The Human Phosphatidylinositol Phosphatase SAC1 Interacts with the Coatomer I Complex*

Received for publication, July 22, 2003, and in revised form, October 1, 2003
Published, JBC Papers in Press, October 3, 2003, DOI 10.1074/jbc.M307983200

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The Saccharomyces cerevisiae SAC1 gene encodes an integral membrane protein of the endoplasmic reticulum (ER) and the Golgi apparatus. Yeast SAC1 mutants display a wide array of phenotypes including inositol auxotrophy, cold sensitivity, secretory defects, disturbed ATP transport into the ER, or suppression of actin gene mutations. At present, it is not clear how these phenotypes relate to the finding that SAC1 displays polyphosphoinositide phosphatase activity. Moreover, it is still an open question whether SAC1 functions similarly in mammalian cells, since some phenotypes are yeast-specific. Potential protein interaction partners and, connected to that, possible regulatory circuits have not been described. Therefore, we have cloned human SAC1 (hSAC1), show that it behaves similar to ySac1p in terms of substrate specificity, demonstrate that the endogenous protein localizes to the ER and Golgi, and identify for the first time members of the coatomer I (COP) complex as interaction partners of hSAC1. Mutation of a putative COP1 interaction motif (KXXXX) at its C terminus abolishes interaction with COP1 and causes accumulation of hSAC1 in the Golgi. In addition, we generated a catalytically inactive mutant, demonstrate that its lipid binding capacity is unaltered, and show that it accumulates in the Golgi, incapable of interacting with the COP1 complex despite the presence of the KXXXX motif. These results open the possibility that the enzymatic function of hSAC1 provides a switch for accessibility of the COP1 interaction motif.

Phosphatidylinositol (PtdIns)₃ phosphates act as signaling components in various intracellular membranes and influence membrane trafficking, cytoskeletal organization, motility, and cellular survival, depending on their subcellular localization and the availability of specialized PtdIns-phosphate-binding proteins, lipases, PtdIns kinases, and PtdIns phosphatases (for reviews, see Refs. 1 and 2). Several PtdIns phosphatases have recently gained much attention, because their loss of function is associated with disease. For example, mutations in the OCRL1 gene, which encodes a phosphatidylinositol-(4,5)-bisphosphate 5-phosphatase of the trans-Golgi network, are responsible for the oculocerebrorenal syndrome of Lowe (3). The tumor suppressor PTEN (phosphatase and tensin homologue deleted on chromosome ten) (for a review, see Ref. 4) functions as a plasma membrane-associated PtdIns-3-phosphatase that negatively regulates the PtdIns-3-kinase/akt pathway (5). Furthermore, myotubularin 1 (MTM1) belongs to a family of PtdIns-3-phosphatases and was originally identified by positional cloning of the gene responsible for X-linked myotubular myopathy (6, 7). Interestingly, PTEN and the MTM1 family harbor a core C₅R/T/S catalytic motif, which was first identified in protein phosphatases (8, 9).

This motif is also embedded in the SAC domain of PtdIns phosphatases like yeast Sac1p (suppressor of actin mutations), Fig4p, Inp51p/Sjl1p, Inp52p/Sjl2p, and Inp53p/Sjl3p (10). Interestingly, ySac1p and its rat homolog, rSAC1 (11), exert a wider substrate specificity than PTEN or MTM1, since they were demonstrated to convert PtdIns(4)P, PtdIns(3)P, and PtdIns(3,5)P₂ to PtdIns (11–13). Inp51p/Sjl1p, Inp52p/Sjl2p, and Inp53p/Sjl3p contain in addition to the SAC domain a C-terminal 5-phosphatase domain and thus constitute homologs of mammalian synaptojanin 1, which is essential for synaptic vesicle recycling during endocytosis. Synaptojanin 1-deficient neurons show accumulation of PtdIns(4,5)P₂, leaving it open whether its SAC domain is enzymatically functional in mammalian cells (14). Synaptojanin 2 displays similar substrate specificity, yet a wider tissue distribution than synaptojanin 1 and appears to act at an earlier step in clathrin-mediated endocytosis (15, 16).

None of the human PtdIns phosphatases containing a SAC domain have been characterized in depth for their biological function. Information about human SAC1 (hSAC1) is hitherto restricted to an EST (KIAA0851), which maps to the C3CER1 segment on chromosome 3p21.3. C3CER1 is commonly eliminated in SCID-derived tumors (17, 18). Human SAC2 was enzymatically characterized and found to exert 5-phosphatase activity specific for PtdIns(4,5)P₂ and PtdIns(3,4,5)P₃ (19).

Yeast strains with mutations in SAC1 exhibit an array of phenotypes including inositol auxotrophy (20), cold sensitivity (21), secretory defects in chitin deposition (13), multiple drug sensitivity (22), and ATP transport deficiencies into the ER (23). Mutations in yeast SAC1 are capable of suppressing le-
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K (hSAC1 interacts with members of the coatomer I complex and Golgi. We also demonstrate for the first time that wild type and PtdIns phosphatase inactive variant by mutation of the core-terminal TM domains (31), whereas rSAC1 apparently only uses the first one (11). In addition to this controversy, the terminal TM domains (31), whereas rSAC1 apparently only uses the first one (11). In addition to this controversy, the terminal TM domains (31), whereas rSAC1 apparently only uses the first one (11). In addition to this controversy, the terminal TM domains (31), whereas rSAC1 apparently only uses the first one (11). In addition to this controversy, the terminal TM domains (31), whereas rSAC1 apparently only uses the first one (11). In addition to this controversy, the terminal TM domains (31), whereas rSAC1 apparently only uses the first one (11). In addition to this controversy, the terminal TM domains (31), whereas rSAC1 apparently only uses the first one (11).

In eukaryotic cells, three vesicle-based protein cargo trafficking systems have evolved, which are defined by a special set of membrane-covering protein coats. Clathrin-covered vesicles allow transport from the plasma membrane to the trans-Golgi network and from the trans-Golgi network to endosomes. Coatomer II (COPII)-coated vesicles have been assigned to ER and Golgi anterograde transport (26, 27). In contrast, coatomer (COPI)-coated vesicles are involved in membrane traffic mainly between Golgi and ER or intra-Golgi compartments (28, 29). Coatomer II (COPII)-coated vesicles have been assigned to ER and Golgi anterograde transport (26, 27). In contrast, coatomer (COPI)-coated vesicles are involved in membrane traffic mainly between Golgi and ER or intra-Golgi compartments (28, 29).

Identification of hSAC1 Interaction Partners—Following SDS-PAGE and Coomassie staining, respective gels were excised and sent to Mobidab Molekularbiologie GmbH (Leipzig, Germany) for MALDI-TOF mass spectrometry analysis. Peptide masses corresponding to human α-COP were covered by 36 peptides (Mwose score 111, p < 0.05). 25 peptide masses matched β-COP (Mwose score 66, p < 0.05).

Phosphatase Activity Assay—To measure phosphatase activity, a modified version of a malachite green assay was used (32). Phospho inositides were purchased from Cell Signals Inc., and phosphatidylinosine was obtained from Sigma (P-1060). 1 µg of recombinant GST-hSAC1wt and GST-hSAC1-C/S in 25 µl of storage buffer were incubated (1 h, 32°C), with 25 µl of liposomes, prepared by sonification of 500 µM phosphatidylserine, 100 µM dilauroylphosphatidylserine, 200 mM sodium acetate, 100 mM Tris-base, 100 mM Bis-Tris, 20 µg/ml porcine gelatin, pH 6.0). Reactions were stopped by the addition of 20 µl of 100 mM N-ethylmaleimide and centrifugation (14,000 × g, 15 min). 25 µl of the supernatant was transferred to a multowell plate, incubated (20 min, room temperature) 50 µl of malachite green solution (33) to quantify released phosphate at 600 nm. Phosphate release was calibrated to a dilution series of KH₂PO₄.

Peptides, Immunization, and Antibodies—Peptides were synthesized according to standard procedures followed by purification on a reverse phase high pressure liquid chromatography. Peptides used for competitive inhibition experiments were hSAC1/378–387 (PRLVKEKID), hSAC1/41–55 (TLAVKDDVPVSATVR), and crocistide (biotin-GRPTTSSFAE). hSAC1 antibodies were raised against recombinant GST-hSAC1 wild type protein in rabbits (Eurogentec). GST-reactive immunoglobulins were removed by adsorption to GST-Sepharose beads (serum 252 and 253). Additionally, polyclonal peptide antisera 69 and 7889 were raised against hSAC1 amino acids 570–587, prepared by adsorption to GST-Sepharose beads (serum 252 and 253). The following antibodies were commercially available and used according to the manufacturer’s instructions: anti-FLAG M2 (F-3165; Sigma), anti-GFP (1814460; Roche Applied Science), anti-COPA (PA1-067; Affinity Bioreagents), anti-COPB, -E, and -G (sc-13335, sc-13345, and sc-13346), respectively.

Molecular Cloning of hSAC1 and Plasmids—The EST KIAA0851 was found to contain an open reading frame that is highly homologous to the yeast enzyme SAC1 (31.8% identity, 46.1% similarity). Human SAC1 cDNA was amplified from brain mRNA (Invitrogen) by reverse transcriptase-PCR using the following primers: primer a, 5′-GAG GAA GAA GGA AGG TGG TGG CA-3′; primer b, 5′-TGT GGA AAA GTA TGC TGT CTA ATA TGT G-3′. PCR products were subcloned into pCR-TOPO vector (Invitrogen) generating pCRII-hSAC1 and confirmed to be identical to KIAA0851 (nucleotide coordinates 40–2272) via sequencing. The hSAC1 ORF is covered by KIAA0851 nucleotides 70–1833.

The hSAC1/C5 mutation was introduced into pCRII-hSAC1 with a Transfection Kit (Promega) according to the manufacturer’s instructions. The hSAC1-KEKID mutation was generated by PCR using the following primers: primer c, 5′-GCA CAA TCC ATG TCG TGG CA-3′; mutagenic primer (primer d), 5′-A TCC TCA GTG TAT CAG CCG TGC CTC GTG CA-3′ with the mutated nucleotides underlined. The respective fragment was cloned into pCR-TOPO, and an EcoRI fragment thereof was swapped into pGFP-hSAC1wt (see below). All mutations were confirmed by sequencing. An N-terminal FLAG tag (amino acids MDYDIDDKAAK, with hSAC1 amino acids 2–5 underlined) was fused to hSAC1wt and hSAC1-C5, using an EcoRI-StuI digestion protocol. Insertion into the mammalian expression vector pcDNA3.1 (Invitrogen) generated pCDNA3-1-FLAG-hSAC1wt and pCDNA3-1-FLAG-hSAC1-C5. To generate Schizosaccharomyces pombe expression vectors pESP-hSAC1wt and pESP-hSAC1-C5, the BamHI site of pESP1 (Stratagene) was exploited to introduce the hSAC1 variants following the PCR-mediated insertion, replacing the BamHI site downstream of the T7 promoter. Products were confirmed by sequencing. BamHI-flanked ORFs from pESP-hSAC1wt and pESP-hSAC1-C5 were used to generate intermediates in pENTR3C prior to Gateway™-mediated shuttling (Invitrogen) into eGFP-A30, an appropriately modified derivative of pEFGP-C2 (Clontech).

Inducible Expression in Saccharomyces cerevisiae—The vectors pYEX-BX, pYEX-BX-hSAC1wt, and pYEX-BX-hSAC1-C5, containing GST-hSAC1 fusion sequences, were transformed into the S. pombe strain SP-401 (Stratagene) and induced as described by the manufacturer (Stratagene). Cells were disrupted by French press in lysis buffer containing 0.3% sorbitol, 0.1 M NaCl, 5 mM MgCl₂, 10 mM Tris/Cl, pH 7.2, and 1% Triton X-100 followed by batch purification of GST fusion proteins with glutathione 4B FF-Septahrose (Amersham Biosciences) according to the manufacturer. Washings were done with lysis buffer and elution of proteins occurred in 10 mM Tris/Cl, 10 mM glutathione, pH 7.5. For storage at 80°C glycol was added to 25% final concentration.

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Identification of Human SAC1 and Generation of a PtdIns Lipid Binding Assay—For assessment of phospholipid binding properties, PIP Strips™ (Echelon, Inc.) were blocked (1 h, room temperature) with 5% skim milk in TBST (50 mM Tris/Cl, pH 7.5, 150 mM NaCl, 0.1% Tween 20) and washed with TBST. Visualization was done using suitable horseradish peroxidase-conjugated secondary antibodies and ECL. For immunoprecipitation, cell lysates were adapted for 24 h to G418-free medium.

RESULTS

Identification of Human SAC1 and Generation of a PtdIns phosphate Inactive Mutant—To clone hSAC1 cDNA, we used a set of primers flanking the ORF found in the EST KIAA0851. A cDNA fragment identical to KIAA0851 nucleotides 40—2272 was amplified via reverse transcriptase-PCR from brain mRNA (see “Experimental Procedures”). Two other human ESTs (KIAA0966 and KIAA0274) also contain a SAC1 homology domain within their sequence. KIAA0966 was recently designated hSAC2 (19), whereas KIAA0274 still awaits characterization.

The hSAC1 ORF (587 amino acids (aa)) exhibits 32% identity to ySac1p and 95% identity to rSac1p (11) and is predicted to code for a protein of 64 kDa with a pl of 6.69. Inspection of the primary amino acid sequence reveals (Fig. 1) two potential TM domains at the C terminus (aa 521—543; aa 550—569). The phosphatase signature motif CX(3R/T/S) (aa 389—396) is indicated by a black bar. Structure predictions were done using the SMART software (EMBL, Heidelberg, Germany). An alignment of sequences flanking the CX(3R/T/S) motif is depicted below. Identical (black), similar (dark gray), and related (light gray) amino acids are highlighted. hSAC1, human SAC1; rSac1, rat SAC1; mSAC1, mouse SAC1; cSAC1, S. cerevisiae SAC1; hSAC2, human SAC2; MTM1, human MTM1; PTEN, human PTEN; TPIP, human TPIP (transmembrane phosphatase with tensin homology)- and PTEN-homologous inositol lipid phosphatase; Syjn1, human synaptojanin 1. The asterisk marks cysteine 389, which was replaced by serine in hSAC1-C/S. B, the leucine zipper motif is conserved in mammalian SAC1 family members. Conserved leucines are in boldface type. The abbreviations are as in A, SAC1; Drosophila SAC1; cSAC1, C. elegans SAC1; spSAC1, S. pombe SAC1.

To assess whether wild type hSAC1 interacts with COPI, we performed a GST pull-down assay using recombinant GST-hSAC1wt, GST-hSAC1-C/S, and GST-hSAC1-C/S, the constructs covering the ORFs for wild type and mutant hSAC1 were subcloned into various expression constructs (see “Experimental Procedures”). The 587-aa ORF contains a SAC1 homology domain (aa 121—500), a putative leucine zipper (L) motif (aa 98—126), and two transmembrane domains (T) at the C terminus (aa 521—543; aa 550—569). The phosphatase signature motif CX(3R/T/S) (aa 389—396) is indicated by a black bar. Structure predictions were done using the SMART software (EMBL, Heidelberg, Germany). An alignment of sequences flanking the CX(3R/T/S) motif is depicted below. Identical (black), similar (dark gray), and related (light gray) amino acids are highlighted. hSAC1, human SAC1; rSac1, rat SAC1; mSAC1, mouse SAC1; cSAC1, S. cerevisiae SAC1; hSAC2, human SAC2; MTM1, human MTM1; PTEN, human PTEN; TPIP, human TPIP (transmembrane phosphatase with tensin homology)- and PTEN-homologous inositol lipid phosphatase; Syjn1, human synaptojanin 1. The asterisk marks cysteine 389, which was replaced by serine in hSAC1-C/S. B, the leucine zipper motif is conserved in mammalian SAC1 family members. Conserved leucines are in boldface type. The abbreviations are as in A, SAC1; Drosophila SAC1; cSAC1, C. elegans SAC1; spSAC1, S. pombe SAC1.

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As shown in Fig. 2, the ΔSAC1 inositol auxotrophy phenotype could be rescued by Cu²⁺-inducible expression of hSAC1 to an extent comparable with expression of wild type ySac1p. In contrast, the hSAC1-C/S mutant was not able to complement inositol auxotrophy similar to the empty vector control. This result demonstrates (i) functional similarity between the yeast and the human SAC1 proteins and (ii) a successful impairment of hSAC1 enzymatic function through mutation of the core phosphatase motif.

The hSAC1-C/S Mutant Protein Displays Reduced Lipid Phosphatase Activity—To assess whether the failure of the hSAC1-C/S mutant to complement a ySac1p-dependent function reflects its inability to process PtdIns phosphates, we expressed and purified GST-conjugated wild type and C/S mutant hSAC1 variants (Fig. 3A). Recombinant proteins were incubated with each of the seven phosphorylated isoforms of PtdIns, and phosphate release was determined employing a malachite green assay (see “Experimental Procedures”).

Fig. 3. Reduced lipid phosphatase activity of hSAC1-C/S. A, recombinant GST-tagged hSAC1 variants (GST-hSAC1wt (lane 2) and GST-hSAC1-C/S (lane 3)) or control protein (GST; lane 1) was expressed in S. pombe, purified by affinity chromatography, and separated using SDS-PAGE. 1 µg of protein was visualized using SimplyBlue™ staining (Invitrogen). The positions of molecular weight standards are indicated on the right. B, phosphatidylserine lipid vesicles carrying the indicated PtdIns phosphate substrate were incubated with 1 µg of GST-hSAC1wt (dark gray), GST-hSAC1-C/S (light gray), or GST proteins. Phosphate release was quantified using a malachite green assay with GST values subtracted (see “Experimental Procedures”).

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incubated (30 min, 32 °C) were seeded. 16 h later, extracts were prepared and normalized, aliquots were removed for input analysis (input, lanes 1–3), and the rest were followed by SDS-PAGE and immunoblotting employing an anti-GFP (WB: α-GFP) or anti-GST (WB: α-GST) antibody. hSAC1wt interacts efficiently with GFP-hSAC1wt, IgG h.c. and IgG l.c., immunoglobulin G heavy and light chain, respectively. hSAC1wt interacts with pcDNA3.1-FLAG-hSAC1wt plus constructs expressing either GFP-hSAC1wt or GFP-hSAC1-C/S and lysed 48 h later. Precleared cell extracts were subjected to immunoprecipitation with either anti-FLAG (α-FLAG) or anti-GFP (α-GFP) antibodies. Molecular weight standards and 10% of starting material (10% input) are depicted.

or rSAC1. There are several possibilities to explain this discrepancy (see “Discussion”). In conclusion, mutation of the core phosphatase motif in hSAC1 abolishes its ability to dephosphorylate PtdIns phosphates.

Wild Type hSAC1 Forms Oligomers—Given that the C/S mutation disables the PtdIns phosphatase function of hSAC1, we tested whether this mutant acts as a classical dominant negative in the sense of forming inactive heterodimers with the wild type enzyme. To this end, we generated extracts from U373MG glioblastoma cells stably expressing GFP-tagged variants of hSAC1wt or hSAC1-C/S (see below; see “Experimental Procedures”). Such clones expressed comparable amounts of wild type and mutant GFP-hSAC1 fusion proteins (see Figs. 4A and 6A). To these extracts, purified GST-hSAC1wt was added and subsequently immunoprecipitated using a GST-specific antibody. Equal amounts of GST-hSAC1wt were detected in the immunoprecipitates as revealed by immunoblotting with GST antibody. Analysis of this material with GST antibody revealed efficient binding of GST-hSAC1wt to GFP-hSAC1wt but strongly reduced binding to GFP-hSAC1-C/S (Fig. 4A). Using GST-hSAC1-C/S in such experiments revealed neither binding to GFP-hSAC1wt nor GFP-hSAC1-C/S (data not shown). To further support these results, we cotransfected expression constructs coding for FLAG-tagged hSAC1wt (pcDNA3.1-FLAG-hSAC1wt; see “Experimental Procedures”) with constructs expressing either GFP-hSAC1wt or GFP-hSAC1-C/S into COS-7 cells. Two days after transfection, cells were lysed and subjected to immunoprecipitation employing an anti-FLAG monoclonal antibody. Equal amounts of FLAG-tagged hSAC1wt were precipitated in each case. Efficient interaction between the FLAG-tagged and the GFP-hSAC1 variant was only detected in GFP-hSAC1wt and not in GFP-hSAC1-C/S samples (Fig. 4B). These results demonstrate that hSAC1-C/S is not likely to function as a dominant negative mutant, because it does not form a complex with the wild type protein. They also show that the wild type hSAC1 protein can form oligomers, although we cannot formally exclude the possibility that a bridging factor(s), which might not be bound by the C/S mutant, is responsible for the interaction between hSAC1wt proteins.

**Fig. 4. Wild type hSAC1 forms oligomers.** A, U373MG cells expressing GFP (clone 3), GFP-hSAC1wt (clone 5), or GFP-hSAC1-C/S (clone 6) were incubated (30 min, 32 °C) with 2 μg of recombinant GST-hSAC1wt. Immunoprecipitation using an anti-GFP antibody (IP: α-GFP, lanes 4–6) was followed by SDS-PAGE and immunoblotting employing an anti-GFP (WB: α-GFP) or anti-GST (WB: α-GST) antibody. hSAC1wt interacts efficiently with GFP-hSAC1wt, IgG h.c. and IgG l.c., immunoglobulin G heavy and light chain, respectively. B, COS-7 cells were transfected with pcDNA3.1-FLAG-hSAC1wt plus constructs expressing either GFP-hSAC1wt or GFP-hSAC1-C/S and lysed 48 h later. Precleared cell extracts were subjected to immunoprecipitation with either anti-FLAG (α-FLAG) or anti-GFP (α-GFP) antibodies. Molecular weight standards and 10% of starting material (10% input) are depicted.

**Fig. 5. Phospholipid binding profile of hSAC1wt and hSAC1-C/S.** Nitrocellulose-immobilized phospholipids (PIP-Strips™, Echelon Inc.; 100 pmol/spot) were incubated after blocking with equal amounts (0.5 μg/ml) of either GST-hSAC1wt, GST-hSAC1-C/S, or GST protein. Bound proteins were detected via Western blot using anti-GST antibody. Only part of the strips is shown. LPA, lysophosphatidic acid; LPC, lysophosphocholine; PI, PtdIns; PI3, PtdIns(3)P; PI4, PtdIns(4)P; PI5, PtdIns(5)P; S1P, sphingosine 1-phosphate; PI3,5, PtdIns(3,5)P2; PI4,5, PtdIns(4,5)P2; PI1,4,5, PtdIns(1,4,5)P3; PA, phosphatidic acid. Similar results were obtained using other batches of PIP-Strips™.

hSAC1wt and hSAC1-C/S Display Equivalent PtdInsP Binding Properties—As stated above, two biochemical features of the wild type enzyme, PtdIns phosphatase activity and self-association, are eliminated in the C/S mutant. To test whether mutation of the core phosphatase motif would also influence the lipid binding capacity of hSAC1, we subjected the respective purified GST fusion proteins to a lipid blot analysis (see “Experimental Procedures”). Both GST-hSAC1wt and GST-hSAC1-C/S protein bound with highly comparable affinity to...
Fig. 6. Subcellular localization of hSAC1. A, Western blot analysis using hSAC1-specific antiserum 252 (upper panel) and anti-actin antibody (lower panel). Normal U373MG cells (lane 6) and U373MG cells stably expressing gEGFP-A30 (GFP; lane 1), GFP-hSAC1wt clone 1 (lane 2), GFP-hSAC1wt clone 5 (lane 3), GFP-hSAC1-C/S clone 1 (lane 4), or GFP-hSAC1-C/S clone 6 (lane 5) are shown next to human kidney

hSAC1 Interacts with COPI
The accumulation of the PtdIns phosphatase inactive mutant in the Golgi as opposed to the wider distribution of the wild type enzyme poses several interesting questions: (i) To what extent is the PtdIns phosphatase function of hSAC1 essential to maintain the wider distribution? (ii) Are differential interactions with ER or Golgi components responsible for the observed subcellular localization of the two hSAC1 variants? (iii) Does hSAC1 influence vesicle transport dynamics between the ER and the Golgi? Some of these questions could be addressed with a better knowledge about hSAC1-binding proteins. To this end, we generated preparative immunoprecipitates from U373MG cells stably expressing GFP-tagged or FLAG-tagged versions of hSAC1wt or hSAC1-C/S, respectively. Silver staining of these immunoprecipitated samples revealed consistently, in addition to the respective hSAC1 fusion proteins, protein species of about 150 and 100 kDa only when using lysates expressing hSAC1wt and not hSAC1-C/S or control proteins (Fig. 7A, data not shown). To identify the proteines interacting with hSAC1wt, respective gel pieces were excised and subjected to MALDI-TOF mass spectrometry analysis (see “Experimental Procedures”). The 100-kDa protein species could unequivocally be identified as β-coatomer protein (β-COP), whereas the 150-kDa protein turned out to be α-coatomer protein (α-COP).

To independently corroborate the interaction, we subjected U373MG cells stably expressing GFP-hSAC1 variants to immunoprecipitation with anti-GFP antibody followed by immunoblotting with antibodies raised against α-COP, β-COP, γ-COP, or ε-COP. In all of these cases, the respective member of the coatomer I complex could be identified in immunoprecipitates containing wild type hSAC1 but not catalytically inactive hSAC1-C/S (Fig. 7B). A virtually identical result was obtained in experiments with stably expressed FLAG-tagged hSAC1 variants, demonstrating that the interaction is clearly not tag-dependent (Fig. 7B). Furthermore, when γ-COP protein was immunoprecipitated from the respective stable U373MG cell line lysates, GFP-tagged hSAC1wt, but not hSAC1-C/S, could be detected (data not shown).

To demonstrate that also endogenous hSAC1 interacts with the COPI complex, human PC-3 prostate carcinoma cells were lysed and immunoprecipitated using our new hSAC1 antibodies 252 and 69. Clearly, both hSAC1 antisera immunoprecipitated hSAC1 protein together with COPI (represented by detection of γ-COP), whereas preimmune serum controls did not (Fig. 7C). Conversely, immunoprecipitation using γ-COP antibody revealed endogenous hSAC1 as a complex partner (Fig. 7D), clearly demonstrating that hSAC1 binds to the coatomer I complex.

A Putative COPI Interaction Motif, KEKID, Is Essential for the hSAC1/COPI Interaction—Inspection of the hSAC1 primary amino acid sequence revealed a motif, KEKID-COOH, at

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**Table I**

| hSAC1 variant | ER | ER + Golgi | Golgi |
|--------------|----|-----------|------|
| U373MG clones |    |           |      |
| GFP-hSAC1 wt 1 | 40.5| 59.5 | 0.0 |
| GFP-hSAC1 wt 5 | 48.3| 48.3 | 3.4 |
| GFP-hSAC1-C/S 1 | 1.8 | 4.2  | 94.0|
| GFP-hSAC1-C/S 6 | 1.5 | 7.3  | 91.2|
| COS-7 transfection |     |         |      |
| GFP-hSAC1 wt | 77.6 | 15.9 | 6.5 |
| GFP-hSAC1-C/S | 9.9  | 17.9 | 72.2|
| GFP-hSAC1-K2A | 13.4 | 12.3 | 74.3|

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lysat (lane 7), PC-3-cells (lane 8), and HCT116 cells (lane 9). For lanes 8 and 9 (upper panel), longer exposures are shown. Positions of endogenous hSAC1 (arrow), GFP-hSAC1 fusions (arrowhead), and molecular weight standards are indicated. B, HeLa cells were grown on glass slides, fixed, and analyzed by double immunofluorescence and confocal microscopy (Leica TCS SP2) using hSAC1-specific antiserum 69 (hSAC1) together with an antibody against the 58-kDa microtubule-binding Golgi-associated protein (p58) or against the ER protein Sec61α. Merge visualizes colocalization. C and D, U373MG clones expressing GFP-hSAC1wt or GFP-hSAC1-C/S were grown on glass slides, fixed, and analyzed by immunofluorescence and confocal microscopy (Zeiss LSM 510 Meta) using Sec61α (C) or golgin-97 (D) antibodies. Labeling is as in B. E, Z intervals were recorded from life U373MG cells expressing GFP-hSAC1wt or GFP-hSAC1-C/S with a Zeiss Axiovert 200M equipped with a Yokogawa confocal scanner unit (CSU 10) and a coolnap HQ Roper Scientific camera. During recording, cells were kept in a humidified chamber at 37 °C with 5% CO2. Recordings at a comparable distance from the glass slide are shown. Where indicated, bars represent 20 μm.
were immunoprecipitated with anti-GFP antibody, subjected to SDS-

**A**

[Image of a gel with bands labeled GFP, GFP-hSAC1wt, GFP-hSAC1-C/S, or GFP proteins.](image)

**B**

[Image of a gel with bands labeled α-COP, β-COP, γ-COP, and ε-COP.](image)

**C**

[Image of a gel with bands labeled γ-COP and hSAC1.](image)

**D**

[Image of a gel with bands labeled γ-COP and hSAC1.](image)

**FIG. 7.** hSAC1 binds to the coatomer I complex.

**A** U373MG cells stably expressing GFP-hSAC1wt, GFP-hSAC1-C/S, or GFP proteins were immunoprecipitated with anti-GFP antibody, subjected to SDS-

**B**

The GFP-hSAC1-C/S mutant displayed also in this cell type strongly reduced COPI binding. These findings demonstrate that hSAC1 harbors a functional COPI interaction motif at its extreme C terminus and that this motif cannot be used efficiently when the PtdIns phosphatase function of hSAC1 is abrogated.

**C**

The KEKID motif is responsible for the hSAC1/COPI interaction, we generated a GFP-hSAC1 mutant in which the two lysines in the KEKID motif were replaced by alanine (resulting in AEAID) to generate the construct GFP-hSAC1-K2A. This mutant was transiently expressed in HCT116 cells. Lysates were immunoprecipitated using GFP-antibody and subjected to γ-COP immunoblotting. Whereas transiently expressed GFP-hSAC1wt interacts with COPI, no binding could be detected using the GFP-hSAC1-K2A mutant (Fig. 8B). The GFP-hSAC1-C/S mutant displayed also in this cell type strongly reduced COPI binding. These findings demonstrate that hSAC1 harbors a functional COPI interaction motif at its extreme C terminus and that this motif cannot be used efficiently when the PtdIns phosphatase function of hSAC1 is abrogated.

**D**

To demonstrate that the KEKID motif of hSAC1 is functional in vivo, we transfected constructs coding for GFP-tagged hSAC1-K2A or for the respective wild type and C/S enzymes into COS-7 cells. Since we reproducibly observed that cells with very high expression of the three GFP-tagged hSAC1 variants tend to (i) form aberrant cytoplasmic aggregates of GFP-positive material and (ii) undergo apoptosis (data not shown), we only evaluated cells that exhibited fluorescence intensity comparable with U373MG clones stably expressing GFP-hSAC1. Each variant displayed a distribution of localization phenotypes (quantified in Table I), which, however, clearly allowed us to conclude that the GFP-hSAC1-K2A mutant accumulates in the Golgi apparatus to an extent very similar to GFP-SAC1-C/S, whereas the wild type protein again mainly displayed ER plus Golgi localization (Fig. 8C).
DISCUSSION

We show that the endogenous hSAC1 protein can be found localized to the ER and the Golgi apparatus in nonsynchronized human cells. Two mutants of hSAC1 were generated, one eliminating the PtdIns phosphatase function (hSAC1-C/S) and the other destroying a putative COPI interaction signal at the extreme C terminus of hSAC1 (hSAC1-K2A). Both mutants showed the same accumulation in the Golgi apparatus when expressed as GFP fusion proteins, whereas the wild type protein, GFP-hSAC1wt, behaved like the endogenous protein and displayed ER plus Golgi localization. We could further demonstrate for the first time that the coatomer I complex interacts with hSAC1, that a peptide harboring the cognate C-terminal KXKXX motif (KEKID) efficiently competes with hSAC1 for binding to COPI and that mutation of the two key lysine residues eliminates the hSAC1/COPI interaction. Surprisingly, this KEKID motif is present in the PtdIns phosphatase inactive hSAC1-C/S mutant, which we show is unable to efficiently interact with COPI.

Lipid Binding and Substrate Specificity—We consider it unlikely that the hSAC1-C/S protein displays aberrant folding due to the point mutation at amino acid 389, since we could demonstrate essentially unaltered PtdIns binding capability when comparing with the respective wild type protein. We have for the first time characterized the lipid binding potential of hSAC1 and show that it binds to monophosphorylated PtdIns phosphates with highest affinity, to PtdIns(3,5)P_2 and PtdIns(3,4,5)P_3 with medium affinity, and to PtdIns(3,4)P_2, PtdIns(4,5)P_2, and unphosphorylated PtdIns with lowest affinity. Analysis of more mutants, however, is necessary to actually map the lipid-binding domain of hSAC1.

We could further demonstrate that hSAC1 dephosphorylates PtdIns(4)P and PtdIns(3)P, whereas PtdIns(3,5)P_2 apparently did not serve as a substrate. This is in contrast to reports using ySac1p or rSAC1 as enzymatic source. This discrepancy could arise from different posttranslational modifications due to the expression systems used (Sf9 insect cells for rSAC1 versus S. pombe for hSAC1). Moreover, we used full-length hSAC1...
protein, whereas other studies analyzed truncated versions of SAC1 (11, 31, 38). Nevertheless, PtdIns(4)P and PtdIns(3)P always were the two phosphoinositides to be converted most efficiently.

In conclusion, hSAC1 appears to display affinity to more phospholipids than it actually uses as substrates. We cannot exclude, however, the possibility that the expanded PtdIns-binding profile as compared with the catalytic profile reflects that hSAC1wt requires slightly distinct hydrolysis conditions for various PtdIns isoforms that we might have missed in our assay.

COPI: First Interaction Partner for SAC1—Despite a history of more than 14 years in SAC1 research, interaction partners for this protein have not been identified. Recently, Bsp1p/Ypr171p was reported to bind the proteins Sjl2p and Sjl3p in a region confined to the SAC homology domain. Bsp1p/Ypr171p might act as an adaptor protein for linking its binding partners to the cortical actin cytoskeleton. However, no interaction was observed with Sjl1p or ySac1p (39).

We demonstrate for the first time that hSAC1 binds to members of the COPI complex (α, β, γ, δ, ε). Although inspection of the human hSAC1 sequence might have implied such an interaction due to the presence of a cognate KK/XX sequence, this motif is not evolutionarily conserved in the SAC1 family. Several studies have documented the interaction between coatomer I and a C-terminal KK/XX motif of transmembrane proteins that have to be retrieved to the ER. It is still controversial which subunit(s) of COPI binds the dilysine signal. Genetic screens revealed that mutations in α, β', γ, δ, and ζ-COP subunits interfere with KKXX retrieval (37, 40). Selective cross-linking of dilysine peptides disclosed γ-COP as the essential subunit (41, 42), whereas α-COP emerged as the major interaction partner for KKXX motifs in a combinatorial screening approach (43). Interestingly, a KKXX motif did not function as a strong ER retrieval motif in this setting. The inconsistencies in identifying a single COPI subunit for dilysine motif binding might be explained by the finding that there are probably at least two dilysine binding sites on the assembled coatomer I complex that might not necessarily lie on the same subunit (44).

Genetic screens in yeast revealed that disruption of SAC1 in a mutant background of SEC21 aggravated the SEC21 phenotype, whereas other mutants in the secretory pathway (like SEC14) were suppressed by a mutant SAC1 allele (24). Sec21p is the yeast homologue of γ-COP. It is, however, currently unknown whether the C-terminal motif of ySac1p (PLKRD), lacking a lysine at the −4 or −5 position, is able to interact directly with Sec21p or other members of the yeast coatomer I complex.

Dynamics of ER/Golgi Localization—It is surprising that we could alter the subcellular distribution of hSAC1 by inactivating its PtdIns phosphatase function. Accumulation of the hSAC1-C/S mutant in the Golgi could be explained by a loss in COPI binding and might reflect that wild type hSAC1 usually functions by assuring its retrograde transport from the Golgi to the ER. The question remains why then inactivation of PtdIns phosphatase activity causes a loss in COPI binding, especially since the cognate retrieval motif, KEKID, is present. It has been reported that α-COP binds PtdIns(3,4)P_2 and to a lesser extent PtdIns(3,4,5)P_3 and PtdIns(4,5)P_2 (other PtdIns derivatives were not tested (45)). Inactive hSAC1 could therefore cause an alteration of the phospholipid composition that directly or indirectly influences attraction of COPI subunits to their target membrane. In this case, hSAC1 would be an essential player in COPI vesicle generation and might impose an additional layer of regulation on top of GTP-bound ARF or the p23/p24 family of coatomer receptors (for a review, see Ref. 30).

This scenario might only apply to our experimental system if the number of hSAC1-C/S molecules per COPI vesicle is high enough to outnumber endogenous hSAC1wt proteins, since the latter might still generate the necessary PtdIns phosphates in the test cells used. In this regard, it is important to keep in mind that the hSAC1-C/S mutant does not act as a classical dominant negative, since it does not form heterodimers (Fig. 4). Determination of PtdIns levels at hSAC1-C/S-containing COPI vesicles might solve these questions, yet this is beyond the scope of this work.

On the other hand, hSAC1 might act as a classical transmembrane cargo without enzymatic impact on coatomer I formation. The fact that endogenous SAC1 protein interacts with members of the coatomer complex I can be taken as a strong support for the notion that hSAC1 does indeed use both TM domains and does acquire a “J”-like topology with the N terminus and the KKXX motif exposed to the cytoplasm. As such, COPI subunits might capture it and route it for antero- or retrograde mode of transport. Since mutation of the two key lysine residues in the hSAC1-K2A mutant disabled interaction with COPI and caused accumulation in the Golgi, the similar behavior of hSAC1-C/S might therefore imply that its KEKID motif is not effectively used. Since motifs of the KKXX type apparently are less efficiently retrieved than polypeptides with a KKXX motif (43), and since coatomer I offers more than two dilysine binding sites (see above), successful retrieval of hSAC1wt might depend on, for example, oligomerization to fully exploit otherwise weak COPI interaction. The failure of the C/S mutant to oligomerize might in this way be at the cost of its efficient retrieval. It is not clear at present whether the C/S mutation directly or indirectly affects the oligomerization competence of hSAC1. In this respect, it might be elucidating to introduce mutations into the leucine zipper of hSAC1 to assess whether this classical dimerization motif is actually used and whether it has an influence on COPI binding and/or subcellular localization.

Alternatively, inefficient binding of the hSAC1-C/S KKXX motif to COPI might be caused by removing it spatially from COPI access. Such removal might be induced by a mutant-specific conformational change with impact on the second TM domain. This impact could be profound in the sense that the whole second TM domain flips through the membrane, thereby rendering the KEKID motif luminal. Such alternative TM usage is reported for Escherichia coli lactose permease LacY, dependent on the phospholipid composition of the inner membrane (46), or other mammalian and viral proteins (47, 48). Alternatively, the impact might be subtle and solely move the second TM domain further into the bilayer. Interestingly, alternative TM predictions for the second TM domain (Fig. 1, data not shown) extend into aa 574, which would leave only 8 amino acids between the bilayer and the KEKID motif, a distance reported to be too short to allow ER retrieval of a KKXX-containing transmembrane protein (49).

We have started to shed new light on the function of the human homologue of ySac1p. We used ectopic expression of mutant proteins and learned about novel interaction partners and a dynamic distribution of hSAC1 between the ER and Golgi. It might also be very interesting to analyze whether other point mutations of ySac1p, like those contributing to multiple drug sensitivity, perform in a similar way in the human counterpart and how they might influence COPI binding or vesicle trafficking.

Acknowledgments—We are grateful to Viola Braschel, Jochen Drescher, Kai Zuckschwerdt, Jennifer Ihe, and Eva Strauss for excellent technical assistance. We thank Thomas Werner for gEGFP-A30 and Horst Ahorn and Erich Spielvogel for peptide synthesis. We are indebted to Peter Downes and Antje Adomeit for valuable discussions,
to Martin Stegmaier and Norbert Kraut for critical reading of the manuscript, and to Wolfgang Rettig for constant support.

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