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Impact of site-specific N-glycosylation on cellular secretion, activity and specific activity of the plasma phospholipid transfer protein

John J. Albers^{*}, Joseph R. Day¹, Gertrud Wolfbauer, Hal Kennedy, Simona Vuletic, and Marian C. Cheung

Northwest Lipid Metabolism and Diabetes Research Laboratories, Division of Metabolism, Endocrinology, and Nutrition, Department of Medicine, University of Washington, Seattle, Washington, USA

John J. Albers: jja@uw.edu; Joseph R. Day: dayjr@comcast.net; Gertrud Wolfbauer: gwolfb@uw.edu; Hal Kennedy: halk@uw.edu; Simona Vuletic: simona@uw.edu; Marian C. Cheung: mccheung@uw.edu

Abstract

The plasma phospholipid transfer protein (PLTP) plays a key role in lipid and lipoprotein metabolism. It has six potential N-glycosylation sites. To study the impact of these sites on PLTP secretion and activity, six variants containing serine to alanine point mutations were prepared by site-directed mutagenesis and expressed in Chinese hamster ovary Flp-In cells. The apparent size of each of the six PLTP mutants was slightly less than that of wild type by Western blot, indicating that all six sites are glycosylated or utilized. The size of the carbohydrate at each N-glycosylation site ranged from 3.14 to 4.2 kDa. The effect of site specific N-glycosylation removal on PLTP secretion varied from a modest enhancement (15% and 60%), or essentially no effect, to a reduction in secretion (8%, 14% and 32%). Removal of N-glycosylation at any one of the six glycosylation sites resulted in a significant 35-78% decrease in PLTP activity, and a significant 29-80% decrease in PLTP specific activity compared to wild type. These data indicate that although no single N-linked carbohydrate chain is a requirement for secretion or activity, the removal of the carbohydrate chains had a quantitative impact on cellular secretion of PLTP and its phospholipid transfer activity.

Keywords

phospholipid transfer protein; N-glycosylation; protein secretion; lipid transfer

1. Introduction

The plasma phospholipid transfer protein (PLTP) is a plasma glycoprotein that facilitates the transfer of phospholipid between lipid containing surfaces such as liposomes, cell membranes and lipoproteins [1 – 3]. PLTP has sequence homology and structural

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^{*}Corresponding Author: John J. Albers, Ph.D., Northwest Lipid Metabolism and Diabetes Research Laboratories, 401 Queen Anne Ave N, Seattle, WA 98109-4517, USA, Tel: 1-206-685-3330, Fax: 1-206-685-3279, jja@uw.edu.

¹Present address: Franklin High School, 3013 S Mt. Baker Blvd., Seattle, WA 98144, USA.

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similarities to several proteins involved in lipid and lipopolysaccharide binding and transfer, such as the bactericidal permeability increasing protein (BPI), the lipopolysaccharide binding protein (LBP) and the cholesteryl ester transfer protein (CETP). Based on other members of this protein's gene family, PLTP is a boomerang-shaped glycoprotein composed of two separate symmetrical barrels with lipid-binding pockets enriched in hydrophobic amino-acids [5, 6]. The predicted mass of the mature secreted protein is 53 kDa (54.7 kDa minus 2.7 kDa 17 amino acid leader peptide), but the apparent molecular weight by sodium dodecyl sulfate polyacrylamide gel electrophoresis is approximately 80 kDa [4]. This difference is due to the glycosylation of PLTP. The most common type of oligosaccharide attachment of a glycoprotein is N-linked and O-linked glycosylation. Based on PLTP's protein sequence [4], it has six potential N-glycosylation sites with the consensus sequence N-X-S/T beginning at residues 47, 77, 100, 126, 228 and 381, respectively. Studies performed by Huuskonen and colleagues showed that nearly all of the differences in molecular weight between the *in vitro* translated PLTP and secreted PLTP are due to N-glycosylation. Also, N-glycans appeared to be mainly of the complex type as the extracellular PLTP was essentially resistant to endoglycosidase H, but was sensitive to the digestion with peptide N-glycosidase F, a glycosidase that removes the entire N-linked oligosaccharide chain of PLTP [7].

N-linked carbohydrate chains are co-translationally added to the amino group of newly synthesized PLTP in the endoplasmic reticulum. The functions of N-linked carbohydrates include improving solubility, ensuring proper folding or enhancing folding efficiency, directing subcellular trafficking, acting as degradation signals and facilitating successful secretion [8]. The relative importance of N-glycosylation varies among proteins and with the physiological context and cell type [9]. Qu and colleagues reported that N-glycosylation was required for secretion of human PLTP [10]. Factors that can modulate PLTP activity, such as the degree of PLTP glycosylation, may have important clinical implications as plasma PLTP activity has been reported to be associated with vascular disease. Plasma PLTP activity in coronary heart disease patients is significantly higher than in controls [11]. Carotid intima-medial thickness in diabetic patients was independently associated with plasma PLTP activity [12]. Also, elevated PLTP activity is associated with diabetes, insulin resistance and obesity, conditions which significantly enhance the risk for vascular disease [13]. However, low plasma PLTP activity was found to be a risk factor for peripheral vascular disease [14]. A genome wide association study has found a PLTP genetic variant associated with higher plasma PLTP activity and carotid artery disease [15]. However, other PLTP SNPs found in this study were not associated with carotid artery disease but were associated with PLTP activity. Findings from a combination of five cardiovascular disease case-control studies showed that genetic variation at the PLTP locus is associated with the incidence of cardiovascular disease [16]. However, the basis for the relationship between the PLTP gene variation and cardiovascular disease remains to be clarified.

Thus far, little work has been done to evaluate the relative importance of individual N-glycosylation sites on PLTP's numerous functions. The aim of the current study was to accurately assess the apparent size of the carbohydrate at each N-glycosylation site and to determine the quantitative impact of each site on PLTP secretion, and on the activity and specific activity of phospholipid transfer by PLTP.

2. Materials and Methods

2.1 Site directed Mutagenesis

Site-directed mutagenesis was conducted using wild-type (WT) PLTP cDNA [4] as a template and the Quick Change site-directed mutagenesis kit (Cat. No. 200514; Stratagene, La Jolla, CA), 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/pcDNATM5/FRT; Cat. No. V6010-20; Invitrogen, Carlsbad, CA) with oligonucleotides complementary to the target template, containing the desired point mutations (see list of primers in Table 1). Mutagenesis was performed under the following conditions: one cycle, 1 minute at 95°C, followed by 30 cycles at 95°C/55°C/65°C (one minute each), and the last (30th) cycle at 95°C/55°C for one minute each and 65°C for 7 minutes. Following the Dpn1 digestion, which substitutes the mutated strand for the original one, the plasmids were grown in XL10-Gold competent cells (Invitrogen) using ampicillin as selection agent. Multiple clones from each plate were selected, grown and double-stranded DNA was prepared using plasmid Qiaprep Spin Miniprep Kit (Cat. No. 27104; Qiagen, Valencia, CA). All created PLTP cDNA constructs were verified by digestion and gel analysis prior to full length double-stranded sequencing. The point mutations created serine-to-alanine substitutions, resulting in following amino acid changes in PLTP: S49A, S79A, S102A, S128A, S230A, and S383A within the putative N-glycosylation motifs (N/X/S-T; numbering based on mature protein without signal peptide). Verified constructs were used for transfection and selection of stable clones.

2.2 In Vitro Expression of PLTP

The Flp-In pcDNA5/FRT System (Cat. No. K6010-01; Invitrogen) was used to generate isogenic stable mammalian expression cell lines. Recombinant PLTP (rPLTP) constructs containing C-terminal 6X His tag were co-transfected with pOG44 (pOG44: PLTP C-His tag/pcDNATM5/FRT ratio = 9:1), which enables correct insertion of the desired construct, into Chinese hamster ovary Flp-in-Ready cells (Cat. No. R758-07; Invitrogen) using Lipofectamine 2000 (Cat. No. 11668-027; Invitrogen). Clones were selected using Hygromycin B (Cat. No. 10687-010; Invitrogen) at 550 µg/ml, based on the selection agent killing curve. Transfected Chinese hamster ovary cells were grown in Ham's F12 Medium (Cat. No. 11765-062; Invitrogen) containing 10% fetal bovine serum (FBS) from HyClone and the selection agent, at 37°C, 5% CO₂ in a humidified incubator.

In order to produce conditioned media, cells were seeded in T25 tissue culture flasks (Corning), and grown to around 90% confluency. After washing the cells with PBS, 5.0 ml of CD CHO-A Medium (Cat. No. 0970182DJ; Invitrogen, Carlsbad, CA) without FBS was added. The optimal harvesting time-point was established through a time course and was 48 h from the start of incubation. To account for differences in cell growth, the cells were analyzed for Lowry protein in order to assess the amount of cell protein per flask.

2.3 PLTP Phospholipid Transfer Activity (PLTA)

PLTA was measured in the conditioned medium samples of Chinese hamster ovary cells, transfected with WT or different mutant PLTP, with our previously reported assay [17]. ¹⁴C-labeled phosphatidylcholine (PC) liposomes were prepared at 1 µmol/ml PC as donor, and HDL₃, purified over Heparin Sepharose (GE Healthcare) column, at 3 µmol/ml phospholipid as acceptor substrate. Samples were assayed in triplicates in a mixture of each 50 µl of donor and acceptor particles, filled to a volume of 0.4 ml with 10 mM Tris, 150 mM NaCl, 1 mM EDTA and 0.01% NaN₃, and placed for 15 min into a shaking 37°C water bath. Following the separation of substrates by precipitation with dextran sulfate and MgCl₂, an aliquot of each supernatant acceptor fraction was counted in a liquid scintillation counter (Beckman).

PLTA was evaluated in 10 µl of each conditioned medium in triplicate, in five different assays, and initially expressed as percent of total radioactivity transferred per tube, minus background transfer from tubes that did not contain any PLTP. The net percent transfer of PC was then converted to µmol PC transferred/h/mg cell protein. To account for inter-assay

variation 1 μ l aliquots of 3 different plasma samples, freshly thawed from -70°C storage, were included, also in triplicate, in each assay.

2.4 PLTP Mass Assay

A sandwich ELISA developed in our laboratories [18] was used to compare the mass of secreted WT and mutant PLTP from Chinese hamster ovary cells. In short: each 96 well plate was coated with 5 $\mu\text{g}/\text{ml}$ of affinity-purified goat anti-PLTP antibody in carbonate buffer and incubated overnight at 4°C . All washes used PBS containing 0.05% Tween-20. Blocking was performed with Starting Block (Pierce/Thermo Scientific) at room temperature (RT) for 2 hours. On each plate 100 μ l aliquots of recombinant PLTP (rPLTP), from 2.5 to 50 ng/ml, were applied in triplicate to establish the standard curve for calculating the amount of PLTP in the samples. Four human plasma samples (2 μ l each) stored at -70°C , were each diluted to 100 μ l and utilized in triplicate to account for variation between assays. One microliter aliquots (diluted separately to 100 μ l each) of serum-free 48h conditioned media from CHO control (Flp-In) cells and cells transfected with PLTP WT or PLTP with different carbohydrate deletions, were assessed in quadruplicate in each of three assays. Incubation was performed at room temperature for 2 h, followed by 4°C overnight. Plates were washed and exposed to the goat anti-PLTP antibody labeled with HRP for 2 h at room temperature. Color was developed with o-phenylenediamine dihydrochloride substrate and measured at 490 nm.

2.5 Western Blot Analysis

Aliquots of approximately 10 μ l of conditioned media from Chinese hamster ovary cells, containing WT or mutant PLTP with regard to carbohydrate, were boiled with DTT in sample buffer (BioRad, Hercules, CA) for 10 min and separated on 4-12% SDS polyacrylamide gels (Criterion, BioRad) together with MagicMark XP (Invitrogen) standards. All protein was transferred completely from gel to PVDF membrane by using a semi-dry transfer (BioRad) with a discontinuous buffer system (BioRad tech note 2134).

The membrane was blocked in TBS (20 mM Tris, 150 mM NaCl, pH 7.6) containing 5% dry milk for 1 h. Antibodies were diluted in TBS-0.05% Tween-20 (TBS-T) with 5% dry milk. First antibody incubation was done with affinity-purified goat anti PLTP at 0.5 $\mu\text{g}/\text{ml}$ for 1 h at 4°C and continued for 2 h at room temperature. After washing in TBS-T, the membrane was exposed to HRP-conjugated rabbit anti-goat IgG diluted 1:15,000 (Calbiochem) for 1h. All procedures were performed at room temperature, unless otherwise noted.

Positive bands were developed with Super Signal West Femto substrate from Pierce/Thermo Scientific and visualized by scanning with Kodak CF400 Gel Imager. The apparent molecular weight of wild type PLTP and each of the PLTP mutants was determined from 6 to 8 Western blots.

3. Results

The six PLTP mutants, in which the serine residue within each of the six putative N-glycosylation sites was replaced by an alanine, were expressed in Chinese hamster ovary cells. The apparent size of wild type and the various mutant PLTPs in the media of the transfected cells was assessed by Western blot after SDS polyacrylamide gel electrophoresis (Figure 1). The apparent size of each of the six PLTP mutants was slightly less than that of wild type PLTP. Computation of the apparent size of each carbohydrate chain by subtracting the apparent size of the mutant from wild type PLTP indicated that the size of carbohydrate at each N-glycosylation site was between 3.14 and 4.20 kDa (Table 2). The 3.24 kDa carbohydrate chain at position 49 was significantly smaller than the 4.16 kDa carbohydrate

chain at position 79 ($p < 0.01$), as was the 4.06 kDa carbohydrate chain at position 102 ($p < 0.02$), and the 4.20 kDa carbohydrate chain at position 230 ($p = 0.03$). Furthermore, the 3.59 kDa carbohydrate chain at position 128 was smaller than the 4.16 kDa carbohydrate chain at position 79 ($p < 0.04$).

The media of the transfected Chinese hamster ovary cells were analyzed for secreted PLTP mass and activity, and the specific activity of the expressed PLTP was computed (Table 2). All but one of the PLTP mutants had a PLTP secretion rate that differed significantly from wild type. Interestingly, two mutants (S79A and S102A) had a PLTP secretion rate greater than wild type, while three mutants (S128A, S230A, S393A) had a somewhat lower secretion rate than that of wild type. These findings indicate that the impact of site specific N-glycosylation on PLTP secretion is modest (Table 2) and varies with the site, ranging from an enhancement of secretion (160% or 115% of wild type) or no effect on secretion, to a slight decrease in secretion (from 68% to 92% of wild type).

Evaluation of PLTP activity in the media indicated that each of the PLTP mutants had significantly lower phospholipid transfer activity than wild type, ranging from 22% of wild type for mutant S49A, to 65% for S230A (Table 2). This finding indicates that removal of carbohydrate from any one of the six glycosylation sites results in a significant decrease in PLTP activity, but the amount of decrease varies with the site. Calculation of specific activity shows that removal of carbohydrate from each of the glycosylation sites resulted in reduction of PLTP specific activity and the impact of carbohydrate removal varied by site, ranging from 20% of wild type for mutant S79A to 72% for mutant S230A (Table 2).

4. Discussion

As glycosylation has been shown to be important for a variety of functions of glycoproteins, including assurance of proper folding and facilitation of solubility and secretion, we wanted to specifically test in PLTP the impact of site specific carbohydrate removal on its secretion rate, phospholipid transfer activity and specific activity. Wild type PLTP and six PLTP mutants, in which one of the putative N-glycosylation sites had been removed by replacing a serine residue with an alanine residue, were expressed in Chinese hamster ovary cells. The medium from each of the transfected cell lines was analyzed for the amount of PLTP secreted, and the phospholipid transfer activity and specific activity of the secreted PLTP.

Examination of the conditioned media of wild type transfected cells by Western blot indicates that these cells expressed essentially a single form of PLTP, which has an apparent molecule weight of approximately 80 kDa. Each of the N-linked deletion mutants also expressed a single form of PLTP, but at a molecular weight 3 to 4 kDa less than wild type PLTP. Our study clearly shows for the first time that all 6 potential N-linked sites are utilized. The sum of the molecular weight of the six N-glycosylated chains is almost 23 kDa. Subtraction of the carbohydrate molecular weight ($80 - 23$ kDa) yields a protein molecular weight of 57 kDa which is 4 kDa more than the calculated molecular weight of 53 kDa of the 476 amino acid mature PLTP without the 17 amino acid signal peptide. This finding supports earlier work showing that most PLTP glycosylation is N-linked glycosylation [7]. Our data is also consistent with the suggestion that a small amount of PLTP glycosylation, up to about 4 kDa, could be O-linked glycosylation, as O-linked glycosylation for PLTP has been reported [7, 19].

Our data demonstrated that no single N-linked carbohydrate chain in PLTP is a requirement for secretion or activity. However, the removal of each one of the carbohydrate chains had a quantitative impact on the secretion of PLTP and its phospholipid activity. It should be noted that although human plasma contains both active and inactive PLTP, these two forms

have the same molecular weight indicating that they are fully glycosylated [20]. We do not have evidence that Chinese hamster ovary cells secrete the inactive form of PLTP, and the evidence from human HepG2 hepatoma cells and other cells supports the notion that cells do not secrete the inactive form of PLTP ([21] and unpublished data).

Although unexpected, deletion of carbohydrate at either residue 77 or 100 appeared to enhance PLTP's secretion rate, while deletion of carbohydrate at three of the remaining four sites reduced PLTP's secretion rate. Perhaps the single carbohydrate chain at position 77 or 100 is not needed for secretion, as N-linked carbohydrate chains at the remaining sites appear to be sufficient to allow proper protein folding through interaction with protein chaperones and processing through the endoplasmic reticulum and Golgi, and for the secretion via the plasma membrane. The fact that each of the PLTP mutants had significantly lower PC transfer activity and specific activity than wild type PLTP, indicates that the N-glycosylation sites contribute to PLTP's ability to transfer phospholipids. The finding that removal of carbohydrate at positions 47 to 77 resulted in the lowest activity and specific activity is not clear. Based on our understanding of PLTP's three-dimensional structure [5, 6] the carbohydrates at these sites are at the outside of the N-terminal barrel. It is possible that these carbohydrate moieties are necessary for proper docking of PLTP on the liposome or lipoprotein surface. Alternatively, carbohydrates may affect PLTP stability.

4.1 Conclusions

Our study indicates that the lack of carbohydrate at any of the six PLTP N-glycosylation sites will have a negative impact on the ability of PLTP to transfer PC.

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Abbreviations

BPI	bactericidal permeability increasing protein
CETP	cholesteryl ester transfer protein
CHO	carbohydrate
FBS	fetal bovine serum
LBP	lipopolysaccharide binding protein
PC	phosphatidylcholine
PLTA	PLTP phospholipid transfer activity
PLTP	phospholipid transfer protein
WT	wild type

Research Highlights

- Six PLTP sites are N-glycosylated at residues 47, 77, 100, 126, 228 and 381.
- The size of these six carbohydrate (CHO) chains ranges from 3.14 to 4.20 kDa.
- CHO removal at each site reduced PLTP activity to 22-65% of wild type.
- CHO removal at each site reduced PLTP specific activity to 20-71% of wild type.

Figure 1. Polyacrylamide gel electrophoresis of wild type and mutant PLTP. Lanes 1 and 8, wild-type PLTP; Lane 2, S49A PLTP mutant; Lane 3, S79A PLTP mutant; Lane 4, S102A PLTP mutant; Lane 5, S128A PLTP mutant; Lane 6, S230A PLTP mutant; Lane 7, S383A PLTP mutant.

List of primers used for site-directed mutagenesis of PLTP's N-glycosylation sites. Numbering is based on sequence without the leader (17 amino acids, 51 nucleotides).

Table 1

Motif	Mutation	Primer	Sequence
NIS	S49A	T232G	5'-CACTTCTACTACAACATC <u>G</u> CTGAGGTGAAGGTCACAGG-3'
NAS	S79A	T322G	5'-AAATCACCAATGCC <u>G</u> CCTTGGGGCTGCGC-3'
NAS	S102A	T391G	5'-GCTACATCAACGCCCGCAGCTGAGGGGTGTG-3'
NVS	S128A	T469G	5'-AAAGTGTCCAAATGTC <u>G</u> CGCTGCCAGGCCCTCTG-3'
NWS	S230A	T775G_G776C	5'-CTGAGAGGGAACCTGG <u>G</u> CCCTCCCCCAACCGGG-3'
NNS	S383A	T1234G	5'-GTTCCGAATCTATTCCAAACCATGCTGCACCTGGAGT-3'

Table 2
Cellular secretion, activity, and average specific activity of PLTP and PLTP mutant in Chinese hamster ovary cells.

PLTP Form	Secretion Rate $\mu\text{g/day/mg}$		Activity $\mu\text{mol/hr/mg}$		Average Specific Activity ^a $\mu\text{mol/hr}/\mu\text{g}$		Carbohydrate removed	
	cell	%	protein	%	PLTP	%	kDa	
WT	18.3 $\pm$ 0.8		7.1 $\pm$ 0.3		0.194			
S49A	19.1 $\pm$ 0.8	104.4	1.6 $\pm$ 0.1 *	22.5	0.042	21.6	3.14 $\pm$ 0.75	
S79A	29.3 $\pm$ 0.4 *	160.1	2.3 $\pm$ 0.1 *	32.4	0.039	20.1	4.16 $\pm$ 0.90	
S102A	21.0 $\pm$ 1.5 *	114.8	3.4 $\pm$ 0.3 *	47.9	0.080	41.2	4.06 $\pm$ 0.94	
S128A	15.7 $\pm$ 0.6 *	85.8	3.4 $\pm$ 0.2 *	47.9	0.108	55.7	3.59 $\pm$ 0.79	
S230A	16.8 $\pm$ 0.6 *	91.8	4.6 $\pm$ 0.2 *	64.8	0.137	70.6	4.20 $\pm$ 0.75	
S383A	12.5 $\pm$ 0.6 *	68.3	2.6 $\pm$ 0.3 *	36.6	0.104	53.6	3.55 $\pm$ 0.69	

* Different from wild type, $p < 0.05$

^a Activity is determined in the medium collected at the 48-hour time point. The mass of PLTP at 48 h = 2 $\times$ secretion rate. Thus, the specific activity = activity/ 2 $\times$ secretion rate.