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



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## 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 selfassociate 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 wthASBT 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 coexpressed 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 OST $\alpha$ -OST $\beta$  [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

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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<sup>+</sup> taurocholate co-transporting polypeptide; OST, organic solute transporter; PAGE, polyacrylamide gel electrophoresis; TM, transmembrane

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	7	14	19			51		69 74			
Human	-MNDPNSCV-	-DNATVCSGA	SCVVPESNEN	NILSVVLSTV	LTILLALVMF	SMGCNVEIKK	FLGHIKRPWG	ICVGFLCOFG	IMPLTGFILS	VAFDILPLOA	97
Rat	-MDNSSVCS-	-PNATFCEGD	SCLVTESNEN	AILSTVMSTV	LTILLAMVMF	SMGCNVEINK	FLGHIKR PWG	IFVGFLCOFG	IMPLTGFILS	VASGILEVOA	97
Mouse	-MDNSSVCP-	-PNATVCEGD	SCVVPESNEN	AILNTVMSTV	LTILLAMVMF	SMGCNVEVHK	FLGHIKRPWG	IFVGFLCOFG	IMPLTGFILS	VASGILPVOA	97
Rabbit	MSNLTVGCL-	-ANATVOEGA	SCVAPESNEN	AILSVVLSTV	LTILLALVMF	SMGCNVEIKK	FLGHIRR PWG	IFIGFLCOFG	IMPLTGEVLA	VAFGIMPIOA	98
Human	-MEAHNAS	APF	NETL PPNEGK	RPTDLALSVI	LVEMLEETML	SLOCTMEESK	TKAHLWKPKG	LATALVAOYG	IMPLTAFVLG	KVFRLKNTEA	90
Rat	-MEVHNVS	APF	NESLPEGER	RATDKALSTT	LVLMLLLTML	SLGCTMEESK	TKAHLWKPKG	VIVALVAOFG	IMPLAAFILG	KTEHLSNIEA	90
Mouse	-MEAUNING	DPF	NEGI DEGECH	PATTYALOUT	LVUMLLITMI	CLOCTMERCK	TKAUFWKDKC	VITATUROVG	IMPLOAFILG	KUFUI TOTEA	90
Noiscoria moningitidie	-MNTL CKTCC	FICKTESIWA	ALFADAFFA	DDTFKWACDY	TOWLLCTIME	CMCLTLKDSD	EDITERADKA	VIIGUIAOFA	TMDATAWLLS	KULNI DAFTA	90
Varcinia fradarikoanii	MIV	VITTOI FOLMA	LICURAVED	DTTTTTCTCDV	VCDIIMITME	AMCUTIPIDD	ENDAI CDDVD	VARATEIUVI	TMDI TAWILA	MI FEMDEDIC	03
TOTOTHING ITCONTINUOUNT	HLD V	INA I INDE E VIIPA	DDD0 VAATEK	LITEIOTOLI	VOE DEHDINE	ANOVIDIODD	ENIVERI	VAAATELETTD	THEPTOP	MBERGIE E DBO	55
105/6 132 144											
Human	VVVLIIGCOP	GGTASNILAY	WVDGDMDLSV	SMTTESTLLA	LGMMPLELLT	YTKMWVDSG-	SIVIPYDNIG	-TSLVALVVP	VSIGMEVNHK	WPOKAKIILK	195
Bat	VVVLTMGCCP	GGTGSNILAY	WIDGDMDLSV	SMTTCSTLLA	LGMMPLCLET	YTKMWVDSG-	TTVTPVDSTG	-TSLVALVTP	VSTGMEVNHK	WPOKAKTTLK	105
Mourse	WWWLTMCCCP	GGTGSNILAY	WIDGDMDLSV	SMTTOSTILLA	LCMMPLCLEV	YTEMWVDSG-	TTVTPVDSTG	-TSLVALVID	VSEGMEVNHK	MDOKRKITIK	100
Debbi =	WWW TMCCCP	COTACNILAY	WUDCDMDICU	CMTTCCTIIA	LCMMDL CL VV	VTEMANDSG-	TIVIPIDOIG	_TCIVALVVD	VOTCMEUNIUK	WDOKRYTTIK	100
Human	LATINGCOCD	CONTRONTERI	AMPCDMNLCT	IMPROPERT	LOMMDILLYT	Venctypopt	VDVUDVVCTU	-TOLVINI TD	PTCTULVEV	PROVMOVUTV	100
nullan Det	LATITOCOCD	CONLON POL	AMACOMULSI	VATIOSTECA	LOMPLIE	VENCTADED	KDKVPIKGIV	-ISLVLVLIP	CTICIVERSK	REVINKIVIK	100
Kat	LAILIGGESP	GGNLSNLFTL	AMAGDMINLSI	VATIOSSESA	LOMMPLILLY	ISKGIIDGDL	KDKVPIKGIM	-ISLVIVLIP	CITICIVERSK	REHIVETILLA	100
Mouse	LAILIUGUSP	GGNLSNLFTL	AMAGDMALSI	VMTTUSSETA	DUMPETER	ISKGIIDGDL	KDKVPIKGIM	-LSLVMVLIP	AIGIFLASA	KPHIVPIVLK	103
Neisseria meningitidis	VGVILVGCCP	GGTASNVMTY	LARGNVALSV	AVTSVSTLIS	PLLTPAIFLM	LAGEMLE	IQAAGML	MSIVKMVLLP	IVEGLIVHKV	LGSKTERLTD	193
iersinia irederiksenii	AGMVLVGSVA	SGTASNVMIY	LAKGDVALSV	TISAVSTLVG	VEATPLETEL	YVDATIS	VDVVGML	KSILQIVVIP	T.LAGLAIHHL.	FURLARKIED	191
							255	270			
Juman	TOSTAGATIT	VITAWCCTI		DVIWTICT	TEDUACVELC	FILAPIACID	255	270	TUOICETE	FINUTEDEDI	201
Human	IGSIAGAILI	VLIAVVGGIL	YQSAWIIA	PKLWIIGT	IFPVAGYSLG	FLLARIAGLP	255 WYRCRTVAFE	270 TGMQNTQLCS	TIVQLSFTPE	ELNVVFTFPL	291
Human Rat	IGSIAGAILI IGSIAGAILI	VLIAVVGGIL VLIAVVGGIL	YQSAWIIA YQSAWIIE	PKLWIIGT PKLWIIGT	IFPVAGYSLG IFPIAGYSLG	FLLARIAGLP	255 WYRCRTVAFE WYRCRTVALE	270 TGMQNTQLCS TGMQNTQLCS	TIVQLSFTPE TIVQLSFSPE	ELNVVFTFPL DLNLVFTFPL	291 291
Human Rat Mouse	IGSIAGAILI IGSIAGAILI IGSITGVILI	VLIAVVGGIL VLIAVVGGIL VLIAVIGGIL	YQSAWIIA YQSAWIIE YQSAWIIE	PKLWIIGT PKLWIIGT PKLWIIGT	IFPVAGYSLG IFPIAGYSLG IFPIAGYSLG	FLLARIAGLP FFLARLAGQP FFLARLAGQP	255 WYRCRTVAFE WYRCRTVALE WYRCRTVALE	270 TGMQNTQLCS TGMQNTQLCS TGMQNTQLCS	TIVQLSFTPE TIVQLSFSPE TIVQLSFSPE	ELNVVFTFPL DLNLVFTFPL DLNLVFTFPL	291 291 291
Human Rat Gouse Rabbit	IGSIAGAILI IGSIAGAILI IGSITGVILI VGSIAGAVLI	VLIAVVGGIL VLIAVVGGIL VLIAVIGGIL VLIAVVGGIL	YQSAWIIA YQSAWIIE YQSAWIIE YQSAWIIE	PKLWIIGT PKLWIIGT PKLWIIGT PKLWIIGT	IFPVAGYSLG IFPIAGYSLG IFPIAGYSLG IFPMAGYSLG	FLLARIAGLP FFLARLAGQP FFLARLAGQP FFLARIAGQP	255 WYRCRTVAFE WYRCRTVALE WYRCRTVALE WYRCRTVALE	270 TGMQNTQLCS TGMQNTQLCS TGMQNTQLCS TGMQNTQLCS	TIVQLSFTPE TIVQLSFSPE TIVQLSFSPE TIVQLSFSPE	ELNVVFTFPL DLNLVFTFPL DLNLVFTFPL DLTYVFTFPL	291 291 291 292
Human Rat Mouse Rabbit Human	IGSIAGAILI IGSIAGAILI IGSITGVILI VGSIAGAVLI GGMIILLES	VLIAVVGGIL VLIAVVGGIL VLIAVIGGIL VLIAVVGGIL VAVTVLSAIN	YQSAWIIA YQSAWIIE YQSAWIIE YQSAWIIE VGKSIMFAMT	PKLWIIGT PKLWIIGT PKLWIIGT PKLWIIGT PLLIATSS	IFPVAGYSLG IFPIAGYSLG IFPIAGYSLG IFPMAGYSLG LMPFIGFLLG	FLLARIAGLP FFLARLAGQP FFLARLAGQP FFLARIAGQP YVLSALF <b>C</b> LN	255 WYRCRTVAFE WYRCRTVALE WYRCRTVALE GRCRTVALE GRCRTVSME	270 TGMQNTQLCS TGMQNTQLCS TGMQNTQLCS TGMQNTQLCS TGMQNVQLCS	TIVQLSFTPE TIVQLSFSPE TIVQLSFSPE TIVQLSFSPE TILNVAFPPE	ELNVVFTFPL DLNLVFTFPL DLNLVFTFPL DLTYVFTFPL VIGPLFFFPL	291 291 291 292 287
Human Rat Mouse Rabbit Human Rat	IGSIAGAILI IGSIAGAILI IGSITGVILI VGSIAGAVLI GGMIIILLO GGMIITFLLS	VLIAVVGGIL VLIAVVGGIL VLIAVIGGIL VLIAVVGGIL VAVTVLSAIN VAVTALSVIN	YQSAWIIA YQSAWIIE YQSAWIIE YQSAWIIE VGKSIMFAMT VGNSIMFVMT	PKLWIIGT PKLWIIGT PKLWIIGT PKLWIIGT PLLIATSS PHLLATSS	IFPVAGYSLG IFPIAGYSLG IFPIAGYSLG IFPMAGYSLG LMPFIGFLLG LMPFSGFLMG	FLLARIAGLP FFLARLAGQP FFLARLAGQP FFLARIAGQP YVLSALF <b>G</b> LN YILSALFQLN	255 WYRCRTVAFE WYRCRTVALE WYRCRTVALE GRCRRTVSME PSCRRTISME	270 TGMQNTQLCS TGMQNTQLCS TGMQNTQLCS TGCQNVQLCS TGCQNVQLCS	TIVQLSFTPE TIVQLSFSPE TIVQLSFSPE TIVQLSFSPE TILNVAFPPE TILNVTFPPE	ELNVVFTFPL DLNLVFTFPL DLNLVFTFPL DLTYVFTFPL VIGPLFFFPL VIGPLFFFPL	291 291 292 287 287
Human Rat Rabbit Human Rat Mouse	IGSIAGAILI IGSIAGAILI IGSITGVILI VGSIAGAVLI GGMIIILLOS GGMIITFLLS AGMIITFSLS	VLIAVVGGIL VLIAVVGGIL VLIAVIGGIL VLIAVVGGIL VAVTVLSAIN VAVTALSVIN VAVTVLSVIN	YQSAWIIA YQSAWIIE YQSAWIIE YQSAWIIE VGKSIMFAMT VGNSIMFVMT VGNSIMFVMT	PKLWIIGT PKLWIIGT PKLWIIGT PKLWIIGT PLLI-ATSS PHLL-ATSS PHLL-ATSS	IFPVAGYSLG IFPIAGYSLG IFPIAGYSLG IFPMAGYSLG LMPFIGFLLG LMPFSGFLMG LMPFTGFLMG	FLLARIAGLP FFLARLAGQP FFLARLAGQP FFLARIAGQP YVLSALFOLN YILSALFOLN YILSALFRLN	255 WYRCRTVAFE WYRCRTVALE WYRCRTVALE GRCRRTVALE PSCRRTISME PSCRRTISME	270 TGMQNTQLCS TGMQNTQLCS TGMQNTQLCS TGMQNTQLCS TGFQNIQLCS TGFQNIQLCS	TIVQLSFTPE TIVQLSFSPE TIVQLSFSPE TIVQLSFSPE TILNVAFPPE TILNVTFPPE TILNVTFPPE	ELNVVFTFPL DLNLVFTFPL DLTVVFTFPL VIGPLFFFPL VIGPLFFFPL	291 291 292 287 287 287
Human Rat Mouse Rabbit Human Rat Mouse Neisseria meningitidis	IGSIAGAILI IGSIAGAILI IGSITGVILI VGSIAGAVLI GGMIIILI GGMIITFLS AGMIITFSLS ALPLVSVAAI	VLIAVVGGIL VLIAVVGGIL VLIAVGGIL VAVTVLSAIN VAVTALSVIN VAVTVLSVIN VLII-GAV	YQSAWIIA YQSAWIIE YQSAWIIE VGSSIMFAMT VGNSIMFVMT VGNSIMFVMT VGSSIMFVMT	PKLWIIGT PKLWIIGT PKLWIIGT PLLI-ATSS PHLL-ATSS SGLLIFAVVV	IFPVAGYSLG IFPIAGYSLG IFPIAGYSLG LMPFIGFLLG LMPFIGFLMG LMPFTGFLMG LMPFTGFLMG	FLLARIAGLP FFLARLAGQP FFLARLAGQP YVLSALFGLN YILSALFQLN YILSALFRLN FFAAKWTGLP	255 WYRCRTVAFE WYRCRTVALE WYRCRTVALE GRCRRTVALE FSCRRTVSME FSCRRTISME YDAQKTLTIE	270 TGMQNTQLCS TGMQNTQLCS TGMQNTQLCS TGMQNTQLCS TGFQNUQLCS TGFQNUQLCS VGMQNSGLAA	TIVQLSFTPE TIVQLSFSPE TIVQLSFSPE TILNVAFPPE TILNVTFPPE TILNVTFPPE ALAAAHFAAA	ELNVVFTFPL DLNLVFTFPL DLNLVFTFPL VIGPLFFFPL VIGPLFFFPL VIGPLFFFPL PVVAVPGA	291 291 292 287 287 287 287
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Human Rat Mouse Rabbit Human Rat Neisseria meningitidis Yersinia frederiksenii Human	IGSIAGAILI IGSIAGAILI IGSITGVILI GGMIIILLGS GGMIITELGS AGMIITESLS ALPLVSVAAI YLPAMSMVGI	VLIAVVGGIL VLIAVVGGIL VLIAVVGGIL VAVTVLSAIN VAVTVLSAIN VAVTVLSVIN VLI-I-GAV LAII-SAV AIFLGFYVAY	YQSAWIIA YQSAWIIE YQSAWIIE VGRSIMFAMT VGRSIMFAMT VGRSIMFVMT VGASKGKIME VAGSQSHIAS 314 KKGHGKNAAE	PKLWIIGT PKLWIIGT PKLWIIGT PLLIATSS PHLLATSS SGLLIFAVVV VGFVVIIAVI IPESKENGTE	IFPVAGYSLG IFPIAGYSLG IFPIAGYSLG IMPFIGFLLG LMPFIGFLLG LMPFIGFLLG LHNGIGYLLG LHNGIGYLLG LHNGIGYLLG	FLLARIAGLP FFLARLAGQP FFLARLAGQP YVLSALFGLN YILSALFGLN FFAAKWTGLP YWGGKLFGFD ANGGFQPDEK	255 WYRGRTVALE WYRGRTVALE GRGRRTVALE GRGRRTVALE GRGRRTVSME PSGRRTISME YDAOKTLTIE ESTGRTLAIE	270 TGMQNTQLGS TGMQNTQLGS TGMQNTQLGS TGMQNTQLGS TGFQNVQLGS TGFQNVQLGS VGMQNSGLAA VGMQNSGLAA	TIVQLSFTPE TIVQLSFSPE TIVQLSFSPE TILNVAFPPE TILNVTFPPE ALAAAHFAAA TLGKIYFS	ELNVVFTFPL DLNLVFTFPL DLTVVFTFPL VIGPLFFFPL VIGPLFFFPL PVVAVPGA PLAALPGA	291 291 292 287 287 287 288 280
Human Rat Mouse Rabbit Human Rat Mouse <i>Verssinia frederiksenii</i> Human Rat	IGSIAGAILI IGSIAGAULI IGSITGVILI VGSIAGAVLI GGMIITFLLS AGMIITFSLS ALPLVSVAAI YLPAMSMVGI IYSIFQLAFA IYTYQLVFA	VLIAVVGGIL VLIAVVGGIL VLIAVGGIL VAUTALSVIN VAVTALSVIN VAVTALSVIN VAUTALSVIN VLII-GAV LAII-SAV AIFLGFYVAY AIFLGFYVAY	YQSAWIIA YQSAWIIE YQSAWIIE VGKSIMFAWI VGNSIMFVWT VGNSIMFVWT VGSQSHIAS 314 KKCHGKNIKAE KKCHGKNIKAE	PKLWIIGT PKLWIIGT PKLWIIGT PLLI-ATSS PHLL-ATSS PHLL-ATSS SGLIFAVVV VGFVVIIAVI IPESKENGTE FLEKTDNDMD	IFPVAGYSLG IFPIAGYSLG IFPIAGYSLG IFPMAGYSLG LMPFIGFLLG LMPFGFLMG LMPFTGFLMG LHNGIGYLLG LHNGIGLLSG PESSFYK PESSFYK	FLLARIAGLP FFLARLAGOP FFLARLAGOP FFLARIAGOP YVLSALFGLN YILSALFRLN YILSALFRLN FFAAKWTGLP YWGGKLFGFD ANGGFQPDEK TNKGFQPDEK	255 WYRGRTVALE WYRGRTVALE GRÖRRTVALE GRÖRRTVSME PSCRRTISME YDAQKTLTIE ESTORTLAIE	270 TGMQNTQLGS TGMQNTQLGS TGMQNTQLGS TGGQNVQLGS TGFQNVQLGS VGMQNSGLAA VGMQNSGLAA 348 348	TIVQLSFTPE TIVQLSFSPE TIVQLSFSPE TILNVAFPPE TILNVTFPPE TILNVTFPPE ALAAAHFAAA TLGKIYFS	ELNVVFTFPL DLNLVFTFPL DLTVVFTFPL VIGPLFFPL VIGPLFFPL VIGPLFFFPL PVVAVGA PLAALPGA	291 291 292 287 287 287 288 280
Human Rat Mouse Rabbit Human Rat <i>Mouse Versinia frederiksenii</i> Human Rat	IGSIAGAILI IGSIAGAILI IGSITGVILI VGSIAGAVLI GGMIITFLS AGMIITFLS ALPLVSVAAI YLPAMSMVOI IYSIFQLAFA IYTVFQLVFA	VLIAVVGGIL VLIAVVGGIL VLIAVGGIL VAVTVLSNIN VAVTVLSNIN VAVTVLSVIN VLII-GAV LAII-SAV AIFLGFYVAY AIILGMYVTY AVILGIYYTY	YQSAWIIA YQSAWIIE YQSAWIIE -YQSAWIIE VGNSIMFAMT VGNSIMFVMT VGASKGKIME VAGSQSHIAS 314 KKCHGKNKAE KKCHGKNKAE KKCHGKNDAE	PKLWIIGT PKLWIIGT PKLWIIGT PLLI-ATSS PHLL-ATSS SGLLIFAVVV VGFVVIIAVI IPESKENGTE FLEKTDNEMD	IFPVAGYSLG IFPIAGYSLG IFPIAGYSLG IFPMAGYSLG LMPFIGFLLG LMPFTGFLMG LHNGIGYLLG LHNGIGLLSG PESSFYK PMPSFQE SRPSFDE	FLLARIAGLP FFLARLAGQP FFLARLAGQP FFLARLAGQP YVLSALFQLN YILSALFQLN YILSALFRLN FFAAKWTGLP YWGGKLFGFD ANGGFQPDEK TNKGFQPDEK TNKGFQPDEK	255 WYRCRTVALE WYRCRTVALE GRÖRRTVALE GRÖRRTVSME PSCRRTISME YDAOKTLTIE ESTORTLAIE	270 TGMONTQLCS TGMONTQLCS TGMONTQLCS TGMONTQLCS TGFONUQLCS TGFONUQLCS VGMONSGLAA VGMONSGLAA VGMONSGLAA 348 348 348	TIVQLSFTPE TIVQLSFSPE TIVQLSFSPE TILNVAFPPE TILNVTFPPE ALAAAHFAAA TLGKIYFS	ELNVVFTFPL DLNLVFTFPL DLTVVFTFPL VIGPLFFFPL VIGPLFFFPL PVVAVPGA PLAALPGA	291 291 292 287 287 287 287 287 288
Human Rat Mouse Rabbit Human Rat <i>Versinia frederiksenii</i> Human Rat Mouse Rabbit	IGSIAGAILI IGSIAGALI IGSITGVILI GGMIITGVILI GGMIITELS AGMIITELS ALPLVSVAAI YLPAMSNVUI IYSIFQLAFA IYTVFQLVFA IYSIFQLAFA	VLIAVVGGIL VLIAVVGGIL VLIAVIGGIL VAUTALSUIN VAVTALSVIN VAVTALSVIN VAUTALSVIN VLI-I-GAV LAII-SAV AIFLGFYVAY AIFLGFYVAY AIFLGYVAY	YQSAWIIA YQSAWIIE YQSAWIIE VGKSIMFAMT VGNSIMFVMT VGSSIMFVMT VGASKGKIMA XKCHGKNKAE KKCHGKNKAE KKCHGKNDAE RKCYGKNDAE	PKLWIIGT PKLWIIGT PKLWIIGT PLLI-ATSS PHLL-ATSS SGLLIFAVVV VGFVVIIAVI IPESKENGTE FLEKTDNEMD FPDIKDTKTE	IFPVAGYSLG IFPIAGYSLG IFPIAGYSLG LMPFIGFLG LMPFIGFLG LMPFIGFLG LHNGIGYLLG LHNGIGLSG PESSFYK SRPSFDE SRPSFDE SRPSFDE	FLLARIAGLP FFLARLAGQP FFLARLAGQP YVLSALFQLN YILSALFQLN YILSALFRLN FFAAKWTGLP WAGGKLFGFD ANGGFQPDEK TNKGFQPDEK NNGFQPDEK-	255 WYRCRTVALE WYRCRTVALE GRORRTVALE GRORRTVSME PSCRRTISME PSCRRTISME PSCRRTISME	270 TGMQNTQLCS TGMQNTQLCS TGMQNTQLCS TGFQNVQLCS TGFQNVQLCS TGFQNVQLCS TGFQNVQLCS VGMQNSGLAA 348 348 348 348	TIVQLSFTPE TIVQLSFSPE TIVQLSFSPE TILNVAFPPE TILNVTFPPE ALAAAHFAAA TLGKIYFS	ELNVVFTFPL DLNLVFTFPL DLNLVFTFPL VIGPLFFFPL VIGPLFFFPL VIGPLFFFPL PVVAVPGA PLAALPGA	291 291 292 287 287 287 287 287
Human Rat Mouse Rabbit Human Rat <i>Mouse Yersinia frederiksenii</i> Human Rat Mouse Rabbit Human	IGSIAGAILI IGSIAGAULI IGSITGVILI VGSIAGAVLI GGMIITFLLS AGMIITFSLS ALPLVSVAAI YLPAMSMVGI IYSIFQLAFA IYTVFQLVFA IYSIFQLAFA LYMIFQLOEG	VLIAVVGGIL VLIAVVGGIL VLIAVGGIL VAVTALSVIN VAVTALSVIN VAVTALSVIN VAVTALSVIN VLII-GAV LAII-SAV AIFLGFYVAY AIILGMYVTY AVILGIVVTY LLIAIFWCY	YQSAWIIA YQSAWIIE YQSAWIIE VGKSIMFAMT VGNSIMFVMT VGNSIMFVMT VGSQSHIAS 314 KKCHGKNKAE KKCHGKNKAE KKCHGKNDAE EKFKTPKDKT	PKLWIIGT PKLWIIGT PKLWIIGT PLLI-ATSS PHLL-ATSS PHLL-ATSS SGLLIFAVVV VGFVVIIAVI IPESKENGTE FLEKTDNEMD FLEKTDNEMD FDDLKDTKTE KMIYTAATTE	IFPVAGYSLG IFPIAGYSLG IFPIAGYSLG IFPMAGYSLG LMPFIGFLLG LMPFGFLMG LMPGIGVLLG LHNGIGVLLG PESSFYK PMPSFQE PESSFYK ETIPQALANG	FLLARIAGLP FFLARLAGQP FFLARLAGQP FFLARLAGQP YVLSALFGLN YILSALFQLN YILSALFQLN YWGGKLFGFD ANGGFQPDEK TNKGFQPDEK TNKGFQPDEK TNKGFQPDES- TYKGEDCSPC	255 WYRCRTVALE WYRCRTVALE GRÖRRTVALE GRÖRRTVSME PSCRRTISME YDACKTLTIE ESTERTLAIE	270 TGMQNTQLGS TGMQNTQLGS TGMQNTQLGS TGGQNVQLGS TGFQNVQLGS TGFQNVQLGS VGMQNSGLAA VGMQNSGLAA VGMQNSGLAA 348 348 348 349	TIVQLSFTPE TIVQLSFSPE TIVQLSFSPE TILQLSFSPE TILNVTFPPE TILNVTFPPE ALAAAHFAAA TLGKIYFS	ELNVVFTFPL DLNLVFTFPL DLTVVFTFPL VIGPLFFFPL VIGPLFFFPL VIGPLFFFPL PVVAVBGA PLAALPGA	291 291 292 287 287 287 287 287
Human Rat Mouse Rabbit Human Rat <i>Yersinia frederiksenii</i> Human Rat Human Rat Mouse Rabbit	IGSIAGAILI IGSIAGAILI IGSITGVILI VGSIAGAVLI GGMIITFLS AGMIITFLS ALPLVSVAAI YLPAMSMVUI IYSIFQLAFA IYTVFQLVFA IYTVFQLVFA IYTVFQLVFA LYMIFQLAEG LYMIFQLAEG	VLIAVVGGIL VLIAVVGGIL VLIAVVGGIL VAVTVLSNIN VAVTVLSNIN VAVTVLSVIN VLI-I-GAV LAII-SAV AIFLGFYVAY AIILGMYVTY AVILGIYVTY AIFLGIYVAY LLILTAIFWCY LLIIIFWCY	YQSAWIIA YQSAWIIE YQSAWIIE VGNSIMFAMT VGNSIMFVMT VGNSIMFVMT VGASKGKIME XAGSQSHIAS 314 KKCHGKNKAE KKCHGKNDAE RKCYCKNDAE RKCYCKNDAE RKCYCKNDAE	PKLWIIGT PKLWIIGT PKLWIIGT PLLI-ATSS PHLL-ATSS SGLLIFAVVV VGFVVIIAVI IPESKENGTE FLEKTDNEMD FLEKTDNEMD FLEKTDNEMD FLEKTDNEMD FLEKTDNEMD	IFPVAGYSLG IFPIAGYSLG IFPIAGYSLG IFPMAGYSLG LMPFIGFLLG LMPFIGFLLG LHNGIGYLLG LHNGIGLLSG PESSFYK PMPSFQE SRPSFDE SRPSFDE ETIFCALCNG DATPALEKG	FLLARIAGLP FFLARLAGQP FFLARLAGQP FFLARLAGQP YVLSALFQLN YILSALFQLN YILSALFRLN FFAAKWTGLP YWGGKLFGFD ANGGFQPDEK TNKGFQPDEK TNKGFQPDEK MNGGFQPD THNGNIPPLQ	255 WYRCRTVALE WYRCRTVALE GRÖRRTVSME PSCRRTISME YDAOKTLTIE ESTORTISME YDAOKTLTIE ESTORTISME YDAOKTLTIE ESTORTISME PSCRRTISME YDAOKTLTIE ESTORTISME PSCRRTISME	270 TGMONTQLCS TGMONTQLCS TGMONTQLCS TGFONUQLCS TGFONUQLCS TGFONUQLCS VGMONSGLAA VGMONSGLAA VGMONSGLAA 348 348 348 348 348 348	TIVQLSFTPE TIVQLSFSPE TIVQLSFSPE TILNVAFPPE TILNVTFPPE ALAAAHFAAA TLGKIYFS	ELNVVFTFPL DLNLVFTFPL DLTVVFTFPL VIGPLFFFPL VIGPLFFFPL PVVAVPGA PLAALPGA	291 291 292 287 287 287 288
Human Rat Mouse Rabbit Human Rat <i>Yersinia frederiksenii</i> Human Rat Rabbit Human Rat Mouse	IGSIAGAILI IGSIAGAULI IGSITGVILI VGSIAGAVLI GGMIITELS AGMIITELS ALPLVSVAAI YLPAMSMVOI IYSIFQLAFA IYTVFQLVFA IYSIFQLAFA IYSIFQLAFA LYMIFQLAEG LYMIFQLAEG	VLIAVVGGIL VLIAVVGGIL VLIAVIGGIL VAVTALSVIN VAVTALSVIN VAVTALSVIN VLII-GAV AIFLGFYVAY AIFLGFYVAY AIFLGIYVAY LLIAIFWCY LLIFIFRCY LLFIIFRCY	YQSAWIIA YQSAWIIE YQSAWIIE VGKSIMFAWT VGNSIMFVWT VGNSIMFVWT VGASQSHIAS 314 KKCHGKNKAE KKCHGKNKAE KKCHGKNDAE RKCYGKNDAE EKKFYENDAT EKIKPENDQT LKIKPQKDQT	PKLWIIGT PKLWIIGT PKLWIIGT PLLIATSS PHLLATSS SGLLIFAVV VGFVVIIAVI IPESKENGTE FLEKTDNEMD FPDIKDTKTE KMIYTAATTE KITYKAAATE	IFPVAGYSLG IFPIAGYSLG IFPIAGYSLG IFPTAGYSLG LMPFIGFLLG LMPFIGFLMG LHNGIGLLSG PESSFYK PMPSFDE SRPSFDE PESSFHQ ETIFGALSNG DATPAALEKG DATPAALEKG	FLLARIAGLP FFLARLAGQP FFLARLAGQP YVLSALFQLN YILSALFQLN YILSALFQLN YILSALFQLP WGGKLFGFD ANGGFQPDEK TNKGFQPDEK MNGGFQPDEK MNGGFQPDE THKGNIPPLQ THNGNIPPLQ	255 WYRCRTVALE WYRCRTVALE WYRCRTVALE GRORRTVSME PSCRRTISME PSCRRTISME PSCRRTISME STORTLAIE	270 TGMQNTQLCS TGMQNTQLCS TGMQNTQLCS TGFQNVQLCS TGFQNVQLCS TGFQNVQLCS VGMQNSGLAA 348 348 348 348 348 348 348 348 348 348	TIVQLSFTPE TIVQLSFSPE TIVQLSFSPE TILNVAFPPE TILNVTFPPE ALAAAHFAAA TLGKIYFS	ELNVVFTFPL DLNLVFTFPL DLTVVFTFPL VIGPLFFFPL VIGPLFFFPL PVVAVPGA PLAALPGA	291 291 292 287 287 287 287 287
Human Rat Mouse Rabbit Human Rat Mouse Versinia frederiksenii Human Rat Mouse Rabbit Human Rat Mouse Neisseria meningitidis	IGSIAGAILI IGSIAGAULI IGSITGVILI VGSIAGAVLI GGMIITFLLS AGMIITFSLS ALPLVSVAAI YLPAMSMVGI IYSIFQLAFA IYTVFQLVFA IYTVFQLVFA LYMIFQLAEG LYMIFQLAEG LYMIFQLAEG LYMIFQLAEG LYMIFQLAEG	VLIAVVGGIL VLIAVVGGIL VLIAVGGIL VAVTALSVIN VAVTALSVIN VAVTALSVIN VAVTALSVIN VLII-GAV LAII-SAV AIFLGFYVAY AIILGMYVTY AVILGIVTY LLIAIFWCY LLIAIFWCY LLFIIFRCY SLL-ATYWAA	YQSAWIIA YQSAWIIE YQSAWIIE VGKSIMFAMT VGNSIMFVMT VGNSIMFVMT VGASQSHIAS 314 KKCHGKNKAE KKCHGKNKAE KKCHGKNDAE EKFKTPKDAT EKIKPPKDQT KAGKHKKPGS	PKLWIIGT PKLWIIGT PKLWIIGT PLLI-ATSS PHLL-ATSS PHLL-ATSS SGLIFAVVV VGFVVIIAVI IPESKENGTE FLEKTDNEMD FLEKTDNEMD FLEKTDNEMT KITYKAAATE KITYKAAATE KITYKAAATE	IFPVAGYSLG IFPIAGYSLG IFPIAGYSLG IFPMAGYSLG LMPFIGFLLG LMPFGFLMG LMPFGFLMG LHNGIGYLLG LHNGIGYLLG PESSFYK PMPSFQE PESSFPQ ETIPGALGNG DATPAALEKG DATPAALEKG	FLLARIAGLP FFLARLAGQP FFLARLAGQP FFLARLAGQP YVLSALFGLN YILSALFQLN YILSALFQLN YILSALFQLD ANGGFQPDEK TNKGFQPDEK TNKGFQPDEK TNKGFQPDET TYKGEDCSPC THNGNIPPLQ THNGNIPPLQ	255 WYRCRTVALE WYRCRTVALE GRÖRRTVALE GRÖRRTVSME PSCRRTISME YDACKTLTIE ESTERTLAIE	270 TGMONTQLCS TGMONTQLCS TGMONTQLCS TGFONVQLCS TGFONVQLCS TGFONVQLCS TGFONVQLCS VGMONSGLAA VGMONSGLAA 348 348 348 349 GQMAN 362 GQMAN 362 GQMAN 362	TIVQLSFTPE TIVQLSFSPE TIVQLSFSPE TILNVAFFPE TILNVTFPPE TILNVTFPPE ALAAAHFAAA TLGKIYFS	ELNVVFTFPL DLNLVFTFPL DLTVVFTFPL VIGPLFFFPL VIGPLFFFPL VIGPLFFFPL PVVAVBGA PLAALPGA	291 291 292 287 287 287 288 280
	Human Rat Mouse Rabbit Human Rat Versinia frederiksenii Human Rat Mouse Rabbit Human Rat Mouse Neisseria meningitidis Yersinia frederiksenii	HumanMNDPNSV- RatMDNSVGS- MouseMDNSVGS- MouseMDNSVGL- HumanMEAHNAS RatMEVHNVS Veisseria meningitidisMEVHNVS NUSEMLV 105/6 Human VVVLINGCOP Rat VVVLINGCOP Rat VVVLINGCOP Rat LAILICGSSP Rat LAILICGSP Rat LAILICGSP Peisseria meningitidis VVVLVSVA	Human - MNDENSUV - DNATVGSGA Rat - MNDENSUCS - PNATFIEGD Mouse - MINDENSUCS - PNATFIEGD - MINENSUCS - PNATFIEGD - MINENSUCE - ANATFIEGD Mouse - MINENUCSAPF Rat - MEAHNASAPF Neisseria meningitidis - MILSKISS FIGKTFSLWA Yersinia frederikseniiMLV KITRLFFVWA MLV KITRLFFVWA APF 	Human - MNDPNSVCS - DNATY GSA SCVVPESNFN Rat - MDNSSVCS - PNATF GGD SCLVTESNFN Mouse - MNDNSSVCS - PNATF GGD SCLVTESNFN Aabbit MSNLTVCC - PNATY GGA SCVVPESNFN Auman - MEAHNASAPF NFLPPNFGK Rat - MSNLTVCC - PNATY GSA SCVAPESNFN Mouse - MEAHNASAPF NFLPPNFGK APF NFLPPNFLPNFLPNFGK APF NFLPPNFGK APF NFLPPNFLPNFLPNFLPNFLPNFLPNFLPNFLPNFLPNFL	Human  -MNDPNSCV-  -DNATYUGSA  SCVVPESNEN NILSVVLSTV    Rat  -MDNSSVCS-  -PNATYUGSA  SCVVPESNEN AILSTVMSTV    Mouse  -MDNSSVCS-  -PNATYUGSA  SCVVPESNEN AILSTVMSTV    Muman  -MENNSVCF-  -NATYUGSA  SCVVPESNEN AILSTVMSTV    Muman  -MEAHNAS APF  NFLPNFGK  RPTDLALSVI    Neisseria meningitidis  -MEHNVS- APF  NFLPNFGK  RPTDLALSVI    Yersinia frederiksenii  -MELHNVS- APF  NFLPNFGK  RATDALSVI    Numan  -MELHNVS- APF  NFLPNFGK  RATDALSVI    Yersinia frederiksenii  -MILSKISS  FIGKTFSLWA  ALFAAAAFFA  PDTFKWAGPY    Numan  VVVLINGCOP  GGTASNILAY  WUDGDMDLSV  SMTTGSTLLA    Mouse  VVVLINGCOP  GGTGSNILAY  WIDGDMDLSV  SMTTGSTLLA    Muman  LAILVGCOP  GGTASNILAY  WUDGDMDLSV  SMTTGSTLA    Mouse  VVVLINCCOP  GGTASNILAY  WUDGDMDLSV  SMTTGSTLA    Rat  VVVLINCCOP  GGTASNILAY  WUTGSTSA  AMTSTSTLA    Rat  LAILUCCOP  GGTASNILAY  WUTGDMDLSV  SMTTGSTLA    Nouse  LAILUCCOP  GGTASNILFTL  AMKODMNLSI  VMTGSSFSA	Human	Human  -MNDENSGV - DNATVESGA SUVPESNEN NILSVVLSTV LTILLALVM F  SMGENVEIKK    Rat  -MNDENSGV - DNATVESGA SUVPESNEN AILSTVMSTV LTILLALVM F  SMGENVEIKK    Kabbit  -MNNSVGS - PNATFEGG SUVPESNEN AILSTVMSTV LTILLALVM F  SMGENVEIKK    Kabbit  MSNLTVGGL - ANATVEGG SUVPESNEN AILSTVMSTV LTILLALVM F  SMGENVEIKK    Human  -MEAHNASAPF NFILPPNFGK  RPTDLALSVI LVFMLFFIML  SLGGTMEFSK    Kat  -MEAHNASAPF NFILPPNFGK  RPTDLALSVI LVFMLFFIML  SLGGTMEFSK    Mouse  -MEAHNASAPF NFILPPNFGK  RPTDTALSVI LVVMLLILML  SLGGTMEFSK    Weisseria meningitidis  -MNULSKISS  FIGKTFSLWA  ALFAAAAFFA  PDTFKWAGPY  IPWLLGIIMF  GMGLTLKPSD    Yersinia frederiksenii  -MULVIIGCOP  GGTASNILAY  WUDGIMDLSV  SMTTGSTLLA  LGMMPLGLFV  YTKMWDSG-    Muman  UVVLIMGCOP  GGTASNILAY  WIDGIMDLSV  SMTTGSTLLA  LGMMPLGLFV  YTKMWDSG-    Mouse  VVVLIMGCOP  GGTASNILAY  WIDGIMDLSV  SMTTGSTLLA  LGMMPLLVY  YKMWDSG-    Kat  VVVLIMGCOP  GGTASNILAY  WIDGIMDLSV  SMTTGSTLLA  LGMMPLLVY  YKMWDSG-    Kat  VVVLIMGCOP  GGTASNILAY  WIDGIMDLSV  SMTTGSTLLA  LGMMPLLVY  YKMWDSG-    Kat  VVVLIMGCOP<	Human  -MNDENSVGS-  -DNATYUGSGA  SGVVPESNEN  NILSVVLSTV  LTILLALVMF  SMCGNVEIKK  FLGHIKRPWG    Rat  -MDNSSVGS-  -PNATFUEGD  SGLVTESNEN  AILSTVMSTV  LTILLAWMF  SMCGNVEIKK  FLGHIKRPWG    Kabbit  -MNDNSSVGS-  -PNATFUEGD  SGLVTESNEN  AILSTVMSTV  LTILLAWMF  SMCGNVEIKK  FLGHIKRPWG    Kabbit  MSNLTVGEG  SGVVPESNEN  AILSTVMSTV  LTILLAWMF  SMCGNVEIKK  FLGHIKRPWG    Human  -MSNLTVGEGA  SGVPESNEN  AILSTVMSTV  LTILLAWMF  SMCGNVEIKK  FLGHIKRPWG    Kat  -MEAHNAS- APF  NFSLPROFGH  RATDRALSII  LVMLLIML  SLGCTMEFSK  IKAHLWKPKG    Mouse  -MEAHNAS- APF  NFSLPROFGH  RATDRALSII  LVMLLLIML  SLGCTMEFSK  IKAHLWKPKG    Yersinia  frederiksenii  -MILSKISS  FIGKTFSLWA  ALFAAAAFFA  PDTFKWAGPY  IPWLLGIIMF  GWGTLKPSD  FULYPUNIG    Muman  105/6  132  144    WuvulinGCCP  GGTASNILAY  WUDGDMDLSV  SMTTSTLLA  IGMPHLGLIY  YKMWVDSG-  TIVIPYDNIG    Kat  VVVLINGCCP  GGTASNILAY  WUDGDMDLSV  SMTTSTLLA  IGMPHLGLY  YKMWVDSG-  TIVIPYDSIG	Human    -MNDPNSVGV - DNATYUGGA SUVPESNEN NILSVVLSTV LTILLALVMF SMGGNVELKK FLGHIKRPMG IFVGFLOPEG      Rat    -MNDSNSVGS - PNATYUGGA SUVPESNEN AILSTVMSTV LTILLALVMF SMGGNVELKK FLGHIKRPMG IFVGFLOPEG      Mouse    -MDNSSVGS - PNATYUGGA SUVPESNEN AILSTVMSTV LTILLALVMF SMGGNVELKK FLGHIKRPMG IFVGFLOPEG      Human    -MEAHNASAPF NSLPPOFGH AILNTVMSTV LTILLALVMF SMGGNVELKK FLGHIKRPMG IFVGFLOPEG      Nouse    -MEAHNASAPF NFLPPNFGK RPTDLALSVI LVEMLFFIML SLGGTMEFSK IKAHLWKPKG LAIALVAQYG      Neisseria meningitidis    -MEAHNASAPF NFSLPPOFGH RATDRALSVI LVVMLLIML SLGGTMEFSK IKAHLWKPKG VIIVAQYGG      Yersinia frederiksenii    -MELKUS - AFF PFSLPPOFGH RATDRALSVI LVVMLLIML SLGGTMEFSK IKAHLWKPKG VIIVAQYGG      Muman    105/6    132      Muman    105/6    132      Muman    VVVLINGCOP GGTASNILAY WVDGDMDLSV SMTTSTLLA LGMMPLGLLI YKKMWVDSG- SIVIPYDNIG -TSLVALVVP      Mouse    VVVLINGCOP GGTGSNILAY WIDGDMDLSV SMTTSTLLA LGMMPLGLEV YTKMWVDSG- TIVIPYDSIG -ISLVALVIP      Muman    LAILVGOS GGTGSNILAY WIDGDMDLSV SMTTSTLLA LGMMPLGLEV YTKMWVDSG- TIVIPYDSIG -ISLVALVIP      Rat    VVVLIMCOP GGTASNILAY WIDGDMDLSV SMTTSTLLA LGMMPLGLEV YTKMWVDSG- TIVIPYDSIG -ISLVALVIP      Rat    VVVLIMCOP GGTASNILAY WIDGDMDLSV SMTTSTLLA LGMMPLGLEV YTKMWVDSG- TIVIPYDSIG -ISLVALVIP      Rat    VVVLIMCOP GGTASNILAY WIDGDMDLSV SMTTSTLLA LGMMPLGLEV YTKMWVDSG- TIVIPYDSIG -ISLVALVIP	Human    -MNDPNSVU - DNATYUSGA SUVPESNEN NILSVVLSTV    LTILLALVM SMCGNVEIKK FLGHIKRPWG    IGVOFLOOFG    IMPLTGFILS      Rat    -MDNSSVGS- PNATFEGD SUVPESNEN ALLSTVRSTV    LTILLALVM SMCGNVEIKK FLGHIKRPWG    IFVGFLOOFG    IMPLTGFILS      Kabbit    -MDNSSVG- PNATFEGD SUVPESNEN ALLSTVRSTV    LTILLALVM SMCGNVEIKK FLGHIKRPWG    IFVGFLOOFG    IMPLTGFILS      Rabbit    MSNITVGEL - ANATVIEGD SUVPESNEN ALLSTVNSTV    LTILLALVMS SMCGNVEIKK FLGHIKRPWG    IFVGFLOOFG    IMPLTGFILS      Rat    -MEAHNAS   AFF NFSLPFOFGR    RATTALSVILSTV    LVMLLLILL    SLGTMEFSK    IKAHLWKPKG    LALVAQVG    IMPLTAFVLG      Nouse    -MEAHNAS   AFF NFSLPFOFGR    RATTALSVIL    LVMLLLIML    SLGTMEFSK    IKAHLWKPKG    VIALVAQVG    IMPLAFVLG      Nouse    -MEAHNAS   AFF NFSLPFOFGR    RATTALSVILVVILLIML SLGTMEFSK    IKAHLWKPKG    VIALVAQVG    IMPLAFVLG      Yersinia frederiksenii    -MULSKISS FIGKTFSLWA ALFAAAAFFA PDTFKWAGPY    IPWLLGIIMF GMGLTLKPSD FDILFKMEKG VIALVAQVG IMPLAAVLLS    IMPLAFVLG      Muman    105/6    132    144    VVVLIIGCOP GGTASNILAY WUDGMDLSV SMTTCSTLLA LGMMPLGLIV    YTKMWDSG- TIVIPYDSIG -ISLVALVIP VSIGMPVNHK    KALUVIV VVLIMGCOP GGTGSNILAY WUDGMDLSV SMTTCSTLLA LGMMPLGLIV YTKMWDSG- TIVIPYDSIG -I	Human    -MNDENSTV - DNATVEGG SUVPESNEN NILSVULSTV    LTILLALVMF SMGENVEIKK FLGHIKRPMG    IEVGFLOPG    IMPLTGFILS    VAFDILPLA      Rat    -MDNSSVGS - PNATFEGG SUVPESNEN ALLSTVMSTV    LTILLAMVMF    SMGENVEIKK FLGHIKRPMG    IFVGFLOPG    IMPLTGFILS    VASGILPVQA      Kabbit    -MDNSSVGS - PNATFEGG SUVPESNEN ALLSTVMSTV    LTILLAMVMF    SMGENVEIKK    FLGHIKRPMG    IFVGFLOPG    IMPLTGFILS    VASGILPVQA      Human    -MEAHNASAPF    FNILPPNFGK    RPTDALSVI    LVILLALVMS    SMGENVEKK    FLGHIKRPMG    IFVGFLOPG    IMPLTGFILS    VASGILPVQA      Nouse    -MEAHNASAPF    NFILPPNFGK    RPTDALSVI    LVFMLFFIML    SLGGTMEFSK    IKAHLMKPKG    LIALVAQYG    IMPLTAFVLG    KVFRLKNIEA      Nouse    -MEAHNASAPF    NFILPPNFGK    RPTDALSVI    LVMLLLIML    SLGGTMEFSK    IKAHLMKPKG    UNLIVALVAQYG    IMPLTAFVLG    KVFRLKNIEA      Yersinia    frederiksenii    -MEAHNASAPF    NFILPPNFGK    PDTFKWAGPY    IPWLLGIIMF    GMGLTLKPSK    IKAHLMKPKG    UINLVALVAQYG    IMPLTAFVLG    KVFRLKNIEA      Waman    105/6    132    144    IMVGDMDLSV    SMTTGSTLLA    IGMMPLCLF

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<sup>+</sup>-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

[<sup>3</sup>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<sub>11</sub>-biotin, dithiobis[succinimidylpropionate] (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  $\times$  10<sup>6</sup> 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

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