ATP-dependent transport of reduced glutathione in yeast secretory vesicles

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Turnover of cellular reduced glutathione (GSH) is accomplished predominantly by export into the extracellular space; however, the plasma membrane transport mechanisms that mediate GSH efflux are not well characterized. The present study examined GSH transport using secretory vesicles isolated from the *sec*6-4 mutant strain of *Saccharomyces cerevisiae*. In contrast with studies in mammalian membrane vesicles, GSH transport in yeast secretory vesicles was mediated largely by an ATP-dependent, low-affinity pathway (K_m 19 ± 5 mM). ATP-dependent [^{3}H]GSH transport was *cis*-inhibited by substrates of the yeast YCF1 transporter, including sulphobromophthalein, glutathione S-conjugates and the alkaloid verapamil, and was competitively inhibited by *S*-(2,4-dinitrophenyl)glutathione (DNP-SG). Similarly, GSH competitively inhibited ATP-dependent [^{3}H]DNP-SG transport, with a K_i of 18 ± 2 mM, but had no effect on ATP-dependent [^{3}H]taurocholate transport. ATP-dependent GSH transport was not affected by either membrane potential or pH-gradient uncouplers, but was inhibited by 4,4′-di-isothiocyanatostilbene-2,2′-disulphonate, probenecid and sulphinpyrazone, which are inhibitors of mrp1 and mrp2, mammalian homologues of the yeast YCF1 transporter. Western blot analysis of the secretory vesicle membrane fraction confirmed the presence of Ycf1p. These results provide the first direct evidence for low-affinity, ATP-dependent transport of GSH, and demonstrate that this ATP-dependent pathway displays kinetic characteristics similar to those of the yeast YCF1 transporter.

INTRODUCTION

Glutathione (GSH) is ubiquitous in eukaryotic cells, and is the major intracellular thiol-containing compound and most abundant low-molecular-mass peptide [1]. Because GSH synthesis occurs in the cytosol, whereas its degradation occurs largely in extracellular spaces, transport across the cell membrane into the interstitial fluid [1], as well as into the intracellular vacuolar space of yeast [2], is required for normal cellular GSH turnover.

GSH transport mechanisms have been characterized in plasma membrane vesicles derived from mammalian liver, kidney and intestine [3], but the transporters have not been identified at the molecular level. Putative clones for hepatic sinusoidal and canalicular GSH transporters were reported by Yi et al. [4,5], but these findings have not been reproduced [6] and appear to be an artifact.

In membrane vesicles derived from mammalian tissues, GSH transport was found to be mediated by ATP-independent, bidirectional systems [3]. Three GSH-efflux mechanisms have been reported in rat hepatocytes: a low-affinity system on the sinusoidal membrane [5,7], and two systems on the canalicular membrane [8], one of low affinity and high capacity ($K_m \approx 16$ mM) and one of high affinity and low capacity ($K_m \approx 0.2$ mM). The low-affinity canalicular transporter appears to be relatively selective for GSH, whereas the high-affinity system may function in transporting glutathione S-conjugates, and perhaps other anions, into bile [8–10].

In contrast with the membrane vesicle studies, recent findings in intact cells suggest that GSH may also be transported by ATP-dependent mechanisms, including possibly the mrp1 and mrp2 transporters [11–14]. However, all of these studies were performed in intact cells or tissues, and none have directly documented ATP-dependent GSH transport. Depletion of GSH in mrp1-overexpressing cell lines led to increased accumulation of drugs such as daunorubicin, vincristine, rhodamine and others [15–21], whereas the transport of calcein, an organic anion, was insensitive to changes in GSH levels [16]. Paul et al. [22] demonstrated that daunorubicin transport by MRP1 was competitively inhibited by GSH; however, no such effect was observed for vincristine transport [18]. Current possibilities are that GSH can be co-transported with natural products [14] (although no increased GSH efflux was observed during daunorubicin transport), or that GSH facilitates transport by inducing either a conformational change in MRP1 [21] or possibly a change in redox status of the protein. A GSH-binding site may exist in MRP1 [23].

To test directly the possibility of ATP-dependent GSH transport, the present study utilized secretory vesicles isolated from the *sec*6–4 mutant strain of *Saccharomyces cerevisiae*. Yeast have an ABC protein (Yfc1p) that shows significant homology ($> 63\%$) with mammalian mrp1 [24] and mrp2 [25]. As found in mammalian cells, *S. cerevisiae* contain high intracellular concentrations of GSH (5–20 mM) [26], and turnover of the tripeptide is mediated predominantly by the ectoproteins γ-glutamyl transpeptidase and cysteinylglycine dipeptidase [27]. These cells should therefore also possess a transport mechanism for export of GSH, although this has not been previously examined. Moreover, yeast secretory vesicles are ideally suited for studies on efflux transporters, because they are of uniform morphology, tightly sealed, can be isolated in relatively high yield and, perhaps most importantly, because they are oriented inside-out. Thus uptake of a solute into the vesicle is equivalent to transport from the cell into the extracellular space, facilitating study of efflux transporters. Previous studies on yeast secretory vesicles have characterized ATP-dependent transport of glutathione S-conjugates and other organic anions (mediated by YCF1) [24] and ATP-dependent transport of bile acids (mediated by BAT1) [28,29]. The findings here indicate that YCF1 may also mediate low-affinity, ATP-dependent transport of GSH.

Abbreviations used: CCCP, carbonyl cyanide *m*-chlorophenylhydrazone; DNP-SG, *S*-(2,4-dinitrophenyl)glutathione; EDAC, 1-ethyl-3-(3-dimethylaminopropyl)carbodi-imide; ethyl-SG, *S*-ethylglutathione; S_1, slow-speed supernatant.

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EXPERIMENTAL

Materials

[Glycine-2-^{3}H]GSH (0.982 Ci/mmol) and [G-^{3}H]taurocholate (2.1 Ci/mmol) were purchased from DuPont NEN. [Glycine-2-^{3}H]*S*-dinitrophenyl glutathione was synthesized as described previously [9]. Gramicidin D and carbonyl cyanide *m*-chlorophenylhydrazone (CCCP) were purchased from ICN (Costa Mesa, CA, U.S.A.). Zymolyase-100T was purchased from Seikagaku America, Inc. (Rockville, MD, U.S.A.). *S*-Dinitrophenyl-*N*-acetylcysteine was synthesized as described previously [30]. All other chemicals and reagents were purchased from Sigma, Aldrich (Milwaukee, WI, U.S.A.), Amersham, J. T. Baker Inc. (Phillipsburg, NJ, U.S.A.) or Fisher Biochemicals (Itasca, IL, U.S.A.). The pH of incubation for solutions containing high concentrations of GSH or other anions was routinely adjusted to 7.5 with Tris base immediately before use.

Secretory vesicle isolation

The *sec6–4* mutant strain of *S. cerevisiae* was provided by Peter Novick (Yale University, New Haven, CT, U.S.A.). Secretory vesicles from cells were isolated by methods developed by Walworth and Novick [31] and Ruetz and Gros [32]. Briefly, NY17 (MAT*a*, *ura*3–52, *sec6–4*) yeast were grown at 25 °C with shaking (Forma orbital shaker, 225 r.p.m.) in a 50-ml culture of YPD medium [1 % (w/v) Bacto yeast extract/2 % (w/v) Bactopeptone/2 % (w/v) glucose] to stationary phase (starter culture). The starter culture was used to inoculate an 800-ml YPD medium fernbach culture, which was then grown under the same conditions to mid-exponential phase (D_{600} 1.6–2.0). Cells were pelleted and then resuspended in 800 ml of YPD medium with 0.2 % (w/v) glucose prewarmed to 37 °C to derepress invertase enzyme synthesis. Cells were grown at the non-permissive temperature of 37 °C for 2 h to accumulate secretory vesicles. NaN_3 (10 mM) was added to cells chilled in an ice bath, and 10 min later the cells were decanted to preweighed tubes and spun down at 8250 ***g*** in a Sorvall GSA rotor for 10 min. Cell pellets were washed with ice-cold 10 mM NaN_3, pelleted as before, weighed and frozen at −80 °C in 3–5 g aliquots. Cell pellets were thawed (at room temperature) in preweighed Sorvall SA-600 rotor tubes with 25 ml of 100 mM Tris/sulphate, pH 9.4, and 40 mM 2-mercaptoethanol for 10 min at room temperature, pelleted at 3000 ***g*** for 10 min and resuspended in spheroplasting buffer (1.2 M sorbitol/20 mM Hepes/Tris, pH 7.5) containing 10 mM NaN_3, 2 mM EDTA, 40 mM 2-mercaptoethanol and Zymolyase 100T (1 mg/g cells). Cells were incubated with gentle shaking for 45 min at 37 °C. Spheroplasts were pelleted in the preweighed tubes at 5000 ***g*** for 10 min, washed twice in spheroplasting buffer with 10 mM $MgCl_2$ to remove all Zymolyase, pelleted as described above and weighed. Pellets were gently suspended with a paintbrush in 25 ml of spheroplasting buffer with 1 mM $CaCl_2$, 5 mM $MnSO_4$ and Concanavalin A (1.5 mg/g cells wet weight), and incubated on ice for 15 min. Lectin-coated spheroplasts were collected by centrifugation (5000 ***g*** for 10 min), washed with ice-cold spheroplasting buffer and pelleted again. Pellets were suspended in lysis buffer (0.8 M sorbitol/20 mM Hepes/Tris, pH 7.0) with 2 mM EDTA, 1 mM PMSF and leupeptin, pepstatin and aprotinin (all at 1 μg/ml) added just before use, and incubated for 10 min on ice. Spheroplasts were disrupted on ice by Dounce homogenization with 20–30 strokes of prechilled loose-fitting pestle A, and lysate was spun down at 10000 ***g*** for 10 min. Secretory vesicles were harvested by ultracentrifugation of slow-speed supernatant (S_1) in a Sorvall T-865 ultacentrifuge rotor at 100000 ***g*** for 45 min. Pellets were then suspended in transport buffer [250 mM sucrose/10 mM Hepes/Tris (pH 7.5)/20 mM KCl], spun at 10000 ***g*** for 10 min, and finally resuspended in transport buffer by passing 10–20 times through a syringe with a 25-gauge needle, at a final concentration of $\approx$ 5 mg/ml.

Electrophoresis and immunoblotting

Spheroplast lysate S_1 fraction and secretory vesicle membrane fractions isolated from heat-shocked NY17 cells were added to an equal volume of sample loading buffer [50 mM Tris/HCl (pH 6.8)/2 % (w/v) SDS/0.1 mM dithiothreitol/10 % (v/v) glycerol] and subjected to SDS/PAGE on 6–20 % (w/v) gradient gels. The separated polypeptides were electrotransferred on to a 0.45-μm nitrocellulose membrane for 2 h at 130 mA using a semi-dry transfer apparatus. The filters were blocked and then incubated for 2 h at room temperature with either a 1:500 dilution of rabbit antisera raised against the yeast Ycf1p antigen (provided by W. Scott Moye-Rowley, University of Iowa, Iowa City, IA, U.S.A.), rabbit antisera against the yeast Bat1p (provided by Daniel F. Ortiz, Tufts University, Boston, MA, U.S.A.), or a 1:1000 dilution of mouse monoclonal antibody against the 100-kDa integral membrane subunit of the V-type ATPase localized in yeast vacuoles (Molecular Probes, Eugene, OR, U.S.A.). Immunoreactive bands were detected by incubating washed membranes with either a 1:2000 dilution of anti-rabbit IgG or anti-mouse IgG antibodies conjugated with horseradish peroxidase enzyme (Sigma), and then detected by enhanced chemiluminescence (ECL) according to manufacturer's instructions (Amersham).

Transport

Transport was measured as uptake of radiolabelled substrate into vesicles collected by rapid filtration on a Millipore 0.45-μm filter under vacuum, essentially as described previously [33]. Secretory vesicles were thawed by immersion in a 30 °C water bath, diluted to 5 mg/ml in transport buffer with an ATP regenerating system consisting of 10 mM phosphocreatine, 10 mM $MgCl_2$, 100 μg/ml creatine phosphokinase, with 0.5 mM acivicin to inhibit resident γ-glutamyl transpeptidase enzyme, and either 5 mM Na_2ATP or 10 mM NaCl. Diluted secretory vesicles were repeatedly passed through a 25-gauge needle ($\times$ 10) and incubated at 30 °C for 15 min before initiation of the transport reaction. Freshly prepared dithiothreitol was added to 5 mM in all transport solutions used to measure GSH uptake in order to maintain glutathione in its reduced form. Transport was started by adding 20 μl of diluted secretory vesicles to 80 μl of incubation transport buffer (with substrate) at 4 °C or 30 °C for timed intervals. Transport was quenched by adding 1 ml of ice-cold stop buffer [300 mM sucrose/10 mM Hepes/Tris (pH 7.5)/20 mM KCl], and secretory vesicles were collected by applying 1 ml of quenched reaction solution to pre-wetted filter under a vacuum, and then washing the filter with an additional 4 ml of ice-cold stop buffer. Filters were collected, dissolved in 5 ml of Opti-Fluor (Packard Instrument Co. Meriden, CT, U.S.A.), and radiolabelled drug uptake was quantified by liquid scintillation measurements. Controls for non-specific binding of drug to filters and vesicles were determined by measuring retention of radiolabelled substrate on filters in the absence of vesicles, or on secretory vesicles incubated in transport buffer at 4 °C for each time point.

Confirmation of [^{3}H]GSH uptake into secretory vesicles

Vesicles were collected on filters after 10 min incubation at 30 °C

by vacuum filtration, as described above, but using 30-fold higher [^{3}H]GSH specific activity (1 mM GSH). Filters and portions of transport reaction solution were added to 200 μl of 1 % (v/v) $HClO_4$, 0.1 mM bathophenanthroline disulphonic acid, and vortexed intermittently for 10 min to lyse vesicles. [^{3}H]GSH was converted into *S*-(2,4-dinitrophenyl)glutathione (DNP-SG) by adding 150 μl of vesicle extract to 110 μl of 77 mM KOH/92 mM $KHCO_3$ with 0.1 % (v/v) 2,4-dinitrofluorobenzene and incubating for 10 min at room temperature. The pH was then lowered to pH 2–3 with 6 M HCl, and samples were analysed by HPLC. The HPLC method described previously [30] was used for the detection, separation and quantification of dinitrophenyl-containing compounds. Metabolites were detected at 365 nm and the data were compared with those obtained from HPLC analysis of DNP-SG standards. HPLC fractions were also collected and analysed for ^{3}H content. The HPLC system consisted of a Varian Model 9012 Liquid Chromatograph System with a Varichrom adjustable-wavelength spectrophotometric detector (Varian Assoc. Inc., Sunnyvale, CA, U.S.A.).

Invertase assay

The amount of invertase enzyme in secretory vesicles was quantified using the enzymic assay developed by Goldstein and Lampen [34] and modified by Walworth and Novick [31].

Statistical analysis

Data from experiments measuring uptake of radiolabelled substrate in secretory vesicles were fitted to the Michaelis–Menten equation by non-linear least-squares regression analysis using Sigmaplot 4.11, and correlated to $P < 0.05$, as determined by paired Student's *t* test. V_{max} and K_m values with standard errors were derived from these curves (see Table 1) and K_i values were calculated using the equation $K_i = [I]/[(K_{mi}/K_{mo}) - 1]$

RESULTS

Functional characterization of yeast secretory vesicles: ATP-dependent, concentrative uptake of [^{3}H]DNP-SG and [^{3}H]taurocholate

Secretory vesicles isolated from the NY17 *sec*6–4 mutant strain of *S. cerevisiae* were enriched with invertase approx. 6-fold, a value comparable with that reported by Walworth and Novick [31]. These secretory vesicles were shown previously to contain distinct ATP-dependent transporters for glutathione S-conjugates, including DNP-SG (mediated by YCF1; [28]), and for the bile salt taurocholate (mediated by BAT1; [28,29]). To confirm these findings and to demonstrate that our secretory vesicles were competent for transport studies, we measured ATP-dependent uptake of 100 μM [^{3}H]DNP-SG (Figure 1A) and 50 μM [^{3}H]taurocholate (Figure 1B). Both of these solutes were accumulated avidly by secretory vesicles incubated in the presence of ATP at 30 °C, whereas uptake was minimal at either 4 °C or at 30 °C in the absence of ATP. Uptake was a linear function of time for approx. 1 min (Figure 1). At the 10 min time point, the intravesicular concentrations of DNP-SG and taurocholate were calculated to be about 5-fold and 10-fold higher than extravesicular concentrations respectively, on the basis of a vesicular volume of 2.8 μl/mg protein [32], indicating concentrative transport. When similar experiments were performed at lower DNP-SG concentrations (50 μM), the apparent concentrative uptake was more dramatic ($\approx$ 20-fold; results not shown).

Kinetic parameters for both ATP-dependent DNP-SG and taurocholate uptake were also measured (Table 1) and are

Table 1 Kinetic characteristics of ATP-dependent GSH, DNP-SG and taurocholate transport in yeast secretory vesicles

K_m and V_{max} values were calculated from data in Figures 4, 5 and 6. Data were fitted by non-linear least-squares regression curves with the Michaelis–Menten equation, and are expressed as means ± S.E.M. K_i values were calculated from these parameters.

	K_m (mM)	V_{max} (nmol · mg^{-1} · 30 s^{-1})	K_i (mM)
GSH	19 ± 5	27 ± 2	
GSH + 0.25 mM DNP-SG	44 ± 5	26 ± 3	0.189
GSH + 0.5 mM DNP-SG	62 ± 7	25 ± 3	0.221
GSH + 1.0 mM DNP-SG	97 ± 17	25 ± 2	0.243
DNP-SG	0.228 ± 0.042	1.040 ± 0.091	
DNP-SG + 5 mM GSH	0.284 ± 0.021	1.009 ± 0.077	20
DNP-SG + 30 mM GSH	0.670 ± 0.032	0.956 ± 0.051	15.5
Taurocholate	0.155 ± 0.035	1.398 ± 0.061	
Taurocholate + 10 mM GSH	0.156 ± 0.044	1.500 ± 0.023	

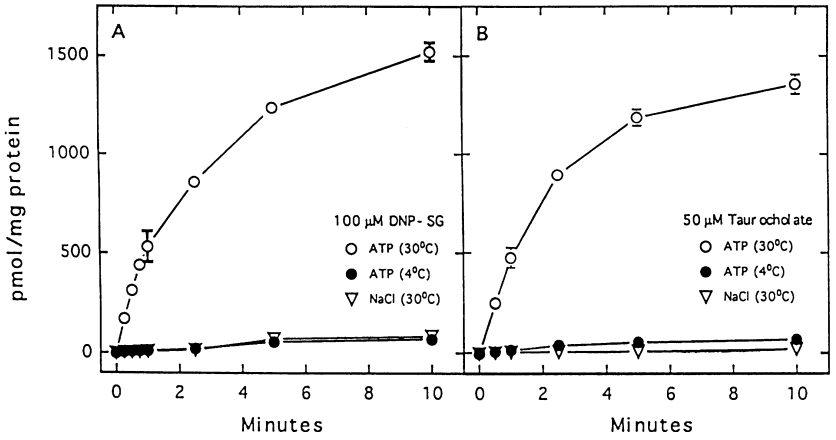

Figure 1 Time course of DNP-SG and taurocholate uptake in yeast secretory vesicles

Secretory vesicles (100 μg) were incubated in medium containing an ATP-regenerating system and 100 μM [^{3}H]DNP-SG (**A**) or 50 μM [^{3}H]taurocholate (**B**) in the presence of 5 mM ATP (○) or 10 mM NaCl (▽) at 30 °C, or 5 mM ATP at 4 °C (●). Each data point represents the mean ± S.E.M. for at least three experiments, each performed in duplicate.

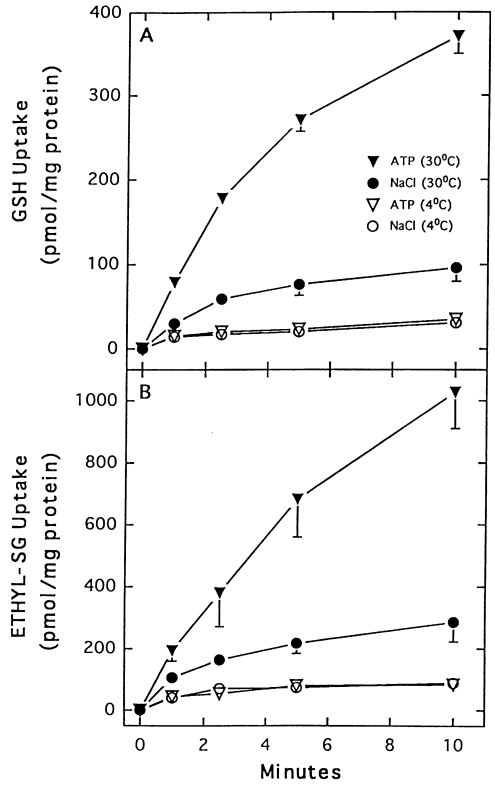

Figure 2 Time course of GSH and ethyl-SG uptake in yeast secretory vesicles

Secretory vesicles pretreated with 0.5 mM acivicin were incubated in medium containing 5 mM dithiothreitol, an ATP-regenerating system and 100 μM [^{3}H]GSH (**A**) or 100 μM [^{3}H]ethyl-SG (**B**) in the presence of 5 mM Na_2ATP (▽,▼) or 10 mM NaCl (○,●) at 30 °C (filled symbols) and 4 °C (open symbols). Each data point represents the mean ± S.E.M. for at least three experiments, each performed in duplicate.

similar to those previously reported [28]. Thus the data confirm previous studies [28], and demonstrate that the secretory vesicles are transport-competent.

ATP-dependent uptake of [^{3}H]GSH and *S*-^{3}[H]ethylglutathione ([^{3}H]ethyl-SG) in secretory vesicles

We next examined whether [^{3}H]GSH and the low-molecular-mass glutathione S-conjugate, [^{3}H]ethyl-SG, were transported by specific carriers in these yeast secretory vesicles. The time course of uptake of 0.1 mM [^{3}H]GSH (Figure 2A) and 0.1 mM [^{3}H]ethyl-SG (Figure 2B) was measured at 4 °C and 30 °C in the presence of either 5 mM Na_2ATP or 10 mM NaCl. The incubation medium also contained 0.5 mM acivicin to inhibit GSH catabolism by γ-glutamyl transpeptidase, and 5 mM dithiothreitol to maintain GSH in its reduced form. Interestingly, uptake of both solutes was dependent largely on the presence of ATP (Figure 2). Uptake in the absence of ATP was comparatively low, and was only slightly higher at 30 °C when compared with 4 °C (Figure 2). These results differ from previous studies in mammalian membrane vesicles, which failed to find an effect of ATP on GSH transport [5,7–10], and found only minimal stimulation of ethyl-SG transport in the presence of ATP [9].

ATP-dependent uptake of 5 mM [^{3}H]GSH was progressively decreased in medium containing increasing concentrations of sucrose, demonstrating [^{3}H]GSH uptake into an osmotically

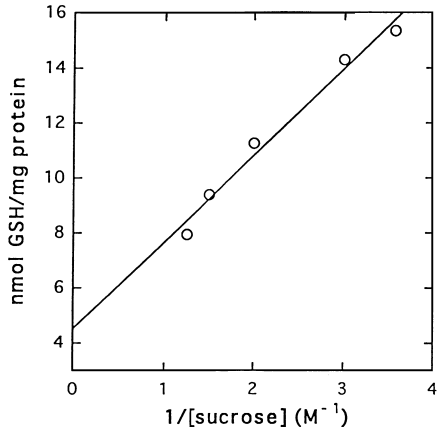

Figure 3 Effect of medium osmolality on ATP-dependent GSH uptake in yeast secretory vesicles

Secretory vesicles were incubated in medium containing 250 mM sucrose, 10 mM Hepes/Tris, pH 7.4, 20 mM KCl, 5 mM dithiothreitol, 0.5 mM acivicin, an ATP-regenerating system, 5 mM Na_2ATP and 5 mM [^{3}H]GSH at either 30 °C or 4 °C for 10 min. The temperature-sensitive uptake after 10 min was plotted versus the inverse concentration of sucrose, and the data were fitted to a least-squares regression curve. Data points represent the means of values measured with two different vesicle preparations, which showed excellent agreement.

sensitive space (Figure 3). Little non-specific binding of GSH to secretory vesicles was observed, and zero time measurements of GSH uptake into the vesicles were similar to values for filter binding (results not shown).

To confirm that [^{3}H]GSH was taken up by these vesicles, we measured [^{3}H]GSH content of lysed secretory vesicles. Intravesicular [^{3}H]GSH was converted into [^{3}H]DNP-SG, and the products were separated on HPLC. A single radiolabelled peak

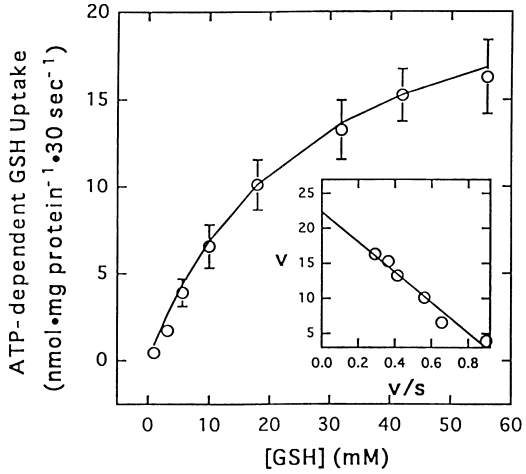

Figure 4 Concentration-dependence of initial rates of ATP-dependent GSH uptake in yeast secretory vesicles

Secretory vesicles were incubated in a buffer containing an ATP-regenerating system and various concentrations of [^{3}H]GSH with either 5 mM Na_2ATP or 10 mM NaCl at 30 °C for 0 s and 30 s. The difference between GSH uptake with 5 mM Na_2ATP and with 10 mM NaCl after 30 s was plotted for each concentration of GSH and the data fitted according to the Michaelis–Menten equation. Each data point represents the mean ± S.E.M. for at least three experiments, each performed in duplicate. Data were analysed by a Woolf–Augustinsson–Hofstee plot (inset).

Table 2 Effects of nucleotides, inhibitors and uncouplers on initial rate of ATP-dependent GSH transport in yeast secretory vesicles

Uptake of 1 mM [^{3}H]GSH in 100 μg of secretory vesicles was measured after 1 min incubation at 30 °C in the presence of various inhibitors. Uptake was measured in the presence and absence of 5 mM ATP, and the difference was considered to be the ATP-dependent component. Rates are expressed as percentages of controls. The mM concentration of inhibitor is given in parentheses. Uptake values represent the mean ± S.E.M. for at least three different secretory vesicle preparations. CCCP, carbonyl cyanide *m*-chlorophenylhydrazone; EDAC, 1-ethyl-3-(3-dimethylaminopropyl) carbodi-imide; AMP-PNP, adenosine 5′-[β,γ-imido]triphosphate; DIDS, 4,4′-di-isothiocyanostilbene-2,2′-disulphonate.

Compound	% Uptake
Nucleotides	
ATP (5)	100 ± 6
CTP (5)	36 ± 4
UTP (5)	38 ± 6
AMP-PNP (5)	3 ± 2
AMP (5)	5 ± 2
Inhibitors	
Vanadate (0.1)	54 ± 5
Sodium azide (1)	82 ± 4
Diethylstilboesterol (0.5)	74 ± 4
EDAC (0.5)	75 ± 8
DIDS (0.5)	30 ± 3
Probenecid (5)	28 ± 3
Sulphinpyrazone (4)	28 ± 5
Uncouplers	
Valinomycin (10 ug/mg)	95 ± 2
NH_4Cl (1)	96 ± 1
CCCP (0.01)	107 ± 2
Gramicidin D (0.01)	89 ± 2
Substrates	
DNP-SG (0.5)	33 ± 7
DNP-SG (0.2)	58 ± 4
Sulphobromophthalein (0.1)	53 ± 6
Taurocholate (0.2)	94 ± 9
S-Ethylglutathione (0.5)	65 ± 9
S-Hexylglutathione (0.5)	37 ± 2
S-Octylglutathione (0.5)	32 ± 4

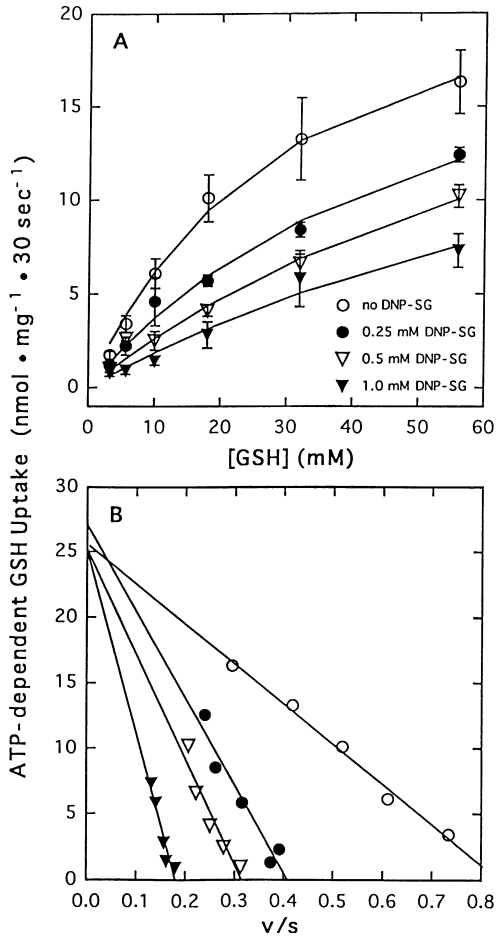

Figure 5 DNP-SG competitively inhibits ATP-dependent GSH uptake in yeast secretory vesicles

(**A**) ATP-dependent GSH uptake was measured in secretory vesicles incubated in a buffer containing an ATP-regenerating system, in the presence of no added DNP-SG (○), or with 0.25 mM (●), 0.5 mM (▽) or 1.0 mM DNP-SG (▼). Each data point represents the mean ± S.E.M. for at least three experiments, each performed in duplicate. (**B**) Data were analysed by a Woolf–Augustinsson–Hofstee plot.

was observed, which co-eluted with the DNP-SG standard (results not shown).

The initial rate of [^{3}H]GSH uptake was measured at increasing extravesicular GSH concentrations (0.56–56 mM) in the presence of either 5 mM Na_2ATP or 10 mM NaCl, at both 30 °C and 4 °C. Without ATP, there was no significant difference between the rate of [^{3}H]GSH uptake at 30 °C and that at 4 °C after 30 s (results not shown). However, there was significant uptake of [^{3}H]GSH in the presence of ATP (Figure 4). When the data were fitted to Woolf–Augustinsson–Hofstee plots (Figure 4, inset), an apparent K_m and V_{max} were measured that were comparable with values obtained from regression curves that followed the Michaelis–Menten equation (Table 1), 19 mM and 27 nmol·mg protein^{-1}·30 s^{-1} respectively.

To determine whether ATP was required directly for GSH transport (primary active transport) or whether uptake was driven indirectly (secondary active) by induced electrical potentials, pH or ion gradients, ATP-dependent GSH uptake was measured in secretory vesicles treated with uncouplers and specific inhibitors (Table 2). Uncouplers of membrane electrical potential (valinomycin, CCCP, gramicidin D) did not reduce GSH transport. Dissipation of the proton gradient with NH_4Cl, CCCP or gramicidin D, or inhibition of mitochondrial, vacuolar V-type and plasma membrane P-type H^+-ATPases [by sodium azide, 1-ethyl-3-(3-dimethylaminopropyl)carbodi-imide (EDAC) and diethylstilboesterol respectively], only showed modest effects (Table 2). The absence of an effect of these uncouplers and inhibitors, along with the finding that a non-hydrolysable analogue of ATP, adenosine 5′-[β,γ-imido]triphosphate, did not stimulate GSH transport (Table 2), is consistent with a direct requirement for ATP hydrolysis in GSH transport. Moreover, the effect of ATP was selective for this nucleotide, because GSH transport was only partially stimulated by UTP and CTP, and not at all by AMP (Table 2). The relative degree of stimulation of GSH uptake by UTP and CTP is similar to that reported for the transport of taurocholate in yeast secretory vesicles under comparable conditions [28].

In contrast, an inhibitor of anion transporters (4,4′-di-isothiocyanatostilbene-2,2′-disulphonate), and inhibitors of MRP-mediated export from mammalian cells (sulphinpyrazone, probenecid) [35], had large effects on ATP-dependent GSH transport (Table 2). Vanadate (0.1 mM) inhibited uptake of both GSH (Table 2) and ethyl-SG (data not shown) by as much as 70%. Putative substrates, many of which have already been identified as substrates of the yeast YCF1 transporter, were also included with 1 mM [^{3}H]GSH to examine whether they inhibited

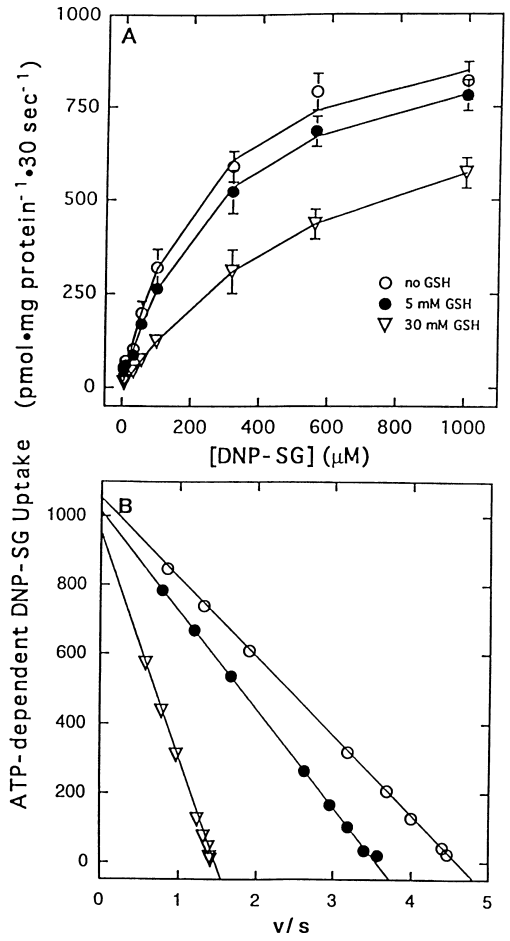

Figure 6 Effect of GSH on ATP-dependent DNP-SG transport in yeast secretory vesicles

(**A**) ATP-dependent uptake of DNP-SG was measured with no added GSH (○), with 5 mM GSH (●) or with 30 mM GSH (▽). Each data point represents the mean ± S.E.M. for at least three experiments, each performed in duplicate. (**B**) Data were analysed by a Woolf–Augustinsson–Hofstee plot.

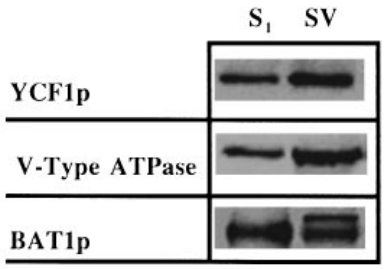

Figure 7 Expression of Ycf1p, V-Type ATPase and Bat1p in S_1 fraction and secretory vesicle fraction isolated from yeast *S. cerevisiae*

Protein (75 μg) from the spheroplast S_1 fraction, and secretory vesicle membrane fractions (SV) isolated after heat-shock treatment of *sec* mutant *S. cerevisiae* NY17 cells, were separated by SDS/PAGE [6–20% (w/v) gradient gels]. Immunoblots using rabbit anti-Ycf1p antisera, mouse monoclonal antibody generated against the 100-kDa integral membrane subunit of the V-Type ATPase or rabbit anti-Bat1p antisera were screened, and immunoreactive bands representing detected antigen are shown. The Ycf1p migrated to about 120 kDa, whereas the Bat1p antigen was detected at 120 and 140 kDa.

GSH uptake. Concentrations of DNP-SG at or above its apparent K_m [24] showed significant inhibition (Table 2). Other glutathione S-conjugates tested also showed lower initial rates of GSH uptake, as did verapamil, whereas the bile acid taurocholate had no effect (Table 2).

The inhibition by DNP-SG was competitive in nature (Figure 5). The kinetic parameters calculated from these data are presented in Table 1; it is noteworthy that the K_i values calculated from these data are similar to the apparent K_m for DNP-SG, suggesting that GSH and DNP-SG are transported by the same carrier.

Likewise, GSH competitively inhibited ATP-dependent [^{3}H]DNP-SG uptake (Figure 6A and Table 1), providing further strong evidence for a shared transport mechanism. K_i values measured for GSH were between 15–20 mM, and are in good agreement with the K_m value determined for GSH (Table 1). In contrast, 30 mM GSH had no effect on ATP-dependent taurocholate uptake (results not shown).

To determine if the yeast ATP-dependent DNP-SG transporter (Ycf1p) was present in our secretory vesicle membrane fraction, we subjected the isolated secretory vesicle membranes and the spheroplast lysate S_1 fraction to Western blot analysis using antisera raised in rabbits against Ycf1p (Figure 7). We observed a band corresponding to Ycf1p at about 120 kDa in both fractions, but the Ycf1p band was enriched in the secretory vesicle membrane fraction (Figure 7). These same cell fractions were also analysed for the presence of the ATP-dependent bile acid transporter (Bat1p) and the vacuolar marker enzyme, V-type ATPase, using rabbit anti-Bat1p antisera and a mouse anti-(V-type ATPase) monoclonal antibody (Figure 7). Both of these antigens were also present in the secretory vesicle membrane fraction (120–140 kDa Bat1p and 100 kDa V-type ATPase). The presence of Bat1p in our secretory vesicle membrane fraction is consistent with the ATP-dependent transport of taurocholate measured in this sample. The positive reaction with the V-type ATPase antibody indicates the presence of vacuolar membrane in our samples.

DISCUSSION

The present study examined the pathway(s) for GSH efflux using secretory vesicles isolated from the *sec* mutant NY17 strain of *S. cerevisiae*. GSH transport in this membrane fraction was both temperature- and osmotically-sensitive, and was mediated largely by a low-affinity, ATP-dependent system. Transport in the absence of ATP or in the presence of a non-hydrolysable ATP analogue or other nucleotides was minimal. ATP-dependent GSH transport was also uncoupler-insensitive, indicating a direct requirement for ATP hydrolysis in transport.

These results are in contrast with studies conducted in mammalian liver, kidney and intestinal plasma membrane vesicles, which uniformly failed to find an effect of ATP on GSH transport [3]. In these systems, GSH transport was found to be both electrogenic and bidirectional, whereas most glutathione S-conjugates were found to be substrates for ATP-dependent transporters, including mrp1 and mrp2 (the latter is also called cMRP or cMOAT) [3]. For example, studies in liver canalicular membrane vesicles indicated the presence of multiple transport mechanisms for GSH and glutathione S-conjugates, and demonstrated that the physicochemical properties of the *S*-substituent are major determinants of transport [9].

However, more recent findings in intact mammalian cells indicate that ATP is required for GSH efflux [11–23]. The reason for this discrepancy between the vesicle studies and those in intact cells is not clear. One possibility is that ATP is modulating indirectly GSH transport in intact cells, an hypothesis that has not yet been ruled out. Alternatively, GSH may be a substrate for an ATP-dependent transporter, but the kinetics of transport preclude detection in the vesicle system. Indeed, hepatic GSH transport exhibits both a low affinity (high K_m) and a low V_{max} in the presence or absence of ATP, and is difficult to measure in plasma membrane vesicles [9]. Moreover, mammalian plasma membrane vesicles tend to be of mixed orientation (both inside-out and right-side-out) and are relatively leaky, limiting both their ability to maintain transmembrane ion or solute gradients and their ability to demonstrate ATP-dependent transport.

On the other hand, membrane vesicles isolated from the *sec*6–4 mutant strain of *S. cerevisiae* are of uniform morphology, tightly sealed, and are oriented almost exclusively inside-out [31,36,37]. Uptake into the vesicles is therefore equivalent to efflux from the cell. The present study demonstrates that a GSH transporter is present in yeast secretory vesicle preparations, and that this transporter is dependent on ATP.

Moreover, the characteristics of GSH transport in yeast vesicles suggest that it is mediated by the YCF1 transporter, the yeast homologue of mammalian mrp1 and mrp2, and thus support the hypothesis that GSH may be a low-affinity substrate for these ATP-dependent transporters. GSH transport in the secretory vesicles was inhibited competitively by DNP-SG, a model substrate for YCF1, mrp1 and mrp2. The K_i value for DNP-SG inhibition of GSH uptake was approx. 0.2 mM, which is similar to the K_m for DNP-SG transport in yeast secretory vesicles (Table 2). ATP-dependent GSH transport was also *cis*-inhibited by other substrates of YCF1, including other glutathione S-conjugates and sulphobromophthalein. Furthermore, the concentration of vanadate that inhibited approx. 50 % of the GSH transport in the secretory vesicles (0.1 mM; Table 2) is similar to the IC_{50} (0.179 mM) reported for Ycf1p-mediated DNP-SG uptake in yeast vacuoles [38]. The kinetic analysis of ATP-dependent, uncoupler-insensitive GSH and DNP-SG transport was supported further by the discovery of Ycf1p in the secretory vesicle membrane fraction of heat-shocked NY17 cells (Figure 7).

These results therefore indicate that our secretory vesicle preparation includes vesicles containing Ycf1p, vacuolar H^+-ATPase (V-type) and Bat1p, which originate from the vacuole and are transported to the plasma membrane via *sec*-facilitated fusion. Alternatively, our secretory vesicles may possess Ycf1p that is either redistributed from its normal vacuolar site of expression [38,39] to facilitate compartmentalization into exocytotic vesicles, or Ycf1p that is destined to be inserted into the plasma membrane. This distribution of Ycf1p to both vacuolar and plasma membranes is consistent with findings of vacuolar accumulation, as well as extracellular efflux of DNP-SG in *S. cerevisiae* [40]. However, additional studies are needed to establish whether GSH is indeed a substrate for Ycf1p, and whether Ycf1p expression and cellular localization are altered in response to stress, such as heat shock. Such studies are currently in progress in our laboratory.

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