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A new role for a classical gene: White transports cyclic GMP

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SUMMARY

Guanosine 3′-5′ cyclic monophosphate (cGMP) and adenosine 3′-5′ cyclic monophosphate (cAMP) are important regulators of cell and tissue function. However, cGMP and cAMP transport have received relatively limited attention, especially in model organisms where such studies can be conducted *in vivo*. The *Drosophila* Malpighian (renal) tubule transports cGMP and cAMP and utilises these as signalling molecules. We show here *via* substrate competition and drug inhibition studies that cAMP transport – but not cGMP transport – requires the presence of di- or tri-carboxylates; and that transport of both cyclic nucleotides occurs *via* ATP binding cassette sub-family G2 (ABCG2), but not *via* ABC sub-family C (ABCC), transporters. In *Drosophila*, the *white* (*w*) gene is known for the classic eye colour mutation. However, gene expression data show that of all adult tissues, *w* is most highly expressed in Malpighian tubules. Furthermore, as White is a member of the ABCG2 transporter class, it is a potential candidate for a tubule cGMP transporter. Assay of cGMP transport in w^- (mutant) tubules shows that *w* is required for cGMP transport but not cAMP transport. Targeted over-expression of *w* in w^- tubule principal cells significantly increases cGMP transport compared with that in w^- controls. Conversely, treatment of wild-type tubules with cGMP increases *w* mRNA expression levels, implying that cGMP is a physiologically relevant substrate for White. Immunocytochemical localisation reveals that White is expressed in intracellular vesicles in tubule principal cells, suggesting that White participates in vesicular transepithelial transport of cGMP.

Key words: Malpighian tubule, functional genomics, cyclic nucleotide, transporters, ABCC, ABCG.

INTRODUCTION

The first *Drosophila* mutation to be identified was *white* (*w*), and it was instrumental to Morgan's description of genes and chromosomes (Morgan, 1910). Despite over 3000 publications on *w* since then (FlyBase, 2007), few have investigated the biological function of White protein, tending instead to concentrate on the genetics of *w*. The most prominent phenotype of *w* mutants is the pronounced lack of eye colour (Fig. 1A). However, eye colour phenotypes can also reflect defects in Malpighian tubule structure and function, because several eye pigment precursors [notably the transport of compounds in the xanthommatin biosynthetic pathway (Sullivan and Sullivan, 1975; Wessing and Eichelberg, 1968)] are stored and processed in the larval tubule before being released into the pupal haemocoel for uptake by the developing adult eye. The Malpighian tubules of w^- *Drosophila* are clear or whitish in appearance due to the absence of tryptophan metabolites and pteridines, unlike wild-type tubules, which are yellow in appearance (Fig. 1B) (Wessing and Eichelberg, 1978).

Insect Malpighian tubules are critical for survival and play essential roles in osmoregulation, homeostasis and immune function (Dow and Davies, 2005). Excess fluid and solutes are transported and excreted largely across these blind-ended tubules, with selective re-absorption occurring in the rectum. *Drosophila* tubules provide a unique model for studying transport in a live polarised epithelial tissue, with added benefits of the availability of genetic tools (Dow et al., 1994a; Dow and Davies, 2003). cGMP and cAMP signalling has been studied in the *Drosophila* Malpighian tubule for over 10 years (Dow et al., 1994b), but the transport mechanisms of these cyclic nucleotides have received rather less attention (Riegel et al., 1998). Nitric oxide (NO)–cGMP signalling was first identified to stimulate fluid transport (Davies et al., 1995), with more recent work implicating NO signalling in the immune response (McGettigan et al., 2005). Furthermore, NO–cGMP signalling can be induced by activation of the capa receptor in Dipteran tubules from several species (Pollock et al., 2004); and so is an important feature of tubule function in insect vectors of disease. Exogenous cAMP also stimulates fluid transport (Davies et al., 1995; Riegel et al., 1998), with fluid transport also being stimulated by corticotropin-releasing factor (CRF)-like peptide (Cabrero et al., 2002) and calcitonin-like peptide (Coast et al., 2001).

Work from other groups has demonstrated that cGMP and cAMP are transported into the Malpighian tubules (Riegel et al., 1998); our laboratory has also recently shown that cGMP transport across the tubule (efflux) is modulated by cGMP-dependent phosphodiesterases (cG-PDEs) (Day et al., 2006). In mammalian systems, cyclic nucleotide transport has been attributed to a number of ATP binding cassette (ABC) transporters and solute carriers (members of the SLC22 family) (Dazert et al., 2003; Koepsell, 2004; van Aubel et al., 2002). These transporters have been widely studied in mammals but equivalent transporters have not previously been identified in dipteran insects.

White is a member of the ABC transporter subfamily G (Shulenin et al., 2001), with greatest sequence similarity to human ABCG2. Investigations of the role of White in Malpighian tubules were carried out ~30 years ago, and tentatively identified location of, and transport substrates for, White. The location of White in the tubules was thought to be either the basolateral membrane (Sullivan et al., 1980) or the pigment storage vesicle membranes (Sullivan et al., 1979). Potential substrates for White included tryptophan (Sullivan et al., 1980), kynurenine (Sullivan and Sullivan, 1975), 3-hydroxykynurenine (Howells et al., 1977), guanine and riboflavin (Sullivan et al., 1979).

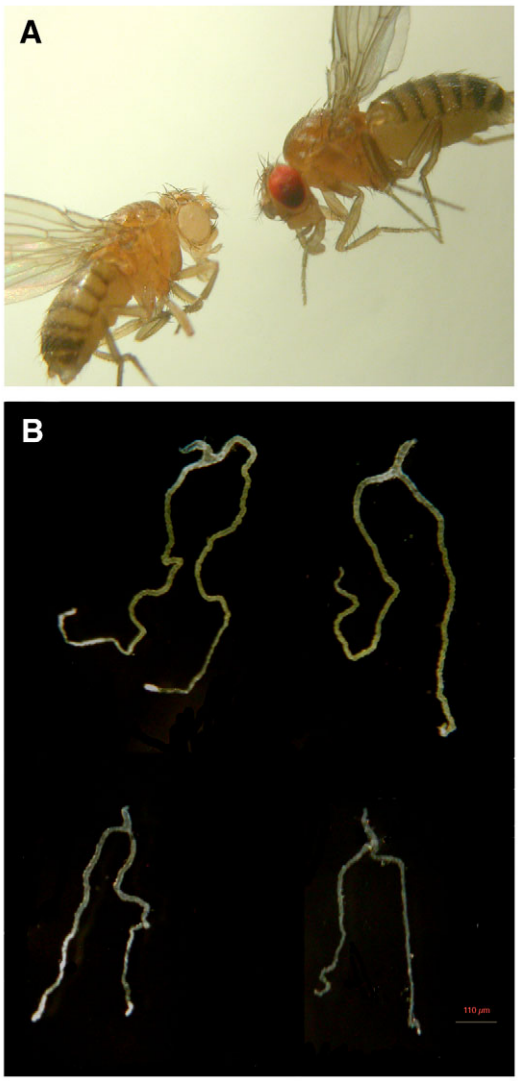

Fig. 1. Phenotypes associated with the *w* gene. (A) The eye colour phenotype: *w* null (w^{1118}; bottom left), and Canton S (top right) adult *D. melanogaster*. Female *w* null is ~3 mm long. (B) Tubule phenotype: Malpighian tubules from Canton S (top) and w^{1118} (bottom) adults. Anterior tubules are on the left, and posterior tubules on the right of the picture. Scale bar, 110 μm.

Here we demonstrate that cGMP transport by the tubule occurs *via* ABCG2 transporters, and that White is required for cGMP but not cAMP, transport across the Malpighian tubule. Localisation of White to intracellular vesicles in the principal cells in the main, fluid-transporting segment of the tubule, indicates a possible storage–excretion mechanism of cGMP transport *via* vesicles.

MATERIALS AND METHODS

Drosophila

Drosophila melanogaster Meigen were reared on standard medium at 25°C, with a 12 h:12 h ligh:dark photoperiod and 55% relative humidity. Lines used in this study were: wild-type, Canton S; *white* mutants: w^{1118} (partial deletion, loss of function: http://flybase.bio.indiana.edu/reports/FBal0018186.html) and w^{H} (hypomorphic allele) (Zachar and Bingham, 1982); tubule principal cell-specific GAL4 driver, c42 (Broderick et al., 2004); transgenic *w*::eYFP lines D4, E5 and H8 (generated for this study).

Mutant *w Drosophila* (w^{1118} and w^{H}) were 'cantonized' by crossing white-eyed flies with isogenized Canton S wild-type flies. The offspring were collected and interbred to produce recessively white-eyed offspring. This process was repeated five times, thus removing 97% of any compensatory mutations that might have accumulated since the mutant stocks were first isolated.

For dissection, flies were anaesthetized by chilling on ice, and decapitated, before removing the tubules in Schneider's medium (Invitrogen Ltd, Paisley, Scotland). All chemicals and drugs were obtained from Sigma (Sigma-Aldrich, Gillingham, Dorset, UK), unless otherwise stated.

Generation of transgenic *Drosophila*

Over-expression lines containing White tagged with enhanced yellow fluorescent protein (eYFP) at the C terminus were generated as follows:

The coding sequence for eYFP (Clontech UK Ltd, Basingstoke, Hampshire, UK) was amplified using primers that incorporated *Not*I and *Kpn*I sites at the 5′ and 3′ ends, respectively (GCGGCC-GCCATGGTGAGCAAGGGCGAGG/GGTACCCTACTTGTAC-AGCTCGTCCATGC). The resulting fragment was cloned into the *Not*I and *Kpn*I sites of *p*P{UAST} using standard methods to form pP{eYFP-UAST}. The *white* open reading frame, excluding the stop codon, was PCR amplified from tubule cDNA template using primers: AGATCTATGGGCCAAGAGGATCAGGAG/GCGG-CCGCCTCCTTGCGTCGGGCCCGAAG, that incorporated *Eco*RI and *Not*I sites at the 5′ and 3′ ends, respectively. This fragment was cloned into *p*P{eYFP-UAST} using the *Eco*RI and *Not*I restriction sites to form plasmid *p*P{w^{-}eYFP-UAST}. The insert was sequenced to check for PCR errors, and the plasmid injected into w^{-} *Drosophila* embryos (w^{1118}) by standard techniques (Vanedis *Drosophila* injections service, www.vanedis.no). Transformants were selected and maintained using standard *Drosophila* genetic techniques.

Fluid transport assays

Fluid transport assays were performed as previously described (Dow et al., 1994b) on intact tubules from Canton S, w^{1118} and cantonised w^{1118} 7-day-old adult flies. Basal rates of fluid transport were established for 30 min, after which 100 μmol l^{-1} cGMP was added to tubules and fluid transport rates measured for a further 60 min. Data are shown as mean fluid transport rates ± s.e.m., N=7.

Cyclic nucleotide transport assays

Transport assays for cGMP and cAMP were based on a modified fluid transport assay; transport rates ratios were calculated as previously described (Day et al., 2006). The transport rate provides a linear measure of basal to apical unidirectional flux, whereas a secreted:bathing ratio of >1 indicates that the transport substrate is being concentrated by the tubules (Maddrell et al., 1974). Maximal rates of cGMP and cAMP transport occurred at 100 μmol l^{-1} (Evans, 2007); thus, all transport assays were conducted with a final concentration of 100 μmol l^{-1} of cyclic nucleotide. The maximal rates of transport of cAMP are significantly higher than that of cGMP: a transport ratio of ~5 at 100 μmol l^{-1} cAMP, *vs* ~3 for cGMP at 100 μmol l^{-1} cGMP.

Tubules were dissected into saline (Dow et al., 1994a) and allowed to recover for 30 min prior to addition of cyclic nucleotides: 'cold' cGMP or cAMP at 100 μmol l^{-1}, and tritiated cGMP or cAMP added as tracer (Amersham Pharmacia, Biotech UK Ltd, Amersham, Bucks, UK). Where competitors or drugs were included, these were added 30 min before the radiolabelled substrate. Where the removal of amino acids and citrate was investigated, a minimal *Drosophila*

saline was used (Linton and O'Donnell, 1999) to which the missing ingredients of *Drosophila* saline (Dow et al., 1994a) were reintroduced at the concentrations normally used.

In all the transport assays, the ratio and rate of transport was measured 1 h after the radiolabelled cyclic nucleotide was added (Evans, 2007). The tubules were allowed to secrete for 1 h before the secreted droplet was measured and removed to Eppendorf tubes containing scintillation fluid (Fisher Scientific, Loughborough, UK). A 1 μl sample of each reservoir droplet was also removed and radioactivity measured in the scintillation counter (Beckman, High Wycombe, UK).

cGMP-dependent kinase bioassay for secreted cGMP

In order to determine if unaltered cGMP is transported through the tubule from the bathing droplet into the lumen, secreted fluid was tested for its ability to stimulate cGMP-dependent protein kinase (cGK) activity *in vitro*. A secretion assay was carried out with 80 tubules in the standard bathing droplet of *Drosophila* saline/Schneiders' medium (control) or saline/Schneiders' medium with 100 μmol l^{-1} cGMP. After allowing the tubules to secrete for 1 h, secreted droplets were pooled (~2 ml in total), removed from the secretion assay dish and placed into an Eppendorf tube. To remove any residual mineral oil derived from the secretion assay, samples were centrifuged and the oil (top layer) was discarded. A cGK assay was then carried out using the *Drosophila* cGK, DG2 (MacPherson et al., 2004), which had been expressed in S2 cells as a source of DG2 and therefore, cGK activity (MacPherson et al., 2004). Standard kinase reactions were set up in a total volume of 44 μl with 5 μl of DG2 protein sample, 39 μl of kinase assay buffer (MacPherson et al., 2004) and either 1 μl of secreted fluid from control samples or 1 μl of secreted fluid from tubules incubated in 100 μmol l^{-1} cGMP. Positive controls were set up by adding 1 μl of 100 μmol l^{-1} cGMP (final concentration 2.2 mmol l^{-1}) to the assay mix as described above. Three separate experiments were performed for each condition and the results expressed in pmol ATP min^{-1} mg^{-1} protein (mean ± s.e.m.).

Real-time quantitative PCR (Q-PCR)

Q-PCR was performed as described previously (McGettigan et al., 2005), using mRNA prepared from tubules from 7-day-old adult *Drosophila*. Where the effect of cGMP on gene expression was being investigated, tubules were incubated with or without 100 μmol l^{-1} cGMP in Schneider's medium for 3 h before the mRNA was extracted. Reverse transcription was carried out using Superscript II (Invitrogen) using oligo(dT) primers. For each sample, 500 ng of cDNA was added to 25 μl of SYBR Green reaction mix (Finnzyme, Oy Espoo, Finland) with an appropriate concentration of the primers – WhiteF: GCCACCAAAA-ATCTGGAGAAGC/WhiteR: CACCCACTTGCGTGAGTTG-TTG. Reactions were carried out in an Opticon 2 thermocycler (MJ Research Inc., Waltham, MA, USA). The ribosomal *rp49* (*rpl32*) gene (primers rp49F: TGACCATCCGCCCAGCATAC/rp49R: TTCTTGGAGGAGGACGCCGTG) was used as a reference standard in all experiments (McGettigan et al., 2005).

Immunocytochemistry

Immunocytochemistry on intact Malpighian tubules was carried out as previously described (MacPherson et al., 2001). A mouse monoclonal primary anti-GFP antibody recognising GFP variants (Zymed, Invitrogen Ltd) diluted 1:1000 in PAT [0.05% (v/v) Triton X-100 and 0.5% (w/v) BSA in PBS with14 mmol l^{-1} NaCl, 0.2 mmol l^{-1} KCl, 1 mmol l^{-1} Na_2HPO_4 and 0.2 mmol l^{-1} KH_2PO_4, pH 7.4], was used; followed by addition of secondary antibody, Alexa Fluor® 568-labelled anti-mouse IgG (Molecular Probes, Invitrogen Ltd), diluted 1:500 in PAT. The nuclear stain 4′,6-diamidino-2-phenylindole, dihydrochloride (DAPI) was applied to the tubules for 1 min at 500 ng ml^{-1} in PBS. Samples were viewed using a Zeiss 510 Meta confocal system and images processed using LMS image software. All images were taken at the same gain and exposure.

RESULTS

cGMP is transported across the *Drosophila* Malpighian tubule

Although cGMP transport has been previously demonstrated in *Drosophila* tubules (Day et al., 2006; Riegel et al., 1998) it has not been clear if transport of cGMP by the tubule occurs as a result of active transport or passive diffusion. Malpighian tubules are an electrogenic epithelial tissue with a resting transepithelial potential difference (TEP) of 50 mV (lumen positive). This is increased to about 60 mV by 100 μmol l^{-1} cGMP and reduced to about 20 mV by the neuropeptide leucokinin (O'Donnell et al., 1996). Leucokinin acts independently of cAMP or cGMP to increase calcium transport and stimulate fluid transport (O'Donnell et al., 1996). Cyclic GMP carries a net negative charge in solution, so concentration in secreted fluid relative to the bathing fluid is not sufficient to assert primary active transport. The Nernst equation predicts that a secreted:bathing droplet ratio of 10 should be achievable passively at a TEP of +60 mV, whereas a smaller ratio of 2.1 should be achievable at a TEP of +20 mV. The observed ratio of cGMP

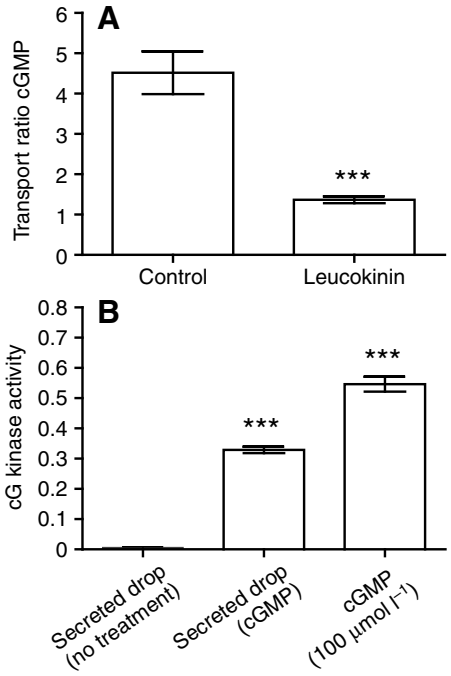

Fig. 2. cGMP is transported by tubules. (A) Effect of depolarisation on the cGMP transport ratio. Leucokinin (100 μmol l^{-1}) was used to depolarise the Malpighian tubule 30 min prior to the addition of cGMP (100 μmol l^{-1}; values are means ± s.e.m.; $N \geq 10$; ***$P<0.0001$, Student's *t*-test). (B) cGMP-dependent kinase (cGK) activity in tubules is stimulated by fluid secreted from cGMP-treated Malpighian tubules. Data are expressed as mean (± s.e.m.) cGK activity, in pmol ATP min^{-1} mg^{-1} protein ($N=3$). cGK activity in tubule extracts is significantly stimulated by secreted drops from cGMP-treated tubules or by 100 μmol l^{-1} cGMP, but not by secreted fluid from non-treated tubules (***$P<0.0001$, Student's *t*-test).

transport after treatment with leucokinin was 1.2 (Fig. 2A). As we were unable to find conditions under which cGMP was concentrated in the lumen beyond the values predicted by the Nernst equation, we cannot assert that transport of cGMP by the tubule reflects primary active transport; it may also take place by secondary active transport, or by facilitated diffusion.

Net transport of the tritium label does not of itself imply that cGMP is transported unmodified by the tubule; for example, cGMP could be cleaved to GMP by cellular phosphodiesterases. In mammals, cGMP is detected in unmodified form in urine (Ashman et al., 1963). In *Drosophila*, cGMP uptake studies in tubule have shown that ~20% of total tubule cGMP remains in unmodified form (Riegel et al., 1998). However, it can be expected that at least some unmodified cGMP is excreted by the *Drosophila* tubule. Here we show that secreted fluid from the tubule can significantly stimulate cGMP-dependent protein kinase (cGK) activity (Fig. 2B). *Drosophila* cGKs are high K_m enzymes, requiring cGMP in the micromolar range for activation (MacPherson et al., 2004). Thus, although the stimulation of cGK activity by secreted tubule fluid does not prove that *all* cGMP is transported in unmodified form, these data strongly suggest that a significant amount of cGMP is secreted by the tubule in unmodified form.

Distinct transport processes for cGMP and cAMP by *Drosophila* tubules; cAMP transport requires di- and tri-carboxylates

In order to determine if tubules transport cGMP *via* a common route for cyclic nucleotides, or if cGMP transport was specific for that cyclic nucleotide, competition assays were carried out for both cAMP and cGMP transport in the presence of the competing cyclic nucleotide (Fig. 3A). The results show that for all concentrations of cyclic nucleotide tested, including the saturating concentration of 100 μmol l^{-1}, no statistically significant effect on cyclic nucleotide transport was determined. This strongly suggests that cAMP and cGMP are differentially transported by the tubule.

Furthermore, transport of cAMP but not cGMP, requires di- and tri-carboxylates. In mammals, it has been shown that both cAMP and cGMP are exchanged for dicarboxylates by organic anion transporter 1 (OAT1), a member of the SLC22 family, at the renal basolateral membrane (Sekine et al., 2000). Fig. 3B shows that cGMP transport is not affected by removal of di- or tri-carboxylates in the assay saline (minimal saline, resulting in a saline containing only salts, sugars and seven of the amino acids) (Linton and O'Donnell, 1999). By contrast, cAMP transport is significantly reduced in minimal saline (Fig. 3B). Re-addition of the most abundant amino acids, including β-alanine, arginine, tryptophan or methionine, revealed that cAMP transport was restored only in the presence of the dicarboxylates aspartic acid or glutamic acid, or the tricarboxylate citrate (Fig. 3C). Thus a dicarboxylate transporter is required for cAMP, but not cGMP, transport.

Identification of cyclic nucleotide transporter families in Malpighian tubules

Putative cyclic nucleotide transporters have yet to be identified in *Drosophila* Malpighian tubules. In this work, identification of putative cGMP or cAMP transporters in the tubule was achieved by using several different substrates and pharmaceutical drugs. As ABCG2 and ABCC4 transporters have been shown to be involved in cyclic nucleotide transport in mammalian cells, known substrates for these were tested (Table 1).

Glibenclamide is a broad-specificity ABC transporter inhibitor. Glibenclamide was previously shown to be a potent inhibitor of

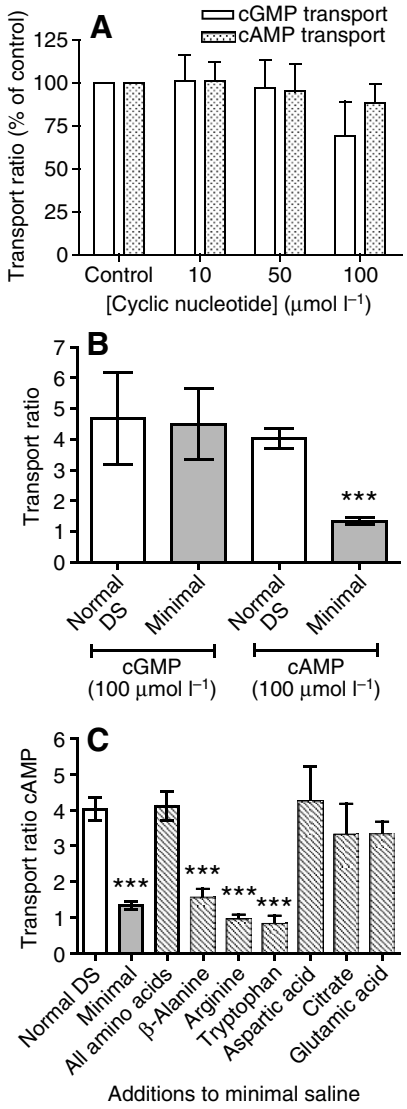

Fig. 3. Transport of cAMP and cGMP by the tubule are distinct processes. (A) cGMP and/or cAMP transport assays were performed on wild-type tubules in the absence (controls=100%) or presence of cyclic nucleotide (cAMP for cGMP transport; cGMP for cAMP transport) Percentage changes in the ratio of cGMP or cAMP transport compared to the controls are shown as mean ± s.e.m. ($N \geq 5$). Statistical significance was verified by Student's *t*-test compared with control (no added drugs), taking $P<0.05$ as the critical level. (B) cGMP and cAMP transport ratios from tubules assayed in either normal *Drosophila* saline (normal DS, unshaded bars) or minimal saline (shaded bars). (C) cAMP transport ratio from tubules assayed in either normal *Drosophila* saline (normal DS, unshaded bars); in minimal saline (shaded bar); or in minimal saline with additions of specific amino acids or other compounds as indicated (hatched bars). Data are mean ± s.e.m. ($N \geq 5$). ***Data significantly different ($P<0.001$; Student's *t*-test) from cAMP transport ratio in normal DS.

cGMP transport in human erythrocyte membrane vesicles, with an IC_{50} of 2.8 μmol l^{-1} (Klokouzas et al., 2003). Although glibenclamide has been shown to inhibit fluid transport by Malpighian tubules, probably by virtue of its action on inwardly-rectifying K^+ channels (Evans et al., 2005), application of glibenclamide to tubules at concentrations far too low to affect fluid transport in tubules (Evans et al., 2005) potently inhibited both cGMP and cAMP transport in tubules (Table 1). This suggests that

Table 1. Effect of ABC transport inhibitors on cyclic nucleotide transport

Compound type	Compound	Concentration (μmol l^{-1})	cGMP transport ratio (% of control)	cAMP transport ratio (% of control)
Broad-specificity ABC transporter inhibitor	Glibenclamide	50	11±2***	1±7***
		10	36±3***	23±9***
		1	64±7*	94±19
ABCG2 substrates	Methotrexate	4000	53±8*	41±5*
		500	71±12	
		100	84±27	91±8
		50	114±15	91±18
	Mitoxantrone	20	27±7**	
		2	63±10	
		0.2	75±17	
ABCC substrates	Dipyridamole	10	95±26	
		1	86±9	
		0.1	79±13	

cGMP and/or cAMP transport assays were performed on wild-type tubules in the absence (controls, 100%) or presence of compounds known to affect specific transporter families.

Values are mean ± s.e.m. ($N \geqslant 5$). Statistical significance was verified by Student's *t*-test compared with control (no added drugs), taking $P<0.05$ as the critical level. *$P<0.05$, **$P<0.01$, ***$P<0.001$.

ABC transporters are involved, but does not indicate a specific ABC sub-family. Thus, substrates specific for sub-families of the ABC transporter class, ABCG and ABCC4, were tested for their effects on cyclic nucleotide transport.

ABCG

To test the involvement of transporters of the sub-family ABCG, the known pharmacological inhibitors methotrexate (Volk and Schneider, 2003) and mitoxantrone (Doyle et al., 1998) were used. Methotrexate has been shown to inhibit cGMP uptake by inside-out human erythrocyte membrane vesicles by 75% at 275 μmol l^{-1} and by 100% at 375 μmol l^{-1} (Klokouzas et al., 2003). In *Drosophila* tubules, both cGMP and cAMP transport were both reduced at high concentrations of methotrexate; this reduction became statistically significant at a concentration of 4 mmol l^{-1}. Although the ABCG inhibitor mitoxantrone has not been shown to affect cGMP transport in erythrocytes (Sundkvist et al., 2002), treatment of tubules with mitoxantrone did impact on cGMP transport; at 20 μmol l^{-1} the ratio of cGMP transport by tubules was reduced to 27% of that of untreated control tubules (Table 1).

ABCC4

In mammalian renal cells, cGMP is transported by ABCC4 (van Aubel et al., 2002); and is potently inhibited by dipyridamole at a concentration of 1.0 μmol l^{-1}. In *Drosophila* Malpighian tubules, dipyridamole did not affect the transport of cGMP (Table 1). ABCC4-associated cAMP transport occurs in mammals (van Aubel et al., 2002), and cAMP-induced inhibition of cGMP transport has been shown to occur at 50 μmol l^{-1} cAMP. However, this is not the case in *Drosophila* tubules because application of cAMP at concentrations between 1–100 μmol l^{-1} does not significantly affect cGMP transport (Table 1). Furthermore, cGMP treatment did not significantly inhibit cAMP transport (Table 1). This suggests that ABCC4-like transporters are not involved in cyclic nucleotide transport in the tubules and that cAMP and cGMP transport do not occur *via* the same transporter.

The Malpighian tubules are the major site of *w* expression in the fly

white (*w*) is famously associated with eye colour in *Drosophila* but also has phenotypic effects in the tubule (Fig. 1). Recently, a comprehensive tissue-based microarray dataset (FlyAtlas.org) has become available for adult *Drosophila* tissues (Chintapalli et al., 2007), allowing sites of gene expression to be determined easily 'in silico'. FlyAtlas expression data reveal that *w* expression is highest in tubules (up by 40× compared with the whole fly), and in relation to other adult tissue in the fly, including the head, which contain the eyes. Furthermore, expression of *w* is very high in larval tubules (56×) compared to whole adults (Table 2). The larval fat body also shows significant expression of *w*.

Table 2. Expression of *w* in adult fly tissues and in two larval tissues

Tissue	mRNA signal	Present call	Enrichment	Affy call
Brain	12.1±1	4/4	0.33	Down
Head	62±5	4/4	1.61	UP
Crop	81±6	4/4	2.07	Down
Midgut	11±2	0/4	0.30	Down
Tubule	**1571±114**	4/4	**40.13**	**UP**
Hindgut	32±5	4/4	0.84	None
Ovary	7±1	0/4	0.18	Down
Testis	38±3	4/4	0.99	None
Male accessory gland	4±2	0/4	0.12	Down
Larval tubule	**2292±94**	4/4	**58.56**	**UP**
Larval fat body	**444±45**	4/4	**11.36**	**UP**
Whole fly	39±7	3/4		

Major sites of expression of *w* are indicated in bold. Data are from flyatlas.org. (Chintapalli et al., 2007). 'mRNA' signal indicates how abundant *w* mRNA is; the Affymetrix 'present call' indicates how many times *w* is detectably expressed out of four arrays; 'mRNA enrichment' indicates comparison to whole flies.

One of the roles of insect Malpighian tubules is the secretion of fluid in the maintenance of homeostasis. Given the very high levels of *w* expression in tubules, investigation of fluid transport rate by wild-type and *w* mutant (w^{1118},

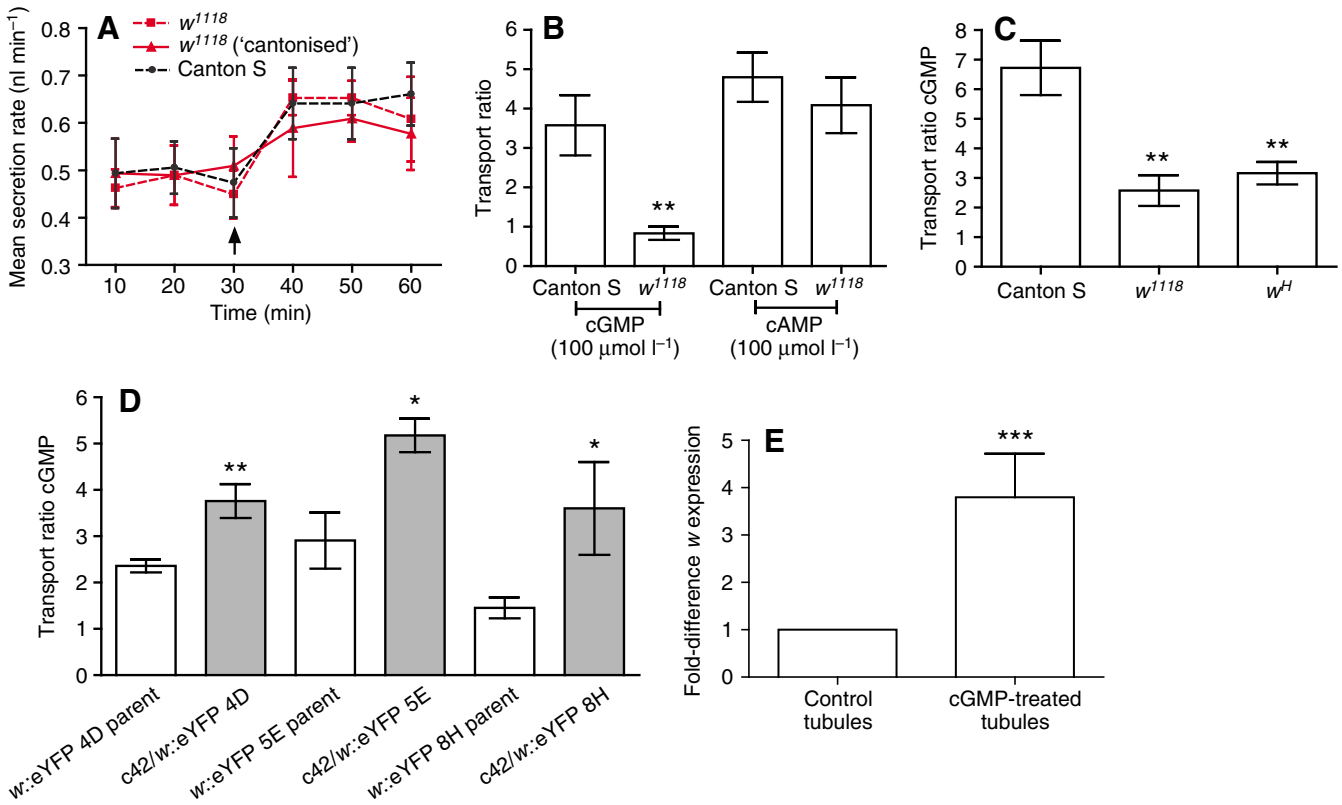

Fig. 4. *w* does not affect fluid transport but has a direct effect on cGMP transport. (A) Fluid transport rates by wild-type (Canton S, black circles) and w^{1118} (Bloomington stock, red squares and 'cantonised' stock, red triangles). Malpighian tubules were stimulated by cGMP (100 μmol l^{-1}, added at 30 min, indicated by arrow). Data are expressed as mean (± s.e.m.) fluid transport rate in nl^{-1} min (N=7). (B) Ratio of cGMP and cAMP transport by Canton S and w^{1118} Malpighian tubules. Data are mean ratios ± s.e.m. (N≥5). (C) Ratio of cGMP (100 μmol l^{-1}) transport by Canton S tubules and by tubules from cantonised w^{1118} and w^{H} lines. Data are mean ± s.e.m. (N≥5). (D) Ratio of cGMP (100 μmol l^{-1}) transport in three independently derived parental transgenic *w*::eYFP lines (4D, 5E, 8H, unshaded bars) and offspring from crosses to a GAL4 driver, c42 (c42/*w*::eYFP; grey bars). Data expressed as a ratio of cGMP transport ± s.e.m. (N≥5). cGMP transport for the c42 GAL4 driver was: 1.9±0.17 (N=7). Significance of data between progeny compared to parental controls of each line is indicated by **P<0.01; *P<0.05, Student's *t*-test. (E) Q-PCR of *w* mRNA levels in untreated (control) tubules; and in tubules treated with 100 μmol l^{-1} cGMP. *w* expression was normalised against a cDNA standard (*rp49*) as previously described. Data are expressed as mean (± s.e.m.) fold-difference of *w* expression of cGMP-treated tubules compared to control tubules (N=4). ***Data significantly different from control (P<0.001; Student's *t*-test).

both extant and cantonised stocks) tubules was carried out. This revealed no significant difference in either basal fluid transport rates, or rates stimulated by cGMP, between these tubules (Fig. 4A). Thus, *w* is not directly involved in fluid transport by the tubule.

White modulates cGMP transport

w is an orthologue of members of the ABCG family of genes, with closest similarity to *ABCG2* (Mackenzie et al., 1999). Given that data in Table 1 show a significant effect on cGMP transport by ABCG inhibitors, and that the *w* gene product is an ABCG2 family member, which is very highly expressed in the tubule, we tested the hypotheses that White acts as a cGMP transporter in the tubule. To initiate these studies, potential substrates of White, including tryptophan, kynurenine and 3-hydroxykynurenine (Howells et al., 1977; Sullivan et al., 1980) were utilised in the cGMP transport competition assays (Table 3). At high concentrations, kynurenine inhibited both cGMP and cAMP transport (Table 3), but tryptophan did not. The mechanism of kynurenine inhibition on cAMP/cGMP transport is unknown. Kynurenine is a large, anionic molecule and may competitively block transport of either cGMP or cAMP. It is also possible that in addition to inhibition of White (and therefore of cGMP transport), a transporter for cAMP is inhibited by kynurenine. Tubule-specific microarray experiments have demonstrated high enrichment of genes encoding monocarboxylic acid transporters, including *CG12286* (Wang et al., 2004), which we argued corresponds to karmoisin (http://flybase.bio.indiana.edu/reports/FBgn0001296.html), a probable kynurenine tranporter. Thus, given the requirement for di- and tri-carboxylates for cAMP transport by tubules, it is possible that the *CG12286*-encoded transporter also transports cAMP; and that this transport of cAMP is inhibited when kynurenine is added exogenously to tubules, thus explaining the data in Table 3. The concentrations of tryptophan and kynurenine used did not affect fluid transport rates by the tubules (data not shown). Hydroxykynurenine was not tested because it is insoluble in aqueous solutions and so could not be added to conventional assays.

As *w* is most abundantly expressed in the tubule, and as substrates for White inhibit cGMP transport by the tubule, cGMP transport in tubules of White mutants was investigated. Such transport assays showed that transport of cGMP was inhibited in tubules from flies that were mutant for *w* (loss-of-function w^{1118}), although cAMP flux was unaffected (Fig. 4B). Furthermore, the ratio of cGMP transport was approximately one, indicating markedly reduced active transport of cGMP. The inhibition of cGMP transport observed in the w^{1118} tubules was not due to genetic modifiers accumulated by this line, as tubules from 'cantonized' *w* mutants (w^{1118} and w^{H}) also showed

Table 3. Effect of known substrates of White on cyclic nucleotide transport

Compound type	Compound	Concentration (μmol l^{-1})	cGMP transport ratio (% of control)	cAMP transport ratio (% of control)
White/scarlet substrates	L-tryptophan	2000	103±24	
		1500		122±8
		1000	100±8	
		500	95±24	135±25
		250	104±23	
	L-kynurenine	2000	48±5**	27±2**
		1000	68±8	81±10
		500	103±20	

cGMP and/or cAMP transport assays were carried out on wild-type tubules in the absence (controls, 100%) or presence of known substrates of White/Scarlet. Percentage changes in the ratio of cGMP or cAMP transport compared to the controls are shown as mean ± s.e.m. ($N \geq 5$). Significance was verified by Student's *t*-test compared with control (no added drugs), taking $P<0.05$ as the critical level. **$P<0.01$.

reduced cGMP transport (Fig. 4C). This demonstrates that cGMP transport is directly associated with functional White.

Overexpression of *w* rescues the cGMP transport defect in *white* mutants

Most transgenic *Drosophila* lines are generated in a w^- background, eg. w^{1118}. Generation of the *w::*eYFP lines in such a background thus allows not only for overexpression of *w* but also for rescue of the *white* phenotype upon targeted overexpression. For these studies, several independently generated *w::*eYFP lines were used, in conjunction with the tubule principal cell-specific GAL4 driver, c42 (Broderick et al., 2004). Q-PCR data for expression of *w* in tubules of c42/*w*::eYFP(D4) and c42/*w*::eYFP(E5) compared to parental UAS-*w*::eYFP lines shows between 15-fold [c42/*w*::eYFP(D4)] and 1.3-fold [c42/*w*::eYFP(E5)] overexpression compared with parental lines (Evans, 2007). Thus, expression levels of *w* are significantly increased upon induction with GAL4 in tubules from these lines.

Furthermore, targeted overexpression of *w* to tubule principal cells resulted in a cGMP transport ratio significantly greater than that observed in tubules from the parental UAS-*w::*eYFP lines (Fig. 4D); and compared to w^{1118} (Fig. 4C). Taken together, these data thus establish a direct role of White in cGMP transport in *Drosophila* tubules.

Exogenous cGMP upregulates expression of *white*

Is transport of cGMP by White incidental to its role in transport of eye pigment precursors, or is White-associated cGMP transport physiologically relevant? In order to test this, possible feedback effects of cGMP on *w* expression were investigated: does an increase in cGMP levels upregulate its expression, so increasing cGMP clearance? Real-time PCR of *w* expression was assessed in control, untreated tubules and in tubules treated with exogenous cGMP for 3 h. In the cGMP-treated tubules, *w* expression increased significantly by 3.8±0.92 fold (Q-PCR data, Fig. 4E). Thus, the cGMP-induced increase in *w* expression supports a role for White in cGMP transport.

Localisation of White in adult tubules

In the *Drosophila* eye, White has been identified in the pigment granule membrane (Mackenzie et al., 2000). In order to resolve the localisation of White in tubules, targeted expression of the *w::*eYFP fusion was driven in tubules using the principal cell-specific GAL4 driver c42. Whilst the fluorescence emitted by eYFP would be expected to be indicative of the expression pattern of White, immunocytochemistry against eYFP using an anti-GFP antibody that recognises GFP derivatives was performed in order to amplify the signal, and to reduce the impact of autofluorescence by the tubule. Results from these experiments in two independently derived UAS-*w::*eYFP lines show that White is expressed in the cytoplasm of the principal cells (Fig. 5A, B). Closer inspection reveals vesicular expression of White in the cytoplasm of principal cells (Fig. 5D, compare to autofluorescence in Canton S tubules, Fig. 5C). Thus, given that White is required for cGMP transport by the tubule, its localisation suggests that trafficking of cGMP can occur through the cell *via* intracellular vesicles.

DISCUSSION

Cyclic nucleotide transport in the Malpighian tubule

Uptake and excretion of cGMP by *Drosophila* Malpighian tubules has been shown previously (Riegel et al., 1998). We show here that the transport of cGMP is sensitive to the electrical gradient, suggesting that a secondary active transport mechanism is involved. We also show here that secreted fluid is capable of stimulating cGMP-dependent protein kinase (cGK) activity, suggesting that as in mammals, cGMP can be transported across the tubule membrane in unaltered form in *Drosophila*. Although primary active transport could not be shown unambiguously, the ABCG transport ATPase, White, is nonetheless necessary for significant cGMP flux; it must thus, at least, facilitate the diffusion of cGMP. In tubules, White is expressed throughout the cytoplasm in large vesicles of the main segment. These vesicles are probably the pigment vesicles which were suspected to be the location of White in a previous study (Sullivan et al., 1980). Other sub-families of ABC transporters also show intracellular localisation: ABCC4 transporters localise to the dense granules of platelets, and not at the plasma membrane (Jedlitschky et al., 2004). Thus, it seems that sub-families of the ABC transporter family, in both vertebrates and invertebrates, can be localised to vesicular structures in the cytoplasm of the cell. The first identified ABCG transporter in *Leishmania*, LiABCG4, has been shown to localise to the plasma membrane and to post-Golgi secretory vesicles when overexpressed in yeast (Castanys-Munoz et al., 2007). However, in the parasite, LiABCG4 is mainly localised to the plasma membrane, with some localisation in flagellar pockets; suggesting that the localisation in secretory vesicles in yeast may be due to over-expression of the ABCG4 transporter in the yeast system. Although the vesicular localisation of White in tubule cells shown in Fig. 5 may be due to targeted over-expression of w^+, localisation of White in non-transgenic tubules as shown by Sullivan et al. (Sullivan et al., 1980) suggests that vesicular localisation for White is not associated with expression artifacts *in vivo*.

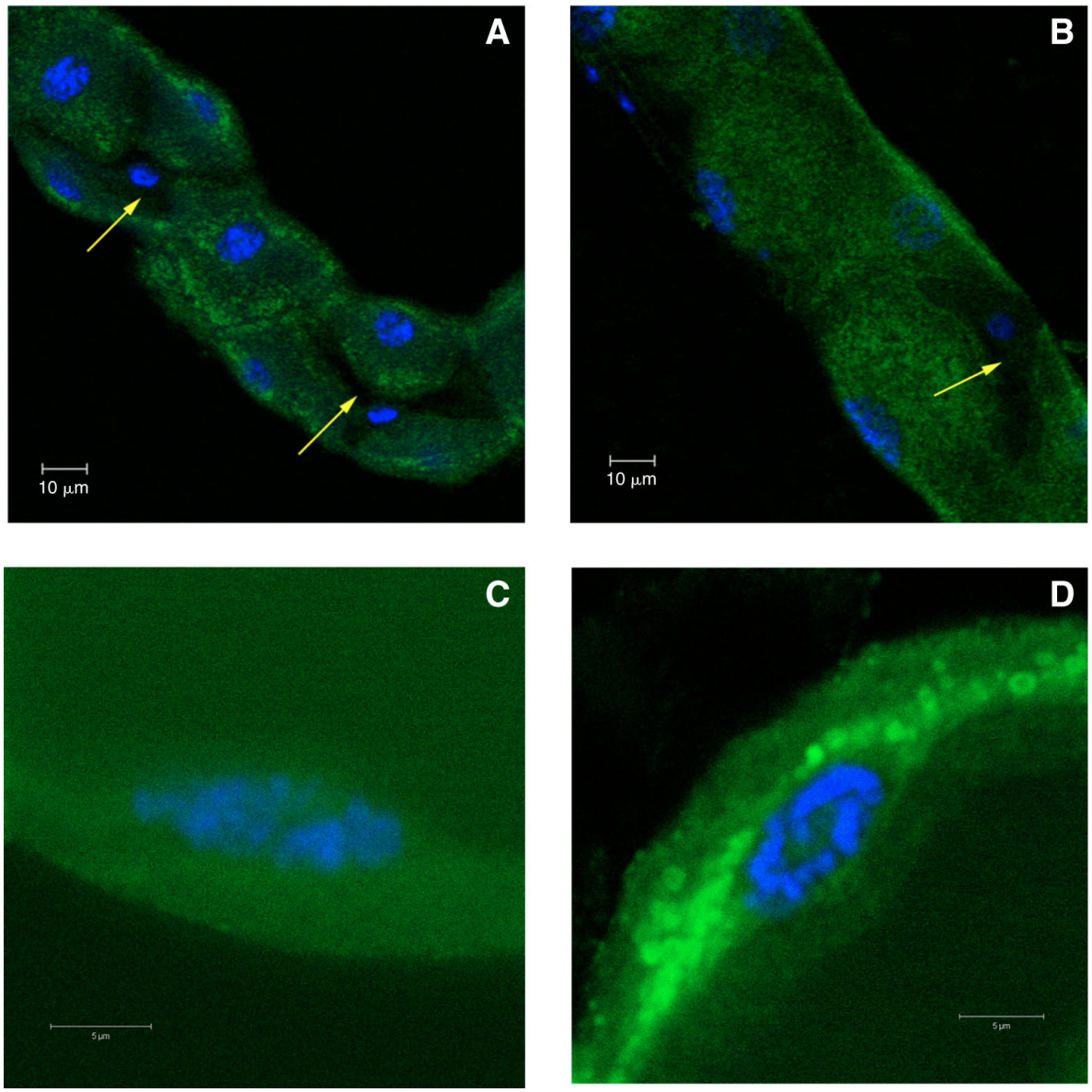

Fig. 5. Expression of White in the *Drosophila* Malpighian tubule. Staining of White expression in c42/*w*::eYFP adult tubules by immunocytochemistry with anti-GFP antibody. Intact tubules from adult progeny of crosses between c42 and two independently derived UAS-*w*::eYFP lines (E5 and H8) were used. (A) Main segment of c42/*w*::eYFP (UAS line E5) tubule showing exclusion of staining in stellate cells, yellow arrows. (B) Main segment of c42/*w*::eYFP (UAS line H8) showing exclusion of staining in stellate cells, yellow arrows. (C) Canton S tubules (high magnification) stained with anti-GFP antibody. Only background fluorescence is observed. (D) Principal cell in c42/*w*::eYFP (UAS line E5) tubule at high magnification showing vesicular staining. Scale bars: A,B 10 μm; C,D 5 μm.

The primarily vesicular localisation of White may also indicate trafficking of cGMP across the Malpighian tubules in vesicles, a novel mechanism of transepithelial cGMP transport that would not compromise the integrity of intracellular cGMP signalling pathways. This would explain why fluid transport assays on tubules from *w* loss-of-function mutants show similar rates of cGMP-induced fluid transport (Davies et al., 1995; Dow et al., 1994b) to wild-type tubules. Our model is thus that cGMP is transported into the cell by a basolateral plasma membrane cGMP transporter that still remains to be discovered; once in the cell, it can act to stimulate fluid transport. However, White sequesters cGMP into vesicles, contributing [perhaps together with the action of *Dm*PDE6 (Day et al., 2006)] to its clearance from the cell; and these vesicles are excreted from the apical surface of the cell, presumably as part of a general purpose organic solute clearance mechanism. In the absence of White, cGMP is still transported into cells but due to reduced uptake into intracellular vesicles, is transported into the lumen at a much reduced rate. It will be interesting in due course to try to identify the plasma membrane transporter for cGMP. Importantly, this multi-stage transport model explains why we were unable to demonstrate accumulation of cGMP beyond Nernst-predicted ratios, despite the involvement of a transport ATPase in the process.

Although the *w* gene has almost exclusively been researched in association with its role as an eye colour marker, recent microarray data of adult fly tissues (Chintapalli et al., 2007) has shown that *w*

is most highly expressed in Malpighian tubule, a tissue enriched for organic anion transporters (Wang et al., 2004). Previous studies have shown that White can act as a heteromeric transporter: with Scarlet, it is a tryptophan transporter, responsible for brown eye colour; with Brown, it transports guanine, the precursor of the red pigment in eye (Dreesen et al., 1988). Mutation of key residues such as glycine 589 in the fifth transmembrane helix of White, significantly reduces guanine transport by White–Brown heterodimers, suggesting the importance of G589 in heterodimerisation and in guanine transport (Mackenzie et al., 1999). Interestingly, mutation of amino acid 553 in TM5 of ABCG2 – a well-conserved residue corresponding to G589 in White – disrupts function and trafficking of ABCG2, implying conservation of dimerisation function of these residues across evolution (Polgar et al., 2006).

Other candidates for cGMP transporters

Even if White does play a key role in cGMP transport, organic solute transporters are heavily represented in the tubule transcriptome (Wang et al., 2004), and so there are other potential candidate transporters. In mammals, transporters of the ABCC class, notably MRP4 (ABCC4) and MRP5 (ABCC5) have been shown to transport cyclic nucleotides out of the cell (Ritter et al., 2005). Treatment of *Drosophila* tubules with glibenclamide, a broad-spectrum inhibitor of ABC transporters, results in inhibition of both cAMP and cGMP transport. However, utilising either known inhibitors or competing substrates for ABBC transporters shows that such transporters are not involved in cAMP/cGMP transport by Malpighian tubules. Interestingly, tubules express five of the seven *Drosophila* homologues of mammalian ABCC transporters (Day et al., 2006); with one gene, *CG9270*, being expressed *only* in tubules of the adult fly. It would be interesting to screen the product of this gene for ABCC transporter function, and to determine its substrate specificity. In any case, the lack of effect of ABCC-specific drugs on the tubule could indicate evolutionary divergence between mammalian and *Drosophila* ABCC transporters, or may simply reflect inaccessibility of these transporters to specific drugs if the transporters are localised to the apical membrane.

Differences between cAMP and cGMP transport

We show that cGMP transport by the tubule is specific and possesses distinct properties from cAMP transport. Previous investigations have suggested that there may be some overlapping function of cyclic nucleotide transporters (Riegel et al., 1998). The different conclusions reached by these different studies may be a reflection of the concentration of each cyclic nucleotide used – the competing cyclic nucleotide was greatly in excess in the Riegel study. We show here that cAMP transport requires the presence of di- or tri- carboxylates; but that cGMP transport is unaffected by these compounds. cAMP transport probably requires an OAT-like transporter at the basolateral membrane, whereas cGMP is transported *via* a different mechanism. Thus in the tubules, the mechanisms of transport of cGMP and cAMP are largely independent and specific. This could reflect the importance of these signalling molecules in the tubules. Alternatively, it could reflect the transport mechanisms necessary in a tissue for which there is a requirement for an established potential gradient to enable solute uptake and excretion.

Overall, though, the results suggest that the extraordinary abundance of White in the adult Malpighian tubule may reflect a novel role that continues beyond the need to handle visual pigment precursors in the larva and pupa. White is thus a more versatile transport protein that previously suspected.

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