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ATP-dependent transport of organic anions in secretory vesicles of *Saccharomyces cerevisiae*

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ABSTRACT **Secretory mutants (*sec1*, *sec6*) of *Saccharomyces cerevisiae* accumulate large pools of secretory vesicles at the restrictive temperature (37°C) because of a block in the delivery of vesicles to the cell surface. We report that secretory vesicles isolated from *sec* mutants exhibit ATP-dependent uptake of two classes of organic anions that are substrates for the canalicular carriers of mammalian liver. Transport of the bile acid taurocholate (TC) and the glutathione conjugate of 1-chloro-2,4-dinitrobenzene (GS-DNP) into vesicles was temperature dependent and saturable and required ATP and Mg^{2+}. Estimates of K_m and V_{max} were 177 μM and 1.2 nmol·min^{-1}·mg^{-1} and 262 μM and 0.53 nmol·min^{-1}·mg^{-1} for TC and GS-DNP, respectively. TC and GS-DNP did not compete for transport. TC transport was sensitive to vanadate and 4,4′-diisothiocyanostilbene-2,2′-disulfonate, inhibited by glycocholate, and retained partial activity when UTP and GTP, but not nonhydrolyzable ATP analogues, replaced ATP. Dissipation of the electrochemical potential with a nitrate buffer and ionophores partially decreased (30–40%) the transport of both anions. Direct testing of the influence of membrane potential was performed in *sec6-4* mutants, in which the expression of electrogenic [H$^+$]ATPase activity is reduced by >85% in glucose-containing medium. Vesicles from *sec6-4* retained full activity for ATP-dependent TC and GS-DNP transport. These results indicate that the transporters operate independently of the membrane potential and that ATP is required. These findings reveal that yeast possess separate ATP-dependent transport mechanisms for elimination of bile acids and glutathione conjugates. The mechanisms are functionally similar to those present in mammalian systems.**

Animals eliminate many exogenous and endogenous substances as anionic conjugates of glutathione (GSH) or glucuronic acid by excreting them into bile by using ATP and membrane potential-dependent transport mechanisms (1–6). Bile acids, the major organic anion in bile, have separate transport systems that also use ATP and transmembrane potential as driving forces (7–11). The regulation of these carriers has not been studied, nor have they been purified or cloned, in part because of their low abundance in the canalicular membrane.

Yeast has proven to be a useful model in which to study transport and secretory processes in higher eukaryotes. Several proteins homologous to the mammalian ATP-binding cassette family of membrane proteins (12) have been identified in *Saccharomyces cerevisiae* (13–15), and, in some cases, mammalian genes have complemented the function of yeast genes (16). Temperature-sensitive secretory (*sec*) mutants of *S. cerevisiae* have been used to dissect the stages of the secretory pathway (17, 18), the organization of which has been conserved in lower eukaryotic and mammalian systems (19). *sec1* and *sec6* mutants exhibit a useful phenotype in that secretory vesicles accumulate at the nonpermissive temperature (37°C) because of a block in delivery and fusion with the plasma membrane (17, 18). Walworth and Novick (20) purified these secretory vesicles and showed that they cotransport newly synthesized proteins destined for the plasma membrane ([H$^+$]ATPase) and for secretion (invertase). Nakamoto *et al.* (21) subsequently showed that membrane-bound proteins are oriented in the membrane of these sealed vesicles such that the catalytic domain is extravesicular with the substrate transported inwardly and that the vesicles are competent for transport studies. Recently, *sec6-4* mutants were used as an expression system for the mammalian multidrug resistance genes and the ATP-dependent transport properties of the gene products were functionally characterized in the isolated secretory vesicles (22).

We have discovered two unusual ATP-dependent transport activities in secretory vesicles of *S. cerevisiae* that result in the intravesicular accumulation of bile acids and GSH conjugates. Although the natural substrates for these transporters and the implications for yeast physiology are not known, the discovery of these carrier systems in yeast may lead to purification and cloning of the elusive mammalian canalicular organic anion transporters.

MATERIALS AND METHODS

Materials. Zymolyase 100T was from ICN; bafilomycin was from Wako BioProducts (Richmond, VA); [^{3}H]taurocholate (TC) (specific activity, 2.6 Ci/mmol; 1 Ci = 37 GBq) and [^{3}H]GSH (specific activity, 43.8 Ci/mmol) were from NEN/DuPont. ^{3}H-labeled *S*-(2,4-dinitrophenyl)glutathione ([^{3}H]GS-DNP) was synthesized enzymatically (16) and specific activity was 1.0 Ci/mmol.

Yeast Cells and Isolation of Secretory Vesicles. The *sec1* mutant strain NY3 (*MATa*, *ura3-52*, *sec1-1*), provided by Peter Novick (Yale University), was grown in 1% Bacto-yeast extract and 2% Bacto-peptone (Difco) and 2% glucose. The *sec6* mutant strain SY4 (*MATa*, *ura3-52*, *leu2*, *his4*, *sec6-4*, *GAL*, *pma1::YIpGAL-PMA1*), obtained from Carolyn Slayman (Yale University), was grown in minimal medium with 2% galactose as described (21). The SY4 strain has its chromosomal PMA1 gene, which encodes the plasma membrane [H$^+$]ATPase, under control of the inducible GAL1 promoter (21). In the presence of galactose, SY4 cells maintain normal [H$^+$]ATPase activity, whereas in glucose-containing medium, expression of the chromosomal PMA1 gene is repressed and the [H$^+$]ATPase activity is reduced by 95% (21). NY3 and SY4 were grown at 25°C to a cell density of 2 OD_{600} units and then subjected to a secretory block (37°C) for 2 hr (17) in galactose or glucose medium to accumulate

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Abbreviations: TC, taurocholate; GS-DNP, *S*-(2,4-dinitrophenyl)-glutathione; GSH, glutathione; AMP-CPP, adenosine 5′-[α,β-methylene]triphosphate; AMP-PCP, adenosine 5′-[β,γ-methylene]-triphosphate; FCCP, carbonylcyanide *p*-trifluoromethoxyphenylhydrazone; CCCP, carbonylcyanide *m*-chlorophenylhydrazone.

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secretory vesicles. Cells were harvested, washed in 10 mM NaN_3, and stored (−80°C). Secretory vesicles were prepared according to a method modified from Nakamoto *et al.* (21). Briefly, cells were digested with zymolyase 100T (1 mg per g of cells) in 1.2 M sorbitol/20 mM Hepes/Tris/1 mM $MgCl_2$, pH 7.5. Spheroplasts were incubated with concanavalin A (15 min) and resuspended in 0.6 M sorbitol buffer containing 2 mM phenylmethylsulfonyl fluoride and pepstatin, leupeptin, and aprotinin (1 μg/ml) and disrupted by Dounce homogenization. After centrifugation at 10,000 × *g*, the supernatant was centrifuged at 105,000 × *g* and the resulting pellet was resuspended in one of three buffers at pH 7.3 (250 mM sucrose/10 mM Tris/Hepes, 50 mM sucrose/100 mM potassium gluconate/10 mM Hepes/Tris, or 50 mM sucrose/100 mM potassium nitrate/10 mM Hepes/Tris) and used immediately.

Transport Assays. Transport was measured by rapid filtration in a medium containing 250 mM sucrose, 10 mM Hepes/Tris, 5 mM MgATP, 10 mM phosphocreatine (Tris salt), and 20 units of creatine phosphokinase per ml. In studies designed to increase the electrochemical potential, the buffer contained 50 mM sucrose and 100 mM potassium gluconate; to dissipate the transmembrane potential, gluconate was replaced with the permeant NO_3^- anion. For TC transport, medium contained [^{3}H]TC (3 μCi/ml), supplemented with 5–1000 μM unlabeled TC, sodium salt; GS-DNP transport was assayed with [^{3}H]GS-DNP (1 μCi/ml) supplemented with unlabeled GS-DNP (1–800 μM).

Transport was initiated by adding 10 μl of vesicle suspension to 90 μl of transport solution at 37°C and then terminated at 0, 0.5, 1, 2, 5, 10, and 30 min by dilution with 2.5 ml of cold stop buffer (100 mM KCl/50 mM sucrose/10 mM Hepes/Tris, pH 7.4) and filtering through nitrocellulose filters (0.22 μm; Millipore) presoaked in the stop buffer. Filters were rinsed with 5 ml and radioactivity was measured in a liquid scintillation counter. Protein was determined by the BCA assay method (Pierce).

Transport of both substrates was linear with time for the first minute. Initial rates (pmol per min per mg of protein) represent uptake measured at 1 min, after correction for nonspecific binding of TC and GS-DNP by subtracting values measured at 0 min. The latter values did not exceed 15 pmol/mg for TC and 10 pmol/mg for GS-DNP. Data represent triplicate analyses of two to four separate preparations and are presented as means ± SD.

RESULTS

Transport of TC and GS-DNP into secretory vesicles of *sec1* mutants was temperature dependent and required ATP and Mg^{2+} (Fig. 1; Table 1). Accumulation was maximal at 10 min and slightly lower at equilibrium (30 min). In the presence of the permeant NO_3^- anion, which lowers the membrane potential in secretory vesicles (21, 22), the initial rates of TC and GS-DNP transport were reduced by 30–40% over control, whereas with the impermeant gluconate anion, a slight increase (20–28%) was observed (Table 1). The transport of both substrates was saturable (Fig. 2). The estimated K_m and V_{max} values for TC were 177 μM and 1.2 nmol per min per mg of protein, respectively; the K_m and V_{max} values for GS-DNP were 262 μM and 0.53 nmol per min per mg of protein. Competition experiments between GS-DNP and TC, presented as a Dixon plot (Fig. 3), showed that transport was not competitive, which suggests that separate carriers exist for each of these anions.

Table 1. Effect of assay conditions on transport of TC and GS-DNP

	Initial transport rate	
	TC	GS-DNP
Effect of anions, pmol·min^{-1}·mg^{-1}		
Control*	41.2 ± 3.1	15.6 ± 3.6
+ potassium gluconate†	52.8 ± 10.1	18.6 ± 1.6
+ potassium nitrate‡	24.9 ± 5.0§	9.7 ± 3.3§
Effect of Mg^{2+}, % of control		
Control + MgATP‡	100	100
+ ATP, − Mg^{2+}	14.6 ± 6.6	12.2 ± 2.2
Effect of temperature, % of control		
37°C	100	100
4°C	5.1 ± 0.8	14.2 ± 2.2

Vesicles were incubated in transport medium as described with 5 mM MgATP and an ATP-regenerating system and 5 μM TC or 1 μM GS-DNP. Data are means ± SD.
*Transport assays performed in sucrose/Hepes buffer.
†Transport assays performed in gluconate buffer.
‡Transport assays performed in nitrate buffer.
§$P < 0.05$ compared to control.

TC (100 μM) uptake was inhibited by increasing concentrations (100–500 μM) of glycocholate (data not shown), suggesting that a single carrier mediates transport of different conjugated bile acids.

The effect of osmolarity on the transport process was tested by adding increasing concentrations of raffinose to the medium. The uptake of TC decreased linearly ($r^2 = 0.92$) with increasing osmolarity (data not shown), which verifies that transport occurs into an osmotically active space.

The relative increase and decrease in transport seen in the presence of gluconate and nitrate anions, respectively, prompted a more direct examination of the role of the transmembrane electrochemical potential as a driving force

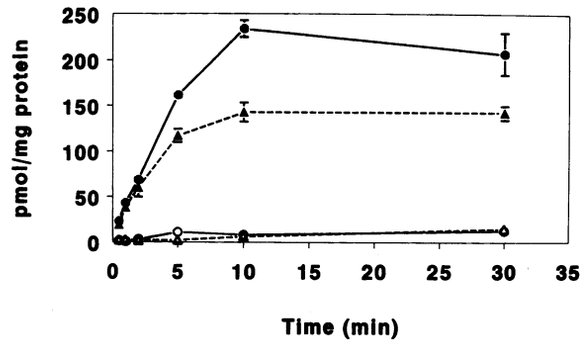

FIG. 1. Uptake of 5 μM TC (● and ○) and GS-DNP (▲ and △) into secretory vesicles of *sec1* in the presence (● and ▲) and absence (○ and △) of 5 mM MgATP and an ATP-regenerating system in sucrose/Hepes buffer. Values are means ± SD (except where smaller than symbols) of three replicate determinations.

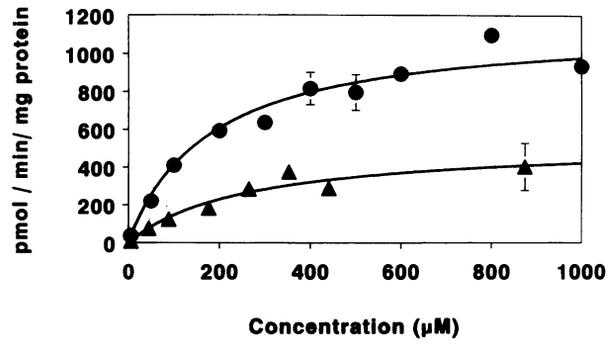

FIG. 2. Initial transport rate (means ± SD, except where smaller than symbols) of TC (●) and GS-DNP (▲) in secretory vesicles of *sec1* as a function of substrate concentration. Lines were generated by computer fitting the Michaelis–Menten equation to the data using the RS/1 program (BBN Software Products, Cambridge, MA).

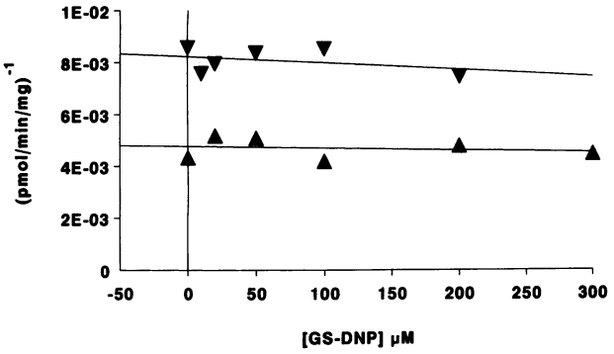

FIG. 3. Dixon plot of transport rate of 50 μM (▼) and 100 μM (▲) TC into secretory vesicles of *sec1* in the presence of increasing concentrations of GS-DNP. Transport of TC and GS-DNP was not competitive.

in the accumulation of TC and GS-DNP within vesicles. Transport was measured in vesicles isolated from *sec6* (SY4) mutants maintained in galactose medium during the temperature shift ($SY4_{GAL}$) and from *sec6* mutants that were transferred to glucose medium during the temperature shift ($SY4_{GLU}$). Vanadate-sensitive ATPase activity in $SY4_{GLU}$ was decreased by 85% compared to that from $SY4_{GAL}$ (data not shown). Such a low expression of electrogenic $[H^+]$ATPase activity in $SY4_{GLU}$ decreased the membrane potential in secretory vesicles by 90% (22). The transport of either anion in the *sec6* mutant was not affected by the large decrease in $[H^+]$ATPase activity (Fig. 4).

The mechanism of transport of TC was further characterized by testing the nucleotide specificity of the process and the effect of several inhibitors. UTP and GTP partially substituted for ATP but no transport occurred in the presence of nonhydrolyzable ATP analogues, adenosine 5′-[α,β-methylene]triphosphate (AMP-CPP) and adenosine 5′-[β,γ-methylene]triphosphate (AMP-PCP), or AMP (Table 2). Transport of TC was sensitive to 4,4′-diisothiocyanostilbene-2,2′-disulfonate, an anion transport inhibitor, and to 200 μM *N*-ethylmaleimide. Vanadate and diethylstilbestrol, which inhibit phosphorylated (P-type) ATPases (23–25), also partially inhibited transport (Table 2). Bafilomycin, a specific V-type ATPase inhibitor (26), had no effect. When the pH gradient was dissipated with NH_4Cl (21), uptake of TC was unaffected (Table 2).

Additional studies were done to test the influence of ionophores on the transport of TC (Table 3). The K^+/H^+ antiporter nigericin decreased maximal TC accumulation (measured at 10 min) by 40% in gluconate but not in nitrate medium. The protonophores carbonylcyanide *p*-trifluoromethoxyphenylhydrazone (FCCP) and carbonylcyanide *m*-chlorophenylhydrazone (CCCP) also inhibited transport by ≈30% but only in gluconate medium (Table 3).

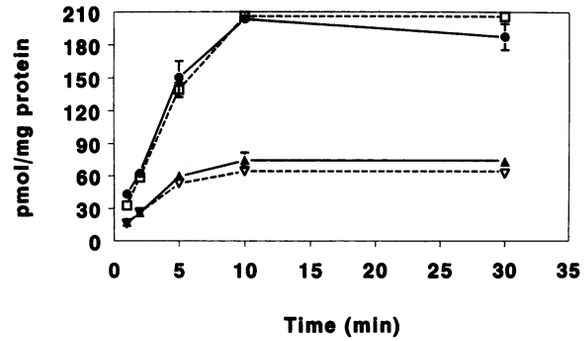

FIG. 4. Comparison of 5 μM TC (● and □) and 5 μM GS-DNP (▲ and ▽) transport in secretory vesicles of *sec6* (SY4) mutants expressing normal levels of $[H^+]$ATPase activity ($SY4_{GAL}$) (● and ▲) and in mutants with $[H^+]$ATPase activity decreased by 85% ($SY4_{GLU}$) (□ and ▽). Transport medium contained 5 mM MgATP and an ATP-regenerating system in sucrose/Hepes buffer. Values are means ± SD (except where smaller than symbols) of three replicate determinations.

Table 2. Effect of nucleotides and various inhibitors on initial transport rates of TC

	Initial transport rate, % of control
Nucleotide	
ATP (5 mM)	100
UTP (5 mM)	28 ± 6
GTP (5 mM)	33 ± 2
CTP (5 mM)	16 ± 0.5
AMP (5 mM)	6 ± 4
AMP-CPP (5 mM)	5 ± 1
AMP-PCP (5 mM)	7 ± 1
Inhibitor	
DIDS (200 μM)	18 ± 2
Vanadate (100 μM)	50 ± 7
Diethylstilbestrol (100 μM)	62 ± 10
N-Ethylmaleimide (200 μM)	63 ± 5
Bafilomycin (0.5 μM)	119 ± 19
NH_4Cl (2 mM)	90 ± 6

ATP was the magnesium salt; UTP, GTP, and CTP were the Tris salts; AMP-CPP was the lithium salt; and AMP and AMP-PCP were the sodium salts. $MgCl_2$ (5 mM) was added to transport media. Initial transport rates were measured at 1 min in sucrose/Hepes with 5 μM TC. DIDS, 4,4′-diisothiocyanostilbene-2,2′-disulfonate.

DISCUSSION

These data demonstrate saturable, ATP-dependent bile acid and non-bile acid organic anion transport in yeast. Related discoveries are the ATP-dependent accumulation of GSH *S*-conjugates (27) and bile acids (28) in plant vacuoles and a vacuolar phytochelatin Cd^{2+} transporter cloned in *Schizosaccharomyces pombe* (29) and shown to be a member of the ATP-binding cassette family of proteins (12). These findings indicate that lower eukaryotes may share the specialized membrane transport functions for organic anions that exist in mammalian systems. Transport in plants (27, 28) and yeast share common features: requirement for MgATP, sensitivity to vanadate, and separate transport activities for bile acids and GSH conjugates. A distinguishing feature is the small influence of the membrane potential noted in yeast vesicles but not in plant vacuoles.

A strong, interior positive membrane potential exists inside secretory vesicles of *S. cerevisiae* and vacuoles of plant and yeast cells due to electrogenic proton ATPases that pump H^+ inward (21, 30). The resulting electrochemical proton gradient is composed of a pH gradient and an electrical potential.

Table 3. Effect of ionophores on transport of TC

	Transport of TC, % of control ± SD			
	Potassium gluconate		Potassium nitrate	
Treatment	2 min*	10 min†	2 min*	10 min†
Nigericin (10 μM)	95 ± 10	61 ± 3	92 ± 14	106 ± 11
FCCP (10 μM)	62 ± 2	70 ± 6	87 ± 10	86 ± 4
CCCP (10 μM)	71 ± 4	66 ± 6	92 ± 6	103 ± 9

Transport of TC was measured in the transport medium indicated, with 5 μM TC, 5 mM MgATP, and an ATP-regenerating system.
*Calculated from accumulation of TC in vesicles at 2 min.
†Calculated from accumulation of TC in vesicles at 10 min.

In secretory vesicles of *sec6* mutants, addition of the permeant NO_3^- anion collapsed the electrical potential (21, 22) but the pH gradient remained intact (22). The transport of TC was lower in the presence of nitrate medium (Table 2), but the addition of nigericin, which decreases the pH gradient by 90% (22), was without effect (Table 3). In contrast, nigericin decreased transport of TC when present in gluconate medium (Table 3). These data imply that the electrical potential and not the pH gradient influences transport. This is consistent with the negligible effects of NH_4Cl and of FCCP and CCCP with KNO_3 (Tables 2 and 3). Because one cannot discount that a high concentration of nitrate anion and ionophores may have nonspecific effects, the influence of membrane potential was measured directly in *sec6* mutants that displayed an 85% decrease in [H^+]ATPase activity ($SY4_{GLU}$). ATP-dependent transport of both anions was equal to that observed in $SY4_{GAL}$, which expresses normal [H^+]ATPase activity (Fig. 4). Additional data show that ATP is the major force driving transport; UTP and GTP supported 30% of TC transport (Table 2), but the activity of the plasma membrane [H^+]ATPase pump decreased to <7% when ATP was replaced (31). In mammalian liver, experiments with canalicular membrane vesicles have shown that both membrane potential and ATP can act as driving forces for the transport of bile acids (9) and other organic anions (32). Whether one or more mammalian carriers exist for each of these transport activities remains unresolved.

These data indicate that ATP-dependent transporters for bile acids and other organic anions are present in yeast secretory vesicles and are functional within the vesicles that are en route to the plasma membrane. Whereas natural substrates for the transporters in yeast are unknown, these studies identify a mechanism whereby organic anions are eliminated. If the carriers in yeast prove to be homologous to those in animals, their discovery may lead to the purification, isolation, and cloning of mammalian equivalents.

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