Sin3A-associated protein, 18 kDa, a novel binding partner of TRIB1, regulates MTTP expression

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Abstract  Mammalian tribbles homolog 1 (TRIB1) is a human locus that has been shown to significantly impact plasma lipid levels across several ethnic groups. In addition, the gene has been associated with the occurrence of nonalcoholic fatty liver disease. In the present study, a yeast-two-hybrid system was used to screen for novel molecular targets of TRIB1 binding. Loci corresponding to clones that were positive for TRIB1 binding subsequently were assessed for roles in lipid metabolism in mice using adenoviral constructs to induce knockdown or overexpression. Sin3A-associated protein, 18 kDa (SAP18) was identified as a novel binding partner of TRIB1. Knockdown of the Sap18 gene in mouse liver decreased plasma lipid levels and increased hepatic lipid levels; Sap18 overexpression showed the opposite effects. Transcriptome analysis of the mouse liver revealed that Sap18 knockdown decreased and Sap18 overexpression increased microsomal TG transfer protein (MTTP) expression levels. Chromatin immunoprecipitation analysis showed that halo-tagged Sap18, halo-tagged TRIB1, and anti-mSin3A antibody enriched precipitates for regulatory sequences of the MTTP gene. Enforced expression of Sap18 enhanced and Sap18 knockdown conversely attenuated the enrichment of MTTP regulatory sequences seen with anti-mSin3A antibody. These studies indicated that Sap18 expression enhanced the recruitment of mSin3A in coordination with TRIB1 to MTTP regulatory elements and increased MTTP expression. —Makishima, S., S. Boonvisut, Y. Ishizuka, K. Watanabe, K. Nakayama, and S. Iwamoto. Sin3A-associated protein, 18 kDa, a novel binding partner of TRIB1, regulates MTTP expression. J. Lipid Res. 2015. 56: 1145–1152.

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Mammalian tribbles homolog 1 (TRIB1) is a human locus that has been shown to exhibit convincing impact on cardiovascular diseases and plasma lipid concentrations across several ethnic groups. These genetic associations have been demonstrated both in genome-wide SNP association studies (1) and in subsequent replication studies (2). In addition, we recently showed the deep association of a TRIB1 regulatory SNP with nonalcoholic fatty liver disease (NAFLD) in individuals of Japanese ancestry (P = 9.39E-7) (3). Association of TRIB1 SNP with NAFLD was replicated in a Japanese population (4) and association with plasma alanine aminotransferase (ALT) was reported in a study of individuals of European ancestry (5), supporting the involvement of TRIB1 in the development of NAFLD. The risk allele of NAFLD decreased transactivation activity (3). Knockdown of the expression of the mouse homolog (Trib1) in mouse liver increased murine plasma and hepatic lipid levels, whereas Trib1 overexpression decreased these parameters (3). The enhanced lipogenesis in mouse liver was estimated to result from the reduced protein decay of carbohydrate response element binding protein [ChREBP (MLXIPL)], which is a master regulator for energy storage, coordinating the switch from carbohydrate to lipid via upregulation of glycolysis and lipogenic genes. While the ChREBP knockout mouse is resistant to dietary induced steatosis (6), ChREBP is not associated with NAFLD in human genetic studies, despite the deep genetic association with plasma TG levels (1). This observation suggested that an undefined pathway might be involved in the pathogenesis of NAFLD associated with TRIB1.

Abbreviations: ChREBP, carbohydrate response element binding protein; HA, hemagglutinin; MTTP, microsomal TG transfer protein; NAFLD, nonalcoholic fatty liver disease; NASH, nonalcoholic steato-hepatitis; Sap18, Sin3A-associated protein, 18 kDa; TRIB1, tribbles homolog 1.

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TRIB1 encodes a human homolog of the *Drosophila* tribbles protein (7). Proteins in the tribbles family include a pseudokinase domain, an E3 ubiquitin ligase (COP1)-binding domain, and a MEK1-binding domain, each of which are involved in the interaction with respective partners (3, 8–10). Depending on the partner, tribbles proteins degrade or support the target proteins. Thus, tribbles proteins are multifunctional proteins, whose functions await further characterization. In the present study, we screened for novel molecular targets of TRIB1 using a yeast-two-hybrid system. Genes corresponding to the positive clones subsequently were screened functionally in mice using adenoviral constructs to deliver shRNA-mediated knockdown or cDNA-mediated overexpression.

**MATERIALS AND METHODS**

**Target screening for TRIB1-interacting proteins**

A human hepatic cDNA library in pGADT7 was screened by a yeast-two-hybrid system (Matchmaker Gold; Clontech, Mountain View, CA) using human full-length TRIB1 (expressed via the pGBKT7 vector) as bait. A total of 10^7 clones were screened; positive clones were picked and extracted from Y2HGold cells. The cDNA inserts of the extracted plasmids were sequenced and characterized using the BLAST program. The repeatedly identified cDNA clones were sequenced and extracted from Y2HGold cells. The positive clones subsequently were screened functionally in mice using adenoviral constructs to deliver shRNA-mediated knockdown or cDNA-mediated overexpression.

**Assays of molecular interaction of TRIB1 with a novel target**

TRIB1-target protein interaction was assessed using HaloTag methodologies (Promega, Madison, WI). The open reading frames (ORFs) of TRIB1 and Sin3A-associated protein, 18 kDa (SAP18) were inserted into the pHNT vector or pCR3 vector with an influenza hemagglutinin (HA) tag for pull-down assays. To search for the interacting domain(s), TRIB1 cDNAs with deleted pseudocatalytic loop (ΔPCL; missing codon 204-210), COP1 (ΔCOP; missing codon 354-372), or MEK (ΔMEK; missing codon 331-372) binding domains in pCR3 vector were also prepared. Constructs were cotransfected into COS7 cells and then HaloTag fusion and associated proteins were extracted from cell lysates using HaloLink resin. Expression of halo-tagged SAP18 and TRIB1 were validated by anti-SAP18 (sc-8473; Santa Cruz, Dallas, TX) and anti-TRIB1 [Abgent (AP7726b; against C-terminal domain) and Abcam (ab89021; against internal peptide 216-265)] antibodies. Molecular interactions were compared by Western blotting, with anti-HA (Sigma-Aldrich) antibodies to the empty pHNT vector as the negative control. For colocalization assays, a TRIB1 cDNA was inserted into the pEGFP vector (Promega) and cotransfected into COS7 cells with pHNT-SAP18 or pHNT-empty vector. Transfected cells were stained 24 h later with HaloTag-TMR ligand followed by DAPI staining and visualized under fluorescence microscope and the associated software (KEYENCE Japan).

**Animal experiments**

The shRNA template against *Sap18* (pAx-shSap18; Table 1) or the nonspecific scramble sequence (pAx-shScrbl) was inserted in pAxcevit (Takara Bio Inc., Otsu, Japan) under control of the U6 promoter. The HA-tagged SAP18 cDNA was inserted in pAXCALNLW2 (Takara). An adenovirus encoding LacZ (pAXCALNLZ2) and the pAx-shScrbl construct were used as control vectors. Purified adenovirus vectors were titrated, and adenoviruses were injected into the tail veins of mice at 2.0 × 10^9 pfu per animal. Adenovirus vectors pAXCALNLW2-SAP18 and pAXCALNLZ2 were co-injected with pAXCALNCre to induce the expression of the cDNA insert. Male 12-week-old C57BL/6 mice (CLEA Japan Inc., Tokyo, Japan) were housed in an air-conditioned environment with a 12 h light-dark cycle. Blood samples were collected from the tail vein during the morning under conditions of food intake ad libitum or after 12 h of starvation. Plasma TG and total cholesterol concentrations were measured using Fuji Dry Chem system (Fujifilm, Kanagawa, Japan). TG and

| Forward Primer | Reverse Primer |
|----------------|---------------|
| **SAP18**      |               |
| **MTTP**       |               |
| **P4HB (PDI)** |               |
| **APOB**       |               |
| **ChIP**       |               |
| -27 kb RXRA    |               |
| -12 kb CEBPB   |               |
| -12 kb Sin3A   |               |
| -2 kb HELT     |               |
| -62 kb Sin3A   |               |
| +40 kb PU.1    |               |
| +59 kb CEBPB   |               |
| -80 kb MAX     |               |
| **SAP18 shRNA template** |         |
| Top sequence   |               |
| Bottom sequence|               |
| **Scrbl shRNA template** |         |
| Top sequence   |               |
| Bottom sequence|               |

**TABLE 1. Oligonucleotide sequences**

ChIP, chromatin immunoprecipitation.
choline levels of hepatic lipid droplets, which reflected the influence of SAP18 expression levels on lipid metabolism in vivo. The expression levels in mouse liver were modulated using a lentivirus gene transfer system. Administration of pAx-shSAP18 reduced the SAP18 mRNA by 72% and the protein by 71% (supplementary Fig. 1). Conversely, the overexpression vector increased SAP18 mRNA by 205% and the protein by 148% (supplementary Fig. 1). X-Gal staining of mouse organs injected with pAx-CALNLZ2 validated that the adenovirus vector system yielded nearly exclusive hepatic induction (supplementary Fig. 2). Plasma total cholesterol and TG on day 8 after vector injection were decreased by knockdown of SAP18, whereas SAP18 overexpression increased these parameters (Fig. 2A). Those parameters on day 4 showed similar trends but failed to achieve statistical significance for the difference of TG levels (supplementary Fig. 3). Lipoprotein profiling showed that the respective changes in cholesterol and TG were mainly observed in LDL and HDL fractions (Fig. 2B). The knockdown of SAP18 led to increased size of the hepatic lipid droplets, which reflected the 5.2-fold increase of TG in the extracts (Fig. 2C, D). In contrast, TRIB1 overexpression yielded a decrease in the number and the area of hepatic lipid droplets, although an associated nominal decrease in the level of extracted TG was not statistically significant (Fig. 2C, D).

SAP18 expression levels influenced MTTP expression levels

To explore the molecular mechanism of the changes in lipid metabolism observed in mice, we performed transcript profiling of the livers of adenovirus vector-injected mice. Although 25 gene sets were affected by SAP18 expression levels, gene sets in pathways for lipid metabolism were not enriched or depleted (supplementary Table 1). Among the genes exhibiting greater than 2-fold changes.

RESULTS

SAP18 is a novel TRIB1-interacting protein

To explore the signaling pathway of TRIB1, we performed a yeast-two-hybrid screen of a human hepatic liver cDNA library using the full-length human TRIB1 cDNA as the bait. Sequence analysis of 117 positive clones in initial screening revealed that three clones encoded full-length SAP18. Interaction of TRIB1 with SAP18 in yeast cells was confirmed by activation of six selection marker genes (QDO/X/A) in Y2HGold cells cotransfected with the cDNAs (Fig. 1A). We next examined the interaction in mammalian cells using a pull-down assay in COS7 cells. Halo-TRIB1 coprecipitated HA-tagged SAP18 protein using halo-ligand-coated resin (Fig. 1B). Molecular binding of TRIB1 to SAP18 was confirmed by pulling-down of TRIB1 with halo-SAP18 (Fig. 1C). Furthermore, deletion of the downstream portion of the plasmid-borne TRIB1 gene diminished this interaction (Fig. 1C), suggesting that the COP-binding domain of TRIB1 is crucial for binding to SAP18. In contrast, a lack of PCL enhanced or stabilized the molecular interaction. A separate experiment using fluorescence microscopy demonstrated EGFP-labeled TRIB1 and halo-SAP18 colocalized in the nucleus (Fig. 1D).

SAP18 interacts with TRIB1 and regulates MTTP expression

To explore the molecular mechanism of the changes in lipid metabolism observed in mice, we performed transcript profiling of the livers of adenovirus vector-injected mice. Although 25 gene sets were affected by SAP18 expression levels, gene sets in pathways for lipid metabolism were not enriched or depleted (supplementary Table 1). Among the genes exhibiting greater than 2-fold changes.

Expression profiling

Hepatic total RNA was extracted immediately after euthanization. Hepatic RNAs of the mice injected with adenovirus vectors were measured by Affymetrix Mouse 430_2 oligonucleotide microarray chip and analyzed using Affymetrix Expression and Transcriptome Console software. Pathways that were significantly enhanced or reduced by altered SAP18 expression were searched using Gene Set Enrichment Analysis (GSEA) software provided by the Broad Institute and the Kyoto Encyclopedia of Genes and Genomes (KEGG) database. The accuracy of the expression levels of individual genes in the microarray were confirmed by quantitative RT-PCR using the primers listed in Table 1. Alternative exon usage was assessed by hybridization to Mouse Exon 1.0 ST oligonucleotide microarray chip (Affymetrix) and analysis on Exon Array Analyzer web-based software (12). The expression level of the candidate genes suspected to be involved in lipid metabolism were further assessed in HepG2 cells transformed by lentiviral vectors (System Biosciences, Mountain View, CA) pSIF-H1-Puro-shSAP18 or pCDF-MCS1-EF1-Puro-SAP18 (for knockdown or overexpression, respectively). HepG2 cells transformed with pSIF-H1-Puro-empty vector were used for negative control.

Chromatin immunoprecipitation analysis

Sheared chromatin of the HepG2 cells transfected with pHTN-SAP18 or pHTN-TRIB1 was precipitated using the HaloCHIP system (Promega). Chromatin of the cell lines transfected with pHTN-empty vector was used for control experiment. Chromatin of lentivirus vector-transformed HepG2 cells also was precipitated using anti-mSin3A antibody (Santa Cruz, Dallas, TX) and chromatin immunoprecipitation (ChIP)-IT Express kit (Active Motif, Carlsbad, CA). Nonspecific rabbit IgG was used for negative control. After referring to the ChIP-seq database in ENCODE for MTTP- and proximal sequences of individual genes in the microarray were confirmed by quantitative RT-PCR using the primers listed in Table 1. Alternately, the expression levels on the SAP18-mSin3A interaction was analyzed by Western Blotting.

Statistical analysis

The experimental studies were at least triplicated and the data were evaluated by Student’s t-test. Statistical analysis was performed using SPSS (SPSS Japan, Tokyo) statistical packages. Data are shown as mean ± SD. Statistical significance was set at the P < 0.05 level.
in expression upon SAP18 overexpression, 1302 and 189 loci exhibited significant ($P < 0.05$) increases or decreases (respectively) in expression. Among the genes exhibiting greater than 2-fold changes upon knockdown of SAP18 expression, 203 and 422 loci exhibited significant ($P < 0.05$) increases or decreases (respectively) in expression. Comparison among these classes revealed 74 genes for which expression correlated (directly or inversely) with SAP18 expression level under the various conditions; these loci are listed in supplementary Table 2. One of these 74 genes, MTTP, was notable; expression of MTTP and SAP18 were correlated in knockdown and overexpression mice (Fig. 3A), and the phenotype of our SAP18 knockdown mice (disturbed hepatic lipid secretion) matched the reported phenotype for MTTP mutant animals (14). In contrast, changes in SAP18 expression did not consistently influence transcript levels for any other loci implicated in lipogenesis, lipolysis, and VLDL assembly (e.g., ACC1, FASN, ACOXI, DGAT, MOGAT, P4HB, APOB, and APOC3). Mouse exon array analysis did not reveal apparent alternative

**MTTP exon use in mouse liver in response to changes in SAP18 expression** (data not shown). Correlation of MTTP mRNA level and negative correlation of P4HB and APOB with that of SAP18 were replicated in HepG2 cells transfected with lentiviral vectors (Fig. 3B).

### SAP18 modulated the interaction of mSin3A with MTTP regulatory sequences

SAP18 was originally identified as a subunit of a transcriptional co-repressor (the Sin3A-HDAC complex). We therefore assessed the binding of SAP18 to the regulatory sequences of the MTTP gene. This assay was performed using ChIP analysis in HepG2 cells. Halo-tagged SAP18 enriched for three regulatory sequences from the regions upstream of MTTP exons 1 and 2 (Fig. 4B). These domains have been shown in the ENCODE database to be enriched by ChIP using anti-CEBPB or anti-mSin3A. The binding partner of SAP18, TRIB1 (halo-tagged), precipitated two of the three fragments that were enriched by SAP18 (Fig. 4B). We next investigated whether SAP18
expression level influenced mSin3A binding to MTTP regulatory sequences, using ChIP analysis in HepG2 cells that had been transformed by lentiviral vectors. Anti-mSin3A antibody enriched the same three DNA fragments that had been enriched by precipitation with halo-tagged SAP18 in HepG2 cells. Enforced expression of untagged SAP18 enhanced the enrichment by anti-Sin3A antibodies; conversely, knockdown of SAP18 diminished this enrichment (Fig. 4C). Additionally, halo-tagged SAP18 pulled down HepG2-intrinsic mSin3A, and the SAP18-mSin3A interaction was attenuated by knockdown of TRIB1 (Fig. 4D). Furthermore, enforced expression of TRIB1 yielded increased accumulation of MTTP mRNA, while TRIB1 knockdown led to decreased MTTP mRNA levels (Fig. 4E). These results indicated that SAP18 binds to the regulatory sequences of MTTP and enhances the binding of mSin3A to the MTTP promoter in coordination with TRIB1, and that TRIB1 is required for the molecular interaction between SAP18 and mSin3A.

**DISCUSSION**

In this study, we demonstrated that the SAP18 protein constitutes a novel binding partner of TRIB1. SAP18 expression levels in mouse liver correlate with plasma and hepatic lipid levels, an effect that we demonstrate to result from modulation of the expression of MTTP. SAP18 bound to the regulatory sequences of the MTTP gene with mSin3A. These studies indicate that SAP18 expression enhances the recruitment of mSin3A to MTTP regulatory elements, thereby yielding increased MTTP expression.
The interaction of SAP18 and TRIB1 is newly identified in this study. SAP18 was initially identified as a component of human Sin3-HDAC complex (15). Following studies revealed a ubiquitin-like domain of SAP18 that has been proposed to be involved in formation of a splicing regulatory multiprotein complex (16), suggesting that SAP18 associates with multiple binding partners in the nucleus. Expression of SAP18 is required for responses to environmental stress in Drosophila (17) and Arabidopsis (18). In human cells, expression of TRIB1 also is induced by stress signals, and TRIB1 has been shown to regulate signal transduction through the MAP kinase pathway (19). The present studies suggest a feasible pathway for the regulatory interaction between SAP18 and TRIB1.

Our work also provides a novel discovery showing that serum and lipid levels in the mouse are modulated by SAP18 expression. Numerous reports have implicated HDAC in hepatic lipogenesis (20) and steatosis (21); thus, our work suggests that mSin3A and SAP18 are potential regulators of lipid metabolism. However, expression of most known lipogenic and lipolytic genes was not affected by changes in SAP18 expression. Nonetheless, increased hepatic lipid levels and decreased plasma lipid levels were observed upon liver-specific knockdown of SAP18; converse changes in lipid levels were observed upon liver-specific overexpression of SAP18. These data suggested that SAP18 expression modulates VLDL secretion from hepatocytes. The Mttp, P4hb (also known as protein disulfide isomerase), Apob gene products and TG are the critical factor for VLDL assembly in the endoplasmic reticulum of hepatocytes (14, 22), among which only the expression level of Mttp was affected by modulation of SAP18 expression. Individuals who are homozygous for loss of function mutation in Mttp develop severely low triglyceridemia and are prone to steatosis (22). Furthermore, the Mttp inhibitor, lomitapide, effectively reduces plasma lipid levels of familial homozygous hypercholesterolemia and severe hypertriglyceridemia, but causes steatosis as an adverse effect (23, 24), supporting the speculation that Mttp expression levels mediate the modulation of plasma and hepatic lipid levels by changes in SAP18 expression. SAP18 could be a novel pharmacological target in the management of dyslipidemia, steatosis, and the adverse effect of lomitapide.

In other work, SAP18 also has been shown to be involved in splicing regulation by serving as a component of the exon-junction complex (16, 25). However, alternative exon use within Mttp was not observed in the liver transcript profile in mice with altered SAP18 expression. Therefore, the change of the expression levels of Mttp in response to SAP18 expression was hypothesized to reflect regulation at the level of Mttp transcription. ChIP studies indicated the direct binding of SAP18 and TRIB1 at the regulatory sequences of Mttp, and SAP18 overexpression enhanced the binding of mSin3A to the same regulatory sequences. These results indicate that SAP18 upregulates Mttp expression by enhancing the binding of mSin3A to these regulatory sequences. Sin3A is usually regarded as transcriptional repressor, but the protein has been shown to activate the transcription of several genes, e.g., Nanog (26) and Hap (27). Mttp could be one of those exceptional targets. TRIB1 was required for the molecular interaction between SAP18 and mSin3A. TRIB1 may provide a scaffold for stabilizing the SAP18-mSin3A interaction and may promote Mttp expression.

NAFLD is the most frequent type of chronic liver disease in developed countries. The disease spectrum varies from simple steatosis to nonalcoholic steatohepatitis. The pathology of hepatic lipid accumulation reflects an imbalance between storage and disposal of TGs in hepatocytes.

**Fig. 3.** SAP18 expression levels affect hepatic transcript levels. A: Volcano plots of the comparison in transcriptome analysis of adenoviral vector-injected mice liver between shSap18 versus shScrbl, shScrbl versus LacZ+Cre, and LacZ+Cre versus SAP18+Cre (n = 4 per group). The fold change (horizontal) and significance (vertical) levels of Mttp, Sap18, P4hb, and Apob expression are shown in each comparison. Dark dots in the volcano plots indicate the genes that exhibited significant (P < 0.05) changes in expression (by factors of 2) in each comparison. B: SAP18, Mttp, P4hb, and Apob expression levels in HepG2 cells transformed with lentiviral vectors. Relative expression levels against internal control 36B4 in three independent transformed cell lines normalized to the mean value of pSIFempty (control) are shown (mean ± SD). pSIF-SAP18, pSIF-H1-Puro-shSAP18; pSIFempty, pSIF-H1-Puro-empty; pCDF-SAP18, pCDF-MCS1-EF1-Puro-SAP18. *P < 0.05, †P < 0.01 (Student’s t-test).
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Reduced VLDL production, discrepancy with our studies about hepatic lipid levels may be based on the difference of expression period, level, and organ specificity throughout the life span and systemic null (29) vs. adult liver specific and 20–40% reduction (3). Further studies are required to clarify the mechanisms for the involvement of TRIB1 in NAFLD.

In conclusion, our study revealed a novel molecular target of TRIB1 that is involved in lipid metabolism. Therapies that target the accumulation and/or activity of TRIB1 or SAP18 are candidates for the prevention and treatment of steatosis.

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