BBA - Biomembranes 1860 (2018) 645–653

Contents lists available at ScienceDirect

BBA - Biomembranes

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

Human bile acid transporter ASBT (*SLC10A2*) forms functional non-covalent homodimers and higher order oligomers

Paresh P. Chothe, Lindsay C. Czuba, Robyn H. Moore, Peter W. Swaan*

Department of Pharmaceutical Sciences, School of Pharmacy, University of Maryland, 20 Penn Street, Baltimore, MD 21201, USA

ARTICLE INFO

Keywords:
Bile acid
Transporter
Epitope tag
Intestinal absorption

ABSTRACT

The human apical sodium-dependent bile acid transporter, hASBT/*SLC10A2*, plays a central role in cholesterol homeostasis via the efficient reabsorption of bile acids from the distal ileum. hASBT has been shown to self-associate in higher order complexes, but while the functional role of endogenous cysteines has been reported, their implication in the oligomerization of hASBT remains unresolved. Here, we determined the self-association architecture of hASBT by site-directed mutagenesis combined with biochemical, immunological and functional approaches. We generated a cysteine-less form of hASBT by creating point mutations at all 13 endogenous cysteines in a stepwise manner. Although Cysless hASBT had significantly reduced function correlated with lowered surface expression, it featured an extra glycosylation site that facilitated its differentiation from wt-hASBT on immunoblots. Decreased protein expression was associated with instability and subsequent proteasome-dependent degradation of Cysless hASBT protein. Chemical cross-linking of wild-type and Cysless species revealed that hASBT exists as an active dimer and/or higher order oligomer with apparently no requirement for endogenous cysteine residues. This was further corroborated by co-immunoprecipitation of differentially tagged (HA-, Flag-) wild-type and Cysless hASBT. Finally, Cysless hASBT exhibited a dominant-negative effect when co-expressed with wild-type hASBT which validated heterodimerization/oligomerization at the functional level. Combined, our data conclusively demonstrate the functional existence of hASBT dimers and higher order oligomers irrespective of cysteine-mediated covalent bonds, thereby providing greater understanding of its topological assembly at the membrane surface.

1. Introduction

Bile acids (BAs) are secreted, after a meal, from the gall bladder into the small intestine to aid in the absorption of lipids and fat-soluble nutrients [1,2]. Whereas most BAs are reabsorbed as mixed micelles with dietary lipids, the human apical sodium-dependent bile acid transporter (hASBT, *SLC10A2*), predominantly expressed in the distal ileum, facilitates the reabsorption of up to 95% of the remaining BAs, thereby efficiently preventing their excretion in the feces. BAs are then returned to the liver via the portal circulation mediated by the basolateral bile acid exporter OSTα-OSTβ [1,3]. Subsequently, the paralogous bile acid transporter NTCP (*SLC10A1*) enables bile acid reentry into the liver. Both ASBT and NTCP have been extensively studied in regards to their relevance to bile acid handling [1,4], cholesterol homeostasis [5,6], and drug delivery applications [7,8]. The recent appreciation that BAs can function as complex signaling molecules that modulate glucose, lipid and energy metabolism [9,10] further necessitates a deeper understanding of BA homeostasis and the physiological role that transporters play in this process.

Our previous work sought to delineate the membrane topology and understand the molecular transport mechanism of hASBT-mediated transport [11–14]. hASBT contains 348 amino acids including 13 cysteines, 12 of which are conserved across mammalian species (Fig. 1). The structural and functional contributions of cysteines in membrane proteins, including transporters, have been well described: they play a role in conformational stability [15,16] through intramolecular disulfide bonds as well as in protein oligomerization [17–19] via intermolecular disulfide linkage. Moreover, cysteine residues engaged in disulfide linkages have shown to be critical for intracellular protein trafficking, stability and –ultimately– for protein function [20,21]. Our previous work suggested that Cys51, Cys105 and Cys255 are critical for hASBT function, while Cys74 may be implicated in protein trafficking

Abbreviations: COS-1, monkey kidney fibroblast cell line; CsA, Cyclosporin A; Cysless, Cysteineless; DSP, dithiobis[succinimidylpropionate]; DTSSP, 3,3′-dithiobis[succinimidylpropionate]; DTT, dithiothreitol; hASBT, human apical sodium-dependent bile acid transporter; NTCP, Na^+ taurocholate *co*-transporting polypeptide; OST, organic solute transporter; PAGE, polyacrylamide gel electrophoresis; TM, transmembrane

* Corresponding author at: Department of Pharmaceutical Sciences, University of Maryland, 20 Penn Street, HSF2-543, Baltimore, MD 21201, USA.
E-mail address: pswaan@rx.umaryland.edu (P.W. Swaan).

https://doi.org/10.1016/j.bbamem.2017.11.016
Received 9 March 2017; Received in revised form 22 November 2017; Accepted 27 November 2017
Available online 01 December 2017
0005-2736/

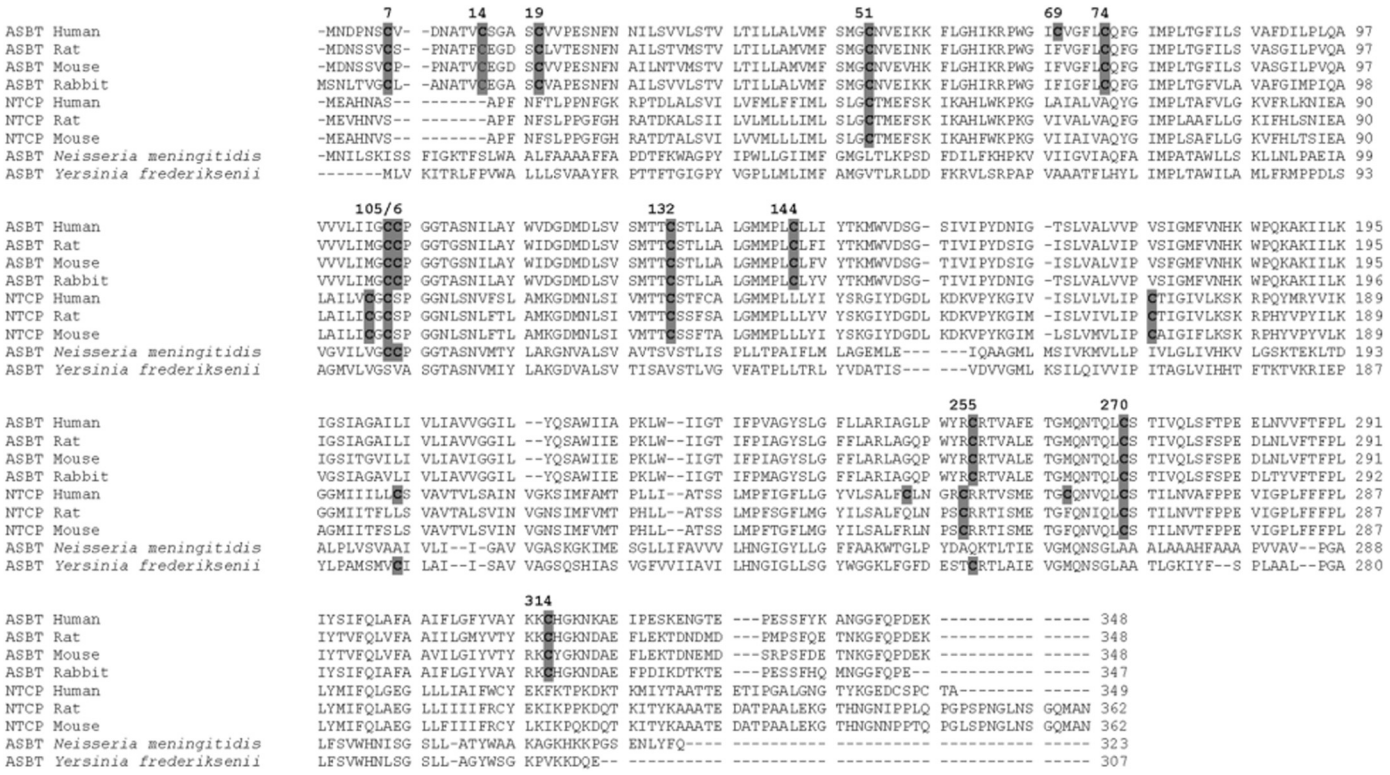

Fig. 1. Multiple protein sequence alignment of ASBT (*SLC10A2*). Protein sequences were retrieved from GenBank in FASTA format and aligned via ClustalW2 with manual adjustment. ASBT, apical sodium-dependent bile transporter; NTCP, Na^+-taurocholate cotransporting polypeptide (*SLC10A1*).

[22]. However, the overall contribution of endogenous cysteines in the oligomeric assembly of functional hASBT is unclear. Previous work had suggested that hASBT presumably functions as a monomer, but that it may also exist in dimeric and higher order oligomeric forms [23,24]. In fact, early studies by Kramer and co-workers [24] using photoaffinity labeling in rabbit intestine correlated a 93 kDa integral membrane protein with sodium-dependent bile acid uptake and this size is in line with a rabbit ASBT dimer. Other *SLC10A* family members, i.e. *SLC10A1*, *SLC10A5* and *SLC10A7*, are known to form dimers as well [25–27]. In fact, it has been suggested that membrane transporters with fewer than 12 transmembrane domains may require oligomerization to be functional [28]. However, there is no direct evidence for hASBT to date to support this notion and the present study sought to close this gap in our understanding of its structure and function.

Given the important role of cysteine residues in maintaining protein structural integrity required for proper assembly and function, we aimed to investigate the relevance of native cysteines to the oligomerization of hASBT. In this report, we used mutational analysis combined with biochemical, immunological and functional approaches to examine the role of endogenous cysteines in the dimer and higher order oligomer formation of hASBT.

2. Materials and methods

2.1. Materials

[^{3}H]-Taurocholic acid (TCA) was procured from Radiolabeled Chemicals, Inc., (St. Louis, MO). Taurocholic acid, tunicamycin, cyclosporin A (CsA) and dithiothreitol (DTT) were from Sigma (St. Louis, MO). MG132 was from Cayman Chemical (Ann Arbor, MI). EZ Link Sulfo-NHS-SS-biotin, maleimide-PEG$_{11}$-biotin, dithiobis[succinimidyl-propionate] (DSP) and 3,3′-dithiobis[succinimidylpropionate] (DTSSP) were purchased from Pierce Biotechnology (Rockford, IL). Cell culture media and supplies were from Invitrogen (Rockville, MD). All other chemicals were of the highest purity available commercially. Goat polyclonal anti-hASBT antibody and Protein G PLUS-Agarose were from Santa Cruz Biotechnology Inc. (Santa Cruz CA). Mouse anti-Flag antibody was from LifeTein LLC (South Plainfield, NJ), mouse anti-HA and mouse anti-calnexin antibodies were from Sigma (St. Louis, MO).

2.2. Cell culture and transfection

COS-1 cells were cultured in Dulbecco's modification of Eagle's medium (DMEM) with 10% FBS, penicillin (100 IU/ml) and streptomycin (100 μg/ml) (Life Technologies, Inc., Rockville, MD). Transient DNA transfection in COS-1 cells was carried out using Turbofect (Thermo Scientific) transfection reagent according to manufacturer's directions. Briefly, COS-1 cells were seeded in 24-well plate at an initial density of 0.065×10^6 cells per well. After 24 h, cells were transfected with WT or Cysless hASBT with Turbofect transfection reagent (1:4). 48 h post-transfection, cells were used for either uptake measurements or surface biotinylation and Western blot analysis.

2.3. Site-directed mutagenesis

hASBT cDNA in pCMV5 vector was used as a template. Site-specific mutations at 13 cysteines to either alanine or threonine were introduced using a site-directed mutagenesis kit from Stratagene (La Jolla, CA). All mutant hASBT were confirmed by sequencing. Primers used for creating these mutations were obtained from our previous studies [22].

2.4. Uptake assay and transport kinetic measurements

COS-1 cells transiently transfected with WT and Cysless ASBT (48 h post-transfection) were used for uptake studies. Uptake was carried out as described previously [29]. Briefly, cells were washed twice with Dulbecco's Phosphate-Buffered Saline (containing calcium and