Volume 304, number 2,3, 256–260 FEBS 11165 June 1992

ATP and GTP as alternative energy sources for vinblastine transport by P-170 in KB-V1 plasma membrane vesicles

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Received 22 April 1992

Purified plasma membrane vesicles isolated from multidrug-resistant human KB-V1 cells accumulate [^{3}H]vinblastine in an energy-dependent manner. The accumulation of [^{3}H]vinblastine in the presence of ATP is a saturable process. ATP can be replaced by other purine nucleotide triphosphates, of which GTP is the most efficient.

Multidrug resistance; Uptake; Vesicle

1. INTRODUCTION

The multidrug resistance (MDR) phenotype, which is characterized by a decreased accumulation of hydrophobic chemotherapeutic drugs by cells, is due to overexpression of P-170 (also known as P-glycoprotein or the multidrug transporter), an intrinsic plasma membrane protein encoded by the *MDR1* gene in human cells [1–5]. The plasma membrane localization of P-170, together with the molecular genetic evidence that transfer of the *mdr* gene is sufficient to confer drug resistance to drug sensitive cells, suggest that P-170 itself is responsible for active drug efflux [6].

The demonstration of the drug transport properties of P-170 has been achieved using sealed plasma membrane vesicles isolated from KB-V1 multidrug-resistant cells [7,9–10]. Previously reported data with this system showed a requirement for ATP, and osmotic sensitivity of the uptake process [7,8]. The population of purified vesicles used to demonstrate transport consists of right-side-out and inverted (inside-out) vesicles. Only the inside-out vesicles can accumulate drugs (e.g. [^{3}H]vinblastine). In these vesicles the orientation of the multidrug transporter is such that the nucleotide binding sites face the incubation medium and therefore are accessible to ATP. This orientation allows the translocation of vinblastine into the intravesicular space. In sealed vesicles, as in intact cells, ATP does not cross the plasma membrane barrier, and therefore cannot drive the pump of right-side-out vesicles, which remain silent in the drug transport assay but may contribute to non-specific uptake by binding drug. Although P-170 has been shown to be an ATP-dependent plasma membrane transporter a possible role for other nucleotides in drug transport has never been examined. We show here that, among numerous nucleotides tested for vinblastine accumulation, GTP was also able to support drug transport in KB-V1 vesicles.

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2. EXPERIMENTAL

2.1. *Cell culture*

The original human KB carcinoma cell line was obtained from the American Type Tissue Culture Collection, and the drug-resistant KB-V1 cell line was selected from the subclone, KB-3-1, as previously described [11]. This vinblastine-resistant cell line was maintained in a constant concentration of the drug (1 μg/ml) during growth as a monolayer at 37°C in 5% CO_2, in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum (Gibco), L-glutamine (2 mM), penicillin (50 U/ml) and streptomycin (50 μg/ml). Cells were grown to confluence on 150 mm dishes (Falcon, Intergrid).

2.2. *Plasma membrane vesicle isolation*

The membrane vesicles were prepared from KB-V1 cells by nitrogen cavitation according to the method of Lever [12], and purified as previously described [7]. Purified vesicles (yield: 10–16 mg of vesicle protein from 60–80 plates) were resuspended in TS buffer (10 mM Tris-HCl, 250 mM sucrose and 50 mM NaCl, pH 7.5) at a protein concentration of 2–3 mg/ml, and stored at −80°C prior to use.

2.3. *[^{3}H]Vinblastine uptake by vesicles (rapid filtration technique)*

Drug uptake was measured in the presence of 5.5 nM [^{3}H]vinblastine according to the protocol described by Horio et al. [7] with the following modifications. All components were prepared in TS buffer. The Millipore filters (HAWP, 0.45 μm size) were pre-soaked in 10 mM Tris-HCl, pH 7.5, containing 10% fetal calf- or calf serum in order to decrease non-specific adsorption of the hydrophobic drug to the filters. Vinblastine was prepared as follows: 50 μCi of [G-^{3}H]vinblastine sulphate (Amersham) in methanol solution was dried under a nitrogen stream and resuspended immediately in 200 μl of dimethylsulfoxide, then diluted to 320 nM in 10 mM Tris-HCl, pH 7.5,

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50 mM NaCl, filtered (0.22 μM, Millipore), and stored in aliquots at −80°C prior to use. The final concentration of the drug in the assay was 5.3 nM. The DMSO concentration was about 0.025%. Non-specific association of vinblastine with the vesicles was determined by omitting the nucleotide in the incubation medium (control). All measurements were carried out in quadruplicate. Filters were dissolved in Aquasol (Du Pont, NEN) prior to counting [^{3}H]vinblastine uptake by the vesicles. All reported [^{3}H]vinblastine uptake values are corrected by subtracting the corresponding 0 min time point. Protein concentrations were determined by the method of Bradford [13] using the Bio-Rad protein kit with bovine serum albumin as standard.

2.4. *Osmosensitivity of the [^{3}H]vinblastine uptake*

For the osmosensitivity experiments freshly thawed vesicles (50–100 μg total protein) were pre-equilibrated for 1 h in TS buffer of the appropriate osmolarity (determined by sucrose concentration) in the absence of nucleotide, $MgCl_2$ and drug [7]. The equilibration time was chosen from the kinetic profiles which showed no leakiness of the vesicles, compared to the control vesicles, during the 90 min incubation. This preequilibration time was also chosen to insure homogeneity in the incubation mixture by allowing equilibration of diffusible molecules. Indeed, during plasma membrane isolation (at 4°C) some of the hydrophobic drug molecules may still be in the membrane, and either become entrapped when vesicles re-seal or remain adsorbed on the membranes and therefore may interfere with the uptake of the radiolabelled vinblastine. The uptake studies were started by adding the appropriate nucleotide and the radioactive drug in solution at the same sucrose concentration, and radioactivity associated with the vesicles was measured 10 min thereafter. A 0 time uptake was obtained by stopping the reaction by a 10-fold dilution with TS buffer and rapid filtration, immediately after addition of the drug. Altogether, the time elapsed from the thawing of the vesicles to the final measurements stayed in the range in which KB-V1 vesicles had been shown to retain their ability to accumulate vinblastine.

2.5. *Materials*

All nucleotides, nucleosides, creatine phosphokinase (EC 2.7.3.2. from rabbit muscle type 1) and creatine phosphate were from Sigma. All other chemicals were of the highest available analytical or spectroscopic grade.

3. RESULTS AND DISCUSSION

3.1. *Optimization of the [^{3}H]vinblastine uptake conditions in KB-V1 vesicles in the presence of ATP to allow screening of other potential energy sources*

Previous studies have demonstrated that the plasma membrane vesicle population isolated from the multidrug-resistant cell line, KB-V1, was able to accumulate vinblastine in a saturable manner, whereas vesicles from the drug-sensitive parent cell line (KB-3-1) showed very low drug uptake [9]. In agreement with Horio et al. [7] we have shown that 0.33 mM ATP, supplemented with the creatine phosphate/creatine phosphokinase system, achieves a steady-state level of vinblastine uptake which reaches a plateau at 20 min (Fig. 1). To be able to screen for other potential energy sources, the regenerative system for ATP has been omitted to avoid interference with the tested nucleotide or with nucleotide combinations. Results shown in Fig. 1 show that omitting the ATP regenerating system in the presence of the low concentration of ATP (0.33 mM) resulted in a substantial decrease in drug uptake compared to levels obtained when the regenerating system was present.

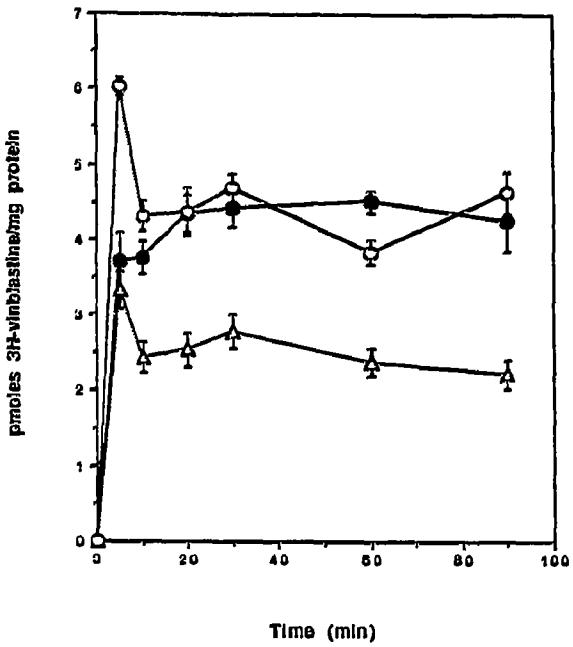

Fig. 1. Time-course of ATP-dependent vinblastine uptake in vesicles from KB-V1 cells. The plasma membrane vesicles were suspended in TS buffer at room temperature. Creatine kinase and creatine phosphate (final concentrations 3 μg/100 μl, and 3 mM, respectively) were added together with ATP (0.33 mM final concentration), and after a 1 min equilibration [^{3}H]vinblastine (5.3 nM final concentration) was added. The final concentration of $MgCl_2$ was 3.3 mM. Plasma membrane protein content was 50–100 μg in the assay (○). Drug uptake without the ATP-regenerative system was tested either in the presence of 0.33 mM ATP (△) or of 3.3 mM ATP (●). Each value is the mean of quadruplicates and is corrected by the respective uptake at 0 min. The overshoot in vinblastine uptake at 5 min was not observed in all batches of plasma membrane vesicles, nor was it seen with the other nucleotides tested.

This very same level of uptake was restored when a 10-fold higher concentration of ATP (3.3 mM) was supplied to the vesicle system. Furthermore the vinblastine accumulation profile seen with the ATP regenerating system is conserved with this high concentration of ATP.

3.2. *Characterization of [^{3}H]vinblastine uptake in the presence of different nucleotides in KB-V1 vesicles*

3.2.1. Effect of GTP on vinblastine uptake

Because GTP has been shown to bind to P-glycoprotein [15] and because it is known to substitute for ATP in other transport processes [16], GTP was tested for its capacity to support vinblastine uptake in KB-V1 vesicles. Fig. 2 shows that drug uptake in the presence of 3.3 mM GTP also reached a plateau after 20 min, with an average for different membrane preparations of 70–80% of the level obtained with the same concentration of ATP.

Osmotic sensitivity studies are a classical approach used to demonstrate the presence of 'true' transport. The increase in the extravesicular osmotic pressure provokes not only a decrease of the intravesicular space

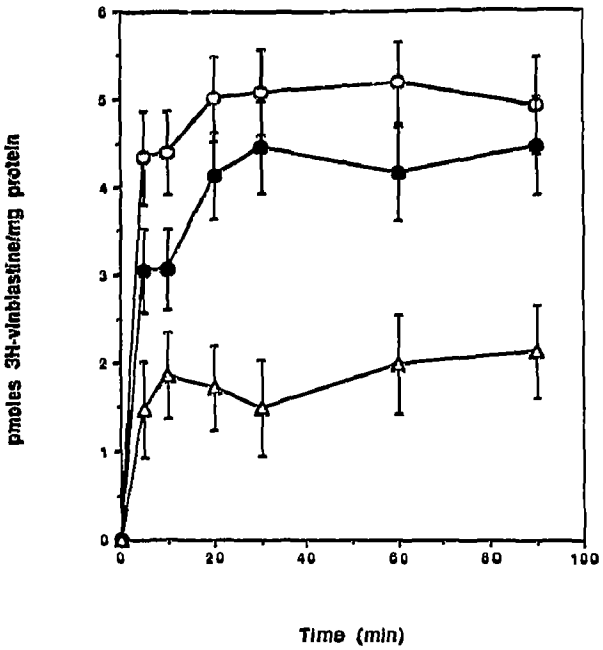

Fig. 2. Time-course of vinblastine uptake in KB-V1 vesicles tested for GTP as energy source. Membrane vesicles were suspended in TS buffer without the ATP regenerating system and assayed for uptake in the presence of 3.3 mM of purine nucleotide as described in Fig. 1. Uptake with ATP (○), GTP (●), and in the absence of nucleotide (control, (△) was measured. The reported values are the mean of quadruplicates and were corrected by subtracting their corresponding vinblastine uptake values at 0 min, but the uptake value of the control vesicles (without nucleotide) was not subtracted.

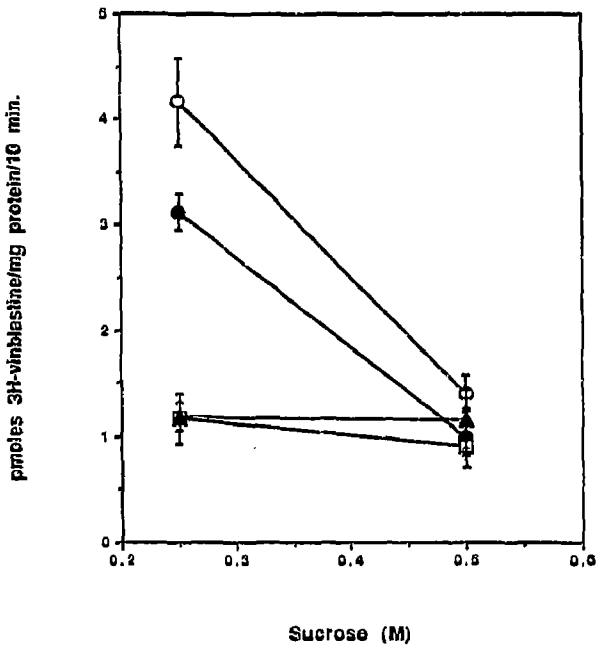

Fig. 3. Osmotic sensitivity of vinblastine uptake in the presence of purine nucleotides. Vesicles from KB-V1 cells were pre-incubated in TS buffer containing different concentrations of sucrose for 60 min at 25°C. The sucrose concentration of the uptake reagents was adjusted to be the same as that of the vesicle suspension. Vinblastine uptake was started after this pre-incubation time by the addition of 5.3 nM [^{3}H]vinblastine with the nucleotide being tested (3.3 mM) and $MgCl_2$ (3.3 mM final concentration). Uptake was measured 10 min thereafter. Uptake was performed either in the presence of ATP (○) or GTP (●) or AMP-PNP (△) or in the absence of nucleotide (□). Experimental data were corrected by subtracting their corresponding vinblastine uptake values at 0 min, but the uptake value of the control vesicles (without nucleotide) was not subtracted.

resulting in lower uptake capacity, but may also induce a collapse of steric interactions in the architecture of the transporter, which in turn alters its function. Under the same conditions the non-specific binding of the lipophilic drugs to the hydrophobic components of the membrane is barely affected. Therefore, the osmotic sensitivity of the [^{3}H]vinblastine accumulation in KB-V1 vesicles was examined to determine whether drug accumulation was due to binding or to transport. This experiment requires preincubation of the vesicles at different sucrose concentrations and the demonstration that the vesicles do not show leakage at room temperature durig this preincubation. The [^{3}H]vinblastine accumulation curves in the presence of ATP (Figs. 1,2) showed no loss of radioactivity associated with the vesicles up to 90 min after addition of the drug. Therefore a pre-incubation of 1 h at different sucrose concentrations in the absence of drug and energy source has been chosen, followed by 10 min in the presence of the drug and of the nucleotide tested. Results obtained under these conditions showed different levels of total vinblastine accumulation compared to our usual uptake conditions, since a pre-incubation in the absence of any nucleotide will deplete the vesicle system of endogenous energy. Fig. 2 shows that GTP increased the amount of radioactivity associated with vesicles incubated with [^{3}H]vinblastine when compared to the control, e.g. in the absence of nucleotide. With increasing osmotic pressure, from 0.25–0.50 M sucrose, both ATP- and GTP-mediated transport showed a dramatic decrease (Fig. 3): from 4.16 ± 0.41 to 1.40 ± 0.17 and from 3.12 ± 0.17 to 0.98 ± 0.27 pmol vinblastine/mg protein, respectively. Under the same conditions no change in the uptake of vinblastine by control vesicles or by vesicles incubated in the presence of AMP-PNP, a non-hydrolyzable analog of ATP, was seen. The strong inhibition obtained by increasing the osmotic pressure, and the absence of uptake in the presence of AMP-PNP, demonstrates that this vinblastine association is related to an energy-dependent transport process.

Among other compounds tested (UTP, CTP, ITP, AMP, ADP, NAD^+, $NADP^+$, NADH and NADPH) only ITP and ADP showed slightly more uptake of [^{3}H]vinblastine into vesicles when compared to the control without any nucleotides at 0.25 M sucrose (Fig. 4). ITP-supported uptake went from 1.22 to 0.40 pmol vinblastine/mg protein in 0.5 M sucrose, and ADP-supported uptake went from 1.24 to 0.05 pmol/mg protein. These results may reflect the activity of ITP to support

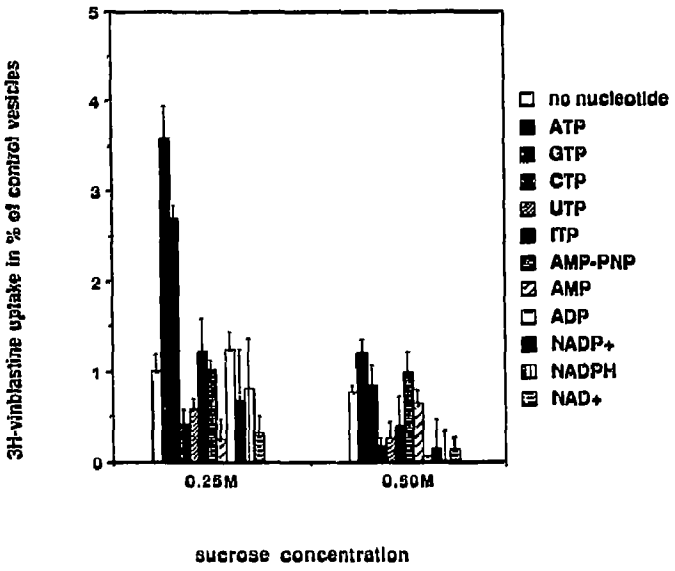

Fig. 4. Uptake of vinblastine in the presence of other nucleotides. Results are given in % of uptake measured in 0.25 M sucrose in the absence of any nucleotide. The uptake of control vesicles (in the absence of nucleotide) was taken as 1 to enable comparison of non-specific vinblastine uptake for different nucleotides in different batches of vesicles. The KB-V1 plasma membrane vesicles were incubated in TS buffer containing either 0.25 or 0.50 M sucrose, and experimental conditions were the same as in Fig. 3. Experimental data were also corrected by subtracting their corresponding vinblastine uptake values at 0 min, but the uptake value of the control vesicles (without nucleotide) was not subtracted.

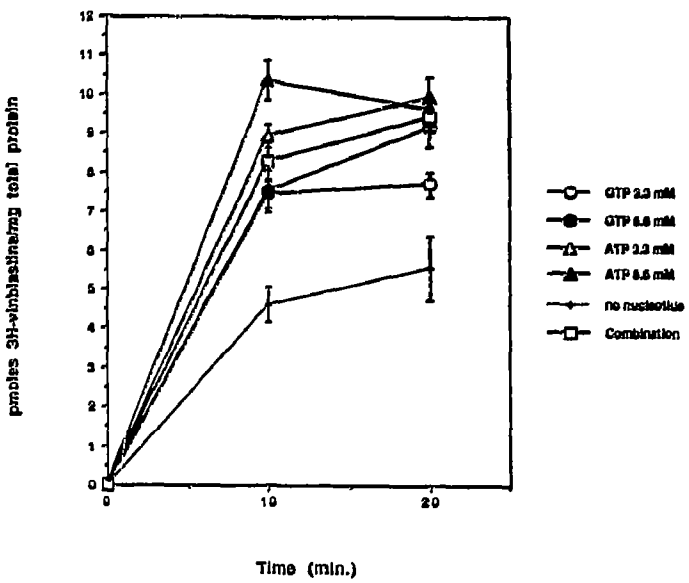

Fig. 5. [^{3}H]vinblastine uptake in KB-V1 vesicles in the presence of different purine nucleotides. Membrane vesicles were assayed for uptake in the presence of 5.3 nM [^{3}H]vinblastine and in the presence of $MgCl_2$ in a molar ratio of 2:1 to the nucleotide(s). Uptake was measured after 10 and 20 min incubations. The control was vinblastine uptake in the absence of any nucleotide. Uptake values are the mean of triplicates and were corrected for their values at 0 min. Accumulation values obtained in the absence of any nucleotide were not subtracted from the plotted values.

transport, and ADP may be able to regenerate ATP via endogenous phosphotransferases. Alternatively it is also possible that ADP and/or ITP have a direct stimulatory effect in this system, perhaps by occupying an allosteric nucleotide-binding site on P-glycoprotein, which also may stimulate a single round of transport.

As noted these osmotic sensitivity experiments gave somewhat different uptake values than the kinetic experiments shown in Figs. 1 and 2. Although the experimental conditions do not allow direct comparison of kinetic data with data obtained in osmosensitivity experiments, results show that the effect of ATP or GTP was quantitatively similar and that in vitro, GTP can substitute for ATP to support vinblastine transport in vitro. In Fig. 4, the level of uptake in the absence of any nucleotide at 0.25 M sucrose was taken as a reference to follow the osmosensitivity of vinblastine uptake with other nucleotides/nucleosides and to be able to compare several batches of membrane vesicles. Indeed, there was some variation in levels of transport activity among the different vesicle preparations, but transport kinetics are consistent within each individual batch of membrane vesicles derived from a single cell population. This kind of variation has been previously reported when cultured cells were used. For instance, variations in purine nucleoside transport from different BHK membrane vesicles may range from 40 to 190 pmol/20min/mg membrane [14]. The relative low yield of purified plasma membrane vesicles (~10 mg total protein deriving from 60 plates of 150 mm diameter) and the amount of protein required for one set (~400–800 μg protein/data point) of experiments limits the number of samples which can be run with a particular batch of vesicles.

3.2.2. [^{3}H]Vinblastine uptake with ATP–GTP

Our results in Figs. 3 and 4 show an energy requirement for a purine nucleotide triphosphate. Further uptake experiments were performed with equimolar concentrations of the combined purine nucleotides. Fig. 5 shows that an increase in the concentration of ATP from 3.3 to 6.6 mM slightly stimulated transport further at 10 min but showed no differences at 20 min, whereas a similar increase in the concentration of GTP resulted in no increase in vinblastine-associated transport at 10 min but showed the same increase at 20 min. This observation is consistent with the slower GTP-associated accumulation also shown in Fig. 2. Several equimolar combinations of both nucleotides have been tested (data not shown), and, as for the 3.3 mM combination depicted in Fig. 5, neither an additive effect nor a synergistic effect was observed.

3.2.3. Substitution of GTP for ATP: general considerations

In all of these experiments (Figs. 2–5) the somewhat lower ability of GTP compared to ATP to support

transport could be related to the fact that for GTP the transport conditions are not optimal for this nucleotide-driven process. This may not be related to the concentration of the nucleotide chosen but rather may reflect either another ion or co-factor requirement for the transport process. Alternatively the lower ability of GTP alone to sustain vinblastine transport could also be related to its intrinsic lower efficiency as an energy source for P-glycoprotein. It is also not excluded that, in vitro, this GTP for ATP substitution acts as a rescue system to prevent ATP depletion. As noted, using several equimolar combinations of both purine nucleotide triphosphates there was neither a synergistic nor an additive effect on uptake.

Based on sequence analysis of P-glycoprotein there are two nucleotide binding sites, both of which are essential for function [17]. One of them could be directly related to drug transport, the second site having a regulatory role. Alternatively, each of them might also support part of the transport system. The inability of GTP to fully replace ATP might therefore be related to the fact that GTP binds either only to one of the nucleotide binding sites or with different affinities to both sites.

In this study we have emphasized the requirement for a purine nucleotide triphosphate in the multidrug transport process. P- Glycoprotein behaves, therefore, like other transporter/permease systems where GTP can substitute for ATP [16]. Our observations are also consistent with the inhibitory effect of ATP or GTP on photoaffinity labelling with 8-azido-ATP of P-glycoprotein performed on KB-V1 vesicles [15]. The use of plasma membrane vesicles is a first step in studying the function of the multidrug transporter. This model enabled the screening of molecules capable of providing energy for drug accumulation, and will serve, as well, for the screening of molecules capable of altering transporter function. Furthermore, this vesicle system constitutes the reference for a more defined model, consisting of purified P-glycoprotein inserted in a known lipid environment. Further studies with such a 'reconstituted system' derived from the in vitro transport system will be needed to determine not ony the stoichiometries of the molecules involved in the transport process, but also the precise mechanism of action of ATP and GTP in providing energy and regulating the transport process.

Acknowledgements: We would like to thank Dr. Gérard Rebel and Dr. Ursula Germann for helpful discussions, and Dwayne Eutsey for secretarial assistance.

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