a considerable fraction of newly synthesized TNSALP (D277A) was also found to aggregate, as found for TNSALP (R54C) (Figure 5A). This probably explains why the extent of the cell-

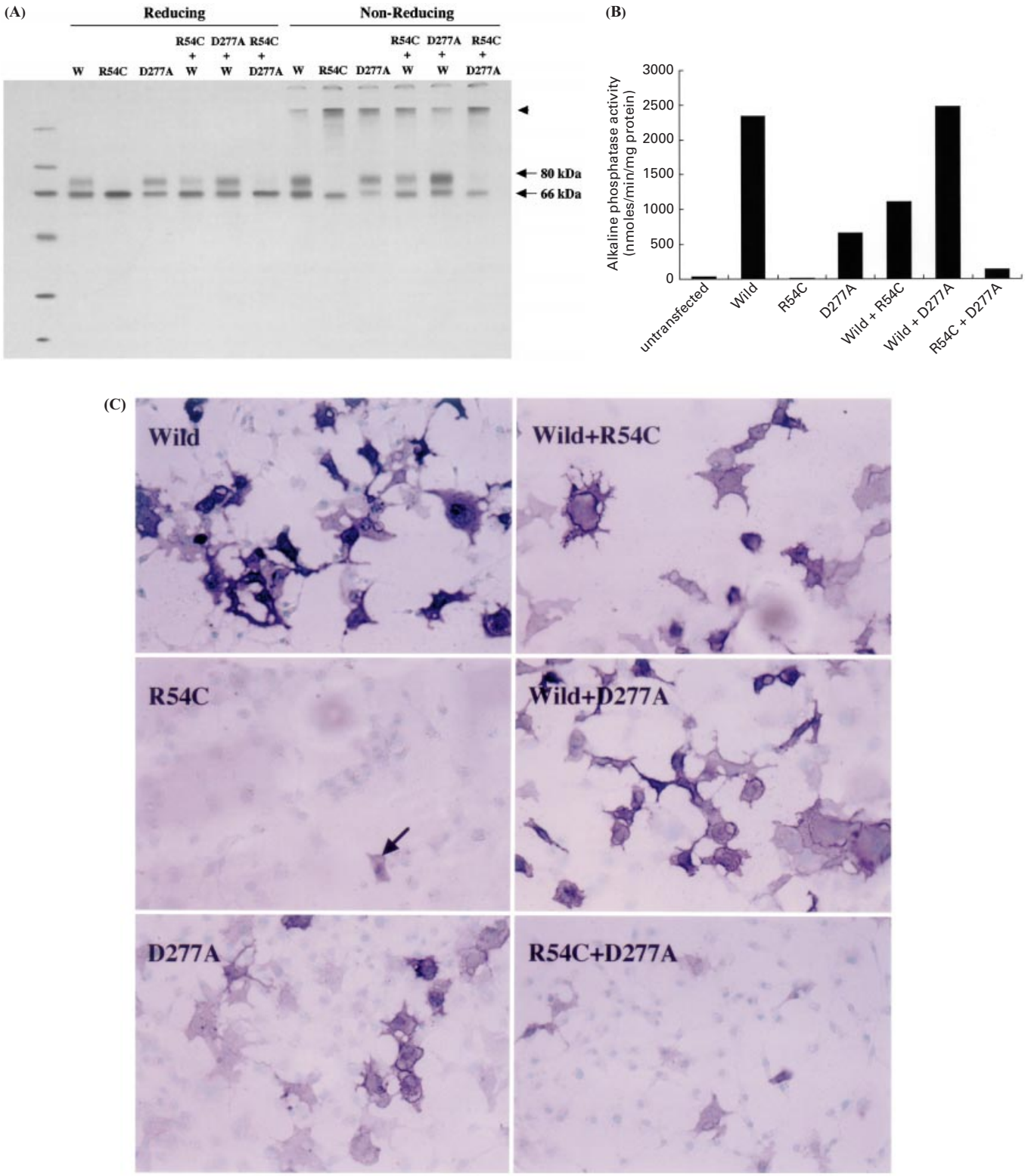


**Figure 5 Co-transfection with the cDNAs for the TNSALP mutants**

COS-1 cells were transfected with the cDNA encoding the WT TNSALP (W), TNSALP (R54C) or TNSALP (D277A), separately or in combination. (**A**) Cells were labelled with [$^{35}$S]methionine 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. When the cells were transfected with the cDNA separately, 1 $\mu$g of plasmid was routinely used, whereas for cells that were co-transfected with two different cDNAs, 0.5 $\mu$g of plasmid was used. Left lane: the same $^{14}$C-methylated protein markers as shown in Figure 2(B). An arrowhead indicates the position of the top of the resolving gel. (**B**) The transfected cells were homogenized and assayed for alkaline phosphatase activity. (**C**) The transfected cells were stained for alkaline phosphatase for 5 min at room temperature, as described in the Materials and methods section. The arrow indicates a single cell showing alkaline phosphatase activity.

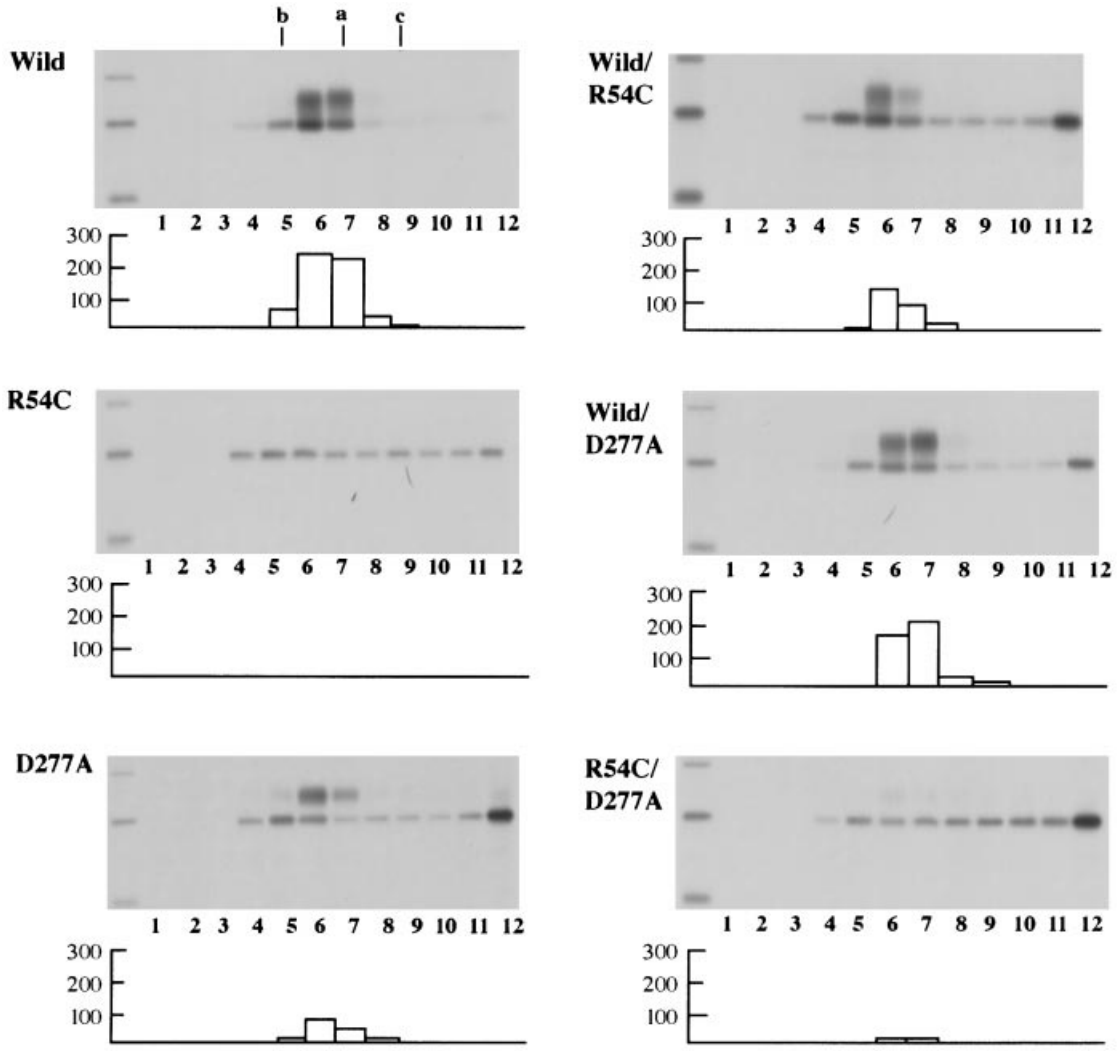


**Figure 6 Sucrose-density-gradient centrifugation**

COS-1 cells were transfected with the cDNA encoding TNSALP (WT) ('Wild'), TNSALP (R54C) or TNSALP (D277A), separately or in combination. Cells were pulsed-labelled with [$^{35}$S]methionine for 0.5 h and chased for 2 h. To prevent the degradation of TNSALP mutants, MG132 (50 $\mu$M) was included throughout the course of the experiments. Cell lysates were prepared and loaded on to a 5–35% sucrose gradient and centrifuged, as described in the Materials and methods section. Twelve fractions were collected from the top (fraction 1) and used for enzyme assays (lower panels of gel/histogram pairings: ordinate, alkaline phosphatase activity expressed as units/ml) and immunoprecipitation (upper panels). The left lane of each gel contains $^{14}$C-methylated protein markers (from the top of the gel, 97.4, 66 and 46 kDa). 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.

surface appearance of TNSALP (D277A) was less than that of TNSALP (WT) (Figures 2 and 3).

When co-transfected with the cDNA constructs for TNSALP (WT) and TNSALP (R54C) (Figure 5A), the 80-kDa mature form appeared in the cells, indicating that the latter does not seriously perturb maturation of the former. Although the level of alkaline phosphatase activity was lower than that of cells transfected by TNSALP (WT) alone (Figure 5B), this is because only half the amount of the WT plasmid (0.5 $\mu$g) was used in the co-transfection experiments. Similarly, the 80-kDa mature form was detected in cells co-transfected with the cDNA constructs for TNSALP (WT) and TNSALP (D277A) (Figure 5A). These findings agree with the notion that these mutation are inherited in a recessive manner [12]. In contrast, there was a marked decrease in the intensity of the 80-kDa mature form of TNSALP (D277A) in cells co-transfected with the cDNA constructs for the two mutants (Figure 5A). Consistent with the decrease in the 80-kDa mature form, alkaline phosphatase activity was also strongly suppressed in cells co-transfected with the cDNAs for the two TNSALP mutants (Figure 5B).

Cytohistochemical staining confirmed the presence of a strong alkaline phosphatase activity on the surface of cells transfected with cDNA for TNSALP (WT) or TNSALP (D277A) (Figure 5C). In contrast, cells transfected with cDNA for TNSALP (R54C) were almost deficient in positive staining, although we occasionally detected cells expressing alkaline phosphatase (Figure 5C, arrow). The strong-activity staining was also observed in the cells co-transfected with the cDNAs for the WT and either TNSALP (R54C) or TNSALP (D277A). Much weaker staining, however, was observed in cells co-transfected with the two mutant cDNAs compared with cells transfected with the cDNAs for TNSALP (WT) and TNSALP (R54C). A diminished cell-

surface appearance of TNSALP (D277A) in the co-transfected cells with the cDNAs for the mutants was supported further by cell-surface biotinylation (results not shown). Taken together, when co-transfected in the cells, TNSALP (R54C) exerts a *trans*-negative effect on TNSALP (D277A), resulting in a marked reduction of the functional enzyme on the cell surface.

### Analysis by sedimentation on sucrose gradient

To examine directly the oligomeric structure of TNSALP molecules in transfected cells, metabolically labelled cells were solubilized and proteins were separated by centrifugation on a 5–35 % sucrose gradient. MG132 was included throughout the pulse–chase experiments to block the degradation of the mutants. The gradient was fractionated and TNSALP was immunoprecipitated, before analysis with SDS/PAGE (Figure 6). TNSALP (WT) appeared in a peak at a position expected for the dimeric form of the 66- or 80-kDa protein. The distribution of alkaline phosphatase activity correlated well with that of the 80-kDa form of TNSALP. Consistent with Figure 5(a), only the 66-kDa form was detected in the cells expressing TNSALP (R54C) and a considerable proportion of newly synthesized TNSALP (R54C) formed high-molecular-mass aggregates (fractions 10–12). Although TNSALP (D277A) gave a similar sedimentation pattern to that of TNSALP (WT), obviously the proportion of aggregates was increased. The active 80-kDa form of the enzyme appeared in the cells co-transfected with the cDNAs for TNSALP (WT) and TNSALP (R54C) or TNSALP (D277A), confirming that the mutants do not seriously impair the oligomeric assembly of the wild-type enzyme. In contrast, there was a marked decrease in the level of the 80-kDa form of TNSALP (D277A) and a concomitant increase in the aggregates in the cells co-transfected with the cDNAs for TNSALP (R54C) and TNSALP (D277A) (compare Wild/R54C with R54C/D277A, top right and bottom right panels of Figure 5C), strongly suggesting that the former interferes with folding and assembly of the latter.

## DISCUSSION

Folding of a protein is thought to be primarily dictated by its amino acid sequence. Thus it is not surprising that replacement of a single amino acid with another could influence to varying degrees the formation of the functional three-dimensional structure of a protein by altering folding and assembly pathway in the ER [6,7].

Both TNSALP (R54C) and TNSALP (D277A) were reported in a hypophosphatasia patient who was a compound heterozygote for these alleles. $Arg^{54}$ is evolutionarily conserved, while $Asp^{277}$ is present in all four human alkaline phosphatase isoenzymes, but not in *Escherichia coli* alkaline phosphatase [37–41]. TNSALP (WT) was synthesized as the 66-kDa Endo H-sensitive form, and underwent processing of N-linked oligosaccharide chains to become the 80-kDa Endo H-resistant form, which was anchored to the cell surface via GPI. In contrast, TNSALP (R54C) remained Endo H-sensitive, formed high-molecular-mass aggregates and was finally degraded in the cell (probably in the ER), reflecting the fact that the replacement of arginine at position 54 with cysteine causes a major effect on protein folding and subunit assembly, such that this mutant failed to acquire transport-competence and formed aberrant disulphide-bonded aggregates. It remains to be determined whether the substituted cysteine residue is directly involved in the formation of interchain disulphide linkages. The aggregation and degradation of the mutant was not due to a defect in GPI-anchor attachment, since the 66-kDa form of the TNSALP mutant was found to be labelled with [$^3$H]ethanolamine. As a result, the cells expressing TNSALP (R54C) showed only negligible enzyme activity.

Although a considerable fraction of newly synthesized TNSALP (D277A) formed aggregates, as in the case of TNSALP (R54C), this mutant also gave rise to the 80-kDa form expressed on the cell surface. Once the mutant succeeded in acquiring a transport-competence structure, the mutant moved to the Golgi at almost the same rate as the WT enzyme, as evidenced by acquisition of Endo H, and exhibited a significant alkaline phosphatase activity. Since the $K_m$ value of TNSALP (D277A) is similar to that of the WT enzyme, the amino acid substitution at position 277 does not appear to grossly change the enzyme structure required for catalytic activity. Thus it is likely that the mutation reduces the probability that TNSALP (D277A) assumes a transport-competent structure, although the precise order of subunit folding and assembly remains unknown. In this context, we cannot exclude the possibility that TNSALP (R54C) also acquires transport-competence and is expressed on the cell surface, albeit at a much reduced level compared with TNSALP (D277A). Cytohistochemically, we detected a few cells exhibiting alkaline phosphatase activity in a large number of cells transfected with the cDNA for TNSALP (R54C), raising the possibility that, once properly folded and correctly oligomerized, an $Arg^{54} \rightarrow Cys$ substitution also does not strongly distort the catalytic domain of TNSALP.

When cells were co-transfected with the cDNA constructs for TNSALP (WT) in combination with either TNSALP (R54C) or TNSALP (D277A), the 80-kDa mature form appeared in the cells, indicating that neither of the mutants seriously perturb the intracellular transport of TNSALP (WT). In contrast, when cells were co-transfected with the cDNAs for the two mutants, the 80-kDa mature form of TNSALP (D277A) almost disappeared, resulting in a decrease of the enzyme activity. These findings strongly suggest that, when expressed in the same cell, TNSALP (R54C) interferes *in trans* with folding and assembly of TNSALP (D277A), thus reducing the conversion rate of TNSALP (D277A) into a transport-competent structure. Taken together, these results suggest that the suppression of alkaline phosphatase activity of TNSALP (D277A) by TNSALP (R54C) provides the molecular basis for lethal hypophosphatasia, found in a patient bearing these two hypophosphatasia alleles.

The possibility exists that low but significant levels of alkaline phosphatase activity detected in the co-transfected cells with the cDNAs for two mutants are ascribed to cells expressing only TNSALP (D277A), since the present experiments were performed on cells transiently expressing the two mutants. Although we have succeeded in cloning CHO cells stably expressing TNSALP (WT), several attempts to establish CHO clones stably expressing TNSALP (R54C) have so far failed, possibly because of the cytotoxity of the aggregates of TNSALP (R54C), which probably overwhelm the capacity of the lactacystin-sensitive degradation pathway in an early stage of the secretory pathway. We are currently establishing cell lines that express the mutant protein only in response to an external stimulus.

Recently, two groups have reported that TNSALP with substitutions of $Phe^{301} \rightarrow Leu$ and $Leu^{272} \rightarrow Phe$ exhibit a level of activity of 70 % and 50 % of the WT enzyme respectively upon their expression in cells [18,42]. However, it has not been determined whether the decrease in enzyme activity of cells expressing these TNSALP mutants is due to a quantifiable or a qualitative defect in TNSALP biosynthesis. So far we have analysed four naturally occurring missense mutations: TNSALP (R54C), TNSALP (D277A), TNSALP (A162T) and TNSALP (G317D), all of which were found in patients

diagnosed with lethal hypophosphatasia (perinatal and infantile hypophosphatasia) [20,21]. Interestingly, the phenotypes exhibited by these mutants are more or less similar. These amino acid changes seem to retard the folding of the enzyme into its normal conformation, resulting in the formation of the aggregates. An amino acid change could destabilize folding intermediates and alter the equilibrium between a proper conformation and aggregates in favour of formation of the aggregates. According to this hypothesis, the equilibrium constants might be different depending upon the site of mutations and the nature of the substituted amino acid. Consequently, the ratio of native enzyme to aggregates varies from one missense mutation to another [TNSALP (WT) > TNSALP (D277A) > TNSALP (A162) > TNSALP (R54C) = TNSALP (G317D)]. These TNSALP mutants were known to be inherited in a recessive manner. Consistent with this, TNSALP (R54C) or TNSALP (D277A) does not seem to significantly affect the folding and assembly of WT enzyme when expressed in the same cell. However, the co-transfection experiments strongly suggested that TNSALP (R54C) interferes *in trans* with the folding of TNSALP (D277A). Although the molecular mechanism underlying this interference event remains unknown, it is possible that unfolded intermediates of TNSALP (R54C) associate with those of TNSALP (D277A), causing a shift of the equilibrium to rapid aggregate formation of the latter. With respect to inheritance, some mild forms of hypophosphatasia are reported to be autosomally dominantly inherited [43]. If these diseases are caused by mutations in the coding region of the TNSALP gene, it will be interesting to examine how the mutants exert dominant-negative effects on the WT enzyme when expressed in the same cell.

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## REFERENCES

1 Harris, H. (1989) Clin. Chim. Acta **186**, 133–150
2 Whyte, M. P. (1994) Endocrine Rev. **15**, 439–461
3 Whyte, M. P., Walkenhorst, D. A., Fedde, K. N., Henthorn, P. S. and Hill, C. S. (1996) J. Clin. Endocrinol. Metab. **83**, 3936–3942
4 Waymire, K. G., Mahuren, J. D., Jaje, J. M., Guilarte, T. R., Coburn, S. P. and MacGregor, G. R. (1995) Nat. Genet. **11**, 45–51
5 Narisawa, S., Frohlander, N. and Millán, J. L. (1997) Dev. Dyn. **208**, 432–446
6 Amara, J. F., Cheng, S. H. and Smith, A. E. (1992) Trends Cell Biol. **2**, 145–149
7 Thomas, P. J., Qu, B.-H. and Pederson, P. L. (1995) Trends Biochem. Sci. **20**, 456–459
8 Helenius, A., Marquaedt, T. and Braakman, I. (1992) Trends Cell Biol. **2**, 227–231
9 Bergeron, J. J. M., Brenner, M. B., Thomas, D. Y. and Williams, D. B. (1994) Trends Biochem. Sci. **19**, 124–128
10 Weiss, M. J., Cole, D. E. C., Ray, K., Whyte, M. P., Lafferty, M. A., Mulivor, R. A. and Harris, H. (1988) Proc. Natl. Acad. Sci. U.S.A. **85**, 7666–7669
11 Weiss, M. J., Ray, K., Fallon, M. D., Whyte, M. P., Fedde, K. N., Lafferty, M. A., Mulivor, R. A. and Harris, H. (1989) Am. J. Genet. **44**, 686–694
12 Henthorn, P. S., Raducha, M., Fedde, K. N., Lafferty, M. A. and Whyte, M. P. (1992) Proc. Natl. Acad. Sci. U.S.A. **89**, 9924–9928
13 Greenberg, C. R., Taylor, C. L. D., Haworth, J. C., Seargeant, L. E., Philipps, S., Triggs-Raine, B. and Chodirker, B. N. (1993) Genomics **17**, 215–217
14 Orimo, H., Hayashi, Z., Watanabe, A., Hirayama, T. and Shimada, T. (1994) Hum. Mol. Genet. **3**, 1683–1684
15 Ozono, K., Yamagata, M., Michigami, T., Nakajima, S., Sakai, N., Cai, G., Satomura, K., Yasui, N., Okada, S. and Nakayama, M. (1996) J. Clin. Endocrinol. Metab. **81**, 4458–4461
16 Orimo, H., Goseki-Sone, M., Sato, S. and Shimada, T. (1997) Genomics **442**, 364–366
17 Goseki-Sone, M., Orimo, H., Iimura, T., Takagi, Y., Watanabe, H., Taketa, K., Sato, S., Mayanagi, H., Shimada, T. and Oida, S. (1998) Hum. Mutat., suppl. 1, S263–S267
18 Sugimoto, N., Iwamoto, S., Hoshino, Y. and Kajii, E. (1998) J. Hum. Genet. **43**, 160–164
19 Mornet, E., Taillandier, A., Peyramaure, S., Kaper, F., Muller, F., Brenner, R., Bussiere, P., Freisinger, P., Godard, J., Le Merrer, M. et al. (1998) Eur. J. Hum. Genet. **6**, 308–314
20 Shibata, H., Fukushi, M., Igarash, A., Misumi, Y., Ikehara, Y., Ohashi, Y. and Oda, K. (1998) J. Biochem. (Tokyo) **123**, 968–977
21 Fukushi, M., Amizuka, N., Hoshi, K., Ozawa, H., Kumagai, H., Omura, S., Misumi, Y., Ikehara, Y. and Oda, K. (1998) Biochem. Biophys. Res. Commun. **246**, 613–618
22 Omura, S., Matsuzaki, K., Fujimoto, T., Kosuge, K., Furuya, T., Fujita, S. and Nakagawa, A. (1991) J. Antibiotics **44**, 117–118
23 Miki, A., Tanaka, Y., Ogata, S. and Ikehara, Y. (1986) Eur. J. Biochem. **160**, 41–48
24 Oda, K., Amaya, Y., Fukushi-Irié, M., Kinameri, Y., Ohsuye, K., Kubota, I., Fujimura, S. and Kobayashi, J. (1999) J. Biochem. (Tokyo) **126**, 694–699
25 Laemmli, U. K. (1970) Nature (London) **227**, 680–685
26 Ikehara, Y., Mansho, K., Takahashi, K. and Kato, K. (1978) J. Biochem. (Tokyo) **83**, 1471–1483
27 Burstone, M. S. (1962) in Enzyme Histochemistry and Its Application on the Study of Neoplasms, (Burstone, M. S., ed.), pp. 275–276, Academic Press, New York
28 Ikezawa, H., Yamanegi, M., Taguchi, R., Miyashita, T. and Ohyabu, T. (1976) Biochim. Biophys. Acta **450**, 154–164
29 Low, M. G. and Finean, J. B. (1977) Biochem. J. **167**, 281–284
30 Field, M. C., Moran, P., Wenlu, L., Keller, G.-A. and Caras, I. W. (1994) J. Biol. Chem. **269**, 10830–10837
31 Oda, K., Ikehara, Y. and Omura, S. (1996) Biochem. Biophys. Res. Commun. **219**, 800–805
32 Oda, K., Wada, I., Takami, N., Fujiwara, T., Misumi, Y. and Ikehara, Y. (1996) Biochem. J. **316**, 623–630
33 Wibourne, B., Nesbeth, D. N., Wainwright, L. J. and Field, M. C. (1998) Biochem. J. **332**, 111–118
34 Ohkubo, A., Langerman, N. and Kaplan, M. M. (1974) J. Biol. Chem. **249**, 7174–7180
35 Oida, S., Sone, M. and Sasaki, S. (1984) Anal. Biochem. **140**, 117–120
36 Hawrylak, K. and Stinson, R. A. (1988) J. Biol. Chem. **263**, 14368–14373
37 Weiss, M. J., Henthorn, P. S., Lafferty, M. A., Slaughter, C., Raducha, M. and Harris, H. (1986) Proc. Natl. Acad. Sci. U.S.A. **83**, 7182–7186
38 Millán, J. L. (1986) J. Biol. Chem. **261**, 3112–3115
39 Henthorn, P. S., Raducha, M., Edwards, Y. H., Weiss, M., Slaughter, C., Lafferty, M. A. and Harris, H. (1987) Proc. Natl. Acad. Sci. U.S.A. **84**, 1234–1238
40 Millán, J. L. and Manes, T. (1988) Proc. Natl. Acad. Sci. U.S.A. **85**, 3024–3028
41 Kishi, F., Matsuura, S. and Kajii, T. (1989) Nucleic Acids Res. **17**, 2129
42 Cai, G., Michigami, T., Yamamoto, T., Yasui, N., Satomura, K., Yamagata, M., Shima, M., Nakajima, S., Mushiake, S., Okada, S. and Ozono, K. (1998) J. Clin. Endocrinol. Metab. **83**, 3936–3942
43 Whyte, M. P., Landt, M., Ryan, L. M., Mulivor, R. A., Henthorn, P. S., Fedde, K. N., Mahuren, J. D. and Coburn, S. P. (1995) J. Clin. Invest. **95**, 1440–1445

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