Characterization of the CLASP2 Protein Interaction Network Identifies SOGA1 as a Microtubule-Associated Protein*§

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CLASP2 is a microtubule-associated protein that undergoes insulin-stimulated phosphorylation and co-localization with reorganized actin and GLUT4 at the plasma membrane. To gain insight to the role of CLASP2 in this system, we developed and successfully executed a streamlined interactome approach and built a CLASP2 protein network in 3T3-L1 adipocytes. Using two different commercially available antibodies for CLASP2 and an antibody for epitope-tagged, overexpressed CLASP2, we performed multiple affinity purification coupled with mass spectrometry (AP-MS) experiments in combination with label-free quantitative proteomics and analyzed the data with the bioinformatics tool Significance Analysis of Interactome (SAINT). We discovered that CLASP2 coimmunoprecipitates (co-IPs) the novel protein SOGA1, the microtubule-associated protein kinase MARK2, and the microtubule/actin-regulating protein G2L1. The GTPase-activating proteins AGAP1 and AGAP3 were also enriched in the CLASP2 interactome, although subsequent AGAP3 and CLIP2 interactome analysis suggests a preference of AGAP3 for CLIP2. Follow-up MARK2 interactome analysis confirmed reciprocal co-IP of CLASP2 and revealed MARK2 can co-IP SOGA1, glycogen synthase, and glycogenin. Investigating the SOGA1 interactome confirmed SOGA1 can reciprocal co-IP both CLASP2 and MARK2 as well as glycogen synthase and glycogenin. SOGA1 was confirmed to colocalize with CLASP2 and with tubulin, which identifies SOGA1 as a new microtubule-associated protein. These results introduce the metabolic function of these proposed novel protein networks and their relationship with microtubules as new fields of cytoskeleton-associated protein biology.

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Microtubules are versatile cytoskeletal structures known to serve the needs of the particular subcellular context they are situated in. Whether they act as a cellular highway tasked with the trafficking of molecular cargo to specific destinations or function to support the structure of the cell, proper microtubule dynamics are essential for normal cell function. The insulin-stimulated glucose uptake system has distinct effects on the cytoskeleton. Insulin mediates acute glucose uptake in part by mobilizing insulin-stimulated glucose transporter 4 (GLUT4)¹ storage vesicles (GSVs) from intracellular pools to the plasma membrane. Insulin stimulates actin to reassemble into filamentous cortical projections, resulting in the physical effect of ruffling the plasma membrane. Insulin signaling proteins such as WASP, Arp3, PI 3-K, Akt, GLUT4, and additional proteins all colocalize with reorganized actin at the membrane ruffle (1–3). Inhibition of actin reorganization with the drugs

¹ The abbreviations used are: GLUT4, glucose transporter 4; AP-MS, affinity purification coupled with mass spectrometry; AGAP1, Arf-GAP with GTPase; ANK, repeat and PH domain-containing protein 1; AGAP3, Arf-GAP with GTPase; ANK, repeat and PH domain-containing protein 3; BLAST, Basic Local Alignment Search Tool; CLASP, CLIP-associating protein 1; CLASP2, CLIP-associating protein 2; CLIP1/CLIP-170, CAP-Gly domain-containing linker protein 1; CLIP2/CLIP-115, CAP-Gly domain-containing linker protein 2; co-IP(s), co-immunoprecipitation(s); FA, formic acid; GAP, GTPase-activating protein; GAS2L1/G2L1, GAS2 like protein 1; GFP, green fluorescent protein; GLYG, glycogenin; GYS1, glycogen synthase; GSV(s), GLUT4 storage vesicle(s); HA, hemagglutinin; ID, identification; INS, insulin; IP(s), immunoprecipitation(s); MARK2, microtubule affinity-regulating kinase 2; MARK3, microtubule affinity-regulating kinase 3; MK, protein ladder marker; MTOC, microtubule organization center; NlgG, nonimmune serum; PI 3-K, phosphoinositide 3-kinase; P-Score, probability score; SAINT, Significance Analysis of Interactome; SD, standard deviation; SOGA1, suppressor of glucose by autophagy; SCP, Spectrum Count Profile; TIRFM, total internal reflection fluorescence microscopy; TGN, trans-Golgi network; TUG/ASPC1, Tether containing UBX domain for GLUT4; WCL, whole cell lysate; YASARA, Yet Another Scientific Artificial Reality Application.
The adipoeyte has the distinct property of storing large amounts of lipid, secretion of several hormones, and in addition, serves as a secondary site for insulin-stimulated glucose uptake and storage (57). Knockdown of CLASP2 protein expression in 3T3-L1 adipocytes inhibited insulin-stimulated glucose transport (16). Here we report label-free quantification of interactome experiments that have been processed with the Significance Analysis of Interactome (SAINT) scoring system (58–61). By performing a series of strategic, successive and confirmatory reciprocal interactome experiments on key novel proteins, we have constructed a 3T3-L1 adipocyte CLASP2 protein network that has led to the identification of suppressor of glucose by autophagy 1 (SOGA1) as a microtubule-associated protein.

EXPERIMENTAL PROCEDURES

Cell Culture, Immunoprecipitation, and Western Blot Analysis—3T3-L1 fibroblasts were cultured in growth media of DMEM (Thermo Fisher Scientific, Waltham, MA, cat. # SH30243.01) with 10% newborn calf serum (Thermo Fisher Scientific, cat. # 16010), 1% penicillin-streptomycin (Thermo Fisher Scientific, cat. # 15140), and 1% glutamox (Thermo Fisher Scientific, cat. # 35050) until confluence. After changing to fresh growth media and culturing for 48 h, differentiation day one was initiated by changing to differentiation media: DMEM with 10% fetal bovine serum (Thermo Fisher Scientific, cat. # 16010), 1% penicillin-streptomycin, and 1% glutamox supplemented with 10.0 μg/ml insulin (Sigma-Aldrich, cat. # I-2643), 0.5 mM IBMX (Sigma-Aldrich, cat. # I-5879), and 1.0 μM dexamethasone (Sigma-Aldrich, cat. # D-1756). After 48 h, differentiation media supplemented with only 10.0 μg/ml insulin was added. After 48 h of culturing in this media, fresh differentiation media with no supplements was added every 24–48 h until lysis, which was performed on day 7 or 8 post-differentiation. For cell treatment experiments, cells were starved for 4 h and then either left untreated or stimulated with 100 μM insulin for 15 min at 37 °C. Cells were lysed with 500 μl of lysis buffer containing 40 mM HEPES (pH 7.6), 120 mM NaCl, 0.3% CHAPS, 10 mM NaF, 10 mM β-glycerophosphate, 1 mM EDTA (pH 8.0), 2 mM sodium orthovanadate, 17 μg/ml aprotinin, 10 μg/ml leupeptin, and 1 mM PMSF. Cell lysates were rotated at 4 °C for 20 min followed by centrifugation (14,000 RPM, 4 °C, 20 min), and the clarified supernatants were used for immunoprecipitation (IP). For each IP, cell lysate from 1 × 150 mm tissue culture dish (3.5–5 mg) was incubated with 5 μg of specific antibodies conjugated to 25 μl protein A or protein G-agarose beads for 3 h at 4 °C with gentle rotation. IPs were washed three times with 1 ml of ice-cold PBS and the proteins bound to beads were eluted by heating at 95 °C for 4 min in 8 μl SDS sample loading buffer (4% SDS, 0.0625% Tris-HCl, 10% glycerol, 0.02% bromphenol blue, 8 μl Urea). The eluate was extracted and the antibody/beads were subjected to a second elution at 95 °C for 4 min in 8 μl SDS sample loading buffer. The second eluate was extracted and the two eluates were combined and separated by 10% SDS-PAGE and the gels were either stained with BioSafe Coomassie G-250 Stain (Bio-Rad, Hercules, CA) or transferred to a nitrocellulose membrane for subsequent Western blotting. For Western blots, proteins were transferred to a nitrocellulose membrane for 1 h at 105 V and blocked with 5% nonfat dry milk in Tris-buffered saline with 0.2% Tween-20 for 1 h at room temperature. Membranes were probed with primary antibodies for 1 h in blocking buffer at room temperature. Blots were washed in Tris-buffered saline with 0.2% Tween-20 (three times for 10 min), and probed with either goat anti-mouse (Santa Cruz Technologies, Santa Cruz, CA) or donkey anti-rabbit (GE Healthcare, Waukesha, WI)
secondary antibodies (both at dilutions of 1:1500) conjugated to horseradish peroxidase and detected with Western Lightning Plus-ECL Enhanced Chemiluminescence Substrate kit (Perkin Elmer, Wal-tham, Massachusetts). Primary antibodies used: anti-CLASP2 “Antibody #1” (the immunogen recognized by this antibody maps to a region between residue 275 and 325 of human CLASP2 using the numbering given in entry BAG11221.1 (GenetID 23122), cat. # 21395, Novus Biologicals, Littleton, CO), anti-CLASP2 “Antibody #2” (immu-nogen not supplied by manufacturer, cat. # ABT263, Millipore, Bil-lerica, MA), anti-CLIP2 (cat. # SABI412760, Sigma-Aldrich), anti-MARK2 (cat. # NBPI–71890, Novus Biologicals), anti-SOGA1 (cat. # SAB3500961, Sigma-Aldrich), anti-AGAP3 (cat. # SAB2700915, Sigma-Aldrich), anti-HA (cat. # 901501, Biologend, San Diego, CA), anti-mCherry (cat. # NB2P–43720, Novus Biologicals), anti-tubulin (cat. # T9026, Sigma-Aldrich and cat. # ab18251, abcam, Cambridge, MA).

In-gel Digestion—Proteins were separated by SDS-PAGE and stained with Bio-Safe Coomassie G-250 Stain. For the interac-tome experiments, each lane of the SDS-PAGE gel was cut into either seven or eight slices (the number of slices was consistent for each specific protein’s interactome), placed in a 0.6 ml LoBind polypyrrole tube (Eppendorf, Hauppauge, NY), destained twice with 375 μl of 50% acetonitrile (ACN) in 40 mm NH4HCO3 and dehydrated with 100% ACN for 15 min. After removal of the ACN by aspiration, the gel pieces were dried in a vacuum centrifuge at 60 °C for 30 min. Trypsin (250 ng; Sigma-Aldrich) in 20 μl of 40 mm NH4HCO3 was added, and the samples were maintained at 4 °C for 15 min prior to the addition of the samples was 0.19%, which is representative of typical rates calcu-lated throughout the remainder of the interac-tome experiments. Probability assessment of peptide assignments and protein identifi-cations were made using Scaffold. Only peptides with ≥ 95% probability were considered. The mass spectrometry proteomics data have been deposited to the ProteomeXchange Consortium (http://protemecentral.proteomexchange.org) via the PRIDE part-ner repository (63) with the data set identifier PXD003674.

Cloning and Subcloning Mouse CLASP2—Total RNA was isolated from the homogenized brain of a C57BL/6J mouse according to the manufacturer’s protocol (RNeasy Mini Kit, cat. # 74104, Qiagen). RT-PCR was utilized to convert mRNA into single-stranded cDNA following the manufacturer’s protocol (LongRange 2Step RT-PCR Kit, cat. # 205922, Qiagen). The total cDNA was used to amplify cDNA with specific primers by PCR and then amplified cDNA was inserted into a vector. For cloning of mouse CLASP2 cDNA, the sense primer was 5'-gggccaggtagcaattgaggctcttcttgggtgt3' and antisense primer 5'–gcaccagctggctcctccagaggtccgq3'. Sequencing results re-veal that the cloned CLASP2 cDNA has the same sequence as NM_001081960.1. This cDNA was subcloned and inserted into the pCMV6–AN-HA plasmid (Origene, Rockville, MD, cat. # PS100013) to generate the construct pCMV-HA-CLASP2. The construct was veri-fied by restriction digestion and DNA sequencing.

Generation of Adenoviruses and Adenoviral Infection of 3T3-L1 Adipocytes—Negative control null adenovirus and GFP/HA-tagged mouse CLASP2 adenovirus (using the aforementioned pCMV-HA- CLASP2 vector to generate N-terminal GFP and C-terminal HA-tagged CLASP2) were created by VECTOR BIOLABS (Malvern, PA). Negative control null adenovirus and mCherry-tagged mouse MARK2 adenovirus (mouse MARK2 cDNA, cat. # MC024604, was purchased from Origene) were also created by VECTOR BIOLABS. Virus infec-tions were performed on 150 mm plates of 3T3-L1 adipocytes after 7–8 days of differentiation as described above. After one PBS wash, 10 ml of serum-free DMEM containing 8.5 × 106 PFU and virus of 5.0 μg/ml were added to each plate followed by incubation at 37 °C for 4 h. Fifteen milliliters of serum-containing DMEM was then added to each plate followed by incubation at 37 °C for 48 h. Cells were then serum-starved for 4 h followed by insulin treatment and cell lysis as described above.

Immunofluorescence and Confocal Microscopy—3T3-L1 adipocytes grown on #1.5H coverslips (Zeiss, Thornwood, NY, cat. # 474030-9000-000) were fixed in methanol at −20 °C for 20 min, permeabilized at room temperature with 1% Tween-20/2% paraformaldehyde solution for 20 min, quenched at room temperature with 0.1 M glycine for 20 min, blocked at room temperature with 1% BSA for 2 × 15 min (IgG-free, protease-free, Jackson ImmunoResearch cat. # 001-000-161), and then either left untreated or incubated with primary antibodies overnight at 4 °C with gentle rocking. After 3 × 10 min 1% BSA washes at room temperature, the samples were incubated with either Alexa Fluor 405 Goat anti-mouse (Thermo Fisher cat. # A31553) or Alexa Fluor 647 Goat anti-rabbit (Thermo Fisher cat. # A21245) for 1 h at room temperature with gentle rocking followed by 2 × 10 min
available CLASP2 antibodies to independently IP endogenous prey proteins, rendering the final interactome sample full of getting regions of the antibody are eluted together with the globally bound to the Sepharose resin, Protein A/G, and nontargeting proteins (64). To reconstruct regions of SOGA1 nonhomologous to 4RH7, BLAST was used to retrieve homologous sequences, create a multiple sequence alignment, and enter the sequences into a “discrimination of secondary structure class” prediction algorithm. The side chain loops were then optimized and added. The resulting structure was subjected to a combined steepest descent algorithm and refined with simulated annealing minimizations within surrounding water.

**Experimental Design and Statistical Rationale**—Using the mean and standard deviations for the spectrum counts of the 39 proteins identified by SAINT analysis as significantly enriched between the CLASP2 Antibody #1 IPs (Group 1, 343 ± 280) and the NlgG IPs (Group 2, 26 ± 21), the effect size is equal to 1.60. With this robust effect size, a sample size of four biological replicates per group provided more than sufficient power to detect differences between groups. Based on the high degree of reproducibility, we also used a sample size of two and three biological replicates. Each IP had a respective NlgG or appropriate tag antibody negative control IP, two to four biological replicates each, and no process and technical replicates because of the strong reproducibility of the data. The varying statistical analyses for the different experiments are listed in the Figure Legends and were justifiable based upon the data and basic experiment performed. For spectral counting measurements, modified peptides, semitryptic peptides, and shared peptides were included.

**RESULTS**

The CLASP2 Interactomes—We devised a streamlined, label-free proteomic technique to identify and quantify protein interaction partners and applied this system to discover proteins that may be linked to regulation of the microtubule-associated protein CLASP2. This method combined cell lysis with a CHAPS detergent-containing buffer together with IP, SDS-PAGE-based fractionation, subsequent in-gel tryptic digestion and bottom-up mass spectrometry analysis (Fig. 1A). This approach possesses the caveat that proteins nonspecifically bound to the Sepharose resin, Protein A/G, and nontargeting regions of the antibody are eluted together with the prey proteins, rendering the final interactome sample full of contaminating proteins which need systematic elimination (65, 66). To avoid artifacts resulting from overexpression of epitope-tagged proteins, we used two different commercially available CLASP2 antibodies to independently IP endogenous CLASP2. On the other hand, because antibodies targeted to endogenous protein can disrupt protein-protein interactions and possess nonspecific cross-reactivity (66, 67), we also adeno-virally overexpressed HA-epitope and GFP-tagged CLASP2 to create an anti-HA antibody GFP-CLASP2-HA interactome. These three independent CLASP2 interactome act as cross references, to narrow down the list of potential CLASP2 interaction partners for follow-up confirmation experiments. Western blot of these CLASP2 protein IPs are shown in Figs. 1B, 1C, and 1D. For a detailed breakdown of the proteome numbers associated with the experimental approach adopted for these interactome studies, using the CLASP2 Antibody #1 experiment numbers as an example, please refer to supplemental Fig. S1 and the accompanying text. For all interactome experiments presented in this study, each gel slice’s protein yield, total number of peptides identified, total number of spectra, and percent identification rate are included in supplemental Tables (CLASP2 Antibody #1 data are in supplemental Tables S1, S2, S3, and S4, GFP-CLASP2-HA data are in supplemental Tables S6, S7, S8, and S9, CLASP2 Antibody #2 data are in supplemental Tables S11, S12, S13, and S14). These numbers are provided throughout the remainder of this report for all interactomes performed. CLIP1 and CLIP2 are the original CLASP associated proteins (17), so we chose to use CLIP2 as a high-confidence CLASP2 interacting partner to test the validity of the current experimental conditions and approach, using the CLASP2 Antibody #1 interactome data as an example. Western blot analysis of CLIP2 in the CLASP2 Antibody #1 basal and insulin IPs showed strong enrichment of CLIP2 in the CLASP2 Antibody #1 IPs, with no effect of insulin on the interaction (supplemental Fig. S2A), and densitometry results from co-IP/Western blot experiments are shown in supplemental Fig. S2B. The spectral count data (supplemental Fig. S2C) is very similar to the results from the co-IP/Western blot experiments, in that CLIP2 shows a strong enrichment in the CLASP2 Antibody #1 basal and insulin IPs over the NlgG negative controls and no effect of insulin on the interaction. CLIP2 averaged 134 spectral counts versus only 13 for CLIP1 (CLIP1 lacked an effect of insulin as well). The strong co-IP of CLIP2 with CLASP2 supports the validity of the experimental conditions used.

We used the established bioinformatic tool Significance Analysis of Interactome (SAINT) (58–61) to analyze our interactome data for protein enrichment. SAINT assigns each protein a SAINT Probability Score (“P-Score”), on a scale of 0 to 1, with 1 being the top score. Based upon review of the literature, we chose 0.65 as a SAINT cut-off score for enrichment (68–73). With the approach used here, all proteins have two P-Scores, one each for basal or insulin enrichment over the respective negative controls. A detailed breakdown of SAINT results typical for our interactome approach are outlined in supplemental Fig. S3, again using CLASP2 Antibody #1 interactome data as an example. Protein SAINT scores and spectrum counts for all interactomes performed in this study are included in Supplemental Tables (CLASP2 Antibody #1 in
We sought to analyze the raw spectral count data of all proteins with SAINT scores greater than a 0.65 in both the basal and insulin samples (which we refer to as “SAINT-qualified”) in a hierarchical manner, with spectral count results from all experiments individually plotted. We therefore devised the Spectrum Count Profile (SCP), an easily readable format for the visualization of whole sets of raw spectral count data for interactome experiments, and applied it to the CLASP2 Antibody #1 interactome data (Fig. 2A). Using the same interactome processing approach, we generated SCPs for the two remaining CLASP2 interactomes we had prepared as cross references, the adenovirally overexpressed HA-epitope and GFP-tagged CLASP2 anti-HA IP (Fig. 2B) and the second, alternative CLASP2 Antibody #2 IP (Fig. 2C). We first searched the three SAINT-qualified CLASP2 interactomes for proteins previously linked to CLASP2 (for reviews on CLASP2 and members of the plus-end tracking microtubule-associated protein family, please refer to (49, 51–54, 74)) and found MARE1/EB1, SLAIN2, MARK2, MARK3, CLIP1, CKAP5/chTOG, PHLDB1/LL5α, MACF1, GCC2, CLASP1, CLIP1, and CLIP2. The CLASP2 Antibody #1 interactome had more

Fig. 1. The CLASP2 immunoprecipitations. A, The focus of this project was to identify new interacting partners for CLASP2 in 3T3-L1 adipocytes. The approach compared different protein IPs (for this figure, CLASP2) against negative control IPs (for this figure, Nonimmune serum “NIgG”), either in the absence or presence of insulin treatment. The IPs were separated by SDS-PAGE, fractionated into gel slices, subjected to trypsin digestion, and analyzed by tandem mass spectrometry. Peptide and protein identification was performed by database searching with Mascot, and the resulting spectral count data was assembled with Scaffold. B, 150 mm plates of serum-starved, differentiated 3T3-L1 adipocytes were either left untreated or treated with 100 nM insulin for 15 mins. The cells were lysed in an isotonic CHAPS lysis buffer and both the NIgG and CLASP2 Antibody #1 IPs were performed as described under Experimental Procedures. The IPs or whole cell lysates (WCL) were resolved by 10% SDS-PAGE and transferred to nitrocellulose membranes. The membranes containing the immunoprecipitated proteins were subjected to Western blot with CLASP2 Antibody #1. The labels on the right side of the blot indicate where CLASP2 and the heavy chain IgG migrate on the gel, and the question mark (“?”) points out a prominent nonspecific band. “MK” stands for protein ladder marker. C, 150 mm plates of differentiated 3T3-L1 adipocytes were infected with either null GFP adenovirus or GFP-CLASP2-HA adenovirus as described under Experimental Procedures. The cells were serum starved and either left untreated or treated with 100 nM insulin for 15 mins. The cells were lysed and the lysates were subjected to anti-HA IP and Western blot and using the same procedure for CLASP2 Antibody #1 as described above. D, The CLASP2 Antibody #2 experiment was performed in a similar manner as described above for the CLASP2 Antibody #1 experiment.
A. For the CLASP2 Antibody #1 IP experiments, the 39 “SAINT-qualified” proteins were ordered in a hierarchical manner, from lowest spectrum counts identified (BNIP2) to highest (CLAP2), and results from all four experiments were individually plotted in a Spectrum Count Profile (“SCP”). Basal NlgG IPs (green), insulin NlgG IPs (magenta), basal CLASP2 Antibody #1 IPs (red), and insulin CLASP2 Antibody #1 IPs (turquoise).

B. Anti-HA antibody IPs for GFP-CLASP2-HA were performed as described in Fig. 1. Tandem mass spectrometry on the IPs was performed as described under Experimental Procedures (n/H10053) and SCP analysis of the 18 SAINT-qualified proteins is shown.

C. CLASP2 Antibody #2 IPs were performed as described in Fig. 1. Tandem mass spectrometry on the IPs was performed as described under Experimental Procedures (n = 4) and SCP analysis of the 17 SAINT-qualified proteins is shown.

Fig. 2. SAINT scoring and Spectrum Count Profile analysis of the CLASP2 interactomes. A, For the CLASP2 Antibody #1 IP experiments, the 39 “SAINT-qualified” proteins were ordered in a hierarchical manner, from lowest spectrum counts identified (BNIP2) to highest (CLAP2), and results from all four experiments were individually plotted in a Spectrum Count Profile (“SCP”). Basal NlgG IPs (green), insulin NlgG IPs (magenta), basal CLASP2 Antibody #1 IPs (red), and insulin CLASP2 Antibody #1 IPs (turquoise). B, Anti-HA antibody IPs for GFP-CLASP2-HA were performed as described in Fig. 1. Tandem mass spectrometry on the IPs was performed as described under Experimental Procedures (n = 3) and SCP analysis of the 18 SAINT-qualified proteins is shown. C, CLASP2 Antibody #2 IPs were performed as described in Fig. 1. Tandem mass spectrometry on the IPs was performed as described under Experimental Procedures (n = 4) and SCP analysis of the 17 SAINT-qualified proteins is shown.
SAINT-qualified proteins, although reciprocal co-IP follow-up interactome experiments were unsuccessful for two of these, namely TUG/ASPC1, which we chose because of a reported role in insulin action (75, 76), and FAM13A because this protein exhibited a significant increase in abundance in the CLASP2 IPs upon insulin stimulation (data not shown). TUG and FAM13A were exclusive to the CLASP2 Antibody #1 interactome, so we took advantage of Cytoscape (77) for the integration and visualization of all three CLASP2 interactomes and followed up on SAINT-qualified proteins that were present in more than one of the individual CLASP2 interactomes (Fig. 3). This approach helps overcome false positives resulting from cross-reactivity or nonspecificity from a single antibody. Proteins with established links to CLASP2 that were present in multiple CLASP2 interactomes include the binding partners SLAIN2 and CKAP5/ch-TOG (78), CLASP1, MARK2 (21), GCC2 (also known as GCC185) (32), and of course CLIP1 and CLIP2 (17). In addition, we present the novel discoveries that CLASP2 co-IPs Arf-GAP with GTPase, ANK repeat and PH domain-containing proteins 1 and 3 (AGAP1 and AGAP3), the microtubule/actin-regulating protein GAS2-like protein 1 (G2L1/G2L1), and the protein “suppressor of glucose by autophagy” (SOGA1), all of which together represent a new subset of CLASP2-associated proteins that do not have any previously established connection to CLASP2.

The AGAP3 and CLIP2 Interactomes—AGAP1, AGAP2, and AGAP3 are GAP proteins with links to membrane traffic and actin (79). Because both AGAP1 and AGAP3 were present in all three CLASP2 interactomes, and GAP proteins are important regulators of cytoskeletal dynamics and vesicle trafficking, we chose to analyze the AGAP3 interactome with a commercially available antibody, using the same SAINT-based interactome approach as described above for CLASP2 (Fig. 4A and accompanying supplemental Tables S16, S17, S18, S19, and S20). SCP analysis of the SAINT-qualified AGAP3 interactome confirmed successful immunoprecipitation of AGAP3 and while CLASP1 but not CLASP2 was detected, CLIP2 was highly enriched, as was AGAP1, albeit to a lesser extent. This finding introduced the possibility that AGAP3 was enriched in the CLASP2 interactomes because of binding CLIP2 rather than direct interaction with CLASP2. To test this, we analyzed the CLIP2 interactome with a commercially available antibody, again, using the same interactome approach (Fig. 4B and accompanying supplemental Tables S21, S22, S23, S24, and S25). We report the novel finding that AGAP3, and to a lesser extent, AGAP1, can strongly co-IP with CLIP2. Because much less CLASP2 was present in the CLIP2 interactome as compared with the CLASP2 interactomes, and the amount of AGAP3 in the CLIP2 interactome was at least triple the detected levels of AGAP3 in the CLASP2 IPs, these findings suggest a preference of AGAP3 for CLIP2 over CLASP2. To visualize interaction partners shared among CLASP2, CLIP2, and AGAP3, we integrated the corresponding SAINT-qualified interactomes with Cytoscape (Fig. 4C). This led to the discovery that G2L1, the novel protein identified in each of the three CLASP2 interactomes, as well as both CKAP5 and SLAIN2, were present in both the CLIP2 and CLASP2 interactomes. This leads to the novel hypothesis that CLIP2-CLASP2-SLAIN2-CKAP5-G2L1 are a protein network, although more evidence is needed supporting a direct link between G2L1, CKAP5 and SLAIN2. Cytoscape integration also revealed that both CLIP2 and AGAP3 share association with CLIP1, CLASP1, and AGAP1, as well as with members of the protein phosphatase 6 holoenzyme, specifically PPP6C, PPM6C, and ANR28. This could hypothetically act as a second protein network for CLIP2, composed of CLIP2-AGAP3-AGAP1-
FIG. 4. SCP analysis for the SAINT-qualified AGAP3 and CLIP2 interactomes and Cytoscape-based CLIP2/AGAP3/GFP-CLASP2-HA interactome integration. A, AGAP3 Antibody IPs and tandem mass spectrometry was performed as described under Experimental Procedures (n = 2) and SCP analysis of the 30 SAINT-qualified proteins is shown. B, CLIP2 Antibody IPs and tandem mass spectrometry was performed as described under Experimental Procedures (n = 2) and SCP analysis of the 58 SAINT-qualified proteins is shown. C, Cytoscape-based integrated visual representation of the AGAP3, CLIP2, and GFP-CLASP2-HA interactomes.
The MARK2 and SOGA1 Interactomes—The MARK family (also known as the Par-1 family) is known to phosphorylate microtubule-associated proteins, which serves to regulate microtubule stability in a negative manner (80), and both MARK2 and MARK3 have been detected by mass spectrometry in GFP-CLASP1 IPs before (21). As a result of the novel discovery of MARK2 in the CLASP2 IPs, we chose to analyze the MARK2 interactome with a commercially available antibody (Fig. 5A and accompanying supplemental Tables S26, S27, S28, S29, and S30). SCP analysis of the MARK2 interactome confirmed the successful immunoprecipitation of MARK2 and reciprocal co-IP of CLASP2. The MARK2 interactome also had strong enrichment of glycogen synthase (GYS1) and glycogenin, (GLYG), two proteins vital to the process of glucose storage as glycogen. At 81 SAINT-qualified proteins, this particular MARK2 antibody had the potential for possessing a high degree of nonspecific cross-reactivity. To avoid potential false positives, we created a cross reference MARK2 interactome derived from an adenovirally overexpressed and mCherry-tagged MARK2 immunoprecipitated with an antibody to the mCherry tag (Fig. 5B and accompanying supplemental Tables S31, S32, S33, S34, and S35). SCP analysis of the mCherry-MARK2 interactome revealed 29 SAINT-qualified proteins and confirmed the successful immunoprecipitation of MARK2. The mCherry-MARK2 SAINT score for BAS and INS CLASP2 enrichment was 1 and 0.64, respectively, whereas the BAS and INS SAINT scores for glycogen synthase were 0.9 and 0.4, respectively. When analyzing the spectrum counts for glycogen synthase in the INS samples, the negative control values were 61 and 52 versus 537 and 364 in the mCherry-MARK2 IPs, representing a very strong enrichment of GYS1 in the mCherry-MARK2 interactome. We modified the SCP of mCherry-MARK2 to include these two proteins for Cytoscape-based integration of the two MARK2 interactomes (Fig. 5C). We confirmed the previous report that MARK2 associates with MTCL1 (81). In addition, suppressor of glucose by autophagy 1 (SOGA1), a protein enriched in two out of the three CLASP2 interactomes, was also identified as a SAINT-qualified protein within both MARK2 interactomes tested. SOGA1 has been linked to adiponectin-mediated inhibition of glucose production by enhancing suppression of autophagy in an insulin-dependent manner in hepatocytes (82), although an association between SOGA1 and microtubules or microtubule-associated proteins has yet to be shown. To test for reciprocal co-IP of CLASP2 and MARK2 with SOGA1, we analyzed the SOGA1 interactome with a commercially available antibody (Fig. 6A and accompanying supplemental Tables S36, S37, S38, S39, and S40). SCP analysis of the SOGA1 interactome confirmed both successful immunoprecipitation of SOGA1 and reciprocal co-IP of both CLASP2 and MARK2. Integration of the GFP-CLASP2-HA, SOGA1, and mCherry-MARK2 interactomes with Cytoscape (Fig. 6B) led to the new finding that SOGA1, like MARK2, can co-IP glycogen synthase (GYS1) and glycogenin, (GLYG). As seen in the SCPs for the MARK2 and SOGA1 interactomes, these proteins are coimmunoprecipitating a relatively large amount glycogen synthase. The fact that each of these three unique antibody IPs (SOGA1, MARK2, mCherry-MARK2) are all enriched in glycogen synthase, independent of each other, suggests specificity of this finding.

SOGA1 Colocalizes with CLASP2 and Microtubules—CLASP2 is known to associate with microtubules, a finding that we also show in adipocytes (Fig. 7, top row). Based on the interactome discovery that SOGA1 and CLASP2 can reciprocally co-IP, we hypothesized that SOGA1 and CLASP2 subcellularly colocalize in adipocytes. As shown in Fig. 7 (middle row), in a cell overexpressing CLASP2, both the GFP-CLASP2 and the anti-SOGA1 antibody signals colocalize within microtubule-like heavy bundles, whereas surrounding, non-CLASP2 overexpressing cells show punctate SOGA1 filamentous structures, suggesting SOGA1 may be microtubule-associated. Further analysis directly comparing microtubules (anti-tubulin antibody) and anti-SOGA1 antibody staining (Fig. 7, bottom row) revealed a high degree of SOGA1 and tubulin colocalization, a discovery which identifies SOGA1 as a new microtubule-associated protein.

To follow up on the interactome discoveries of both SOGA1 and CLASP2 potentially linked to the microtubule-associated protein kinase MARK2, we tested for colocalization of MARK2 with CLASP2 and SOGA1. Although GFP-CLASP2 and mCherry-MARK2 showed similar localization, there was a strong loss of microtubule-like structure for CLASP2 (Fig. 8, first row). Similar findings were seen for the anti-SOGA1 and anti-tubulin stains within MARK2 overexpressing cells as well (Fig. 8, second and third rows). These results agree with others who have reported that overexpression of MARK2 disrupts the microtubule network, from a complete loss of microtubules to varying stages of microtubule disruption (80), a phenomenon we reproduced in adipocytes. The lack of SOGA1 colocalizing with tubulin in cells with MARK2 overexpression-induced microtubule destabilization is further evidence that SOGA1 is a microtubule-associated protein. Interestingly, as seen in the overexpressed mCherry-MARK2 interactome (Fig. 5B), even with the loss of microtubule integrity, mCherry-MARK2 was still able to strongly co-IP SOGA1 and GYS1, which questions the necessity of intact microtubules for MARK2 to network with SOGA1 and GYS1.

To better understand the SOGA1 structure-function relationship, we developed a homology model of full-length SOGA1 protein structure. Due to the absence of an X-ray crystallography structure for SOGA1, we utilized the YASARA Structure software package (83) to perform three-dimensional homology modeling of SOGA1 (Uniprot Accession E1U8D0) in silico. Screening of SOGA1 against the Protein Data Bank...
Fig. 5. SCP analysis for the SAINT-qualified MARK2 and mCherry-MARK2 interactomes and Cytoscape-based MARK2/mCherry-MARK2 interactome integration. A, MARK2 Antibody IPs and tandem mass spectrometry was performed as described under Experimental Procedures (n = 2) and SCP analysis of the 81 SAINT-qualified proteins is shown. B, Anti-mCherry Antibody IPs for mCherry-MARK2 and tandem mass spectrometry was performed as described under Experimental Procedures (n = 2) and SCP analysis of the 31 SAINT-qualified proteins is shown. C, Cytoscape-based integrated visual representation of the MARK2 and mCherry-MARK2 interactomes.
resulted in a best alignment/coverage score for the Cytoplasmic Dynein 2 Motor Domain (PDB ID 4RH7) (64). Using the dynein 2 domain as a template, the complete structure of SOGA1 was built in silico. Our analysis of the resulting structure indicates that SOGA1 shares a close similarity to the main features of dynein such as microtubule binding, stalk and strut domains (Fig. 9). SOGA1 sequence length is less than dynein 2, because of SOGA1 lacking the massive linker, neck, and tail portions of the dynein 2 N terminus. The SOGA1 structure predicts long N-terminal alpha-helixes of structural similarity to Tropomyosin chains, which could hypothetically interact with actin fibers after release from the observed “clamp”
structure (Fig. 9, top right inset). Further structure-function experiments for SOGA1 will be the focus of future follow up studies. The molecular modeling data indicating SOGA1 is structurally like a known microtubule binding protein like dynein 2 is in agreement with the findings that SOGA1 colocalizes with CLASP2 and tubulin and supports the identification of SOGA1 as a new microtubule-associated protein.

DISCUSSION

We have developed and successfully executed a streamlined interactome approach to characterize the CLASP2 interactome in a 3T3-L1 adipocyte system. We compared IgG antibody negative control IPs against two different CLASP2 antibody IPs and combined these interactomes with one null anti-HA antibody IP versus a GFP-CLASP2-HA anti-HA antibody IP and analyzed the raw interactome data with SAINT to identify proteins enriched in the CLASP2 IPs. To aid in the visualization of the raw spectral count data from the interactome experiments, we developed the Spectrum Count Profile, which allowed for facilitated interpretation of the raw spectral count results. By performing these in-depth, multiple antibody interactome experiments, we discovered that different anti-bodies for the same protein, in this case CLASP2 and MARK2, can present individually diverse interactomes, while also containing cross-correlating proteins as well. Taking this into account for the CLASP2 data, we narrowed down our focus to AGAP3 as one of the proteins of interest in the CLASP2 interactome. Subsequent analysis of the AGAP3 interactome revealed a strong enrichment of CLIP2 but not CLASP2, opening the likelihood that AGAP3 appeared in the CLASP2 interactome as a result of associating with CLIP2 (which was highly present in all three CLASP2 interactomes tested). In support of this, successive experiments performed to characterize the CLIP2 interactome revealed robust enrichment of AGAP3. AGAP3 coimmunoprecipitated AGAP1 very strongly, which may support the proposed functional cooperativity between AGAP family members (84), as AGAP1 and AGAP3 were also detected in tandem in the CLIP2 interactome and all three CLASP2 interactomes. The AGAPs, also referred to as the centaurins (85) and the GGAPs (86), have been shown to act as GAPs for Arf family members (79, 84) and have intrinsic GTPase property as well (85, 86), although GTPase activity has been questioned (79, 84). The fact that AGAP1 and AGAP3 consistently co-immunoprecipitated with the microtubule-associated proteins CLIP2 and CLASP2 now places these two proteins in position to be tested for roles in regulating microtubule dynamics. TBC1D4/AS160 is a GAP whose...
inhibitory control of Rab function in GLUT4 translocation is deactivated by insulin-stimulated AKT phosphorylation (87). Based on our interactome findings, we hypothesize that both AGAP1 and AGAP3 are linked to CLASP2 through CLIP2, and because CLASP2 undergoes strong insulin-stimulated phosphorylation (16), it will be of interest to explore whether the proposed GTPase activity of the AGAP proteins is under the control of insulin-stimulated AGAP phosphorylation.

Another novel finding was that G2L1 (Uniprot gene name GAS2L1; protein name also known as GAR22) exhibited enrichment in all three CLASP2 interactomes as well as in the CLIP2 interactome. G2L1, like CLASP2, contains microtubule-tip localization sequences, which supports likely colocalization and possible cooperativity between these two proteins, and perhaps CLIP2 as well. G2L1 has been shown to bind with both filamentous actin (F-actin) and microtubules (88–91) to support microtubule and actin coalignment (88, 92). G2L1 mediates actin and microtubule crosstalk by promoting microtubule guidance along actin, which is of interest to insulin action because it has been proposed that the GSV could at some point transfer from microtubules to actin during the vesicle translocation process (93). This hypothesis is supported by the findings that knockdown of the actin-based motor proteins Myo1c and Myo5, which are responsible for cargo transport along actin, reduced cell surface levels of GLUT4 (94–96). It is also worth noting that both CLASP1 and CLASP2 have been found to interact with actin filaments (97).

Future studies will investigate the functional consequences for the proposed CLASP2-CLIP2-G2L1 protein network, and the importance this complex has in regulating the function of these individual proteins.

With regards to the known associating partners for CLASP2, CLIP1, and CLIP2, both had excellent enrichment in the CLASP2 IPs, although there was no observable insulin effect on the co-IP of the two CLIPs with CLASP2 as tested, nor did insulin affect CLASP2 (or CLASP1) co-IP in the CLIP2 interactome. There has been a reported association between insulin action and the CLIP proteins, in that CLIP1 restores insulin-stimulated GLUT4 translocation in TSC2<sup>−/−</sup> cells, a mechanism postulated to involve mTOR-regulated microtubule organization (98) because mTOR can phosphorylate CLIP1 (99). CLIP2 and CLASP2 both shared association with CLIP1 and CLASP1, as well as with the binding partners SLAIN2 and CKAP5/ch-TOG, which is agreement with previous findings implicating that the microtubule plus end-tracking proteins SLAIN2 and CKAP5/ch-TOG, which is agreement with previous findings implicating that the microtubule plus end-tracking proteins SLAIN2, CKAP5, CLIP2, and CLASP2 can complex together (78). Another novel finding resulting from this study was the shared association of CLIP2 and AGAP3 with
ANR28, PP6R3 and PPP6, all of which are subunits of the serine/threonine-protein phosphatase 6 holoenzyme complex. Seeing as phosphorylation is a known modulator of GAP activity, the protein phosphatase 6 holoenzyme complex may contribute to the dynamic regulation of AGAP function.

We present the discovery that SOGA1, a protein with no previous connection to cytoskeletal elements, is enriched in the CLASP2 interactome. This finding was strengthened by successive analysis and confirmation of the presence of CLASP2 in the reciprocal SOGA1 interactome. SOGA1 is a relatively uncharacterized protein that has been linked to the regulation of autophagy in hepatocytes (82). In response to nutritional deprivation, autophagy can initiate the release of glucose from the liver by promoting the hydrolysis of proteins, glycogen, and triglycerides (100–102). SOGA1 expression was shown to be increased upon adiponectin-stimulated activation of the insulin signaling pathway in hepatocytes, and this increase in SOGA1 protein levels contributes to the reduction of glucone production by inhibiting autophagy through an as-yet unknown mechanism (82). These previous findings highlight the importance of our discovery that glycogen synthase (GYS1), the key biosynthetic enzyme for the synthesis of glycogen (103), is enriched in the SOGA1 interactome. Although the role of glycogen autophagy in adipocytes was only recently examined (104), it is possible to hypothesize that SOGA1 may regulate glucose and glycogen metabolism by directly cooperating with glycogen synthase and the glycogen synthase-associated protein glycogenin, which was also detected in the SOGA1 interactome. Because increased expression of SOGA1 was found to enhance inhibition of glycogen autophagy (82), SOGA1 binding to glycogen synthase and glycogenin could hypothetically participate in the protection of glycogen from autophagy, although these new proposed interactions from our proteomics studies will require further validation. In addition, this mechanism likely involves a microtubule element because SOGA1 subcellular localization studies presented here revealed SOGA1 as a new...
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microtubule-associated protein and molecular modeling studies predict SOGA1 to have a structure similar to the known microtubule binding protein dynein. This hypothesis is further strengthened by the additional discovery of MARK2 and MARK3 in the SOGA1 interactome, both of which are kinases known to regulate microtubule dynamics. Whereas CLASP2 promotes microtubule stability, MARK family members can disrupt microtubule growth (105), a phenomenon we showed that also results in the subcellular displacement of both SOGA1 and CLASP2 in adipocytes. The MARK family of kinases do have an established connection to metabolism, as MARK2, MARK3, and MARK4 knockout mice each exhibit enhanced peripheral insulin sensitivity and resistance to high-fat diet induced obesity (106–108). In addition, the MARK family of kinases are closely related to the energy sensor and metabolic regulator AMPK, as these kinases share a common consensus phosphorylation motif (109). These observations, together with the discovery presented here that MARK2, like SOGA1, can co-IP glycogen synthase and glycogen, introduce the proposed SOGA1–MARK2–GYS1–GLYG network and microtubules as targets for studies aimed at determining the metabolic function of this potentially novel protein complex.

By taking advantage of proteomics and the advancements that are being made in interactome studies and bioinformatic resources, this study has made progress in identifying proteins that potentially associate with CLASP2, which has allowed us to propose a series of novel protein networks (Fig. 10). The discoveries presented here will lead to future studies aimed at further confirming these findings and understanding the purpose of the functional relationship these new protein networks have with microtubule dynamics, actin reorganization, glucose and glycogen metabolism, autophagy, and insulin action.

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DATA AVAILABILITY

The .RAW files and Scaffold data (in mzIdent format) for all experiments have been deposited to the ProteomeXchange Consortium (http://proteomecentral.proteomexchange.org) via the PRIDE partner repository with the data set identifier: PXD003674.

* The content is solely the responsibility of the authors and does not necessarily represent the official views of the National Institutes of Health.

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