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REGULAR ARTICLE

Megan McNulty · Michael Puljung · Greg Jefford
Ronald R. Dubreuil

Evidence that a copper-metallothionein complex is responsible for fluorescence in acid-secreting cells of the *Drosophila* stomach

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Abstract Copper cells were originally identified in *Drosophila* midgut epithelium by their striking orange fluorescence in copper-fed larvae. Here, we examined copper cell fluorescence in light of the previous observations that (1) a similar fluorescent signal in yeast is produced by a complex between copper and metallothionein, and (2) metallothionein is expressed constitutively in the copper cell region and inducibly in other regions of the *Drosophila* midgut. Pulse-feeding experiments with 1 mM $CuCl_2$ revealed that fluorescence appeared rapidly in copper cells (<5 min) and slowly in other cells of the midgut (days), suggesting a constitutive cofactor in the former and an inducible cofactor in the latter. Fluorescence was also detected in *Drosophila* S2 tissue culture cells after induction of metallothionein synthesis by addition of $CuCl_2$ to the growth medium. Thus, fluorescence coincided spatially and temporally with the expression of metallothionein. Fluorescence was also linked to the acid-secreting activity of copper cells. Fluorescence was not observed when acid secretion was inhibited by a mutation in the α spectrin gene and acidification was blocked in copper-fed wild-type larvae. However, acidification was restored after a 1-day chase period in which the fluorescent signal became sequestered within a vesicular compartment. We therefore conclude that copper cell fluorescence is most probably attributable to a cytoplasmic copper-metallothionein complex, suggesting an unanticipated role for metallothionein in acid-secreting cells.

Keywords Copper-metallothionein complex · Fluorescence · Acid-secreting cells · Digestive system · *Drosophila*

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M. McNulty · M. Puljung · G. Jefford · R. R. Dubreuil (✉)
Department of Neurobiology, Pharmacology, and Physiology, University of Chicago, 947 East 58th Street, Chicago, IL 60637, USA
e-mail: ron@drugs.bsd.uchicago.edu
Tel.: +1-773-7029154

Introduction

The larval digestive tract in *Drosophila* is divided into several discrete regions of morphology and function (Skaer 1993). The longest of these is the midgut, which is an epithelial tube that extends from the proventriculus at the anterior to the Malpighian tubule junction at the posterior. The midgut is further subdivided into a short anterior region, a longer middle midgut, and a much longer posterior midgut where further digestion and absorption are thought to occur. The distinguishing feature of the middle midgut is its extremely acidic pH, which, by analogy with the vertebrate stomach, is thought to contribute to the digestive process. Recent evidence implicates copper cells as the source of midgut acid secretion (Dubreuil et al. 1998).

Copper cells are the most conspicuous cell type in the middle midgut. They were originally named calycocytes on the basis of their morphology and acidophilic secretion (Strasburger 1932). They have also been refered to as cup-shaped cells because of their peculiar invaginated morphology (Filshie et al. 1971). The apical surface of these cells forms a flask-shape that is connected to the gut lumen by a narrow canal. The name copper cells (or cuprophilic cells) comes from their unique orange fluorescence in copper-fed larvae (Poulson and Bowen 1952). The ability of these cells to take up copper was initially presumed to reflect a role in the detoxification of dietary metal. However, subsequent studies have revealed that most dietary copper is sequestered in other regions of the gut (Lauverjat et al. 1989). Thus, the functional significance of the brilliant fluorescence that is uniquely observed in copper cells remains an open question.

Recent studies in rat kidney (Okabe et al. 1996) and in yeast (Presta and Stillman 1997) have revealed a copper-induced fluorescence that is remarkably similar to that originally observed in *Drosophila* copper cells. Fluorescence in these systems has been shown to result from a complex between copper and metallothionein. Interestingly, metallothionein is constitutively expressed in

the middle midgut of *Drosophila* larvae (Durliat et al. 1995), raising the possibility that the fluorescence observed in *Drosophila* copper cells is also attributable to a copper-metallothionein complex.

There are two known metallothionein genes in *Drosophila*: *mtn* and *mto*. Both genes encode small (~40 amino acid) proteins with an unusually high content of cysteine residues (Lastowski-Perry et al. 1985; Mokdad et al. 1987). Genetic evidence supports the hypothesis that *Drosophila* metallothionein has a role in metal detoxification (Maroni et al. 1987). Fly strains carrying a duplication of the *mtn* gene that results in increased *mtn* transcription can survive higher dietary doses of copper and cadmium than can control flies. However, although much attention has been focused on *Drosophila* metallothioneins as stress-response proteins, relatively little is known about the functions of any metallothioneins under stress-free conditions (Vallee 1995).

The finding that midgut acidification and fluorescence in response to copper feeding are both attributable to copper cells raises the possibility that these functions are in someway related. Here, we have tested this possibility by using several recently developed tools for studying larval gut function. First, we have employed pulse-chase copper-feeding to compare the spatial and temporal patterns of copper-dependent fluorescence with the previously described patterns of metallothionein expression. Second, pH-sensitive dyes have been used to examine the effect of copper feeding and consequent flourescence on the acid-secreting activity of copper cells. Third, the contribution of acidification to the development of fluorescence has been examined by feeding copper to α spectrin mutant larvae, in which acid secretion is inhibited. The new results, together with data from several previous studies, support the hypothesis that metallothionein has a role in the normal physiology of acid-secreting copper cells.

Materials and methods

Fly stocks

The α-*spec*^{rg41} and α-*spec*^{rg35} mutants were maintained in a *yellow*$^{-}$ background as balanced heterozygous stocks with a *TM3, y*$^{+}$ chromosome as previously described (Lee et al. 1993). Homozygous mutant larvae were identified by their *yellow*$^{-}$ mouth hooks; wild-type siblings carrying the balancer chromosome were *yellow*$^{+}$; *yw* larvae were used in all other experiments. All stocks were maintained at room temperature (21–23°C) on standard cornmeal-molasses agar medium.

Copper feeding

Copper-enriched medium was made by supplementing melted cornmeal-molasses medium with 1 mM $CuCl_2$. After being thoroughly mixed, the medium was poured into 60-mm plates for collecting larvae. Adults were allowed to feed, mate, and lay eggs on the plates. Larvae were collected at the indicated times and dissected directly in 3.7% formaldehyde in phosphate-buffered saline (pH 7.2). Dissected guts were mounted in Vectashield medium (Vector Laboratories). Pulse-chase experiments were carried out by transferring copper-fed *yw* larvae to apple-juice agar plates without copper before dissection at the indicated timepoints. Preliminary experiments revealed that 1 mM $CuCl_2$ was toxic on apple-juice agar plates, presumably because the free Cu^{++} concentration was higher on the simpler medium. Therefore, all copper treatments were carried out with cornmeal-molasses medium. Copper-dependent fluorescence was viewed and photographed on a Jenalumar fluorescence microscope with a DAPI filter set.

Tissue culture

Drosophila S2 tissue culture cells (Schneider 1972) were grown at 25°C in Schneider's medium (Sigma) supplemented with 10% fetal calf serum (Gibco/BRL). A previously described S2 cell line expressing *Drosophila* neuroglian under control of the metallothionein promoter (Hortsch et al. 1995) was used to monitor activation of the promoter in response to copper treatment. Neuroglian expression was detected by staining the cells with anti-neuroglian antibody followed by rhodamine-conjugated secondary antibody, as previously described (Dubreuil et al. 1996). For induction, a sterile solution of 0.7 M $CuCl_2$ in water was added to the growth medium at a final concentration of 3.5 mM.

Gut acidification assays

One-day-old copper-fed larvae were transferred to apple-juice agar plates without copper and fed with yeast paste containing bromphenol blue or phenol red to assess midgut pH (Dubreuil et al. 1998). After 1–2 h, larvae were dissected as above and immediately scored for acidification or alkalinization.

All samples were photographed on either TMAX400 or Ektachrome400 (Kodak) film and digitized by using a Polaroid SprintScan scanner. Images were edited by using Adobe Photoshop 5.0.

Results

Appearance and fate of copper-dependent fluorescence

Pulse-chase copper-feeding experiments were used to analyze the spatial and temporal parameters of copper fluorescence in the larval midgut. Dissected midguts from control larvae grown on standard medium with no added copper exhibited a weak blue fluorescence, but no significant orange fluorescence, when examined under UV excitation (Fig. 1A). However, after as little as 5 min of feeding on medium containing 1 mM copper, a conspicuous orange fluorescence was observed in the copper cells (Fig. 1B). After 1 day of feeding, the copper cell fluorescence intensified significantly (Fig. 1C). Thus, the initial appearance of fluorescence was rapid and increased significantly over time.

Whereas fluorescence was always most conspicuous in the copper cell region, other cells began to fluoresce with increased copper-feeding times. By day 2 of copper-feeding, bright orange fluorescence was detectable in the anterior midgut (Fig. 2) and in the posterior iron cell region (not shown), which separates the middle and posterior midgut regions (Filshie et al. 1971). Weaker fluorescence was also visible in the gastric caeca, posterior midgut, and Malpighian tubules at later times. Interestingly, fluorescence was never observed in the interstitial cells (between copper cells) or in the large flat cells found between the copper and iron cell regions. Thus, inducible fluorescence was a property of a subset of cells in the midgut.

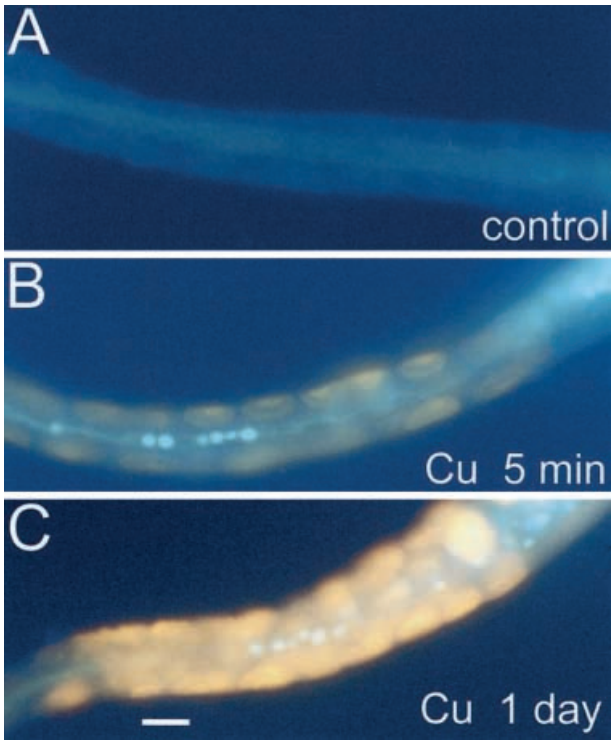

Fig. 1 Development of copper cell fluorescence in copper-fed larvae is rapid. Dissected midguts from control larvae (**A**) and 1 mM $CuCl_2$-fed larvae (**B** 5 min, **C** 1 day) were examined by fluoresence microscopy under 365-nm peak illumination. Orange fluorescence was exclusively observed in the copper cells of copper-fed larvae. Additional blue fluorescence was primarily attributable to food in the lumen of the gut. *Bar* 10 µm

The fate of the fluorescent signal over time was examined by transferring larvae to copper-free medium after 1 day of copper-feeding. There was a marked change in the distribution of fluorescence within 1 day of chase (Fig. 2). The bright signal throughout the copper cell cytoplasm became concentrated in small vesicular structures, yielding an increasingly speckled appearance over time. The speckled pattern was first visible in the anterior-most copper cells (Fig. 2). However, the same pattern was eventually observed in all of the copper cells before fluorescence gradually disappeared altogether after 5–7 days of copper-free chase. The speckled pattern was consistent with previous electron-microscopic studies showing the accumulation of copper-containing lysosomal profiles in the midgut of copper-fed larvae (Filshie et al. 1971; Lauverjat et al. 1989). Fluorescence outside the copper cell region (shown here in the anterior midgut; Fig. 2) was also observed in pulse-chase experiments. Once induced, the fluorescent pattern in the anterior midgut underwent the same progression observed in copper cells, gradually acquiring a speckled appearance during the chase and disappearing altogether by day 6–8.

The fluorescent cell patterns produced during feeding experiments in vivo were not observed when fixed midguts were treated with copper in vitro. Instead, a bright pattern of labeled nuclei was found throughout the gut epithelium (not shown). No significant fluorescence was detected in the cytoplasm of copper cells. Thus, the conditions that yielded cytoplasmic fluorescence in feeding experiments were not preserved in fixed tissue.

Copper induction of metallothionein in S2 tissue culture cells induces fluorescence

The distribution of fluorescence in the larval midgut paralleled the previously described pattern of metallothionein expression (Durliat et al. 1995). As a further test of the link between metallothionein expression and copper-dependent fluorescence, metallothionein synthesis was induced in *Drosophila* S2 tissue culture cells by adding 3.5 mM $CuCl_2$ to the growth medium. Significant metallothionein mRNA synthesis is induced in S2 cells within 1 day of copper treatment (Bunch et al. 1988). Orange fluorescence, comparable to that observed in larva feeding experiments, was detectable by 1 day of copper treatment, and the level of fluorescence increased substantially over several days of metallothionein induction (Fig. 3B). No orange fluorescence was observed prior to copper treatment (Fig. 3A,C), although a faint green fluorescent signal was detectable in this channel. To demonstrate directly the coincidence of metallothionein induction and copper fluorescence, we utilized S2 cells tranfected with a reporter gene construct (Hortsch et al. 1995). Expression of *Drosophila* neuroglian under the control of the *Drosophila* metallothionein promoter was monitored by staining cells, after copper induction, with a monoclonal antibody against this cell adhesion molecule (Fig. 3F). Expression of neuroglian caused cells to aggregate, and orange fluorescence was conspicuous in the aggregates (Fig. 3E). No orange fluo-

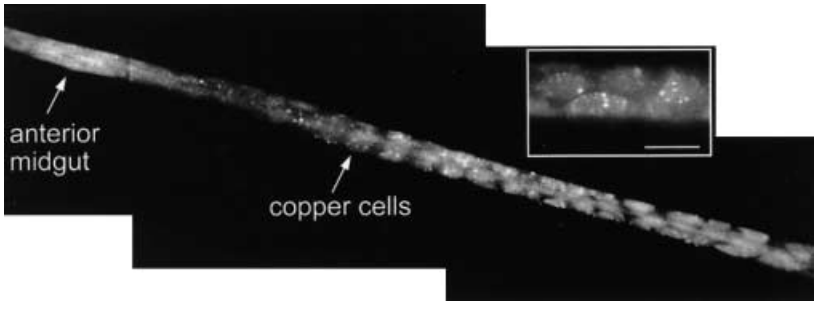

Fig. 2 Fate of copper cell fluorescence over time. Larvae were collected and allowed to feed on medium containing 1 mM copper chloride for 1 day and then transferred to medium without added copper for a 1 day chase before dissection. *Bar* 10 µm

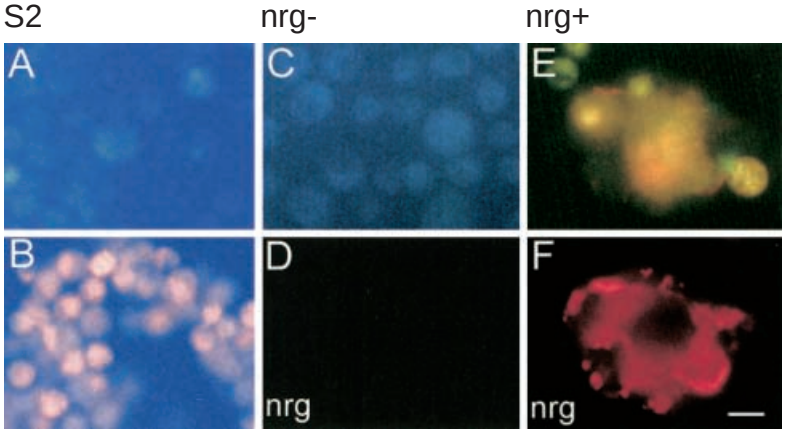

Fig. 3A–F Copper-dependent fluorescence is observed in *Drosophila* S2 tissue culture cells. S2 cells were grown in Schneider's medium with (**B**, **E**, **F**) or without (**A**, **C**, **D**) induction of metallothionein synthesis by the addition of $CuCl_2$ to the growth medium. Control S2 cells (**A**, **B**) and neuroglian-transfected S2 cells (**C–F** *nrg*) were examined for copper-dependent fluorescence as in Fig. 1 (**A–C**, **E**) or for neuroglian expression detected with a mouse anti-neuroglian primary antibody and rhodamine-conjugated secondary antibody (**D**, **F**). Cells were treated for 5 days with 7 mM $CuCl_2$ (**B**) or for 3 days with 3.5 mM $CuCl_2$ (**E**, **F**). *Bar* 10 μm

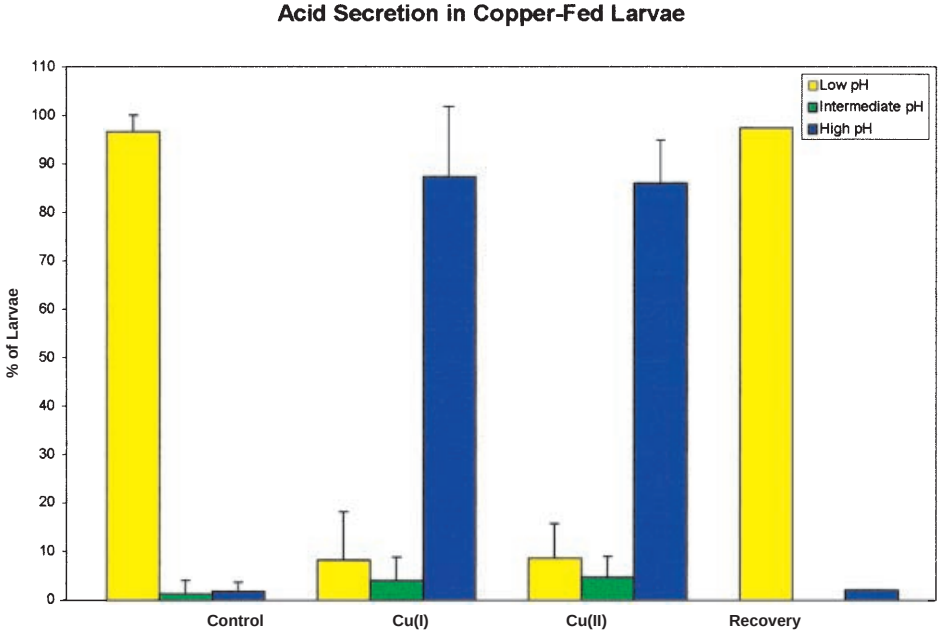

Fig. 4 Copper feeding inhibits larval midgut acidification. Control and copper-fed larvae were scored for stomach acid secretion in a bromphenol blue feeding assay. The dye turned yellow in the middle midgut of control larvae but remained blue in larvae that lacked significant stomach acid secretion. Stomach acid secretion was effectively blocked by feeding 1 mM CuCl or 1 mM $CuCl_2$. However control-level stomach acid secretion was restored by a 1-day chase after copper feeding (as in Fig. 2) during which the fluorescent copper signal became sequestered in a cytoplasmic compartment. Results, presented as the mean ± standard deviation, are from three or more independent experiments with more than 100 larvae. Recovery data shown are from a single representative experiment with 43 larvae

rescence (Fig. 3C) or neuroglian expression (Fig. 3D) was observed without addition of $CuCl_2$ to the medium. Thus, copper-induced fluorescence appeared to be correlated with the induction of metallothionein expression and was not dependent on any unique property of the larval midgut epithelium.

Relationships between copper fluorescence and acid secretion

The constitutive expression of metallothionein in the middle midgut suggests that it has a role in normal cell function, perhaps independent of its role in metal detoxification. Since midgut acidification is the only known role of copper cells, we examined the relationship between copper-dependent fluorescence and midgut pH. Larvae were initially fed for 1 day on medium containing 1 mM $CuCl_2$ and were then were transfered to copper-free medium containing bromphenol blue. Loading of the digestive tract with bromphenol blue allows the detection of a discrete acidified zone of the middle midgut by a change in dye color from blue to yellow (Dubreuil et al. 1998). As indicated by the finding that the dye remained blue throughout the midgut region, acidification was inhibited in copper-fed larvae (Fig. 4). Identical results were obtained in feeding experiments with Cu(I)Cl and $Cu(II)Cl_2$. However, when copper-fed

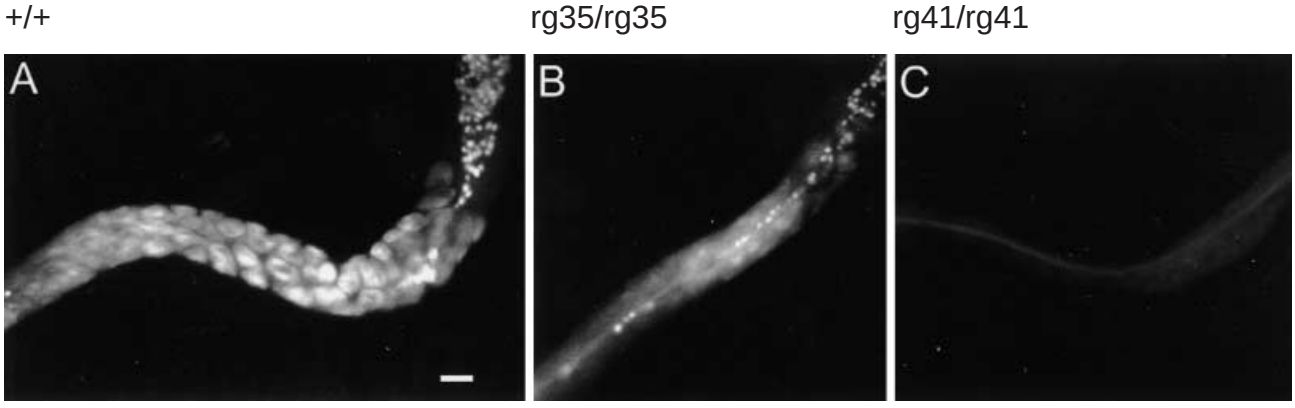

Fig. 5A–C Mutations of α spectrin that block acid secretion also block copper-dependent fluorescence. Wild type (**A**), α-*spec*^{rg35}/α-*spec*^{rg35} (**B**), and α-*spec*^{rg41}/α-*spec*^{rg41} (**C**) larvae were grown on medium containing 1 mM $CuCl_2$ for 1 day before the midgut was dissected for fluorescence microscopy. *Bar* 10 μm

larvae were chased for 1 day in copper-free medium prior to loading with bromphenol blue dye, a return of midgut acidification comparable to that of control larvae was observed. Thus, inhibition of acid secretion was overcome at the timepoint during the chase at which the fluorescent copper complex appeared to be sequestered within a lysosomal compartment.

The effect of copper on midgut acidification did not appear to be attributable to a pleiotropic toxic effect on all midgut activities. Phenol red was used to detect the potent midgut alkalinizing activity immediately posterior to the acidified region of the gut. The dye detected a transition near pH 7 as a change from yellow in the middle midgut to crimson in the posterior midgut in controls. This transition was observed, even when midgut acidification was blocked by copper feeding, because food was somewhat acidic before entering the digestive tract (pH ~5). The alkalinizing activity was not detectably altered by copper-feeding (not shown).

We also examined the possibility that copper-induced midgut fluorescence depended on gut acidification. Previous studies have demonstrated that *labial* mutant larvae, in which copper cells fail to differentiate, lack midgut fluorescence after copper feeding (Hoppler and Bienz 1994). Copper cells differentiate in α spectrin mutant larvae, but they are abnormally shaped and defective in acid secretion (Dubreuil et al. 1998). Copper-feeding experiments revealed that fluorescence was virtually undetectable in homozygotes carrying the most severe α spectrin allele (α-*spec*^{rg41}; Fig. 5C). Weak fluorescence was detectable in homozygotes expressing the milder α-*spec*^{rg35}allele (Fig. 5B), which exhibits moderate acid secretion (Dubreuil et al. 1998), but the signal was greatly reduced relative to wild-type controls (Fig. 5A). Thus, the degree of copper-dependent fluorescence was related to the degree of midgut acidification in copper-fed α spectrin mutants.

Discussion

The striking fluorescence of midgut epithelial cells in copper-fed *Drosophila* larvae was first observed by Poulson and Bowen (1952) nearly 50 years ago. The same fluorescent pattern is also induced by copper-feeding in *Drosophila* adults (R.R. Dubreuil, T. Grushko, O. Baumann in preparation). The fluorescent signal has proven to be a useful marker of cell differentiation in studies of midgut development (Hoppler and Bienz 1994). However, this observation has otherwise remained little more than a curiosity of the literature. Here, we bring together several independent lines of evidence that suggest copper cell fluorescence is caused by a cytoplasmic copper-metallothionein complex.

Metallothionein and copper cell fluorescence

A search of the literature for copper-induced fluorescence in other systems has turned up two examples that bear interesting similarity to the fluorescence observed in copper cells. The Long Evans cinnamon coat rat exhibits age-dependent copper accumulation with ensuing organ damage. Copper accumulation in the kidneys of these rats is paralleled by accumulation of an orange fluorescent signal (Okabe et al. 1996). Protein fractionation experiments have revealed that the fluorescent signal is attributable to a complex between copper and metallothionein. An orange fluorescent signal has also been found in yeast cells grown in the presence of 2 mM $CuCl_2$ (Presta and Stillman 1997). A copper-metallothionein complex has been shown to be responsible for fluorescence. Thus, orange fluorescence in response to UV excitation appears to be a unique signature of copper-metallothionein complexes in diverse cell types (Presta and Stillman 1997).

The expression patterns of the two known metallothionein genes in *Drosophila*, *mtn* and *mto*, make them attractive candidates to explain copper-dependent fluorescence in *Drosophila*. Both genes are constitutively expressed in the middle midgut of larvae and adults and are inducibly expressed in many other regions of the gut (Durliat et al. 1995). The demonstration here of a close

correspondence between sites of metallothionein expression and orange fluorescence both in the gut and in S2 tissue culture cells provides strong, albeit indirect, evidence implicating metallothionein. It will be important to establish a more direct link in future studies. Previous attempts to purify the major form of metallothionein from *Drosophila* larvae have been unsuccessful (Silar and Wegnez 1990), suggesting that a biochemical approach to the problem may not be feasible. A more practical approach may be to use genetic methods to demonstrate that metallothionein expression is necessary and sufficient to explain copper-induced fluorescence.

Copper cell fluorescence and acid secretion

The recent demonstration that copper cells are important for midgut acidification in *Drosophila* (Dubreuil et al. 1998) prompted us to ask what is the relationship, if any, between fluorescence and acid secretion? Our results suggest that these processes are interrelated. First, copper cells do not fluoresce in response to copper-feeding when acid secretion is blocked by an α spectrin mutation. One possible explanation of this result is that the formation of the fluorescent copper complex is somehow physiologically coupled to acid secretion, and consequently, the complex may not form when acid secretion is blocked. Alternatively, copper-dependent fluorescence may depend directly on spectrin function. Spectrin is known to affect the accumulation of interacting membrane activities such as the Na,K ATPase (Dubreuil et al. 2000), voltage-dependent sodium channels, and the cell adhesion molecule neurofascin (Zhou et al. 1998). There may be a similar effect of α spectrin mutations on the accumulation of an apical copper transporting activity that is required for copper uptake into the copper cell cytoplasm. Antibody staining of metallothionein in copper cells from α spectrin mutants and wild-types would shed light on this issue. Unfortunately, antibodies that react with *Drosophila* metallothioneins are not available.

Second, fluorescent copper cells lose their ability to secrete stomach acid. Therefore, there is also a spectrin-independent link between copper-feeding and acid secretion. One possible explanation of the effect is that copper-free cytoplasmic metallothionein is essential to the acid secretion process. The restoration of acid secretion in chase experiments may then be attributable to either the recycling of metallothionein after dissociation of copper and/or the synthesis of new metallothionein molecules. Alternatively, copper may inhibit acid secretion independently of metallothionein, perhaps by directly interfering with the activity of apical proton pumps or other enzyme activities that contribute indirectly to acid secretion. For example, copper toxicity is known to include effects on the activity of Na,K ATPase and Mg-ATPase (Vasic et al. 1999). Tests of direct copper toxicity must await the identification and characterization of the enzyme activities that are responsible for acid secretion in *Drosophila* copper cells.

Biological roles of metallothionein

Metallothionein is induced by exposure to heavy metals, it binds metal ions, and overexpression of metallothionein confers increased resistance to metal toxicity. For these reasons, metallothionein is thought to have a biological role in metal detoxification (for a review, see Hamer 1986). However, the role of metallothionein under non-stressed conditions remains poorly understood (Vallee 1995). Descriptive studies in many systems suggest a broad range of possible cellular roles for metallothionein, any of which may be relevant to understanding the link between copper cell fluorescence and acid secretion.

Recent data support a role for metallothionein in controlling the distribution of zinc in cells (Jiang et al. 1998; Jacob et al. 1998). However, this role is not likely to be relevant to metallothionein function in *Drosophila*, since *mtn* and *mto* are not induced by zinc (Bonneton et al. 1996) and copper cell fluorescence is neither induced by nor inhibited by the presence of zinc (Poulson and Bowen 1952).

Metallothionein is also thought to function as a free-radical scavenger and is induced by oxidative stresses (Sato and Bremner 1993). For example, overexpression of metallothionein in the mouse heart confers resistence to oxidative damage (Kang 1999). Free radicals are generated as byproducts from a variety of oxidation-reduction reactions that take place within the cell, including those involved in energy generation. The free radical scavenging activity of metallothionein prevents DNA degradation in vitro (Abel and deRuiter 1989) and may contribute to its ability to prevent cardiotoxicity associated with the anticancer agent doxorubicin (Kang 1999). Direct interactions between metallothionein and free radicals have been demonstrated in vitro (Thornalley and Vasak 1985). There are likely to be significant oxidative stresses in acid-secreting cells that could lead to constitutive metallothionein expression in the *Drosophila* stomach. Interestingly, metallothionein is also known to be expressed constitutively in the acid-secreting cells of dog stomach (Shimada et al. 1997).

Further evaluation of the role of metallothionein in the *Drosophila* midgut must await the production and characterization of metallothionein mutants. Standard genetic approaches have failed to produce these mutants thus far. However, this obstacle is likely to be overcome by the current rapid progress in the *Drosophila* genome project. Once available, metallothionein mutants will provide a powerful experimental tool with which to refine our understanding of the intriguing relationships between copper-dependent fluorescence, metallothionein expression, and stomach acid secretion in the *Drosophila* midgut.

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