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PLTP is present in the nucleus, and its nuclear export is CRM1-dependent

Simona Vuletic [a,1], Weijiang Dong [a,b,1], Gertrud Wolfbauer [a], Joseph R. Day [a,2], John J. Albers [a,*]

[a] Department of Medicine, Northwest Lipid Metabolism and Diabetes Research Laboratories, University of Washington, 401 Queen Anne Ave N, Seattle, WA 98109, USA
[b] Xi'an Jiaotong University School of Medicine, Department of Human Anatomy and Histology and Embryology, Yanta West Road 76, Xi'an 710061, People's Republic of China

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ABSTRACT

Phospholipid transfer protein (PLTP), one of the key lipid transfer proteins in plasma and cerebrospinal fluid, is nearly ubiquitously expressed in cells and tissues. Functions of secreted PLTP have been extensively studied. However, very little is known about potential intracellular PLTP functions. In the current study, we provide evidence for PLTP localization in the nucleus of cells that constitutively express PLTP (human neuroblastoma cells, SK-N-SH; and human cortical neurons, HCN2) and in cells transfected with human PLTP (Chinese hamster ovary and baby hamster kidney cells). Furthermore, we have shown that incubation of these cells with leptomycin B (LMB), a specific inhibitor of nuclear export mediated by chromosome region maintenance 1 (CRM1), leads to intranuclear accumulation of PLTP, suggesting that PLTP nuclear export is CRM1-dependent. We also provide evidence for entry of secreted PLTP into the cell and its translocation to the nucleus, and show that intranuclear PLTP is active in phospholipid transfer. These findings suggest that PLTP is involved in novel intracellular functions.

1. Introduction

Phospholipid transfer protein (PLTP) is a versatile transfer protein with predicted protein molecular mass of approximately 55 kDa with signal peptide, or 53 kDa without [1]. Posttranslational modifications, such as N-glycosylation, account for the difference between the predicted molecular mass, 53 kDa, and observed mass of 81 kDa of the secreted protein [1]. Secreted PLTP is bound to lipoprotein particles, and associates with apolipoprotein A-I (apoA-I) and E (apoE), and with lipoproteins containing other apolipoproteins [2,3]. PLTP functions have been associated with the metabolism of lipoproteins, particularly high-density lipoproteins (HDL) (reviewed in [4]). PLTP has been shown to modulate phospholipid and cholesterol transfer between lipoproteins, as well as between lipoproteins and cells [5–7]. PLTP also transfers apolipoprotein E (apoE), α-tocopherol and other molecules such as cerebrosides and diacylglycerides [7–11].

PLTP is expressed by nearly all types of cells and tissues, with wide variation in expression patterns ([5,12] and unpublished data). For example, neighboring neurons in Cornu Amonis (CA) layers 1 and 2 in human hippocampus differ significantly in PLTP expression [12]. Interestingly, neurons in the CA1 layer, which express significantly lower levels of PLTP than other hippocampal neurons, are particularly sensitive to oxidative stress [12,13], and PLTP is one of the key molecules for maintenance of the brain anti-oxidative properties through regulation of tissue levels of α-tocopherol [11]. These findings, along with nearly ubiquitous, yet differential expression of PLTP in other cells and tissues suggest that PLTP has physiological roles other than purely extracellular functions related to lipoprotein metabolism. Although the role of PLTP as a secretory protein has been extensively studied, nothing is known about the possible intracellular functions of this protein.

In our immunohistochemical studies of the human brain [12], we noticed intranuclear staining in neuronal cells using several different antibodies against PLTP (unpublished data). We tested these initial observations in order to establish whether PLTP is indeed present in the nucleus. In this study we provide evidence for PLTP presence and activity in the nuclear compartment and discuss motifs that could be involved in PLTP nuclear import and export, implying that PLTP has novel intracellular functions beyond that of extracellular lipid transport.

2. Materials and methods

2.1. Materials

Dulbecco modified cell culture media (DMEM) were purchased from Lonza (Walkersville, MD). Chinese hamster ovary cells prepared for Flp-In vectors, Flp-In vectors and specialized growth media were purchased from Invitrogen (Carlsbad, CA). Fetal calf serum was obtained from HyClone (Logan, UT). Mutagenesis kits were purchased from Qiagen (Valencia, CA). Leptomycin B (LMB), bovine skin collagen, Triton X-100 and antibodies against lamin A were purchased from

* Corresponding author. Tel.: +1 206 685 3330; fax: +1 206 685 3279.
E-mail address: jja@u.washington.edu (J.J. Albers).
[1] These authors contributed equally to this study.
[2] Currently at McClure Middle School; 1915 1st Ave W, Seattle, WA 98119, USA.

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Sigma-Aldrich (St. Louis, MO). Nunc Lab-Tech chamber slides and other cell culture plastic-ware were purchased from VWR (West Chester, PA). Methanol-free formaldehyde (16%) was purchased from Polysciences (Warrington, PA). Monoclonal antibody against PLTP was produced at the Northwest Lipid Research Laboratory, in collaboration with Dr. Marcovina [12]. Secondary antibodies for Western blotting were purchased from eBioscience (San Diego, CA). Antibodies against TATA binding protein (TBP) for Western loading control were purchased from Abcam (Cambridge, MA). Biotinylated secondary antibodies (horse anti-mouse), Vectastain ELITE ABC, NovaRED and DAB kits, horse serum, hematoxylin, AquaMount and VectaShield mounting media were purchased from Vector Laboratories (Burlingame, CA). Fluorescently labeled secondary antibodies, chicken anti-mouse AlexaFluor 488 and goat anti-rabbit AlexaFluor 594 antibodies, and Image-iT FX signal enhancer were from Molecular Probes/Invitrogen (Eugene, OR). PLTP tagged with enhanced green fluorescent protein (PLTP-EGFP) and mock vectors were a kind gift from Dr. Vesa Olkkonen, National Public Health Institute, Helsinki, Finland [14]. Cytoplasmic and nuclear proteins were isolated using NE-PER kit with addition of protease inhibitor cocktail, Halt (Pierce, Rockford, IL).

2.2. Cell culture

Experiments were conducted in human neuroblastoma cells (SK-N-SH), human cortical neurons (HCN2; ATCC CRL-10743), Chinese hamster ovary (CHO) and baby hamster kidney (BHK) cells. Cells were incubated in 5% CO_2, at 37 °C, supplemented with 5% (SK-N-SH), 10% (CHO and BHK) or 15% (HCN2) serum in growth media. For standard immunocytochemistry and fluorescent live-cell imaging cells were plated in 8-chamber slides, while cells prepared for confocal microscopy were grown on collagen-treated coverslips in six-well plates. Cells were incubated without or with 10 ng/ml of LMB for up to 24 h. All experiments were performed in serum-free media, thus eliminating potential external source of PLTP, conducted in triplicate, and replicated multiple times.

2.3. PLTP mutagenesis

Site-directed mutagenesis was conducted using wild-type (WT) PLTP cDNA [1] as a template, using QuickChange site-directed mutagenesis kit (Stratagene) according to the manufacturer's instructions. Site-directed *in vitro* mutagenesis was accomplished by hybridizing a plasmid containing the PLTP insert (human PLTP C-his tag/pcDNA5/FRT) to oligonucleotides which are complementary to the target template except for a region of mismatch near the center, containing the desired serine-to-alanine mutations. We mutated the following residues in PLTP: S49A, S79A, S102A, and S128A within the putative N-glycosylation motifs (N/X/S-T; numbering based on mature protein without signal peptide). All created cDNA were verified by digestion and sequencing prior to transfection.

2.4. PLTP expression

Recombinant PLTP (rPLTP) constructs containing His tag were transfected into CHO Flp-In-Ready cells (Invitrogen) using Lipofectamine 2000, and clones were selected using Hygromycin B (0.6 mg/ml; Invitrogen). PLTP-EGFP constructs were transfected into BHK cells using Lipofectamine 2000, and positive clones were selected using G418 (1 mg/ml; Invitrogen). Highest expressing clones for PLTP-EGFP were selected by measurement of fluorescence (excitation/emission 488/535 nm), and all PLTP clones (wild-type His-tagged and PLTP-EGFP, as well as mutant PLTP) were confirmed by Western blot analysis using PLTP-specific monoclonal antibodies, and by measurement of PLTP phospholipid transfer activity in an *in vitro* assay [15].

2.5. Immunocytochemistry

Following incubation, cells prepared for standard, chromogenic ICC were fixed with 95% ethanol for 10 min, washed and incubated for 30 min with 3% hydrogen peroxide to reduce endogenous peroxidase. Slides were blocked with horse serum for 20 min, and incubated with mouse anti-PLTP antibody (20 μg/ml) overnight at 4 °C, followed by biotinylated horse anti-mouse antibody (5 μg/ml) for 30 min. The signal was amplified using Vectastain ELITE ABC reagents for 30 min, and stained using DAB or NovaRED kits. The slides were counter-stained with hematoxylin, coverslipped and observed under Olympus microscope. Digital images were created using Nikon E990 digital camera. Cells prepared for confocal microscopy were grown on coverslips, incubated without or with 10 ng/ml LMB for up to 24 h, fixed using 3.7% formaldehyde for 15 min, and incubated with primary antibodies against PLTP (mouse; 20 μg/ml) and Lamin A (rabbit; 2.5 μg/ml), as a nuclear marker, at 4 °C overnight. Alexa Fluor secondary antibodies (chicken anti-mouse Alexa Fluor 488, 5 μg/ml; and goat anti-rabbit Alexa Fluor 594, 3 μg/ml) for 45 min, rinsed with PBS and mounted using VectaShield HardSet mounting medium. The slides were observed under confocal Zeiss LSM 510 Meta microscope, using water-immersion objective, and recorded using Zeiss LSM 510 software, version 4.1 SP1.

2.6. Live cell imaging

Live cells transfected with PLTP-EGFP and grown in 8-chamber slides in phenol red-free media, incubated without or with LMB (10 ng/ml), were observed under Zeiss Axiovert inverted fluorescent microscope, using QColor 3 digital camera and QCapture Pro software.

2.7. PLTP re-entry experiment

Phenol red-free media from control BHK cells and cells transfected with PLTP-EGFP were collected after a 24-hour incubation, centrifuged to remove cells, concentrated using 30 kDa cut-off Amicon Ultra-4 centrifugal filter device, and added to control BHK cells incubated with vehicle (70% methanol) or 10 ng/ml LMB for 24 h. Following

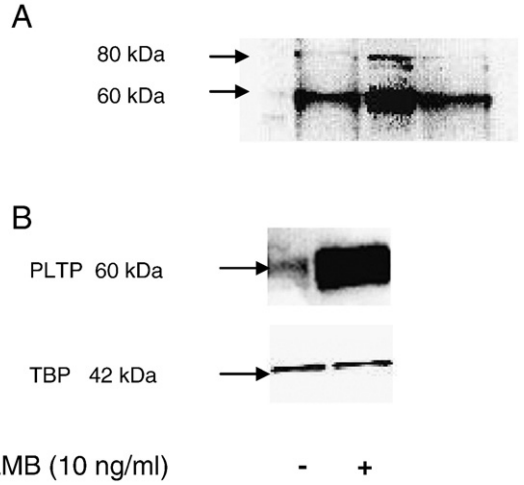

Fig. 1. PLTP is present in nuclear protein extracts of SK-N-SH neuroblastoma and human cortical neurons, HCN2 cells, and its nuclear export is chromosome region maintenance 1 (CRM1) dependent. (A) Isolated SK-N-SH nuclear proteins from three different samples were resolved using SDS-PAGE, and visualized using Western blot analysis with monoclonal anti-PLTP antibody. (B) PLTP Western blot analysis of nuclear proteins isolated from HCN2 cells incubated without (−) or with (+) 10 ng/ml LMB, a specific inhibitor of nuclear export mediated by CRM1, for 24 h under serum-free conditions. PLTP was identified using monoclonal anti-PLTP antibody. Loading control was evaluated using anti-TATA binding protein (TBP). Secondary antibody controls were negative (not shown).