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Defective Intracellular Transport of Tissue-Nonspecific Alkaline Phosphatase with an Ala^{162}→Thr Mutation Associated with Lethal Hypophosphatasia[1]

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We have studied the biosynthesis and intracellular transport of tissue-nonspecific alkaline phosphatase (TNSALP) transiently expressed in COS-1 cells. Mutations were introduced into TNSALP to examine the effects of a single amino acid substitution on the activity and biosynthesis of TNSALP. The cells expressing wild-type TNSALP exhibited more than 200-fold higher alkaline phosphatase activity than untransfected ones. Pulse-chase experiments showed that TNSALP was synthesized as a 66-kDa endoglucosaminidase H (Endo H)-sensitive form and converted to EndoH-resistant forms with heterogenous molecular masses (~80 kDa), which finally appeared on the cell surface as judged by digestion with phosphatidylinositol-specific phospholipase C (PI-PLC). In contrast, a TNSALP with a Glu^{218}→Gly mutation exhibited no phosphatase activity at all and the 66-kDa Endo H-sensitive form was the only molecular species throughout the chase in the transfected cells. In accordance with this finding, digestion with PI-PLC and immunofluorescence observation confirmed that this mutant was never expressed on the cell surface. Another mutant with a Ala^{162}→Thr substitution, which naturally occurs in association with a lethal hypophosphatasia, exhibited a low activity and only a small fraction of the 66-kDa form acquired Endo-H resistance and reached the cell surface. Since the wild-type and the mutant TNSALPs were labeled with [^{3}H]ethanolamine, a component of glycosylphosphatidylinositol (GPI), it is unlikely that the impaired intracellular transport of the two mutants is due to a failure in their modification by GPI. Interestingly, the 66-kDa Endo H-sensitive form of the TNSALP mutants but not that of the wild-type, was found to form an interchain disulfide-bonded high-molecular-mass aggregate within the cells. These results suggest that impaired intracellular transport of the TNSALP (Ala^{162}→Thr) molecule caused by its aggregation is the molecular basis for the lethal hypophosphatasia carrying this mutation.

Key words: aggregation, defective intracellular transport, glycosylphosphatidylinositol, hypophosphatasia, tissue-nonspecific alkaline phosphatase.

In humans, there are four isoenzymes of alkaline phosphatase: tissue-nonspecific (TNSALP), intestinal, placental (PLAP), and germ cell (placental-like) alkaline phosphatase, each encoded by a separate gene (*1*). The latter three enzymes are distributed essentially in a tissue-specific manner, while TNSALP is expressed in a wide variety of tissues and cells and is abundant in liver, bone and kidney. Thus, TNSALP is also called liver/bone/kidney alkaline phosphatase. Since the isozymes are known to be anchored on the outer leaflet of plasma membranes *via* glycosylphosphatidylinositol (GPI) (*2-5*), it seems likely that the enzymes catalyze the hydrolysis of phospho-compounds at the extracellular surface, though the natural substrates for each isoenzyme remain to be unequivocally unidentified.

Hypophosphatasia is an inborn error of metabolism characterized by a reduced TNSALP activity in serum and tissues with a concomitant increase in the levels of several phospho-compounds such as phosphoethanolamine, inorganic pyrophosphate, and pyridoxal 5′-phosphate in the serum and urine of affected individuals (*6*). In spite of the ubiquitous distribution of TNSALP, clinical symptoms are restricted to failure of mineralization of bone and dentition,

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Abbreviations: DMEM, Dulbecco's modified MEM; Endo H, endoglucosaminidase H; ER, endoplasmic reticulum; GPI, glycosylphosphatidylinositol; MEM, minimum essential medium; PAGE, polyacrylamide gel electrophoresis; PNGase F, Peptide:*N*-glycosydase F; PBS, phosphate-buffered saline; PI-PLC, phosphatidylinositol-specific phospholipase C; PLAP, placental alkaline phosphatase; SDS, sodium dodecyl sulfate; TNSALP, tissue-nonspecific alkaline phosphatase.

leaving a question as to the physiological importance of TNSALP in other tissues and organs. Patients with hypophosphatasia manifest a wide range of symptoms from death *in utero* with an unmineralized skeleton to premature loss of deciduous teeth in childhood and pseudofracture first presenting in adult life, indicating that TNSALP is closely involved in the mineralization of bone and tooth, though the precise mechanism whereby TNSALP participates in the formation and maintenance of hydroxyapatites remains to be clarified. Several mutations have been identified in the TNSALP gene associated with a wide clinical spectrum of hypophosphatasia. In most cases they are missense mutation in a homozygous or compound heterozygous state (*7-10*), though, recently a frame-shift mutation has also been reported (*11*). Severe forms of the disease are inherited in an autosomal recessive manner, while modes of transmission of mild forms are obscure. Weiss *et al.* (*7*) first reported a single base change (G711A) in exon 6 of the TNSALP gene, which converts Ala162 to Thr, of an affected infant with severe skeletal hypomineralization. The patient was homozygous for the mutant allele and died at the age of 3 months. When expressed in NIH 3T3 cells, the mutant cDNA (G711A) gave rise to mRNA of the predicted size and produced an immunologically crossreacting material (*7*); however, it has not been elucidated how this particular missense mutation affects the TNSALP molecule and causes severe hypophosphatasia.

Relatively little is understood about the biosynthesis of TNSALP, in contrast to PLAP, which has been extensively studied, particularly in the context of GPI-anchoring (*12-16*). In order to define the molecular defect of TNSALP mutants, we expressed the wild-type TNSALP and two mutants, including one with the Ala^{162}→Thr substitution, in a heterologous expression system and examined their biosynthesis. Here we report that the missense mutations profoundly affect the intracellular transport of TNSALP molecules, probably by causing their aggregation in the endoplasmic reticulum (ER).

EXPERIMENTAL PROCEDURES

Materials—Trans^{35}S-label (>1,000 Ci/mmol) was obtained from ICN Biomedicals (Boston, MA, USA), [1-^{3}H]-ethanolamine hydrochloride (12.0 Ci/mmol) and ^{14}C-methylated proteins were from Amersham (Arlington Heights, IL, USA); Protein A-Sepharose CL-4B and Cy5 AutoRead Sequence Kit from Pharmacia-LKB Biotechnology (Uppsala, Sweden); Lipofectin Reagent from Gibco-BRL (Gaithersburg, MD, USA); immobilized streptavidin from Pierce (Rockford, IL); aprotinin from Sigma Chemical (St. Louis, MO, USA); Pansorbin from Calbiochem-Novabiochem (La Jolla, CA, USA); rhodamine-conjugated goat anti-(rabbit IgG) from Cappel Laboratories (Malvern, PA, USA); Peptide:N-glycosydase F (*Flavobacterium meningosepticum*, PNGase F), various DNA-modifying enzymes and restriction endonucleases from New England Biolabs (Beverly, MA, USA) and Takara Shuzo (Kyoto); antipain, chymostatin, elastatinal, leupeptin, and pepstatin A from Protein Research Foundation (Osaka); tunicamycin from Wako Pure Chemical (Osaka); phosphatidylinositol-specific phospholipase C (PI-PLC) from Funakoshi (Tokyo); sulfosuccinimidyl *N*-(D-biotinyl)-6-aminohexanoate from Dojindo Laboratories (Kumamoto); endo-β-*N*-acetylglucosaminidase H (*Streptomyces griseus*, Endo H) from Seikagaku Kogyo (Tokyo); neuraminidase (*Arthrobacter ureafaciens*) from Nacalai (Kyoto). TNSALP was purified from rat ascites hepatoma (AH-130) expressing a high level of TNSALP and antiserum against TNSALP was raised in rabbits as described previously (*17*). Antiserum against human placental alkaline phosphatase (PLAP) was raised in rabbits as described previously (*17*). COS-1 cells were cultured in Dulbecco's modified Eagle's minimum essential medium (DMEM) supplemented with 10% fetal bovine serum (*18*). SAOS-2 cells were cultured in α-MEM supplemented with 10% fetal bovine serum.

Plasmids and Transfection—A full-length cDNA for human TNSALP (*19*), which was originally deposited by Weiss, was obtained from the Japanese Cancer Research Resources Bank at the National Institute of Health and subcloned into a unique *Eco*RI site of the mammalian expression vector pSG5 (Stratagene, San Diego, USA). Since this original cDNA clone (pSG5-TNSALPGlu^{218}→Gly, E218G) has a single base change of adenine at 880 to guanine (*20*), a mutation was introduced to create a cDNA for a wild type-TNSALP (pSG5-TNSALP). Another plasmid encoding a TNSALP mutant (pSG5-TNSALPAla^{162}→Thr, A162T), in which guanosine of 711 was changed to adenosine, was also created. Point mutations were introduced at desired sites using a Takara Mutan K kit (Takara Shuzo, Kyoto). Oligonucleotides used were: 5′-GATGTGGAGTATGAGAGT-3′ for (G880→A) and 5′-CGCCTACACCCACTCGGC-3′ for (G711→A). Mutations were verified by restriction enzyme digestion and nucleotide sequences of mutation sites were further determined by the dideoxynucleotide chain termination method using a Cy5 AutoRead Sequence Kit. Construction of pSG5-PLAP and transfection of the plasmid were performed essentially as described previously (*18*). Each purified plasmid (4-5 μg) in 50 μl of phosphate-buffered saline (PBS) was mixed with 4-5 μg of Lipofectin Reagent in 50 μl of PBS and the mixture was incubated at room temperature for 15 min. COS-1 cells, which had been inoculated (1.0×10^5 cells/35-mm plastic dish) ~24 h before transfection, were washed three times with DMEM and incubated with the plas- mid-Lipofectin complex in 1 ml of DMEM in a humidified CO_2 incubator. After 6-8 h incubation the medium was replaced with 2 ml of DMEM supplemented with 10% fetal bovine serum and the cells were incubated for 24 h before each experiment.

Metabolic Labeling and Immunoprecipitation—For pulse-chase experiments, cells were preincubated for 0.5-1 h in the methionine-free MEM and metabolically labeled with 25-50 μCi of [^{35}S]methionine for 0.5-3 h in the fresh methionine-free MEM. After a pulse period, cells were washed and chased in the MEM as described previously (*18*). In some cases, pulse-labeling was stopped by the addition of cold methionine (2 mM) and a chase was conducted to see if newly synthesized TNSALP was secreted into the medium. Where indicated, cells were preincubated for 1 h in the presence of 2 μg/ml of tunicamycin and labeled with [^{35}S]methionine in its presence. For specific labeling of the GPI-anchor moiety of TNSALP, cells were preincubated in the MEM for 1 h and labeled with 300 μCi of [^{3}H]ethanolamine in the MEM for 16 h. The medium was removed, and cells were washed with cold PBS and lysed in 0.5 ml of lysis buffer [1% TritonX-100/

0.5% deoxycholate/0.05% sodium dodecyl sulfate (SDS) in PBS] as described previously (*18*). A protease inhibitor cocktail (antipain, aprotinin, chymostatin, elastatinal, leupeptin, and pepstatin A) was added to cell lysates and media (10 μg of each/ml). The lysates were incubated for 20 min at 37°C to extract TNSALP according to Brown and Rose (*15*). The lysates and media were centrifuged at 15,000×*g* for 10 min to remove insoluble materials and incubated with 30-40 μl of a 10% suspension of Pansorbin (fixed *Staphylococcus aureus* cells) for 30 min. The precleared supernatants were mixed with 50 μl of powdered skim milk (5% in PBS) and incubated with 2-3 μl of anti-rat TNSALP serum for 3-16 h. The immune complexes were collected on 30-40 μl of protein A-Sepharose (50% suspension in PBS) by shaking for 1 h and washed once with the lysis buffer and then twice with a high salt washing buffer [10 mM Tris/HCl (pH 7.5) containing 500 mM NaCl, 0.5% Nonidet P-40, and 1 mM EDTA], followed by washing twice with a low salt washing buffer [10 mM Tris/HCl (pH 7.5) containing 150 mM NaCl, 0.5% Nonidet P40, and 1 mM EDTA] (*21*). The immune complexes/protein A beads were used for enzyme digestion or directly boiled in Laemmli's sample buffer (*22*) and analyzed by SDS-polyacrylamide gel electrophoresis (PAGE, 9% gel). Gels were fixed and equilibrated in 1 M sodium salicylate prior to fluorography according to Chamberlain (*23*).

Enzyme Digestions—For PI-PLC digestion, metabolically labeled cells were washed three times with MEM and subsequently incubated with 0.1-0.2 unit of PI-PLC in 0.7 ml of MEM for 1 h in a CO_2 incubator (*18*). Media were harvested and centrifuged at 5,000×*g* for 5 min to remove detached cells. The resultant supernatants were subjected to immunoisolation as described above. For digestion with Endo H or neuraminidase, immune complexes collected on protein A beads were washed once with distilled water, divided into two equal parts, then suspended in 50 μl of 50 mM acetate buffer (pH 5.5) for Endo H digestion or 50 μl of 50 mM acetate buffer (pH 5.0) for neuraminidase digestion in the presence of the protease inhibitor cocktail. Enzyme digestions were carried out in the absence or presence of 0.2 unit/ml of Endo H or 0.5 unit/ml of neuraminidase at 37°C for 16 h. Immune complex/protein A beads were washed once with distilled water and boiled in Laemmli's sample buffer (*22*). For digestion with PNGase F, immunocomplexes were dissociated from protein A beads by boiling in PBS containing 1% SDS for 3 min, and then diluted 10-fold with 1% NP-40. Unless otherwise stated, digestion was carried out in the presence of 500 units/ml of the enzyme and the protease inhibitor cocktail at 37°C for 1 h according to the manufacturer's protocol. Proteins were precipitated with cold acetone containing 0.1 M HCl and dissolved in Laemmli's sample buffer.

Cell Surface Biotinylation—Biotinylation was performed essentially according to Lisanti *et al.* (*24*). Metabolically labeled cells were washed three times with 20 mM Hepes buffer (pH 7.3) containing 150 mM NaCl and incubated at 4°C with 0.5 mg/ml of sulfosuccinimidylbiotin in the same buffer for 20 min. After the biotinylation reagent was removed, the cells were incubated with the same buffer containing 10 mM glycine at 4°C for 10 min. Cells were scraped off using a rubber policeman and centrifuged at 10,000×*g* for 5 min. The resulting pellets were lysed in 0.5 ml of the lysis buffer containing 10 mM glycine and the protease inhibitor cocktail. The lysates were warmed at 37°C for 20 min to solubilize TNSALP and centrifuged at 15,000×*g* for 10 min. Total TNSALP molecules (intracellular and cell surface) were immunoprecipitated as described above. The immune complexes/protein A-Sepharose were divided into two equal parts. One was directly boiled in Laemmli's sample buffer to analyze total TNSALP. The other was boiled in 50 μl of PBS containing 1% SDS and centrifuged. The resultant supernatant was adjusted to final concentrations of 1% NP40 and 0.05% SDS and further incubated with 40 μl of streptavidin beads (50% slurry) at 4°C overnight. The biotinylated TNSALP/streptavidin beads were extensively washed, then boiled in Laemmli's sample buffer (*22*).

Determination of Protein and Enzyme 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 and protein. Alkaline phosphatase was assayed using *p*-nitrophenyl phosphate as a substrate (*25*). Proteins were assayed using a Bio-Rad protein assay kit (Hercules, CA, USA).

Immunofluorescence—COS-1 cells grown on cover slips in a 35-mm dish were transfected with 5 μg of plasmid as described above. After 24 h, the cells were fixed with 4% paraform-aldehyde in PBS for 20 min at 4°C. The fixative was aspirated and the cells were rinsed with cold PBS. The cells were further incubated with PBS in the absence or presence of 0.1% NP40 for 20 min. Then intact cells and permeabilized cells were incubated with PBS containing 5% skim milk in the absence or presence of 0.1% NP40 overnight. The cells were incubated first with anti-rat TNSALP serum, and then with rhodamine-conjugated goat anti-rabbit IgG. The cover slip was washed with PBS, mounted on a slide glass with 90% glycerol in PBS and subjected to microscopic observations.

RESULTS

Expression of the Wild-Type TNSALP in COS-1 Cells—COS-1 cells transiently expressing human TNSALP were labeled with [^{35}S]methionine and lysed, then TNSALP molecules were immunoisolated with anti-rat TNSALP antiserum and analyzed by SDS-PAGE, followed by fluorography. As shown in Fig. 1A, two major bands (66 and ~80 kDa) were detected in the cells transfected with a plasmid encoding human wild-type TNSALP (lane 2), but not in untransfected cells (lane 1). Anti-rat TNSALP antiserum also crossreacted with a 42-kDa unidentified protein endogenously expressed in COS-1 cells and SAOS-2 cells (lanes 1 and 4). Upon incubation with PI-PLC, mainly ~80-kDa band was found to be released into the medium (lane 3), indicating that TNSALP molecules expressed in the COS-1 cell are anchored on the cell surface *via* GPI, as are those expressed endogenously in SAOS-2 osteosarcoma cells (lanes 4 and 5), from which the original cDNA clone of TNSALP was isolated (*19*). Similarly, PI-PLC treatment released PLAP expressed in the COS-1 cells (lanes 6 and 7) into the medium as reported previously (*13, 18*). As opposed to the sharp band in SAOS-2 cells, a broad band of TNSALP is presumably due to underglycosylation in the transfected COS-1 cells, where the synthesis of a large amount of TNSALP might overwhelm the capacity of the cellular glycosylation machinery. After

digestion with PNGase F, which removes both high mannose-type and complex-type *N*-linked oligosaccharides from glycoproteins, the two TNSALP molecules expressed in the different cell lines became indistinguishable and migrated to the same position as that of TNSALP synthesized in the presence of tunicamycin, which inhibits core glycosylation of proteins in the ER (Fig. 1B). The major ~80-kDa form of TNSALP was at least in part terminally glycosylated in the transfected cells as evidenced by digestion with neuraminidase (Fig. 1A, lanes 8 and 9). The nucleotide sequence of the human TNSALP cDNA encodes a 524 amino acid polypeptide containing 5 potential *N*-linked glycosylation sites (*19*), while that of human PLAP cDNA encodes a 534 amino acid polypeptide with two putative *N*-linked glycosylation sites (*26*). To estimate the extent to which TNSALP bears *N*-glycan, pulse-labeled TNSALPs were incubated with various concentrations of PNGase F. As shown in Fig. 2A, two bands were detected during the deglycosylation reaction catalyzed by PNGase F (lanes 4 and 5), indicating that at least 3 putative sites of TNSALP are *N*-glycosylated. The molecular mass of unglycosylated TNSALP synthesized in the presence of tunicamycin was 55 kDa (Fig. 2B, lane 4), compatible with that estimated from its amino acid sequence (57.2 kDa) (*19*), taking the cleavage of a 17 amino acid signal sequence into account. Transiently expressed TNSALP was not detected in the medium throughout the chase time either in the absence or presence of tunicamycin (Fig. 2B, lanes 2 and 5). Interestingly the unglycosylated TNSALP was also expressed on the cell surface *via* GPI as judged from the result of PI-PLC digestion (Fig. 2B, lane 6).

Expression of TNSALP Mutants in COS-1 Cells—To see

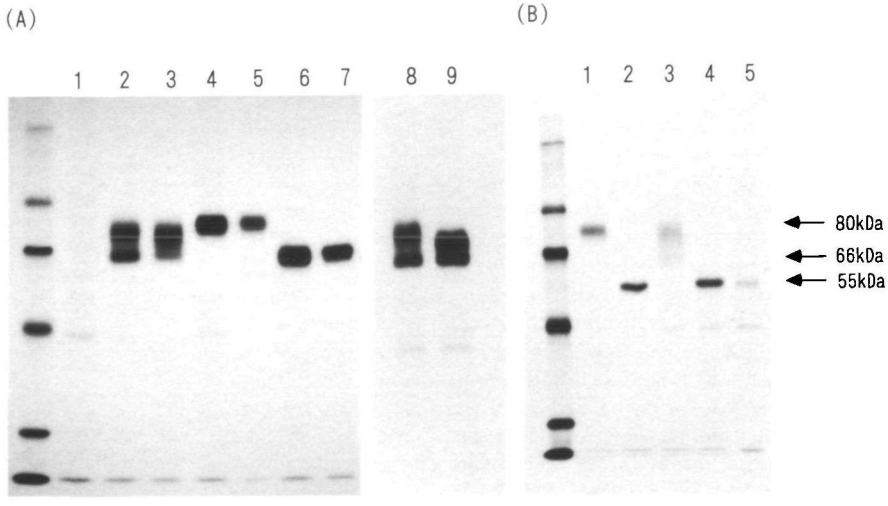

Fig. 1. **Expression of TNSALP in COS-1 cells.** (A) Untransfected COS-1 cells (lane 1), COS-1 cells expressing either the wild-type TNSALP (lanes 2, 3, 8, and 9) or PLAP (lanes 6 and 7), or SAOS-2 osteosarcoma cells (lanes 4 and 5) were labeled with [^{35}S]-methionine for 4 h. The cells were further incubated with PI-PLC for 1 h (lanes 2–7). The cell lysates (lanes 2, 4, 6, 8, and 9) and PI-PLC digestion media (lanes 3, 5, and 7) were subjected to immunoisolation using anti-rat TNSALP antiserum (lanes 1–5, 8, and 9) or anti-PLAP (lanes 6 and 7). Immune complexes were analyzed by SDS-PAGE, followed by fluorography. Some immune complexes were further incubated in the absence (lane 8) or presence (lane 9) of neuraminidase. Left lane: ^{14}C-methylated proteins (from the top of the gel, 200, 97.4, 69, 46, 30, and 14.3 kDa). (B) SAOS-2 osteosarcoma cells (lanes 1 and 2) or COS-1 cells expressing the wild-type TNSALP (lanes 3, 4, and 5) were labeled with [^{35}S]methionine for 4 h in the absence (lanes 1–4) or presence (lane 5) of tunicamycin. The cell lysates were prepared and subjected to immunoisolation. The immune complexes were further incubated in the absence (lanes 1 and 3) or presence (lanes 2 and 4) of PNGase F as described under "EXPERIMENTAL PROCEDURES." Each sample was analyzed by SDS-PAGE/fluorography. Left lane: the same ^{14}C-methylated proteins as shown in 1A.

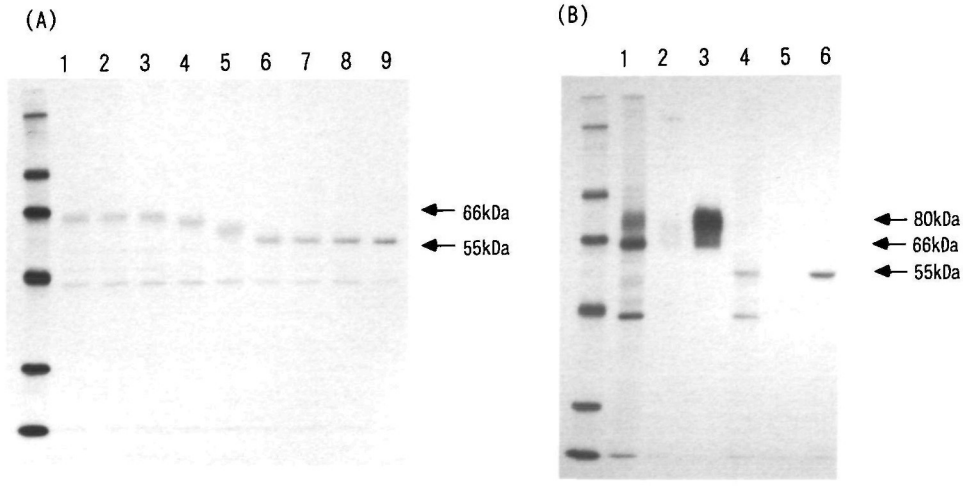

Fig. 2. ***N*-linked oligosaccharide chains in the TNSALP molecule.** (A) COS-1 cells expressing wild-type TNSALP were labeled with [^{35}S]methionine for 30 min. The cell lysates were prepared and subjected to immunoisolation. Immune complexes were dissociated by boiling and further incubated at 37°C for 1 h in the absence (lane 1) or presence of 5 U/ml (lane 2), 10 U/ml (lane 3), 50 U/ml (lane 4), 100 U/ml (lane 5), 500 U/ml (lane 6), 1,000 U/ml (lane 7), 5,000 U/ml (lane 8) of PNGase F. Lane 9, TNSALP immunoisolated from tunicamycin-treated cells. Left lane: the same ^{14}C-methylated proteins as shown in Fig. 1A. (B) COS-1 cells expressing the wild-type TNSALP were labeled with [^{35}S]methionine in the absence (lanes 1–3) or presence (lanes 4–6) of tunicamycin. Following a 2-h pulse cells were chased for 4 h by the addition of cold methionine. After the chase, the media were harvested, and the cells were further incubated with PI-PLC. The cell lysates (lanes 1 and 4), chase media (lanes 2 and 5), and PI-PLC digestion media (lanes 3 and 6) were subjected to immunoisolation. Each sample was analyzed by SDS-PAGE/fluorography. Left lane: the same ^{14}C-methylated proteins as shown in Fig. 1A.

the effects of a single amino acid change on the expression of TNSALP, two mutants were analyzed. A mutant with Glu^{218}→Gly substitution was identified in the original cDNA clone isolated by Weiss *et al.* (*19, 20*). They assumed that this mutation was introduced erroneously by reverse transcriptase during cDNA cloning or represents a null allele in SAOS-2 cells (*20*). Another mutant, Ala^{162}→Thr, was found in an infant associated with lethal hypophosphatasia (*7*). Enzyme activity was assayed in cells transiently expressing each of the TNSALPs. Cells expressing the wild-type TNSALP exhibited the highest alkaline phosphatase activity among the three TNSALPs (Fig. 3). Activity was negligible in the cells expressing TNSALP (Glu^{218}→Gly), while a low activity was detected in those expressing TNSALP (Ala^{162}→Thr). These results suggest that Glu218 is essential for enzyme activity, as reported by Weiss *et al.* (*20*), while Ala162 is not absolutely required.

Figure 4 shows the biosynthesis of TNSALP molecules expressed in COS-1 cells. After labeling with [^{35}S]methionine, cells were further incubated with PI-PLC to release TNSALP on the cell surface (lane 3). The cells were lysed and subjected to immunoprecipitation (lane 1). The 66-kDa and ~80-kDa bands were detected in the cells transfected with the wild-type TNSALP cDNA (Fig. 4A, lane 1). Under this labeling condition, most of the ~80-kDa species reached the cell surface as shown in Fig. 4A (lane 3). In the cells expressing TNSALP (Ala^{162}→Thr), both the 66-kDa and ~80-kDa bands were also detected, but, the amount of the ~80-kDa band was markedly reduced compared with that in the cells expressing the wild-type protein (Fig. 4, A and B, lane 1). Furthermore PI-PLC digestion showed that the surface expression of TNSALP (Ala^{162}→Thr) was far less efficient than that of the wild-type TNSALP (Fig. 4B, lane 3). Remarkably, the 66-kDa band was the only form found in the cells expressing TNSALP (Glu^{218}→Gly) (Fig. 4C, lane 1) and was never expressed on the cell surface, as judged by PI-PLC digestion (Fig. 4C, lane 3). Neither TNSALP (Ala^{162}→Thr) nor TNSALP (Glu^{218}→Gly) was secreted into the medium (Fig. 4, B and C, lane 2).

The wild-type TNSALP migrated exclusively as a monomeric form on SDS-polyacrylamide gel, irrespective of non-reducing or reducing conditions (Fig. 4A, lanes 1 and 4). Interestingly, a significant amount of the newly synthesized TNSALP (Ala^{162}→Thr) was found on top of the gel under non-reducing conditions (Fig. 4B, lane 4), indicating that this mutant forms disulfide-bonded high-molecular-mass aggregates within the cell. Note that the ~80-kDa form was monomeric, while the 66-kDa form was present in disulfide-bonded aggregates within the cell. Furthermore all TNSALP (Glu^{218}→Gly) molecules were found on top of the gel under non-reducing conditions (Fig. 4C, lane 4). These findings imply that the formation of high-molecular-

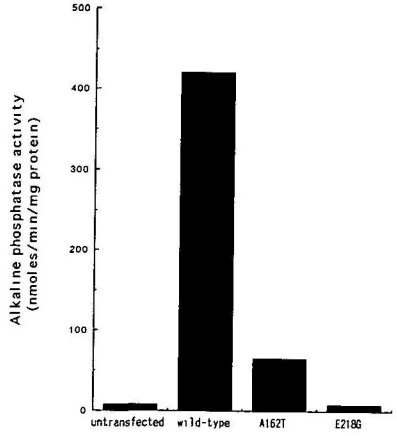

Fig. 3. **Alkaline phosphatase activity of the TNSALP mutants expressed in COS-1 cells.** Untransfected COS-1 cells, and COS-1 cells expressing either the wild-type TNSALP, TNSALP (Ala^{162}→Thr) and TNSALP (Glu^{218}→Gly) were homogenized and assayed for alkaline phosphatase activity. Values are averages of two experiments.

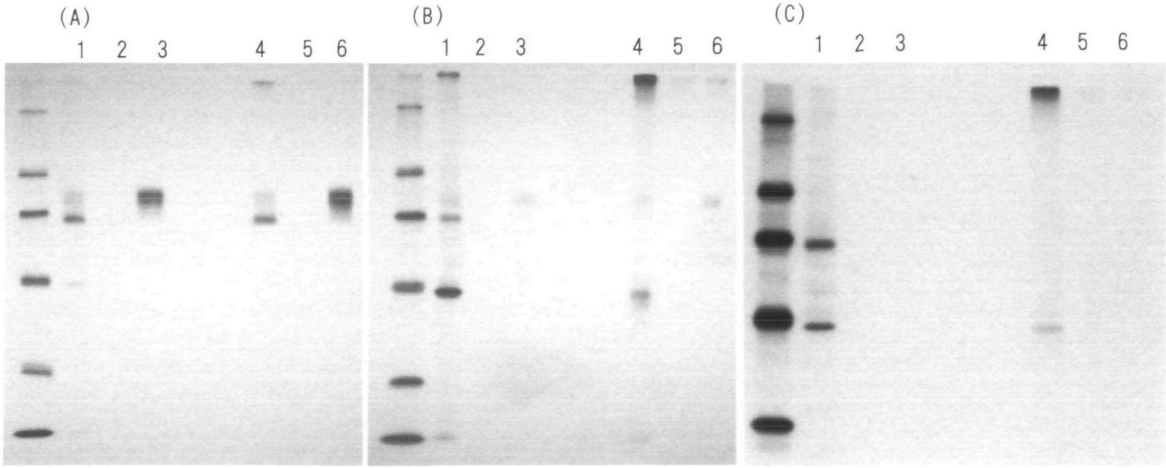

Fig. 4. **Expression of the TNSALP mutants in COS-1 cells.** COS-1 cells expressing the wild-type TNSALP (A), TNSALP (Ala^{162}→Thr) (B), or TNSALP (Glu^{218}→Gly) (C) were labeled with [^{35}S]-methionine. Following a 3-h pulse period, cells were chased for 4 h by addition of cold methionine. After the chase, the media were harvested, and the cells were further incubated with PI-PLC. The cell lysates (lanes 1 and 4), chase media (lanes 2 and 5), and PI-PLC digestion media (lanes 3 and 6) were subjected to immunoisolation. The immune complexes were dissolved in Laemmli's sample buffer, boiled in the absence (lanes 4-6) or presence (lanes 1-3) of 1% β-mercaptoethanol and then analyzed by SDS-PAGE/fluorography. Left lane: the same ^{14}C-methylated proteins as shown in Fig. 1A.

mass aggregates is related to the reduced surface expression of TNSALP mutants. The 42-kDa protein was also found on top of the gel under non-reducing conditions (Fig. 4A, lanes 1 and 4). The 42-kDa protein itself tends to form disulfide-linked high-molecular-mass aggregates in untransfected COS-1 cell (data not shown).

Pulse-Chase Experiment—In order to study the intracellular transport of TNSALPs in detail, pulse-chase experiments were performed and immunoisolated TNSALP molecules were analyzed for sensitivity to Endo H, which removes high mannose-type oligosaccharide chains from glycoproteins (Fig. 5). Following a 30 min labeling, the 66-kDa Endo H-sensitive form was the only wild-type TNSALP molecule in the cell (Fig. 5A, lanes 1 and 2). As chase time elapsed, the ~80k-Da Endo H-resistant form progressively increased and the newly synthesized proteins largely became Endo H-resistant by 4 h of chase (Fig. 5A, lanes 7 and 8), though a small fraction of TNSALP was still Endo H-sensitive. From these results together with the data in Figs. 1-4, we conclude that the wild-type TNSALP is synthesized as the 66-kDa form with high mannose-type oligosaccharides and converted to the ~80-kDa form bearing complex-type carbohydrate chains, which is eventually expressed as an active enzyme on the cell surface *via* GPI.

In the cells expressing TNSALP (Ala^{162}→Thr), the ~80k-Da Endo H-resistant form was found, though most TNSALP (Ala^{162}→Thr) remains Endo H-sensitive even after a 4 h chase (Fig. 5B, lanes 7 and 8). These results strongly suggest that only a small fraction of the newly synthesized TNSALP (Ala^{162}→Thr) gains access to the Golgi, undergoes processing of the carbohydrates and reaches the cell surface, while most of the TNSALP (Ala^{162}→Thr) molecules are retained as Endo H-sensitive form within the cells, presumably due to the formation of aggregates. In accordance with the results in Fig. 4C, the ~80-kDa molecular species was totally absent in the cells expressing TNSALP (Glu^{218}→Gly) and the only molecular form was the 66-kDa Endo H-sensitive form throughout the chase time (Fig. 5C), indicating that transport of this molecule is arrested before it reaches the medial Golgi, where *N*-linked oligosaccharide chains acquire Endo H-resistance.

Modification by GPI—GPI attachment to proteins occurs in the ER immediately after the synthesis of polypeptides (*2-5*). GPI-anchored proteins are thought to be initially synthesized as proforms with a COOH-terminal peptide extension. This propeptide, which serves as a GPI-anchor signal, is cleaved with a half life of ~5 min after its synthesis, concomitantly with transfer of a preformed GPI moiety to a newly exposed COOH-terminal amino acid residue (*27*). Failure to cleave the polypeptide and GPI-anchor addition often results in the retention of precursor molecules in a pre-Golgi compartment (*28-33*). To determine if the single amino acid substitution described above might affect GPI addition to the TNSALP mutants, cells expressing different TNSALPs were labeled overnight with [^{3}H]ethanolamine, a component of GPI. In agreement with its sensitivity to PI-PLC (Fig. 4, A and B, lane 3), the ~80-kDa species of both the wild-type TNSALP and the TNSALP (Ala^{162}→Thr) were labeled with [^{3}H]ethanolamine (Fig. 6, lanes 4 and 5). Furthermore, the 66-kDa molecular species of TNSALP (Ala^{162}→Thr) and TNSALP (Glu^{218}→Gly), which presumably exist as high-molecular-mass disulfide-bonded aggregates within the cells (Fig. 3, B and C), were also found to be labeled (Fig. 6, lanes 5 and 6), like that of the wild-type protein (Fig. 6, lane 4), arguing against the possibility that failure of GPI attachment is a cause of cellular retention of the TNSALP mutants. The lower intensity of labeled bands in the cases of mutant TNSALPs is probably due to degradation of the molecules.

Cell Surface Expression—Since PI-PLC digestion is not capable of removing all the GPI-anchored molecules expressed on the cell surface under our experimental conditions (*18*), the cell surface appearance of TNSALPs was studied more quantitatively by cell surface biotinylation (Fig. 7) or qualitatively by immunofluorescence microscopy (Fig. 8). As shown in Fig. 7, the ~80-kDa form was heavily

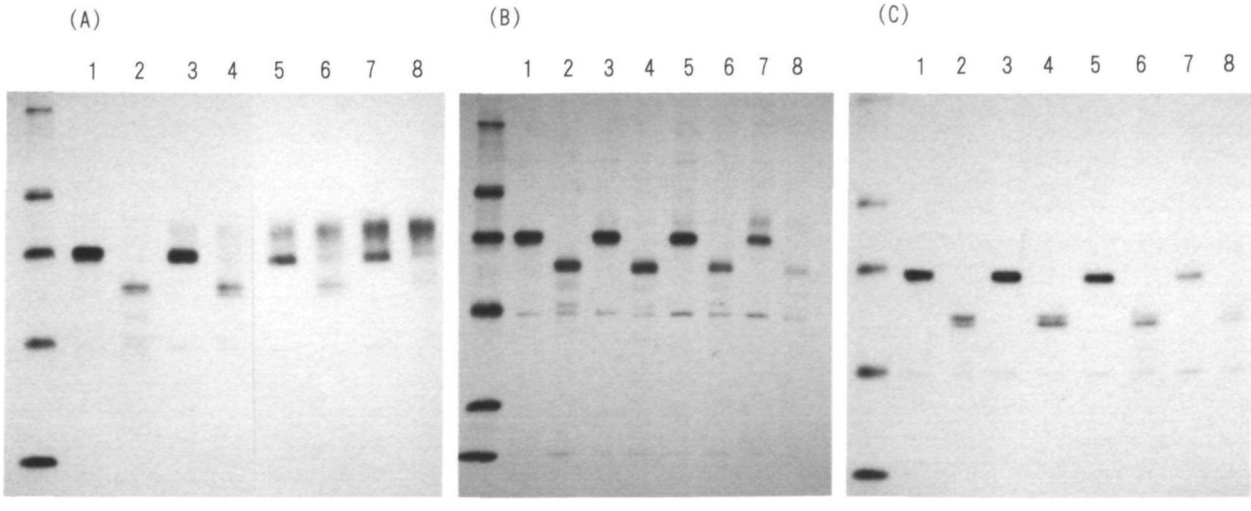

Fig. 5. **Acquisition of Endo H resistance of the TNSALP mutants.** COS-1 cells expressing the wild-type TNSALP (A), TNSALP (Ala^{162}→Thr) (B), or TNSALP (Glu^{218}→Gly) (C) were pulse-labeled with [^{35}S]methionine for 30 min (lanes 1 and 2) and chased for 1 h (lanes 3 and 4), for 2 h (lanes 5 and 6) or 4 h (lanes 7 and 8). Cell lysates were prepared and subjected to immunoisolation. The immune complexes were incubated in the absence (lanes 1, 3, 5, and 7) or presence (lanes 2, 4, 6, and 8) of Endo H. Each sample was analyzed by SDS-PAGE/fluorography. Left lane: the same ^{14}C-methylated proteins as shown in Fig. 1A.

labeled with biotin succinimidyl-ester in COS-1 cells expressing the wild-type TNSALP (lane 4). This is in accordance with the observed release of the ~80-kDa molecular species by PI-PLC digestion (Figs. 1 and 4). The ~80-kDa form was also detected in the cells expressing TNSALP (Ala^{162}→Thr) (lane 5), but to a much lesser extent. The relative intensity of the bands in lane 5 with respect to lane 4 was estimated to be one-sixth by scanning the fluorogram. In contrast to the ~80-kDa form, the 66-kDa form was not labeled with biotin in the cells expressing the TNSALP mutants (lanes 5 and 6). The 42-kDa protein was also not labeled with biotin (lanes 1 and 4).

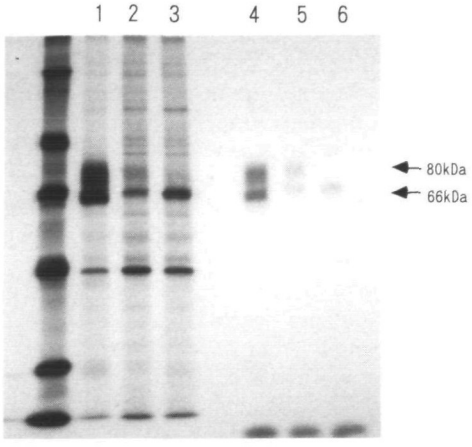

Fig. 6. **Metabolic labeling of the TNSALP mutants with [^{3}H]-ethanolamine.** COS-1 cells expressing the wild-type TNSALP (lanes 1 and 4), TNSALP (Ala^{162}→Thr) (lanes 2 and 5), or TNSALP (Glu^{218}→Gly) (lanes 3 and 6) were metabolically labeled with [^{35}S]-methionine (lanes 1-3) for 6 h or [^{3}H]ethanolamine (lanes 4-6) for 16 h. Cell lysates were prepared and subjected to immunoisolation. Each sample was analyzed by SDS-PAGE/fluorography. The dried gel was exposed for a month. Left lane: the same ^{14}C-methylated proteins as shown in Fig. 1A.

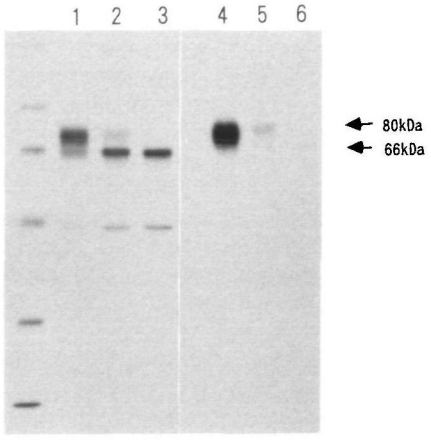

Fig. 7. **Cell surface biotinylation.** COS-1 cells expressing the wild-type TNSALP (lanes 1 and 4), TNSALP (Ala^{162}→Thr) (lanes 2 and 5) or TNSALP (Glu^{218}→Gly) (lanes 3 and 6) were labeled with [^{35}S]methionine for 6 h. The cells were further incubated with biotin succiniimidyl-ester at 4°C as described under "EXPERIMENTAL PROCEDURE." Cell lysates were prepared and subjected to immunoisolation with anti-rat TNSALP. Each sample was boiled and divided into two parts. One part was directly analyzed by SDS-PAGE (lanes 1-3). The other was further incubated with streptoavidin beads in order to collect biotin-labeled TNSALP molecules before analysis (lanes 4-6). Left lane: the same ^{14}C-methylated proteins as shown in Fig. 1A.

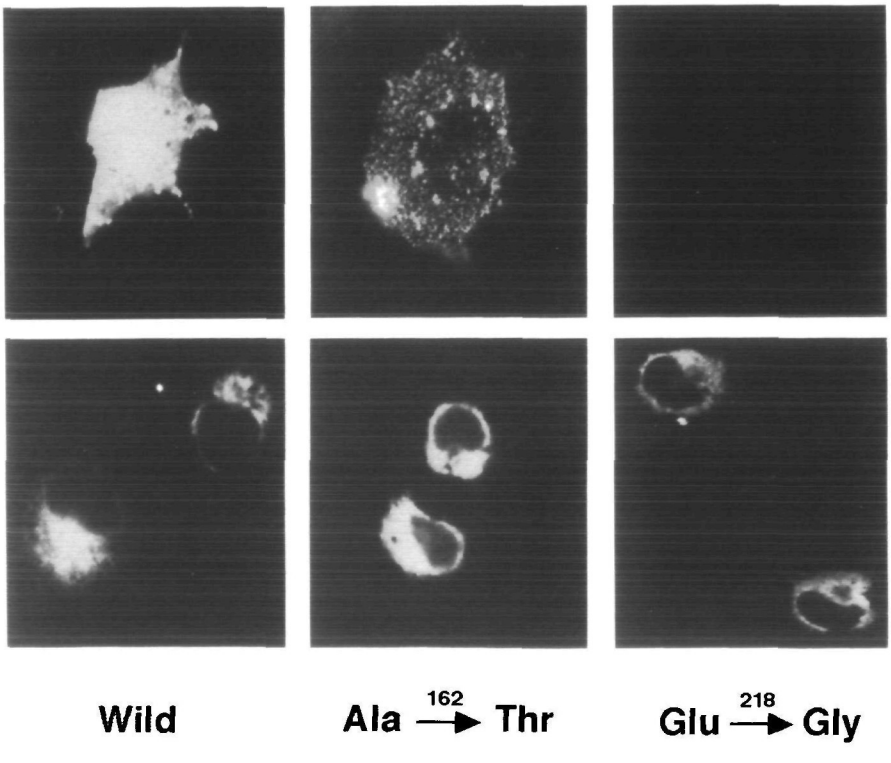

Fig. 8. **Immunolocalization of the TNSALP mutants expressed on COS-1 cells.** COS-1 cells expressing the wild-type TNSALP, TNSALP (Ala^{162}→Thr), or TNSALP (Glu^{218}→Gly) were fixed and incubated with anti-TNSALP serum in the absence (upper panels) or presence (bottom panels) of 0.1% NP40, followed by rhodamine-conjugated anti-(rabbit IgG).

Strong immunofluorescence was observed on the cell surface of cells transfected with the wild-type TNSALP cDNA (Fig. 8, upper panel). We also detected cell surface fluorescence in the cells expressing TNSALP (Ala^{162}→Thr) (Fig. 8, upper panel), though the fluorescence was much lower than that of the cells expressing the wild-type protein. In contrast, we failed to observe definite appearance of TNSALP (Glu^{218}→Gly) on the cell surface (Fig. 8, upper panel). These observations are quite compatible with the results of cell surface biotinylation described above. In agreement with biochemical studies, extensive reticular staining patterns were observed in the permeabilized cells expressing the TNSALP mutants (Fig. 8, bottom panels), suggesting that the mutant proteins are largely localized in the ER. Anti-rat TNSALP antibody, though it coprecipitated the 42-kDa protein in the labeling experiments, did not give a definite staining pattern in untransfected COS-1 cells (data not shown).

DISCUSSION

Hypophosphatasia is caused by mutations in the gene encoding TNSALP. In order to define the molecular defect of TNSALP mutants, we transfected COS-1 cells with corresponding cDNAs and examined their biosynthesis. Since the COS-1 cells neither produce TNSALP nor exhibit measurable alkaline phosphatase activity, this heterologous expression system allows us to analyze precisely mutant TNSALP molecules introduced exogenously. However, the disadvantage of this system is that overexpression results in incomplete processing, giving rise to underglycosylated proteins. The wild-type protein expressed in the transfected cells migrated as a broad band on the SDS-polyacrylamide gel, moving faster than the band due to the protein synthesized endogenously in SAOS-2 cells. Despite this drawback, pulse-chase experiments revealed that by 4 h of chase the majority of newly synthesized wild-type TNSALP molecules had acquired Endo H-resistance. Furthermore the wild-type TNSALP is expressed as the ~80-kDa form on the cell surface as judged by PI-PLC digestion, cell surface biotinylation and immunofluorescence observations, indicating that the secretory pathway is not seriously perturbed by overexpression. It should also be pointed out that overexpression did not lead to the secretion of TNSALP molecules.

Weiss *et al.* (*7*) first identified a homozygous missense mutation TNSALP (Ala^{162}→Thr) in the TNSALP gene of a patient associated with lethal hypophosphatasia. Biosynthetic studies showed that when transfected with cDNA for TNSALP (Ala^{162}→Thr), only a small fraction of the newly synthesized molecules was expressed as the ~80-kDa form on the cell surface, while most of them remained as the 66-kDa Endo H-sensitive form even after 4 h of chase. We also detected a low level of alkaline phosphatase activity in the cells expressing TNSALP (Ala^{162}→Thr), suggesting that replacement of Ala162 with Thr does not lead to complete inactivation of the enzyme. In contrast to our results, however, Weiss *et al.* (*7*) reported that TNSALP (Ala^{162}→Thr) exhibited no activity when expressed in NIH3T3 cells. The reason for this discrepancy is not known at present. In this regard Chaidaroglou and Kantrowitz (*34*) recently reported that substitution of Ala161 (corresponding to Ala162 of human TNSALP) with Thr does not change the catalytic properties of *E. coli* alkaline phosphatase, ruling out a catalytic role of Ala161. Considering that this amino acid residue is highly conserved among different alkaline phosphatase from *E. coli* to human, with a few exceptions (*7, 34, 35*), it seems reasonable that TNSALP (Ala^{162}→Thr) has a basic catalytic activity, but not full activity. We must await a crystallographic analysis of human TNSALP to determine the structural effects of the Ala^{162}→Thr mutation. Based on the results presented here, we suggest that the molecular basis of this hypophosphatasia is defective intracellular transport of the mutant protein with reduced phosphatase activity.

We do not know the reason why not all the newly synthesized TNSALP (Ala^{162}→Thr) molecules are blocked in transport to the cell surface, in contrast to the complete block of TNSALP (Glu^{218}→Gly). This is reminiscent of what is known about the secretion of α_1-antitrypsin Z (PiZ variant) in which Glu342 is substituted with Lys. Only 10-15% of the newly synthesized PiZ is secreted and the rest is retained in the ER (*36*). Although the PiZ has antiprotease activity, a reduced plasma level of α_1-antitrypsin Z causes hereditary emphysema and intracellularly accumulated PiZ causes chronic liver disease in susceptible individuals (*37*). Similar transport-defective phenotypes are found in human genetic diseases such as familial hypecholesterolemia (low-density lipoprotein receptor), cystic fibrosis (cystic fibrosis transmembrane conductance regulator), and leprechaunism (insulin receptor) (*38-42*).

There is increasing evidence that newly synthesized proteins are scrutinized and assessed for proper folding and assembly in the ER in a process collectively referred to as quality control (*43-45*). Incompletely assembled proteins, misfolded proteins and aggregates are selectively retained in the ER and eventually degraded *via* a nonlysosomal degradation pathway (*44, 46*). Thus the ER not only synthesize proteins, but also control the quality of proteins, thereby ensuring that only correctly folded and assembled proteins leave the ER for their destination. Purified soluble TNSALP is a non-covalently associated homodimer and TNSALP probably exists as a tetramer on the membranes (*46*). As expected, both the 66-kDa and ~80-kDa forms of the wild-type TNSALP migrated as a monomer on SDS-polyacrylamide gel, irrespective of non-reducing or reducing conditions. In contrast, the 66-kDa form of both TNSALP (Ala^{162}→Thr) and TNSALP (Glu^{218}→Gly) was exclusively present in a disulfide-linked high-molecular-mass aggregate, implying that each single amino acid change might bring about conformational alterations of the TNSALP mutants such that they form an interchain disulfide-bonded aggregate. Immunofluorescent localization, in addition to their Endo H sensitivity, suggests that the mutant proteins are largely retained in the ER (or a subcompartment). Importantly, since the ~80-kDa form of TNSALP (Ala^{162}→Thr) migrated as a monomer in SDS-PAGE as observed for the counterpart of the wild-type, selective aggregation must have occurred by segregating the misfolded form (the 66-kDa form) from a small fraction of properly folded form (~80-kDa form), which leaves the ER and is transported through the Golgi apparatus to the cell surface. Similar selective aggregation was suggested for a mutant chimeric protein with an uncleavable GPI-anchor signal. Field *et al.* (*32*) have found that the mutant chimeric protein formed a high-molecular-

mass disulfide-bonded aggregate and proposed that the uncleavable GPI-anchor signal is responsible for the aggregation and retention of the protein in the ER. However, this is unlikely for the TNSALP mutants analyzed here, since both TNSALP (Ala^{162}→Thr) and TNSALP (Glu^{218}→Gly) as well as the wild-type protein were modified by GPI as evidenced by their labeling with [^{3}H]ethanolamine, a component of GPI. Information directing GPI attachment is believed to reside on the peptide extension at the COOH-terminus of the newly synthesized polypeptide since this peptide extension, a GPI-anchor signal, is implantable. Proteins fused with the intact GPI-anchor signal are usually expressed on the cell surface *via* GPI (*2-5, 18*). So it is not unexpected that a missense mutation located in other sites than a GPI-anchor signal itself does not affect the GPI attachment, though the cleavage/attachment site of GPI for TNSALP is still undetermined. PNGase F digestion revealed that at least three of five putative *N*-glycosylation sites of TNSALP were glycosylated in the COS-1 cells. Since *N*-linked oligosaccharides are reported to be absolutely essential for the enzyme activity of TNSALP, but not that for placental and intestinal enzymes (*48*), it is of interest to know which *N*-glycosylation site(s) is involved in the enzyme activity. It is noteworthy that unglycosylated TNSALP synthesized in the presence of tunicamycin was also expressed on the cell surface *via* GPI, indicating that a possible structural change of the enzyme resulting from loss of all the *N*-linked oligosaccharides does not affect GPI attachment.

TNSALP (Glu^{218}→Gly) has not been reported as a naturally occurring mutant, though *a priori*, it seems likely that we will encounter some naturally occurring mutants that lose not only catalytic activity, but also transport competence, like TNSALP (Glu^{218}→Gly). Detailed analysis of mutations of TNSALP at the molecular level in combination with determination of its three-dimensional structure is expected to contribute to an understanding of the structure and function of TNSALP.

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