Molecular Genetics and Metabolism 115 (2015) 180–185

Contents lists available at ScienceDirect

Molecular Genetics and Metabolism

journal homepage: www.elsevier.com/locate/ymgme

Molecular phenotype of tissue-nonspecific alkaline phosphatase with a proline (108) to leucine substitution associated with dominant odontohypophosphatasia

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ARTICLE INFO

Article history:
Received 11 March 2015
Received in revised form 11 May 2015
Accepted 11 May 2015
Available online 12 May 2015

Keywords:
Autosomal dominant
Cell surface localization
Dimerization
Hypophosphatasia
Tissue-nonspecific alkaline phosphatase

ABSTRACT

Hypophosphatasia (HPP) is a genetic disease characterized by defective calcification of hard tissues such as bone and teeth accompanying deficiency of serum alkaline phosphatase (ALP) activity. Its development results from various mutations in the *ALPL* gene encoding tissue-nonspecific ALP (TNSALP). HPP is known to be transmitted in an autosomal recessive or autosomal dominant manner. A point mutation (c.323C>T) in the *ALPL* gene leading to a proline to leucine substitution at position 108 of TNSALP was first reported in a patient diagnosed with odonto-HPP (M Herasse et al., *J Med Genet* 2003;40:605–609), although the effects of this mutation on the TNSALP molecule have not been elucidated. To understand the molecular basis of this dominantly transmitted HPP, we first characterized TNSALP (P108L) by expressing it in COS-1 cells transiently. In contrast to wild-type TNSALP (WT), TNSALP (P108L) showed virtually no ALP activity. When coexpressed with TNSALP (WT), TNSALP (P108L) significantly inhibited the enzyme activity of TNSALP (WT), confirming that this mutant TNSALP exerts a dominant negative effect on TNSALP (WT). Using immunofluorescence and digestion with phosphatidylinositol-specific phospholipase C, we demonstrated that TNSALP (P108L) was anchored to the cell surface via glycosylphosphatidylinositol-like TNSALP (WT) in a Tet-On CHO cell expression system. Consistent with this, TNSALP (P108L) acquired endo-β-*N*-acetylglucosaminidase H resistance and sialic acids, as evidenced by glycosidase treatments. Importantly, TNSALP (WT) largely formed a functional dimeric structure, while TNSALP (P108L) was found to be present as a monomer in the cell. This indicates that the molecular structure of TNSALP is affected by a missense mutation at position 108, which is in contact with the active site, such that it no longer assembles into the functional dimeric form. Collectively, these results may explain why TNSALP (P108L) loses its ALP activity, even though it is able to gain access to the cell surface.

1. Introduction

Hypophosphatasia (HPP) is an inherited disease resulting from nearly 300 mutations of the *ALPL* gene encoding tissue-nonspecific alkaline phosphatase (TNSALP) [1–6], which resides on the outer leaflet of the cell membrane and is believed to hydrolyze phosphomonoesters such as inorganic pyrophosphate, phosphoethanolamine, and pyridoxal phosphate [1–6]. The severity of the disease is well correlated with in vitro alkaline phosphatase activity of the mutant proteins [3,6]. HPP is classified into five principal forms: 1) perinatal, 2) infantile, 3) childhood, 4) adult, and 5) odonto-HPP [1–4]. It is clinically broad-ranging, encompassing a wide range of presentations from death in utero with severe skeletal hypomineralization in perinatal HPP to premature loss of deciduous teeth and dental caries without bone symptoms in odonto-HPP [1,3,4]. Severe forms (perinatal and infantile) of HPP are inherited in an autosomal recessive manner, while milder forms manifest both autosomal recessive and autosomal dominant inheritance patterns [1–6].

While being cotranslationally translocated into the lumen of the endoplasmic reticulum (ER), TNSALP acquires high-mannose-type N-linked oligosaccharides and is anchored to the ER at its C-terminus via glycosyl- phosphatidylinositol [GPI] [2]. Then, properly folded TNSALP moves to the Golgi apparatus, undergoes oligosaccharide processing, and is finally exposed on the outer surface via GPI as a cell-surface enzyme. TNSALP on matrix vesicles buds from hypertrophic chondrocytes and osteoblasts promotes the growth of hydroxyapatite crystals by hydrolyzing inorganic pyrophosphate, an inhibitor of mineralization [1,2,5,6]. A total of 300 mutations in this gene have been reported worldwide as of 2015 (http://www.sesep.uvsq.fr/03_hypo_mutations.php).

Abbreviations: ALP, alkaline phosphatase; ER, endoplasmic reticulum; Endo H, endo-β-*N*-acetylglucosaminidase H; DOX, doxycycline; GPI, glycosylphosphatidylinositol; HPP, hypophosphatasia; PI-PLC, phosphatidylinositol-specific phospholipase C; PNGase F, peptide-N-glycosidase F; TNSALP, tissue-nonspecific alkaline phosphatase; TNSALP (P108L), TNSALP with a proline to leucine substitution at position 108; TNSALP (A116T), TNSALP with an alanine to threonine substitution at position 116; TNSALP (WT), wild-type TNSALP.

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http://dx.doi.org/10.1016/j.ymgme.2015.05.006
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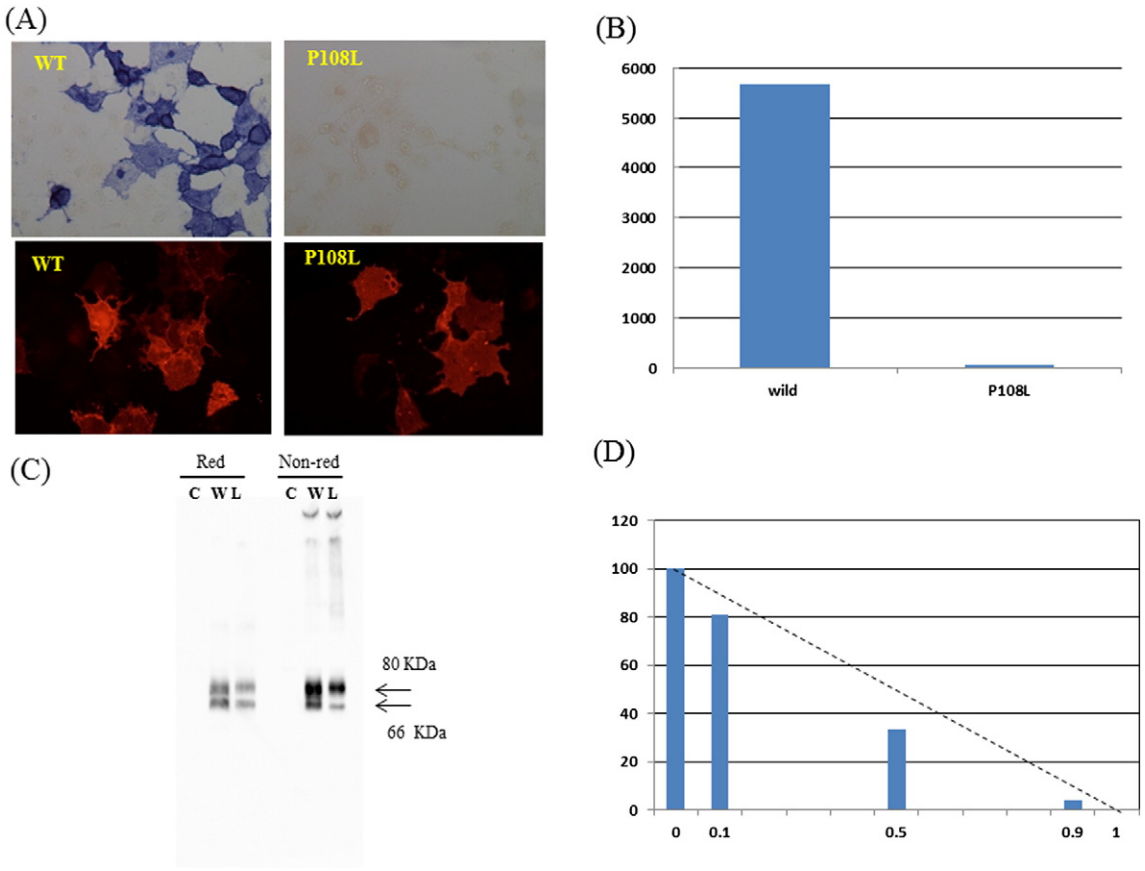

Fig. 1. Expression of TNSALP (P108L) transiently transfected in COS-1 cells. Cells were transfected with a plasmid encoding TNSALP (W) or TNSALP (P108L), cultured for 24 h, and then stained for ALP for 5 to 10 min at room temperature (A, upper panel) or processed for immunofluorescence with anti-TNSALP antibody (A, lower panel). TNSALP (P108L) gained access to the cell surface like TNSALP (WT), but failed to show ALP activity. WT, TNSALP (WT); P108L, TNSALP (P108L). In (B), cells transfected as in (A) were homogenized and assayed for ALP activity (B). y-axis: ALP activity (unit: mg protein). Only trace ALP activity was detected in the cells expressing TNSALP (P108L). WT, TNSALP (WT); P108L, TNSALP (P108L). In (C), cells that were untransfected or transfected with a plasmid encoding TNSALP (WT) or TNSALP (P108L) were homogenized and analyzed by SDS-PAGE under reducing or non-reducing conditions, followed by western blotting using anti-TNSALP antibody. TNSALP (P108L) was indistinguishable from TNSALP (WT) in SDS-PAGE. C, Untransfected control; W, TNSALP (WT); L, TNSALP (P108L). In (D), cells were cotransfected with the plasmids encoding TNSALP (WT) and TNSALP (P108L) in various ratios (1:0, 0.9:0.1, 0.5:0.5, 0.1:0.9. 0:1, x-axis). Cells were assayed for ALPase activity (expressed as a percentage of the wild-type enzyme, y-axis). Instead of the dotted line expected in the recessive model, ALP activities were below the line, indicating a dominant negative effect of TNSALP (P108L).

Most of them are missense mutations (75%) with deletions, splicing mutations, nonsense mutations, and insertions. In theory, when the transcription of the *ALPL* gene is not impaired, these loss-of-function mutations could affect any biosynthetic step of TNSALP from the ER to the cell membrane, resulting in diminished and/or altered cell surface ALP activity. Studies in vitro in cultured cells have indicated that the cell surface localization of TNSALP mutant proteins is retarded to various degrees due to improper folding and resultant perturbed protein trafficking [7–14]. Some TNSALP mutant proteins are retained in the ER or cis-Golgi and eventually subjected to ER-associated degradation [10,11,13]. Even though some TNSALP mutants are able to reach the cell membrane like TNSALP (WT), it is likely that they exhibit diminished ALP activity and/or altered catalytic properties [15,16]. Of interest is a frame-shift mutation that converts TNSALP to a GPI-anchorless form; this frame-shift mutant protein no longer resides on the cell membrane, but is largely degraded within the cell [17].

An increasing number of TNSALP mutations associated with dominant HPP have been identified [6,14,18]; however, the molecular characterization of these TNSALP mutant proteins has remained limited [19–22]. TNSALP (P108L) was first reported in a 9-year-old boy with dominantly transmitted odonto-HPP [23]. A 3D model of TNSALP revealed that proline at position 108 is located in close proximity to the active center of the molecule (serine at position 110) essential for phosphate binding, suggesting that the replacement of proline with leucine may have a direct effect on the catalytic activity [23]. Here, we report that TNSALP (P108L) no longer assumes the dimeric structure that is a prerequisite for its enzymatic activity, although it is able to gain access to the cell surface like TNSALP (WT).

2. Materials and methods

2.1. Plasmids and transfection

pSG5 vector (Stratagene, La Jolla, CA, USA) encoding TNSALP (WT) was created as described previously [7]. A point mutation was introduced to convert the proline at position 108 of TNSALP to a leucine [TNSALP (P108L)] using QuikChange Lightning Site-Directed Mutagenesis Kit (Stratagene, La Jolla, CA, USA). The oligonucleotides used were: 5′-ACCAATGCCCAGGTCC**T**TGACAGCGCCGGCA-3′ and 5′-TGCCGGCGCT GTC**A**AGGACCTGGGC-ATTGGT-3′. The full coding sequence of TNSALP (P108L) was verified by DNA sequencing.

TNSALP (P108L) was further subcloned into pTRE2-hyg (Invitrogen, Carlsbad, CA, USA). For transient expression, COS-1 cells, inoculated at a density of 8×10^4 cells/35-mm dish 24 h before transfection, were each transfected with 0.5 μg of plasmid using Lipofectamine Plus reagent (Invitrogen, Carlsbad, CA, USA). COS-1 cells were cultured as described previously [7]. For establishing cells, CHO K1 Tet-On cells (Invitrogen, Carlsbad, CA, USA) were transfected with 0.5 μg of pTRE2-hyg encoding TNSALP (P108L) using Lipofectamine Plus reagent and were screened by immunofluorescence to establish cell lines essentially according to