J. Biochem. 126, 694-699 (1999)

A General Method for Rapid Purification of Soluble Versions of Glycosylphosphatidylinositol-Anchored Proteins Expressed in Insect Cells: An Application for Human Tissue-Nonspecific Alkaline Phosphatase[1]

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Received June 22, 1999; accepted July 22, 1999

A soluble form of tissue-nonspecific alkaline phosphatase was purified to apparent homogeneity from the culture media of Sf9 cells which had been infected with recombinant baculoviruses encoding human tissue-nonspecific alkaline phosphatase (TNSALP). To facilitate purification, an oligonucleotide consisting of 6 tandem codons for histidine and a stop codon was engineered into the TNSALP cDNA. The molecular mass of the enzyme purified through a nickel-chelate column was estimated to be 54 kDa by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis. That of the native enzyme was 90 kDa as estimated by gel filtration, indicating that the purified soluble TNSALP is dimeric. The enzyme was used for production of antibodies specific for human TNSALP. Immunoblotting analysis showed a single 80-kDa band in the cell homogenate prepared from Saos-2 (human osteosarcoma) cells. However, upon digestion with peptide: *N*-glycosidase F, the 80-kDa TNSALP of human origin and the soluble enzyme of insect origin migrated to the same position on SDS-polyacrylamide gel, indicating that the size difference between the two enzymes is ascribed to *N*-linked oligosaccharides. The antibodies prepared against the purified TNSALP were found to be useful also for immunoprecipitation and immunofluorescence studies.

Key words: baculovirus, glycosylphosphatidylinositol, *N*-linked oligosaccharide, Sf9 cells, tissue-nonspecific alkaline phosphatase.

Tissue-nonspecific alkaline phosphatase (TNSALP) is expressed in a broad range of tissues and cells, while tissue-specific isozymes such as placental, intestinal, and germ cell alkaline phosphatase are localized to placenta, intestine, and germ cells, respectively. Each isozyme is encoded by a separate gene (*1*). TNSALP is also called liver/bone/kidney-type, since it is abundant in liver, bone, and kidney. Like other isozymes, TNSALP is anchored to the cell membrane *via* glycosylphosphatidylinositol (GPI) (*2-4*), and thus it is believed to function as an ectoenzyme. Although its physiological substrate has been not unequivocally identified, pyrophosphate, phosphoethanolamine, and pyridoxal 5′-phosphate are candidates, since these phospho-compounds are reported to accumulate in serum and urine of patients with hypophosphatasia, which is caused by mutations of the TNSALP gene (*5*). Levels of these three compounds were also significantly elevated in TNSALP-deficient mice as compared with control mice (*6*).

Clinical symptoms of hypophosphatasia vary widely from stillbirth with almost an unmineralized skeleton to premature loss of deciduous teeth and pseudofracture first appearing in adult life. Severe forms of hypophosphatasia are inherited in an autosomal recessive manner, while some of mild forms are reported to be dominantly inherited. So far more than 40 mutations have been reported in the TNSALP gene of patients suffering from hypophophatasia. Most of them are missense mutations. (*7-15*), though deletion mutations (*11, 16*) and mutations in the consensus sequences at the splice junctions and in promoter region have also been reported (*15*). An Ala^{162}→Thr substitution (*7*) and a Gly^{317}→Asp substitution (*10*), have been found to cause lethal hypophosphatasia in human patients. In previous experiments we ectopically expressed TNSALP

[1] This work was supported in part by a Grant-in-Aid for Scientific Research from the Ministry of Education, Science, Sports and Culture of Japan (to K. Oda) and by Enhancement of Center of Excellence, Special Coordination Funds for Promoting Science and Technology, Science and Technology Agency, Japan (to J. Kobayashi).

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Abbreviations: DMEM, Dulbecco's modified MEM; MEM, minimum essential medium; GPI, glycosylphosphatidylinositol; Ni-NTA, nickel-nitrilotriacetic acid; PAGE, polyacrylamide gel electrophoresis; PBS, phosphate-buffered saline; PI-PLC, phosphatidylinositol-specific phospholipase C; PNGase F, Peptide: *N*-glycosidase F; SDS, sodium dodecyl sulfate; TNSALP, tissue-nonspecific alkaline phosphatase; $TNSALP_{his_6}$, TNSALP with a histidine-tag at the COOH terminus.

(Ala^{162}→Thr) (*17*) and TNSALP (Gly^{317}→Asp) (*18*) in COS-1 cells in order to define the molecular defect of these mutated TNSALPs. We found that a considerable fraction of TNSALP (Ala^{162}→Thr) and most of TNSALP(Gly^{317}→Asp) synthesized in COS-1 cells formed a disulfide-bonded high-molecular-mass aggregate and was eventually degraded, probably in the endoplasmic reticulum, leading to a decreased number or total loss of functional TNSALP molecules on the surface of cells expressing the TNSALP mutants. These findings indicate that the single amino acid change due to a one-point mutation profoundly affects the proper folding and oligomeric assembly of TNSALP, and thereby impairs or almost completely inhibits ER-to-Golgi transport of the TNSALP mutants with only a small amount of enzyme gaining access to the Golgi and beyond as a functional enzyme (*17*).

Ala^{162} of TNSALP is conserved in *Escherichia coli* alkaline phosphatase, human placental alkaline phosphatase, and human TNSALP, while Gly^{317} is present only in the latter enzymes (*19, 20*). To understand the precise roles of the mutated amino acid residues at a molecular level, detailed structural information on TNSALP is indispensable. However, X-ray diffraction structure data are available only for *E. coli* alkaline phosphatase and overall homology between human TNSALP and *E. coli* enzyme is only 25-30% (*19, 21*). As a first step to obtain purified TNSALP in sufficient quantity for X-ray diffraction study, we started the purification of human TNSALP. Here, we report a one-step purification method for a soluble form of TNSALP from culture media of insect cells infected with recombinant baculovirues encoding human TNSALP.

EXPERIMENTAL PROCEDURES

Materials—Express ^{35}S ^{35}S protein labeling mix (>1,000 Ci/mmol) was obtained from Dupont-New England Nuclear (Boston, MA, USA) and ^{14}C-methylated proteins, ECL western blotting detection reagent, protein A-Sepharose CL-4B, HiPrep16/60 Sephacryl S-300HR column, rainbow protein marker, Thermo sequenase fluorescence labeled primer cycle sequencing kit, and anti-rabbit IgG, horseradish peroxidase-linked whole antibody were from Amersham Pharmacia Biotech (Arlington Heights, IL, USA); Lipofectamine Plus reagent, Lipofectin, and Sf-900II serum free medium from Gibco-BRL (Gaithersburg, MD, USA); pSG5 from Stratagene (San Diego, CA, USA); Ni-NTA (nickel-nitrilotriacetic acid) resin and plasmid Midi kit from Qiagen (Hilden, Germany); peptide:*N*-glycosidase F (*Flavobacterium meningosepticum*, PNGase F) and various DNA-modifying enzymes and restriction nucleases from New England Biolabs (Beverly, MA, USA); rhodamine-conjugated goat anti-(rabbit IgG) from Cappel Laboratories (Malvern, PA, USA); phosphatidylinositol-specific phospholipase C (PI-PLC) from Funakoshi (Tokyo). COS-1 cells were cultured in Dulbecco's modified Eagle's minimum essential medium (DMEM) supplemented with 10% fetal bovine serum (*17*). Saos-2 cells were cultured in α-MEM supplemented with 10% fetal bovine serum (*17*).

Plasmid Construction and Transfection—Construction of mammalian expression vector (pSG5-TNSALP) encoding a wild-type TNSALP was described previously (*17*). To introduce a histidine (his)-tag into TNSALP, site-directed mutagenesis was performed using a Takara Mutan K kit (Takara Shuzo, Kyoto). The oligonucleotide used was: 5′-TGTGCTCCTGCCAGCCATCACCATCACCATCACTAGGCAGGCAGCCTTGCTGC-3′. The nucleotide sequence of the mutation site of pSG5-TNSALP$_{histag}$ was verified by dideoxynucleotide chain termination using a Thermo sequenase fluorescence labeled primer cycle sequencing kit with 7-deaza-dGTP. For transfection, plasmids were purified using a Qiagen plasmid Midi kit according to the manufacturer's protocol. COS-1 cells ($1.2\text{-}1.3\times10^5$) were inoculated into 35-mm dishes ~24 h before transfection. Cells were transfected using Lipofectamine Plus, essentially according to the manufacturer's protocol, with 1.0 μg of plasmid for each transfection. After incubation for 24 h in a CO_2 incubator, the cells were used for metabolic labeling and immunofluorescence studies.

Construction of Recombinant Baculovirus and Expression in Sf9 Cells—pSG5-TNSALP$_{histag}$ was digested with *Eco*RI and *Sma*I and subjected to agarose electrophoresis. The cDNA fragment (1.8 kb) was excised from the agarose gel and purified. The cDNA was further blunt-ended and ligated into pAcUW51 Baculovirus Transfer vector plasmid (Pharmingen, San Diego, CA, USA), which was previously digested with *Eco*RI and blunted, to express TNSALP$_{histag}$ under the control of the strong AcNPV p10 gene promoter (pAcTNSALP$_{histag}$). The direction of the cDNA was determined by sequencing of the plasmid. For obtaining recombinant AcNPV, the Lipofectin method (*22*) was applied to Sf9 cells with slight modifications. Briefly, 5 μg of pAcTNSALP$_{histag}$ and 0.5 μg of BaculoGold Baculovirus DNA (Pharmingen, San Diego, CA, USA) were mixed with 8 μl of Lipofectin reagent in 1 ml of serum-free TC-100 medium and added to 1×10^6 Sf9 cells seeded in a well of a 6-well plate. After 4 h of incubation at room temperature, the Lipofectin/DNA mixture was replaced with 2 ml of TC-100 medium supplemented with 10% bovine serum. The cells were further incubated for 1 week at 27°C. Then the culture supernatant containing recombinant viruses was harvested and subjected to 2 cycles of plaque purification, essentially as described by Maeda (*23*) using Sf9 cells. For affinity purification, Sf9 cells cultured in SF-900II serum-free medium were infected with the recombinant viruses at a multiplicity of infection (MOI)= 1.0 plaque forming unit (pfu)/cell. The culture supernatants were harvested at 9 days post-infection.

Purification of Enzyme—Purification of the soluble human TNSALP with a histag was carried out using Ni-NTA resin essentially according to the instructions provided by the manufacturer. A 120-ml portion of Sf9 medium was extensively dialyzed against 50 mM Na_2HPO_4/NaH_2PO_4 buffer (pH 8.0), then centrifuged at $5{,}000\times g$ for 15 min to remove insoluble material. The supernatant was adjusted to a final concentration of 20 mM imidazole and applied on a Ni-NTA column (bed volume 0.8 ml), preequilibrated with the buffer containing 20 mM imidazole. The resin was washed with 50 ml of the buffer containing 20 mM imidazole, then TNSALP$_{histag}$ was eluted with the buffer containing 250 mM imidazole. Fractions of 1 ml were collected and assayed for alkaline phosphatase activity. Usually fractions 2-4 were combined and dialyzed against 10 mM Tris/HCl (pH 7.5)/150 mM NaCl/1 mM $MgCl_2$. The purified enzyme was stable for at least 3 months at 4°C.

Preparation of Antibody—A 100-μg portion of the purified protein was mixed with an equal volume of Freund's complete adjuvant to form a complete emulsion. This mixture was subcutaneously injected at multiple sites on the backs of rabbits. Two and 4 weeks after the first immunization, 100 μg of the purified protein was mixed with Freund's incomplete adjuvant and subcutaneously injected. Boosters (100 μg of the purified protein) were intraperitoneally injected at weekly to monthly intervals after the last immunization. The rabbits were bled 10 days after each booster.

Metabolic Labeling and Immunoprecipitation—COS-1 cells transfected with pSG5-TNSALP were labeled with 50 μCi of [^{35}S]methionine in the methionine-free MEM for 3 h and chased for another 3 h to exhaust ^{35}S-labeled secretory proteins from the cells as described previously (*17*). The cells were washed with MEM and further incubated in the absence or presence of 0.2 unit of PI-PLC. COS-1 cells expressing TNSALP$_{histag}$ were labeled with 50 μCi of [^{35}S]-methionine for 4 h. The media were harvested and cells were lysed in a lysis buffer as described previously (*17*). The cell lysates and media were subjected to immunoisolation using the antiserum produced against the soluble human TNSALP. Immune complexes/Protein A-Sepharose were extensively washed, dissolved in Laemmli's sample buffer (*24*), and analyzed by SDS-PAGE (9% gel)/fluorography as described previously (*17*). In one experiment the cell lysate and medium were incubated with Ni-NTA resin to isolate TNSALP$_{histag}$. The TNSALP$_{histag}$/resin complex was extensively washed with 10 mM Tris/HCl (pH 7.5) containing 0.5% NP-40 and 10 mM Tris/HCl (pH 7.5) containing 0.5% NP-40 and 150 mM NaCl, and then dissolved in Laemmli's sample buffer.

PNGase F Digestion—For ^{35}S-TNSALP, immune complexes/protein A beads were boiled in phosphate-buffered saline (PBS) containing 1% SDS for 3 min, centrifuged to remove protein A beads, then the supernatants were diluted 10-fold with 1% NP-40. For the purified TNSALP$_{histag}$ and cell homogenates, the samples were denatured by boiling in PBS containing 1% SDS for 3 min. Denatured samples were adjusted to a final concentration of 1% NP-40 and digested with 1,000 U/ml of PNGase F at 37°C for 16 h as described previously (*17*).

Western Blotting—A cell homogenate prepared from Saos-2 cells and the purified soluble TNSALP were dissolved in Laemmli's sample buffer (*24*), boiled and run on SDS-polyacrylamide gel. After electrophoresis, proteins were transferred to Immobilon P (Millipore, Bedford, USA) using a semi-dry transfer apparatus. The membrane was incubated first with 4,000-fold diluted antiserum against the soluble TNSALP$_{histag}$, then with 4,000-fold diluted anti-rabbit IgG conjugated with horseradish peroxidase. Proteins were detected with ECL western blotting detection reagents.

Immunofluorescence—Saos-2 cells and COS-1 cells expressing TNSALP on cover slips were fixed with 4% paraformaldehyde. After blocking with skim milk in PBS, the cells were incubated first with 400-fold diluted antiserum against the soluble TNSALP, then with rhodamine-conjugated goat anti-rabbit IgG as described previously (*17*).

Determinations of Protein and Enzyme Activity—Proteins were assayed using a Bio-Rad protein assay kit (Hercules, CA, USA) with bovine serum albumin as a standard. Alkaline phosphatase activity was assayed using 10 mM *p*-nitrophenyl phosphate as a substrate in 0.1 M 2-amino-2-methyl-1,3-propanediol/HCl buffer (pH 10.5) containing 5 mM $MgCl_2$ and 0.1% Triton X-100 (*25*). One unit of enzyme activity was defined as 1 nmol substrate hydrolyzed/min at 37°C.

Amino Acid Sequence Determination—The purified TNSALP$_{histag}$ was dialyzed against distilled water containing 0.1% SDS and its amino acid sequence was determined by use of an Applied Biosystems Model 492 Protein Sequencer.

RESULTS AND DISCUSSION

TNSALP is anchored to the cell membrane *via* GPI. GPI-anchored proteins are synthesized as precursor forms with a COOH-terminal peptide extension containing a stretch of hydrophobic amino acid residues, which serves as a GPI-anchor signal (*2, 3*). Modification by GPI occurs in the endoplasmic reticulum. Shortly after their synthesis, these peptide extensions of precursor molecules are thought to be cleaved and simultaneously replaced with GPI. We reasoned that if the translation of TNSALP were prematurely terminated at a position N-terminal to a GPI-anchor signal, TNSALP would fail to be attached by GPI and consequently be secreted into the medium as a soluble

Wild-type human tissue-nonspecific alkaline phosphatase (TNSALP)

⬇

481 482 483 484 485 486 487 488 489 490 491 492 493 494 495 496 497 498 499 500 501 502 503 504 505 506 507
GCT CCT GCC AGC TCG GCA GGC AGC CTT GCT GCA GGC CCC CTG CTG CTC GCT CTG GCC CTC TAC CCC CTG AGC GTC CTG TTC TGA
Ala Pro Ala Ser Ser Ala Gly Ser *Leu Ala Ala Gly Pro Leu Leu Leu Ala Leu Ala Leu Tyr Pro Leu Ser Val Leu Phe* ---

Soluble form of human tissue-nonspecific alkaline phosphatase (TNSALPhistag)

481 482 483 484 (485)(486) (487) (488) (489) (490)
GCT CCT GCC AGC CAT CAC CAT CAC CAT CAC TAG
Ala Pro Ala Ser His His His His His His ---

Fig. 1. **Construction of the cDNA encoding a soluble form of human TNSALP.** An oligonucleotide containing six tandem codons for histidine and a stop codon was introduced into the wild-type human TNSALP cDNA by site-specific mutagenesis as described under "EXPERIMENTAL PROCEDURES" to convert GPI-anchored TNSALP to a soluble secretory protein (TNSALP$_{histag}$). Italic letters denote a stretch of hydrophobic amino acid residues of a putative GPI-anchor signal of TNSALP. The GPI attachment site of human TNSALP has not been identified. An arrow indicates the position where tandem histidine residues were introduced.

enzyme. As shown in Fig. 1, we introduced into the TNSALP cDNA an oligonucleotide comprising six consecutive codons for histidine, which is expected to facilitate purification of a putative soluble enzyme, and a stop codon. A preliminary experiment showed that when COS-1 cells were transfected with this cDNA encoding $TNSALP_{hlstag}$, alkaline phosphatase activity in the culture medium remarkably increased (data not shown), suggesting that $TNSALP_{hlstag}$ is secreted as a soluble enzyme, which was further confirmed by the immunoprecipitation experiment described below (Fig. 4).

Next we constructed a recombinant baculovirus encoding $TNSALP_{hlstag}$ to express a soluble form of TNSALP in Sf9 cells. $TNSALP_{hlstag}$ was found to be a major protein secreted from Sf9 cells infected with the recombinant baculoviruses encoding $TNSALP_{hlstag}$ (Fig. 2, lane 1). $TNSALP_{hlstag}$ was purified from the culture supernatants using a Ni-chelate resin as shown in Table I. The specific activity of the soluble enzyme ranged from 1,000,000 to 1,500,000 units/mg protein from experiment to experiment. These values of specific activity are comparable to those of TNSALP purified from rat liver, swine kidney, and rat hepatoma cells (*26-28*). The enzyme migrated as a single band with a molecular mass of 54 kDa on SDS-polyacrylamide gel (Fig. 2, lane 3). The purified enzyme was eluted at a position corresponding in size to 90 kDa when analyzed by gel filtration (Fig. 3), suggesting that the soluble TNSALP expressed in Sf9 cells is a homodimer. N-terminal peptide sequence analysis indicated that the structure of $TNSALP_{hlstag}$ is as follows: NH_2-L-V-P-E-K-E-K-D-P-K-, which is identical to the reported amino acid sequence of human liver TNSALP (*19*). K_m values determined by the method of Lineweaver and Burk for the purified $TNSALP_{hlstag}$, a cell homogenate prepared from COS-1 cells expressing TNSALP, and a cell homogenate of Saos-2 (osteosarcoma) cells were 1.5×10^{-4}, 1.6×10^{-4}, and 4×10^{-4} M, respectively, indicating that the purified soluble enzyme has a catalytic property similar to those of authentic GPI-anchored TNSALPs. Thus, by the present method, we were able to circumvent the laborious and time-consuming purification steps following solubilization by detergent, *n*-butanol or PI-PLC, that have been employed by others to purify TNSALP (*26-29*).

COS-1 cells transiently expressing the wild-type TNSALP were metabolically labeled with [^{35}S]methionine, and TNSALP molecules were immunoprecipitated using antiserum raised against the purified $TNSALP_{hlstag}$. Since COS-1 cells produce no endogenous TNSALP, this heterologous expression system is appropriate to examine ectopic expressions of TNSALP mutants (*17*). In previous experiments we showed that TNSALP is synthesized as a 66-kDa form with high mannose-type oligosaccharide chains and becomes a 80-kDa mature form with complex-type oligosaccharide chains, which is finally exposed on the cell surface *via* GPI (*17, 18*). In accordance with these reports, the polyclonal antibodies precipitated the 66- and 80-kDa forms of TNSALP (Fig. 4, lane 1). After incubation with PI-PLC, which is known to release GPI-anchor proteins from the cell surface (*2-4, 17*), the 80-kDa form, but not its precursor form, was found in the medium (Fig. 4, lane 4), indicating that the antibodies are capable of immunoprecipitating the GPI-anchored TNSALP as well as the anchor-less TNSALP. When transfected with a plasmid encoding $TNSALP_{hlstag}$, $TNSALP_{hlstag}$ was found in the medium without PI-PLC treatment (Fig. 4, lane 6), indicating that $TNSALP_{hlstag}$ lacks GPI and is secreted. Ni-NTA resin was useful for the purification of ^{35}S-$TNSALP_{hlstag}$ from the culture media (Fig. 4, lane 7), but not from cell lysates (Fig. 4, lane 8).

We noticed that when expressed in COS-1 cells, TNS-

TABLE I. **Purification of soluble human tissue-nonspecific alkaline phosphatase.**

Purification step	Protein (mg)	Specific activity (units/mg protein)	Recovery (%)	Purification (-fold)
Sf9 media (dialyzed)	14.0	538,795	100	1
Ni-NTA resin	2.5	1,269,065	42.0	2.3

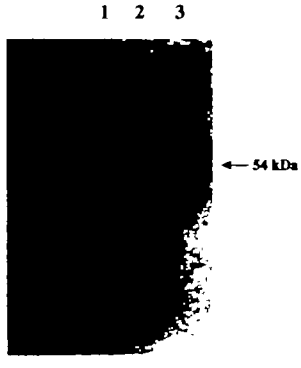

Fig. 2. **Purification of $TNSALP_{hlstag}$ from culture media of Sf9 cells.** The culture media of Sf9 cells infected with recombinant baculoviruses encoding $TNSALP_{hlstag}$ were extensively dialyzed and applied on a Ni-NTA resin column as described under "EXPERIMENTAL PROCEDURES." The dialysate (lane 1, 12 μg), flow-through fraction (lane 2, 10 μg) and eluate (lane 3, 1.5 μg) were analyzed by SDS-PAGE. The gel was stained with Coomassie Brilliant Blue. Left lane: molecular markers (from the top of the gel, 220, 97.4, 66, 46, 30, 21.5 kDa)

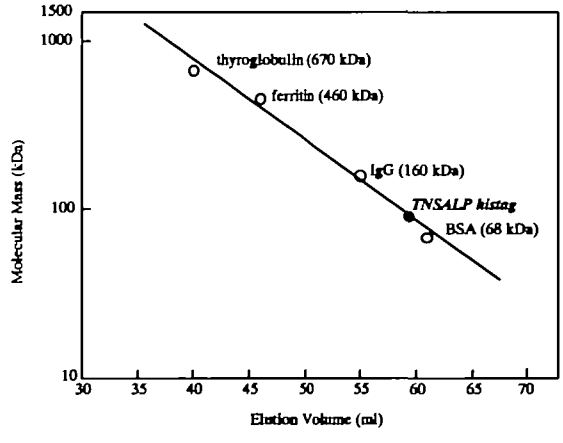

Fig. 3. **Gel filtration of the purified $TNSALP_{hlstag}$.** A 50 μg sample of the purified $TNSALP_{hlstag}$ was applied onto HiPrep16/60 Sephacryl S-300HR column (1.6×60 cm), equilibrated with 10 mM Tris/HCl (pH 7.5) containing 150 mM NaCl. Fractions of 1 ml were collected and assayed for alkaline phosphatase activity. Thyroglobulin, ferritin, IgG, and bovine serum albumin (BSA) were applied separately to the same column for calibration.

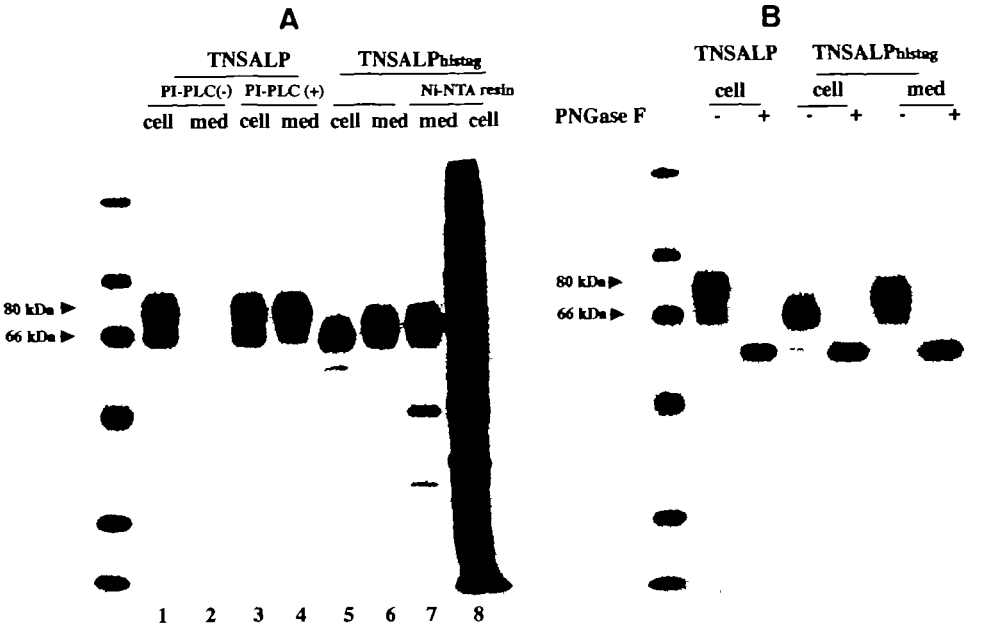

Fig. 4. **Expression of the wild-type TNSALP and $TNSALP_{histag}$ in COS-1 cells.** (A) COS-1 cells transfected with pSG5-TNSALP (lanes 1-4) or pSG5-$TNSALP_{histag}$ (lanes 5-8) were labeled with [^{35}S]methionine for 3-4 h. The cells were further incubated in the absence (−) (lanes 1 and 2) or presence (+) (lanes 3 and 4) of PI-PLC. The cell lysates (lanes 1 and 3) and media (lanes 2 and 4) were subjected to immunoisolation using antiserum against the purified $TNSALP_{histag}$ (lanes 1-6), while the cell lyate (lane 8) and medium (lane 7) were incubated with Ni-NTA resin. Each sample was analyzed by SDS-PAGE/fluorography. Left lane: ^{14}C-methylated protein markers (from the top of the gel, 200, 97.4, 66, 46, 30, and 14.3 kDa). (B) TNSALP and $TNSALP_{histag}$ immunoprecipitated from the cell lysate (A, lane 1 and lane 5) or medium (A, lane 6) were incubated in the absence (−) or presence (+) of PNGase F and analyzed by SDS-PAGE/fluorography. Left lane: the same ^{14}C-methylated protein markers as shown in A.

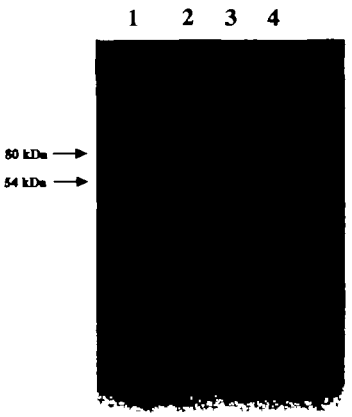

Fig. 5. **Immunoblotting of TNSALP.** A Saos-2 cell homogenate (lanes 1 and 2, each 5 μg) and the purified $TNSALP_{histag}$ (lanes 3 and 4, each 47 ng) were boiled in PBS containing 1% SDS and incubated in the absence (lanes 1 and 3) or presence (lanes 2 and 4) of 1,000 U/ml of PNGase F as described under "EXPERIMENTAL PROCEDURES." Each sample was run on SDS-polyacrylamide gel, transferred to Immobilon P, and detected using ECL reagents.

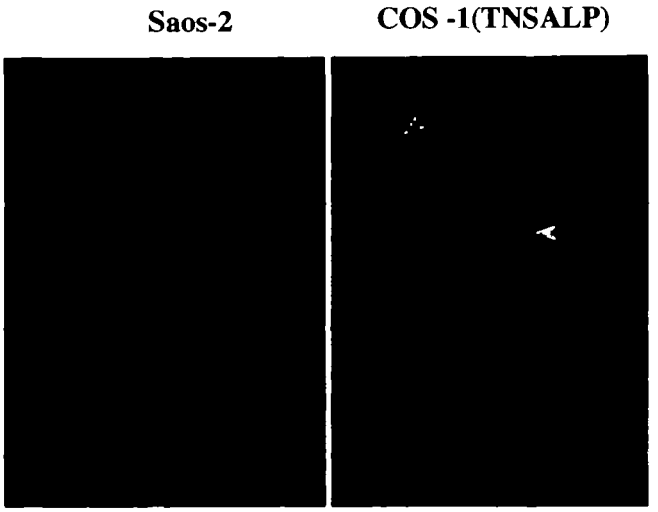

Fig. 6. **Detection of TNSALP by immunofluorescence.** Saos-2 cells and COS-1 cells transiently expressing the wild-type TNSALP were fixed and incubated with antiserum against $TNSALP_{histag}$, followed by rhodamine-conjugated anti-(rabbit IgG). Arrow heads indicate COS-1 cells not expressing TNSALP.

ALP_{histag} migrated faster than the wild-type TNSALP on SDS-polyacrylamide gel (Fig. 4A, lanes 1 and 6). Upon digestion with PNGase F, which cleaves between the innermost *N*-acetylglucosamine and asparagine residues of high mannose, hybrid and complex oligosaccharides from *N*-linked glycoproteins, the two proteins became indistinguishable on the gel (Fig. 4B), suggesting that the extent of terminal glycosylation causes the size difference of the two enzymes. It is likely that after $TNSALP_{histag}$ acquired terminal sugars, a mature medium-form was quicky released from the cells, since this form was not detectable in the cells (Fig. 4A, lanes 5 and 6). Although the purified $TNSALP_{histag}$ is significantly smaller than the authentic TNSALP expressed in Saos-2 cells, as shown in Fig. 5, again the two proteins comigrated on the gel after PGNase F digestion, indicating that the observed difference in molecular mass between the two enzymes from different origins is presumably due to the differential processing of *N*-linked oligosaccharides. Analysis of *N*-linked oligosaccharides of the purified $TNSALP_{histag}$ is currently being carried out.

Immunofluorescence observation revealed that Saos-2 cells endogenously expressing TNSALP were strongly stained with the antibodies against $TNSALP_{histag}$ (Fig. 6). TNSALP was found over the cell surface, especially in cell-cell contact regions. The antibody also stained the cell

surface of COS-1 cells expressing TNSALP.

In conclusion, $TNSALP_{histag}$ expressed in insect cells was secreted into the culture media, from which we were able to obtain mg amounts of the soluble form of human TNSALP by one-step purification using a Ni-NTA column. Considering that the purified enzyme has N-terminal amino acids identical to the membrane-bound TNSALP and is capable of binding to Ni-NTA resin, it is reasonable to conclude that $TNSALP_{histag}$ expressed in the insect cells comprises 484 amino acids covering most of the amino acid sequence of the mature TNSALP and COOH-terminal hexa histidine residues. Analysis of the specificity of the polyclonal antibodies raised against $TNSALP_{histag}$ guarantees that the antibodies are useful for immunoprecipitation, Western blotting, and immunofluorescence studies. The gene construction designed to facilitate purification of the soluble form of human TNSALP can be applied for purification of any GPI-anchored proteins.

We thank Drs. Masahiro Ito and Jun Cheng for their help in preparation of the manuscript.

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