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Essential role for diacylglycerol in protein transport from the yeast Golgi complex

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Abstract

Yeast phosphatidylinositol transfer protein (Sec14p) is required for the production of secretory vesicles from the Golgi. This requirement can be relieved by inactivation of the cytosine 5′-diphosphate (CDP)-choline pathway for phosphatidylcholine biosynthesis, indicating that Sec14p is an essential component of a regulatory pathway linking phospholipid metabolism with vesicle trafficking (the Sec14p pathway[1–6]). Sac1p (refs 7 and 8) is an integral membrane protein related to inositol-5-phosphatases such as synaptojanin[9], a protein found in rat brain. Here we show that defects in Sac1p also relieve the requirement for Sec14p by altering phospholipid metabolism so as to expand the pool of diacylglycerol (DAG) in the Golgi. Moreover, although short-chain DAG improves secretory function in strains with a temperature-sensitive Sec14p, expression of diacylglycerol kinase from *Escherichia coli* further impairs it. The essential function of Sec14p may therefore be to maintain a sufficient pool of DAG in the Golgi to support the production of secretory vesicles.

The Sec14p requirement for yeast Golgi function is bypassed by mutations in seven genes[1–6]. Recessive ‘bypass Sec14p’ mutations define genes encoding negative regulators of the Sec14p pathway. Examples include the structural genes for enzymes of the CDP-choline pathway, *KES1* and *SAC1* (refs 3, 4, 6, 7, 10). *SAC1* encodes an integral membrane protein (Sac1p) of the Golgi and endoplasmic reticulum, and Sac1p defects result not only in a ‘bypass Sec14p’ phenotype, but also: (1) a cold-sensitivity for growth; (2) a relief of specific yeast actin defects; and (3) a new inositol auxotrophy[6–8].

Sac1p shares primary sequence homology with the non-catalytic domains of phosphatidylinositol-4,5-bisphosphate ($PtdInsP_2$)-active inositol-5-phosphatases such as yeast Inp5p (J. York and P. Majerus; personal communication) and rat brain synaptojanin[9]. Sac1p also shares homology with ORF YNL325c, ORF106c and ORF YOR109w identified by the Yeast Genome Project. Although Sac1p-inactivating mutations involve residues conserved among these various homologues (Fig. 1a), deletion of the structural genes for

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Inp5p, ORF YNL325c, ORF106c or ORF YOR109w failed to 'bypass Sec14p'. Moreover, increased gene dosage of either ORF YNL325c or ORF106c failed to relieve the 'bypass Sec14p' phenotype associated with Sac1p dysfunction (not shown). These data indicate a unique functional interaction between Sac1p-and the Sec14p pathway.

[^{32}P]Orthophosphate radiolabelling experiments demonstrated altered inositol-sphingolipid metabolism in *sac1* strains. Yeast spingolipids are inositol-phosphoceramides whose headgroups are donated by phosphatidylinositol (PtdIns) in two distinct DAG-producing reactions. The terminal PtdIns-hydrolytic reaction is contained within the Golgi complex[11 – 13] (Fig. 1b). Remarkably, *sac1* strains experienced a sixfold increase in flux through the inositol-sphingolipid pathway with a commensurate accumulation of mannosyldiinositoldiphosphorylceramide (M(InsP)$_2$C), the most highly phosphoinositolylated sphingolipid form (Fig. 1c). The altered inositol sphingolipid metabolism experienced by *sac1* strains, that is itself accompanied by accelerated rates of Golgi PtdIns turnover (as shown by the overproduction of M(InsP)$_2$C; Fig. 1c), provides the first demonstration that Sac1p regulates inositol phospholipid metabolism[6,8]. The homology of Sac1p with inositol phospholipid-5-phosphatases is independently consistent with the data.

To examine further the relationship between inositol-sphingolipid metabolism and the 'bypass Sec14p' condition, we took advantage of a particular Sac1p dysfunction caused by the *sac1–22* allele. This Sac1p defect is unique on two counts. First, it does not render yeast auxotrophic for inositol. Second, the accelerated flux through the inositol-sphingolipid pathway in *sac1–22* yeast is inositol-dependent (not shown). Notably, the *sac1–22* allele corrected growth defects resulting from Sec14p deficiency only when yeast were grown in the presence of inositol (Fig. 2). This inositol effect was Golgi-directed as indicated by *sac1–22*-mediated relief of vacuolar proteinase trafficking defects in Sec14p-deficient strains grown in inositol-replete, but not in inositol-free, media (not shown).

Inositol-sphingolipid biosynthesis generates two moles DAG per mole M(InsP)$_2$C[11 – 13] (Fig. 1b). [^{14}C]Acetate pulse-radiolabelling experiments demonstrate that *sac1–22* strains experienced a threefold elevation in bulk cellular DAG content relative to wild-type strains when growth medium contained inositol (the 'bypass Sec14p' condition). This differential in bulk DAG was eliminated when *sac1–22* and wild-type strains were grown in inositol-free medium (not shown): the condition that fails to support *sac1–22*-mediated 'bypass Sec14p' (see above), The relationship between DAG and Sec14p function was further strengthened by the demonstration that di-C8 DAG: (1) effected a 2.S-fold increase in invertase secretion efficiency at 37 °C in yeast strains producing a temperature-sensitive Sec14p (*sec14–1^{ts}*) (Fig. 3a), and (2) enhanced the efficiency with which the *sac1–22* allele improved secretion in Sec14p-deficient strains (Fig. 3a). These effects were specific because short-chain ceramide provided no such enhancement. Finally, di-C8 DAG did not improve invertase secretion in other *sec^{ts}* strains *(sec62–1^{ts}, sec18–1^{ts}, sec7–1^{ts}, sec4–1^{ts}* and *sec9–4^{ts}*).

The reciprocal test of a DAG requirement for yeast Golgi function was to determine whether DAG depletion exacerbated Sec14p defects. Thus, *E. coli* DAG-kinase[14] (DGK) was expressed in *sec14–1^{ts}* strains. As expected, such DGK expression effected elevation of bulk

membrane phosphatidic acid (PA) with an accompanying reduction in DAG (not shown). DGK expression clearly reduced the threshold growth temperature for yeast strains producing a temperature-sensitive Sec14p (Fig. 3b), thereby demonstrating that shunting of DAG to PA was detrimental to Golgi function. DGK expression-mediated enhancement of *sec14–1*^{ts} defects was also specific as it had no effect on other *sec*^{ts} strains (*sec62–1*^{ts}, *sec18–1*^{ts}, *sec7–1*^{ts}, *sec4–1*^{ts} and *sec9–4*^{ts}; not shown).

DGK expression was also predicted to compromise the ability of Sac1p deficiency to effect 'bypass Sec14p'. Constitutive expression of DGK from the powerful yeast phosphoglycerate kinase structural gene (*PGK*) promoter strongly diminished the ability of Sac1p-deficiency to alleviate Sec14p defects (Fig. 3c). Thus, contrary to the proposed action of PA as a downstream effector of ADP-ribosylation factor in a mammalian Golgi vesicle assembly pathway[15], DGK-mediated PA production impaired Sec14p-dependent Golgi function. These collective data indicate that reduction of Golgi DAG both: (1) specifically exacerbated Sec14p insufficiencies, and (2) impaired the ability of Sac1p deficiency to effect its usual 'bypass Sec14p' phenotype.

We suggest that Sec14p functions to preserve an essential Golgi DAG pool (Fig. 4). Sec14p-independent expansion of this DAG pool will result in 'bypass Sec14p'. In constructing models for how Sac1p dysfunction effects 'bypass Sec14p', particularly regarding the involvement of accelerated inositol sphingolipid biogenesis, we wish to address a topological consideration. Mammalian sphingolipids are assembled in the lumen of intracellular organelles[16]. If true in yeast, this topology would be seemingly inconsistent with the cytosolic residence of Sec14p; a disposition that limits Sec14p action (and presumably the critical DAG pool) to the cytosolic leaflet of Golgi membranes[1,3]. However, as DAG flip-flops at appreciable rates between membrane leaflets[17], even an expanded DAG pool generated in the lumenal leaflets of Sac1p-deficient Golgi membranes could potentially gain access to the cytosolic leaflet of Sec14p-deficient Golgi and effect 'bypass Sec14p'.

The concept of a Sec14p-dependent DAG pool has several attractive features. First, it identifies a biochemical signal required for progression through the Sec14p pathway. Second, it suggests unique and cooperative functions for both the PtdIns- and phosphatidylcholine (PtdCh)-bound forms of Sec14p in Golgi DAG maintenance (Fig. 4). Third, it accounts for the specific toxicity of the CDP-choline pathway to Golgi function on the basis of its DAG consumption properties[3]. By focusing on consumption of DAG by the CDP-choline pathway, this model shifts the emphasis from the notion of toxic accumulation of PtdCh; a tenet originally proposed to reconcile the opposing actions of Sec14p and the CDP-choline pathway on Golgi secretory function[3,4,6].

In yeast, DAG might represent a precursor for the genuine lipid effector of the Sec14p pathway (which is not PA; Fig. 3b, c). A downstream component of that pathway (for example, Keslp (ref. 10)) may serve as a Golgi DAG sensor to regulate secretory membranes so as to allow membrane transformation events required for secretory vesicle budding and scission reactions. These concepts emphasize the dynamic interface between phospholipid metabolism and Golgi function.

Finally, Sac1p is also required for efficient ATP transport into the endoplasmic reticulum lurnen[18]. This raises the issue of how loss of such an activity might relate to 'bypass Sec14p'. We found that endoplasmic reticulum microsomes prepared from *sac1–22* yeast strains exhibited wild-type rates of ATP import, and that over-expression of this defective Sac1p gene product resulted in a proportional overproduction of ATP import activity. Yet, over-expression of the *sac1–22* gene product did not diminish the 'bypass Sec14p' phenotype normally associated with it (not shown). The uncoupling of 'bypass Sec14p' from *sac1*-associated ATP import defects indicates that the relationship between Sac1p and inositol phospholipid metabolism is directly relevant to Sec14p pathway activity whereas the ATP import function of Sac1p is not. □

Methods

Yeast strains.

Wild-type strain CTY182 (*Mat***a** *ura3–52, Δhis3–200, lys2–801, SEC14, SAC1*)*; Δsacl* strain CTY244 (CTY182 *Δsac1*)*; sec14–1^{ts}*, strain CTY1–1A (*Mat***a** *ura3–52 Δhis3–200, lys2–801, sec14–1^{ts}*); *sec14–1^{ts}, sac1–22* strain CTY165 *(Mat***a** *ura3–52, Δhis3–200, ade2–101, sec14–1^{ts}, sac1–22); sec14–1^{ts}, sac1–22*/YCp*SEC14* strain CTY947 (CTY165/YCp*SEC14*); *sec14–1^{ts}*, sac1–22, *Δ*cki strain CTY952 (CTY165 *Δ*cki1); *sec14–1^{ts}*, sac1–22, *Δ*kes1 strain CTY953 (CTY165 *Δkes1*); *SEC14*/YEp*URA3* strain CTY948 (CTY182/ YEp*URA3*); *SEC14*/YEpP_{INO1}*::DGK* strain CTY949 (CTY182/YEp$P_{INO1::}$*DGK*)*; sec14–1^{ts}*/YEp*URA3* strain CTY950 (CTY1–1A/YEp*URA3*); *sec14–1^{ts}*/YEp$P_{INO1::}$*DGK* strain CTY951 (CTY1–1A/YEp*URA3); sec14–1^{ts}*/YEpP_{INO1}*::DGK* strain CTY949 (*CTY182*/YEpP_{PGK}*::DGK*)*; sac1–22, sec14–1^{ts}*/YEp*URA3* strain CTY991 (CTY165/YEp*URA3*); *sac1–22, sec14-t^{ts}*/ YEpP_{PGK}*::DGK* strain CTY992 (CTYl65/YEpP_{PGK}*::DGK*).

Media and genetic techniques.

The minimal defined media and complex media (YPD; supplemented with glucose to a final concentration of2%) used in all of the experiments in this work have been described[4]. Yeast transformation[19] and gene disruption[20] methods are standard. The abilities of individual or combinatorial gene disruption mutations to effect 'bypass Sec14p' were assessed by plasmid shuffle as described in detail elsewhere[21]. Sac1p-inactivating mutations were recovered from *suc1* strains by gap repair[22].

Lipid analyses and quantification.

In experiments where phospholipid species were identified and quantified, cells were grown to mid-logarithmic phase in inositol-containing minimal medium and radiolabelled with [^{32}P]orthophosphate for 20 min at 25 °C[4]. Lipids were extracted and resolved by two-dimensional paper chromatography using previously described solvents[11,12]. Extracts derived from equal numbers of cells were loaded on each chromatogram, and specific [^{32}P]-radiolabelled phospholipids were identified, excised, and quantified by scintillation counting[4].

Radiolabelled acetate was used in experiments where DAG and PA were monitored. To this end, cells were grown to mid-logarithmic growth phase (absorbence at 600 nm = 0.6–1.0) in

minimal medium with or without inositol and radiolabelled with [2-^{14}C]-acetate (2 μCiml^{-1}) for 20 min at 25 °C with shaking[4]. DAG was resolved by thin-layer chromatography as previously described[23,24]. [^{14}C]-Radiolabelled lipids were quantified by phosphorimaging[4].

Invertase secretion index.

Yeast strains were grown in YPD medium[4] at 25 °C and incubated with 200 μM 1,2-dioctanyl-*sn*-glycerol (diC_8-DAG) or di-C_6-ceramide in dimethylsulphoxide (DMSO) for 15 min before simultaneous shift to 37 °C (to impose Sec14p deficiency) and low glucose (0.1 %) YPD (to induce synthesis of secretory invertase). Mock controls were treated with the same amount of DMSO as diC_8-DAG-challenged cultures. Invertase secretion indices were determined as described[25].

Construction of P_{INO}::*DGK and* P_{PGK}::*DGK*.

Polymerase chain reaction (PCR) technology (using the appropriate primers and *E. coli* genomic DNA as template) was used to place expression of the *E. coli DGK* gene under yeast promoter control. The primers were designed to flank the entire *DGK* open reading frame, including the cognate initiating ATG and terminating TAA codons, and were clamped with *Hin*dIII and *Sph*I sites at the 5′ ends of the forward and reverse primers, respectively. The resulting PCR fragment (representing *DGK* nucleotide sequence from −1 to +337; ATG initiation codon defining base +1) was digested with *Hin*dIII and *Sph*I and ligated into the corresponding half-sites of pRE247 (ref. 26) to generate plasmid pRE626. The *INO1* and *PGK* promoters were PCR-amplified from total yeast genomic DNA such that the amplified promoters were flanked with an upstream *Eco*RI site and a downstream *Hin*dIII site, respectively. The promoter fragments (*INO1* bases −330 to −1; *PGK* bases −840 to −1) were restricted with *Eco*RI and *Hin*dIII and individually ligated into the cognate half-sites of pRE626. The corresponding *DGK* expression cassettes were then excised as *Eco*RI-*Sph*I restriction fragments and ligated singly into the multi-copy yeast shuttle vector YEp1ac195 (ref. 27) generating pCTY84 (P_{INO1}::*DGK*) and pCTY85 (P_{PGK}::*DGK*). Translational termination of the expressed DGK polypeptide is, in both cases, mediated by the native *DGK* termination codon.

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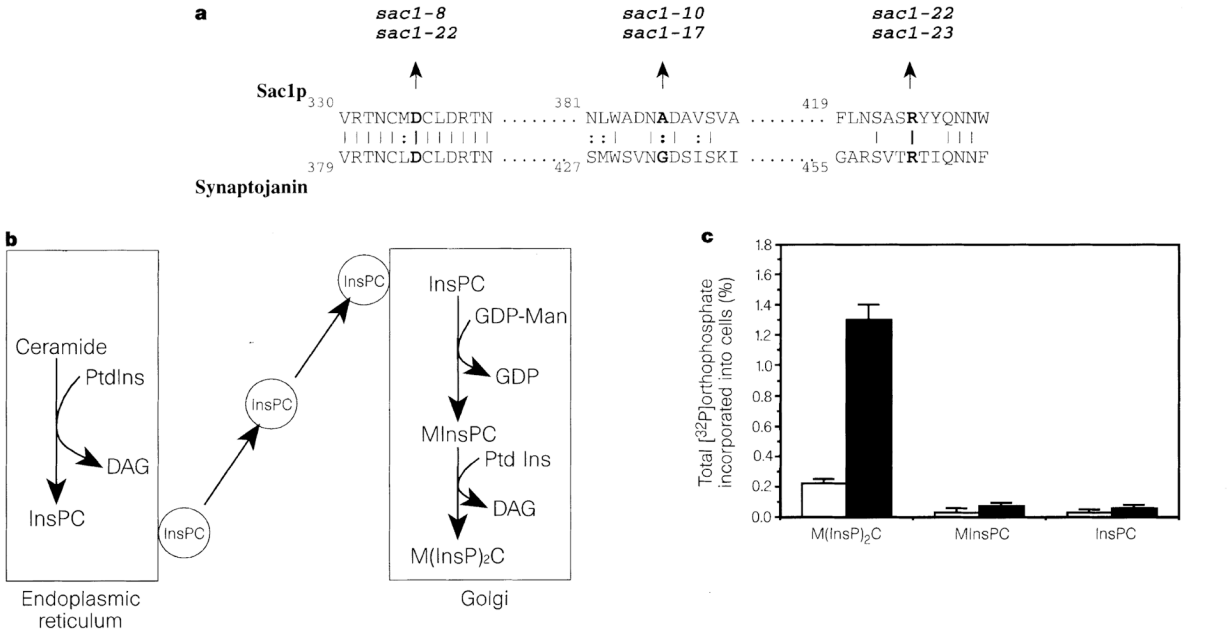

Figure 1.

a Sac1p-inactivating mutations. Designations for *sac1* mutations expressing full-length, but nonfunctional, Sac1p[7,8] are indicated at top with the corresponding amino-acid substitutions. Cognate regions of rat brain synaptojanin are given. Identities (vertical lines) and similarities (double dots) are shown. Mutations represent: $D_{337}N$, GAT to AAT; $A_{337}V$, GCA to GTA; and $R_{425}H$, CGT to CAT. **b**, Yeast inositol-sphingolipid pathway[11–13]. Ceramide is decorated with phosphoinositol donated by phosphatidylinositol (PtdIns) in the endoplasmic reticulum to generate inositolphosphorylceramides (InsPC) and diacylglycerol (DAG). InsPCs are mobilized through transport vesicles to the Golgi. InsPC is mannosylated, with GDP-mannose (GDP-Man) as sugar donor, to yield mannosylinositolphosphorylceramide (MInstPC). Some MInsPC is further phosphoinositolylated in the Golgi to yield mannosyldiinositoldiphosphorylceramide ($M(InsP)_2C$); generating one mol Golgi DAG per mol $M(InsP)_2C$. **c**, Quantification of inositol sphingolipids as a percentage of total [^{32}P] incorporated into cells from [^{32}P]orthophosphate pulse-radiolabelling experiments ($n > 3$) is presented (wild-type, empty bars; *sac1–22*, black bars). The *sac1–22* strain exhibited 70% of wild-type efficiency for [^{32}P] incorporation (per cell basis), and the marked increase in bulk $M(InsP)_2C$ was also readily measured in steady-state radiolabelling experiments (not shown). Strains used: wild-type, CTY182; *sac1–22*, CTY165 (see Methods for detailed genotypes).

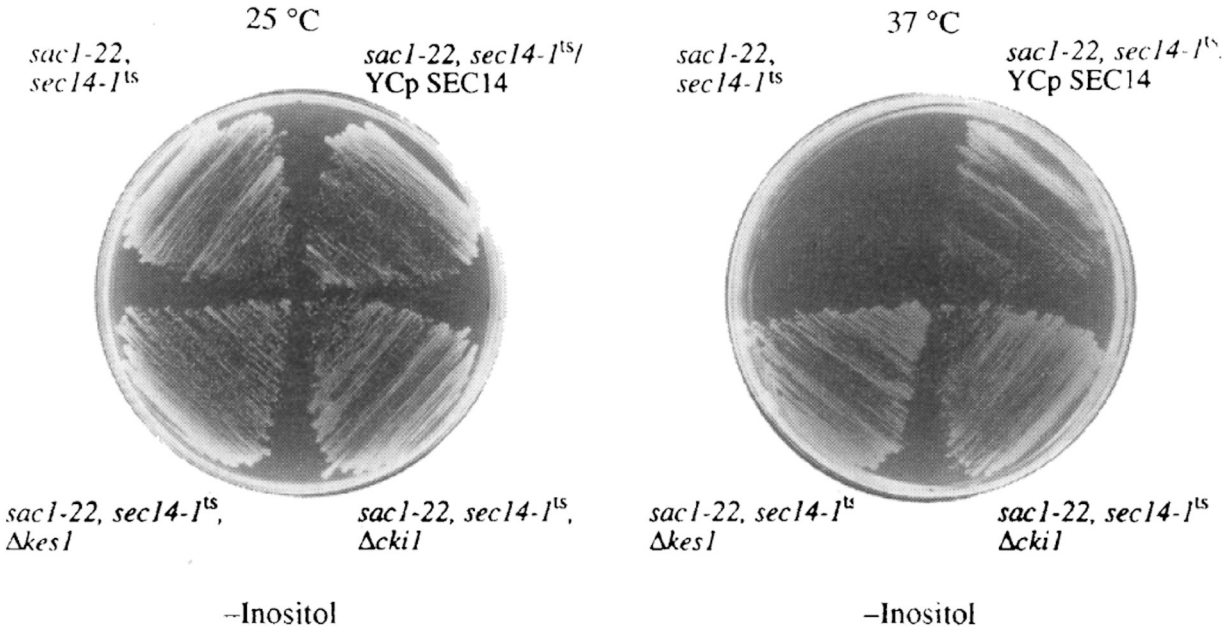

Figure 2.

sac1–22 fails to effect 'bypass Sec14p' when inositol is withheld from the medium. Yeast were incubated at the indicated temperatures for 96 h. *sac1–22, sec 14-1ts* strains failed to grow in '-Inositol' medium at a restrictive temperature for *sec14–1ts* (37 °C), but exhibited inositol protrophy at a permissive temperature (25°C). Temperature-sensitive inositol auxotrophy of *sac1–22, sec14-/1ts* strains was remedied by *SEC14* (for example, YCp(*SEC14*)) or by other 'bypass Sec14p' mutations, such as *Δcki1* and *Δkes1*, the structural genes for choline kinase and a yeast homolgoue of human oxysterol binding protein[3,10]. Strains used: *sec14– 1ts, sac1 −22*. CTY165; *sec14–1ts, sac1–22/*YCp*SEc14*, CTY947; *sec14–1ts, sac1–22, Δcki1* CTY952; *sec 14–1ts, sac1–22, Δkes1*, CTY953 (see Methods for detailed genotypes).

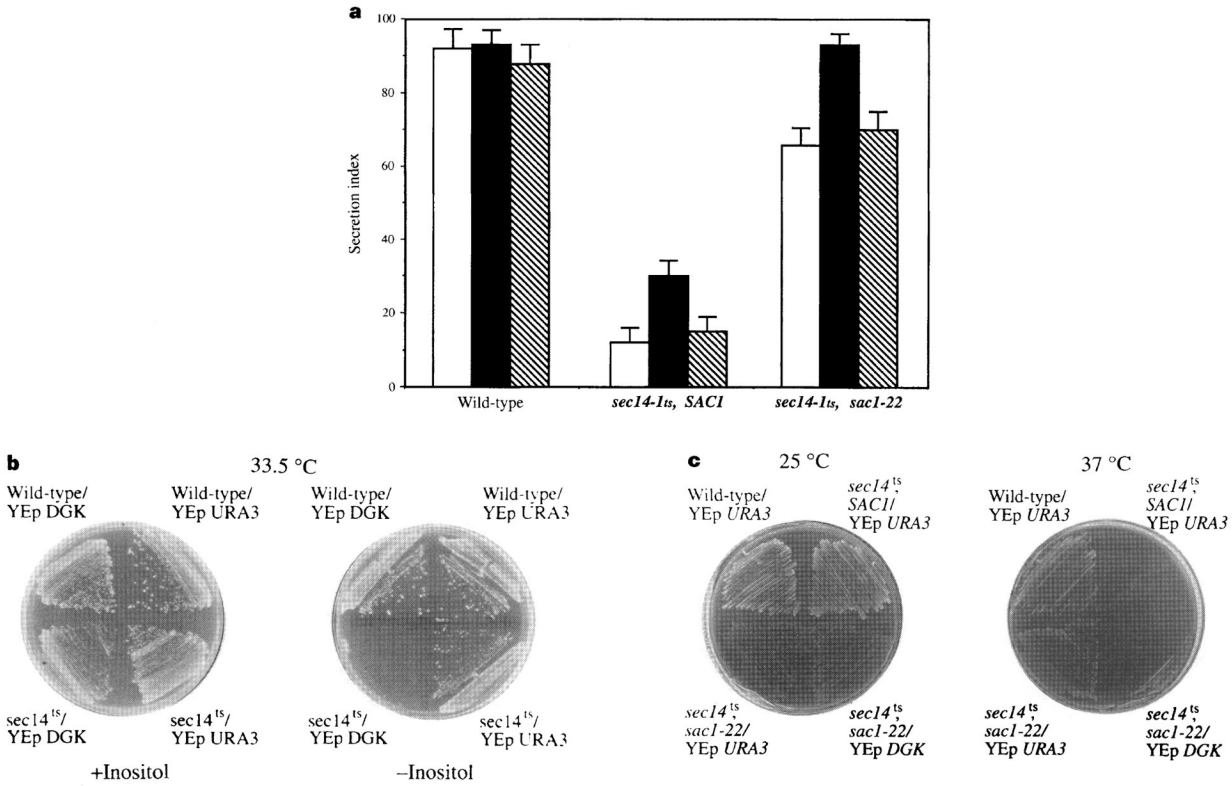

Figure 3.

a, Invertase secretion as a function of short-chain DAG. Wild-type and *sec14–1^{ts}* strains indicate secretory efficiency (as measured by secretion index at 37°C[25]) under Sec14p-proficient and deficient conditions, respectively. *sac1–22, sec14–1^{ts}* strains exhibited an intermediate secretion index. These controls are indicated by empty bars. Exogenous di-C_8 DAG (200 μM; black bars) stimulated secretion from *sec14–1^{ts}* and *sac1–22, sec14–1^{ts}* strains at 37°C, but had no effect on wild-type strains (n = 5). Stimulation was significant (99% confidence level by Student's t-test). Di-C_6 ceramide(200 μM; hatched bars) failed to stimulate secretion. Strains used: wild-type, CTY182;*sec 14–1^{ts}*, CTY1–1A;*sec14–1^{ts}* ,*sac1–22*, CTY165 (see Methods for detailed genotypes). **b**, DGK exacerbates *sec14–1^{ts}* defects. Yeast were transformed with the insert-less parental plasmid (YEp*URA3*). Transformants were streaked for isolation on minimal defined medium with inositol (+Inositol; DGK expression repressed) or without inositol (-Inositol; DGK expression derepressed), and incubated at 33.5 °C for 96 h. This temperature is permissive for both *sec14–1^{ts}* and *sec14–1^{ts}*/YEpP_{INO1}::*DGK* strains that do not express DGK. DGK expression prohibits growth of *sec14–1^{ts}* strains at 33.5 °C, indicating enhancement of *sec14–1^{ts}* defects. Strains used: wild-type / YEp*URA3*, CTY948; wild-type/YEp*DGK*, CTY949; *sec14–1^{ts}* /YEp*URA3*, CTY950; sec *14–1^{ts}* / Yep*DGK*, CTY951 (see Methods for detailed genotypes). c, DGK compromises suppression of *sec14–1^{ts}* by *sac1–22*. Yeast were transformed with a plasmid driving DGK expression from the powerful and constitutive yeast *PGK* promoter (YEpP_{PGK}::*DGK*), or with the insert-less parental plasmid (YEp*URA3*). Transformants were incubated on glucose minimal medium lacking uracil at 25 °C or 37 °C for 72h. DGK expression rendered *sac1–22, sec14–1^{ts}* strains incapable of growth at 37°C, a

nonpermissive temperature for *sec14–1*^{ts}. *SEC14* strains expressing DGK grew well at 37 °C. YEp*URA3* did not compromise growth of *sac1–22, sec14–1*^{ts} strains. Strains used: wild-type /YEp*URA3*, CTY948; *sec14–1*^{ts} , *SAC1/*YEp*URA3*, CTY950; *sec14–1*^{ts} , *sac1–22/* YEp*URA3*, CTY991; *sec14–1*^{ts}, *sac1–22*/YEp*DGK*, CTY992 (see Methods for detailed genotypes).

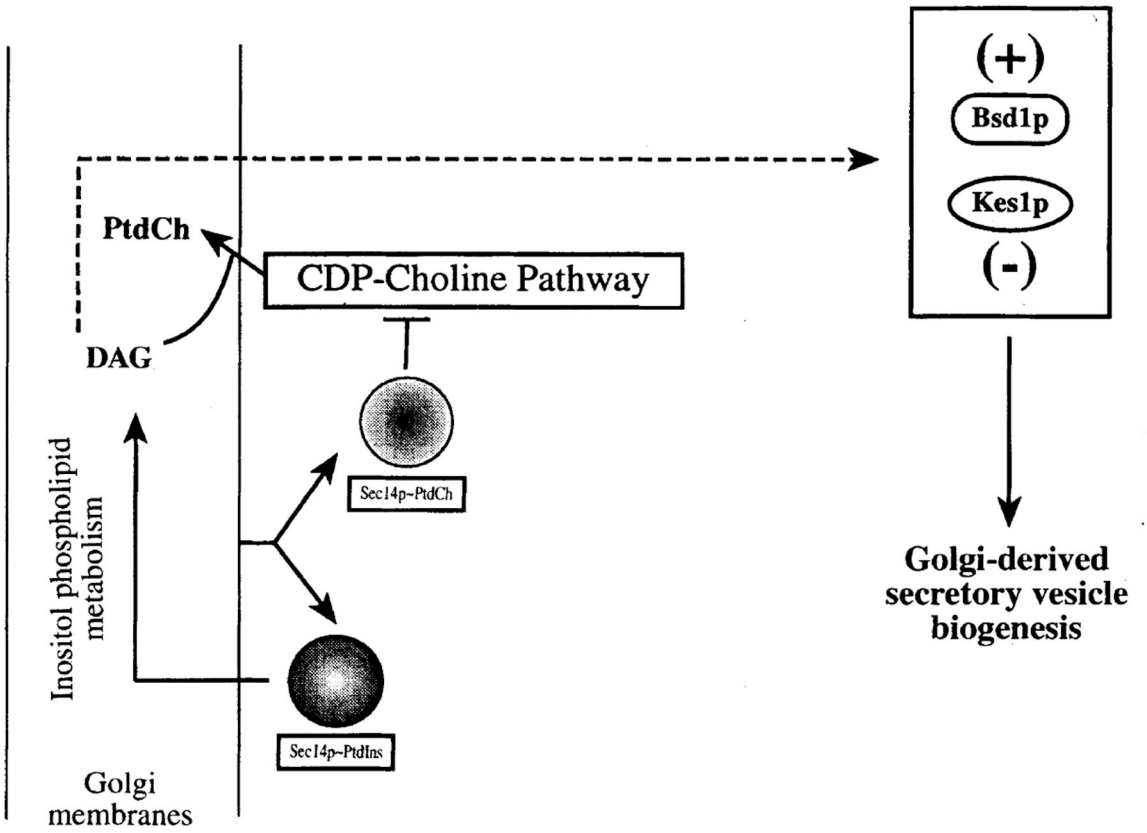

Figure 4.

Sec14p preserves Golgi DAG. PtdCh-bound Sec14p (Sec14p~PtdCh)·negatively regulates Golgi CDP-choline pathway activity and prevents DAG consumption by this PtdCh biosynthetic pathway[4,5]. PtdIns-bound Sec14p (Sec14p~PtIns) stimulates PtdIns metabolism through which Golgi DAG may be replenished. This latter action of Sec14p-PtdIns is consistent with the requirement for PtdIns-transfer activity in mammalian PtdInsPs-mediated rescue of *sec14–1*^{ts} defects[29], and with accelerated PtdIns-turnover representing a mechanism for 'bypass Sec14p'[30]. Downstream regulators of the Sec14p pathway, the *BSD1* and *KES1* gene products (positive (+) and negative (−) effectors in the Sec14p pathway, respectively[10]) represent candidate DAG--responsive activities.