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METAL IONS SUPPRESS THE ABNORMAL TASTE BEHAVIOR OF THE *DROSOPHILA* MUTANT *malvolio*

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Summary

A mutation in the *malvolio* (*mvl*) gene affects taste behavior in *Drosophila melanogaster*. The *malvolio* gene encodes a protein (MVL) that exhibits homology to the mammalian natural resistance-associated macrophage proteins. It is also homologous to the Smf1 protein from *Saccharomyces cerevisiae*, which we have recently demonstrated to function as a Mn^{2+}/Zn^{2+} transporter. We proposed that the *Drosophila* and mammalian proteins, like the yeast SMF1 gene product, are metal-ion transporters. To test this hypothesis, *malvolio* mutant flies were allowed to develop, from egg to adulthood, on a medium containing elevated concentrations of metal ions. Mutant flies that were reared in the presence of 10 mmol l^{-1} $MnCl_2$ or $FeCl_2$ developed into adults with recovered taste behavior. $CaCl_2$ or $MgCl_2$ had no effect on the mutant's taste perception. $ZnCl_2$ inhibited the effect of $MnCl_2$ when both ions were supplied together. Similar suppression of the abnormal taste behavior was observed when *mvl* mutants were fed $MnCl_2$ or $FeCl_2$ only at the adult stage. Furthermore, exposure of adult mutant flies to these ions in the testing plate for only 2 h was sufficient to restore normal taste behavior. The suppression of the defective taste behavior suggests that MVL functions as a Mn^{2+}/Fe^{2+} transporter and that Mn^{2+} and/or Fe^{2+} are involved in the signal transduction of taste perception in *Drosophila* adults.

Key words: metal ions, transporters, *Drosophila melanogaster*, taste behavior, bacterial resistance.

Introduction

The gustatory pathway of *Drosophila melanogaster* is a useful model for the analysis of gene function in the nervous system. An assay which measures the ability of flies to detect and respond to sugars has been used to isolate mutations in several genes affecting taste perception (Isono and Kikuchi, 1973; Falk and Atidia, 1975; Tompkins *et al.* 1979; VijayRaghavan *et al.* 1992; Inamdar *et al.* 1993). Among them, a mutation in the *malvolio* (*mvl*) gene affects taste behavior in *Drosophila*. The gene is expressed in mature neurons in the central and peripheral nervous system as well as in macrophages (Rodrigues *et al.* 1995). It was shown that the electrophysiological responses of the peripheral neurons to taste stimuli are normal in these flies. This suggests that the abnormal taste behavior of the mutant resulted from a defect in information processing rather than in the reception of the stimulus (Vidal *et al.* 1993; Rodrigues *et al.* 1995). Since the amino acid sequence of the malvolio protein (MVL) is 65 % identical to that of the mammalian natural resistance-associated macrophage protein (NRAMP) (Vidal *et al.* 1993, 1995*b*; Cellier *et al.* 1995), it is likely that they both have a similar function. These two proteins show a very limited homology to a 22-amino-acid sequence of the nitrate transporter CRNA from *Aspergillus nidulans* (Vidal *et al.* 1993; Rodrigues *et al.* 1995). Consequently, it was proposed that the NRAMP protein, as well as the MVL protein, may function as nitrite (NO_2^-) or nitrate (NO_3^-) transporters. These ions are subsequently converted to nitric oxide (NO) by dismutation (Rodrigues *et al.* 1995). It was suggested that the NO produced in this way may function in the signal transduction of taste perception in *Drosophila*. The discovery of a yeast protein (Smf1p) that is 30 % identical in its amino acid sequence to NRAMP and MVL, and which was shown to function as a metal-ion transporter, raised the possibility that the mammalian and *Drosophila* proteins also have a similar function (Supek *et al.* 1966). According to this hypothesis, metal-ion homeostasis is impaired in the MVL mutant, resulting in a loss of taste perception for sugars. To assess this hypothesis, we attempted to suppress the abnormal taste perception phenotype of the *malvolio* mutant by adding metal ions to the growth medium.

Materials and methods

Drosophila *strains*

Unless otherwise specified, flies were reared on molasses–cornmeal–yeast medium at 25 °C, under constant

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illumination. The P-element insertional mutant *Mvl*^{97f} was kindly provided by Dr William Chia (Rodrigues *et al.* 1995). The Canton-S strain (CS) was used as a wild-type control, since the *Mvl* mutant was isogenized to the CS genetic background.

Feeding preference assay

Flies were used for the experiments 3–5 days after eclosion. Flies were collected upon eclosion and aged on medium containing Mn^{2+}. Prior to the test, flies were starved for 20 h by transferring them into bottles containing no food but supplied with distilled water soaked in 3MM paper. The feeding preference test was carried out as described by Tanimura *et al.* (1982) with some modifications according to Rodrigues *et al.* (1995). Alternate wells of a 6×10 microtiter plate (Nunc, Denmark) were filled with 10 µl of 1 % agar (Difco, Noble) containing 100 mmol l^{-1} trehalose (Sigma) as a stimulus. The rest of the wells contained 0.2 % Acid Red ($C_{27}H_{29}O_7N_2Na$, Sigma) in 1 % agar (Fig. 1A). The control experiment ascertained that Acid Red dye at the concentration used (0.2 %) was neither toxic nor metabolized and did not interfere with the test. Approximately 100–150 flies were introduced into each plate and were left to feed for 2 h in the dark at 25 °C. Thereafter, flies were immobilized by cooling and were visually scored for the color in their abdomens. All the values were obtained by blind scoring. The acceptance response of the stimulus was calculated as the percentage of flies with uncolored abdomens in the population. Means and standard deviations of each data point were obtained from at least six independent experiments.

Results

In the work described in this paper, we attempted to use the feeding preference assay in order to try to recover the phenotypic expression of the taste mutant *malvolio*. In this assay, flies are presented with an option to choose between food containing an attractant, usually a sugar, and medium containing only a food dye (Acid Red) which is neither attractive nor repellent to the flies. Wild-type flies will almost always prefer food containing the sugar over food containing the Acid Red dye. Fig. 1B depicts two flies, one with an uncolored abdomen, since it was able to taste the sugar and preferred it, and the second with a red abdomen, resulting from consumption of the red dye. The ability of flies to discriminate between food containing sugar and food lacking sugar is manifested in the percentage of flies with an uncolored abdomen in the population tested.

Adult wild-type (Canton-S) and mutant (homozygous *Mvl*^{97f}) *Drosophila* were transferred to bottles containing a series of media with increasing concentrations of $MnCl_2$, allowed to lay eggs for 4 days and then removed. The eggs developed into adult flies on this medium. The resulting adult flies were then tested for their behavioral response in a feeding preference assay of sugar *versus* dye solution. When the flies developed in the absence of Mn^{2+}, the control wild-type flies had an acceptance rate of more than 40 %, whereas the *Mvl* mutants chose randomly (acceptance rate of approximately 2 % only). When wild-type and *Mvl* mutants were reared throughout development on standard food supplemented with different concentrations of Mn^{2+} and

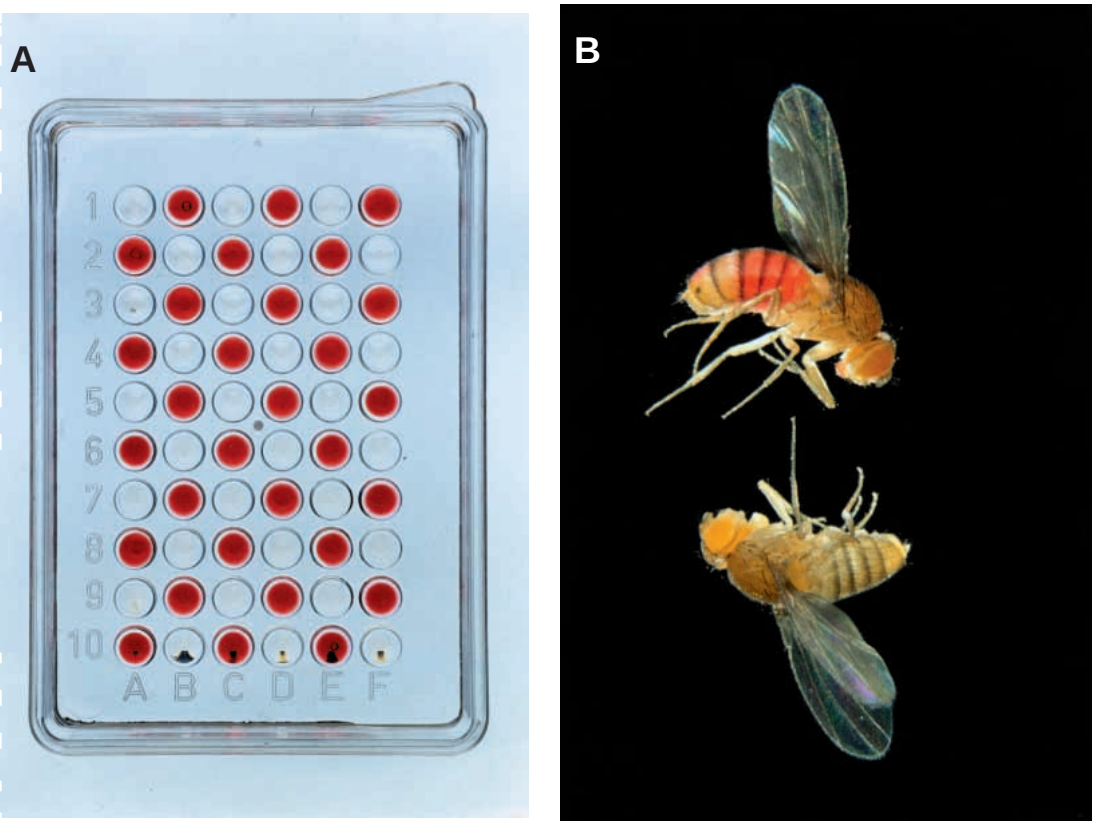

Fig. 1. The results of a feeding preference assay demonstrated by the two flies. (A) A typical plate for assaying feeding preference of flies. Red wells contain agar Acid Red dye. Uncolored wells contain agar with sugar. (B) The (upper) fly with the red-colored abdomen is unable to taste the sugar, and the lower fly is able to taste the sugar; as a result, the lower fly has an uncolored abdomen.

then tested for trehalose acceptance, Mn^{2+} was able to reverse the mutant phenotype in a dose-dependent manner (see Fig. 2). When the same experiment was repeated, but this time by adding Ca^{2+}, Mg^{2+} or Zn^{2+} as a replacement for Mn^{2+} in the medium, there was no reversion of the mutant phenotype.

It has previously been shown that, in *S. cerevisiae*, Zn^{2+} inhibits Mn^{2+} transport by Smf1p, which is a yeast homolog of *malvolio* (Supek *et al.* 1996). In flies too, when Zn^{2+} is present in the growth medium together with Mn^{2+} throughout development the ameliorating effect of Mn^{2+} on the mutant flies was suppressed, and these flies again lacked taste preference. At the same Zn^{2+} concentrations, the taste behavior of wild-type flies was normal (Fig. 3). Recently, we observed that Smf2p and Smf3p, which are two yeast homologs of Smf1p, may function in the transport of additional metal ions other than Mn^{2+} and Zn^{2+} (A. Kahan, A. Cohen and N. Nelson, unpublished observations). We therefore tested whether Fe^{2+} affects the taste behavior of the *Mvl* mutant. As shown in Fig. 3, Fe^{2+} had a similar effect to Mn^{2+} in suppressing the mutant taste behavior. Moreover, when both Fe^{2+} and Mn^{2+} were present in the medium throughout development, an additive effect on the taste preference of the mutant flies was observed.

We next set out to determine whether normal taste perception requires the presence of metal ions throughout the development of the nervous system in the fly. Alternatively, it may be sufficient for these metal ions to be supplied to the adult flies, in which the nervous system has already developed. If so, these metal ions may be implicated in signal transduction

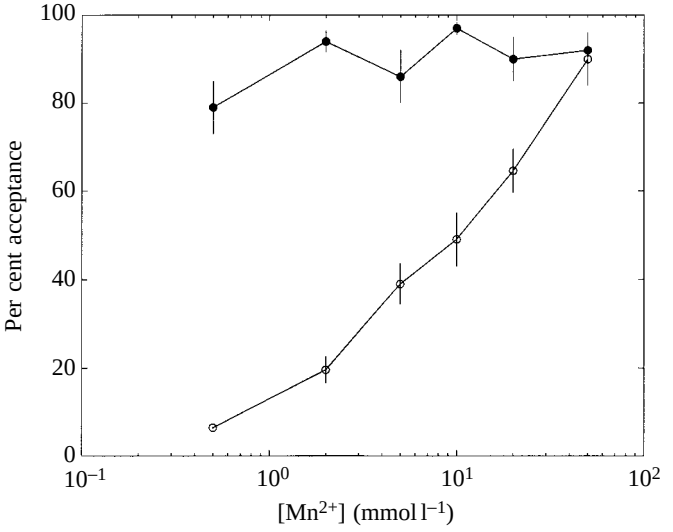

Fig. 2. Taste responses of adult wild-type (Canton-S) and *Mvl*^{97f} mutant *Drosophila* flies to Mn^{2+}. The flies were reared throughout development in the presence of increasing Mn^{2+} concentrations. In this and the following figures, 0 % acceptance represents a random choice between sugar and dye. In medium without Mn^{2+} supplementation, the acceptance for wild-type flies was 43 %; that for *Mvl*^{97f} flies was 2 %. (○) Homozygous *Mvl*^{97f} flies; (●) Canton-S (wild-type) flies. Values are means ± S.D., $N \geqslant 6$.

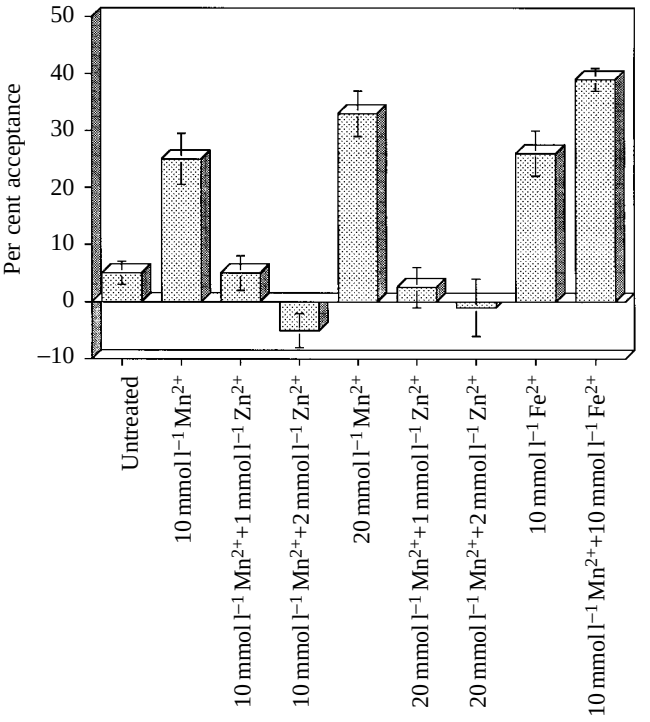

Fig. 3. Behavioral response of *Mvl*^{97f} mutant flies in the feeding preference assay. Flies were reared throughout development on food containing the indicated ions and were tested using the feeding preference assay under similar conditions to those described by Tanimura *et al.* (1982). Under these conditions, wild-type (Canton-S) flies had an acceptance level of over 40 %. Values are means ± S.D., $N \geqslant 6$.

of taste perception in the adult. To test this, mutant flies that had developed on standard medium (no elevated concentration of any metal ions) were transferred, upon eclosion, to vials containing food supplemented with Mn^{2+}, Fe^{2+} or a combination of Mn^{2+} and Zn^{2+} for 3 days prior to testing. As shown in Fig. 4, these flies had the same pattern of behavior as flies that had been reared throughout development in the presence of the same metal ions. These findings indicate that the metal ions are not required for correct development of the nervous system components involved in taste perception. To corroborate these results, mutant flies that had developed on standard medium were tested on plates in which all the wells, those containing sugar and those containing dye, were supplemented with Mn^{2+}, Fe^{2+} or a combination of Mn^{2+} and Zn^{2+}. The suppression of the abnormal taste perception of the *Mvl* mutant by Mn^{2+} and Fe^{2+} was even stronger in this case than when the mutant flies had developed in the presence of the ions or had been fed these metal ions for 3 days prior to testing. Under these conditions, the inhibitory effect of Zn^{2+} was almost undetectable (Fig. 5). These results implicate Mn^{2+} and Fe^{2+} in a signal transduction pathway underlying taste perception. The lack of effect of Zn^{2+} may be explained by the slow accumulation of this ion and the consequent failure to reach the required critical amounts.

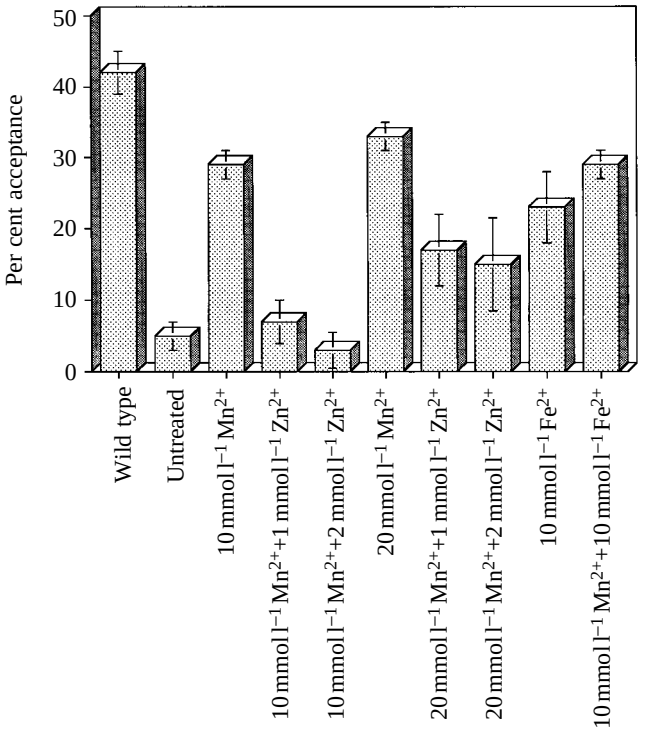

Fig. 4. Effect of metal ions provided to adult flies on their behavioral response. *Mvl*^{97f} mutant flies were reared throughout development on standard medium. Upon eclosion, the adult flies were transferred to vials containing food supplemented with the indicated ions for 3 days, starved for 18 h, and then tested in the feeding preference assay as described in Fig. 3. Values are means ± S.D., $N \geqslant 6$.

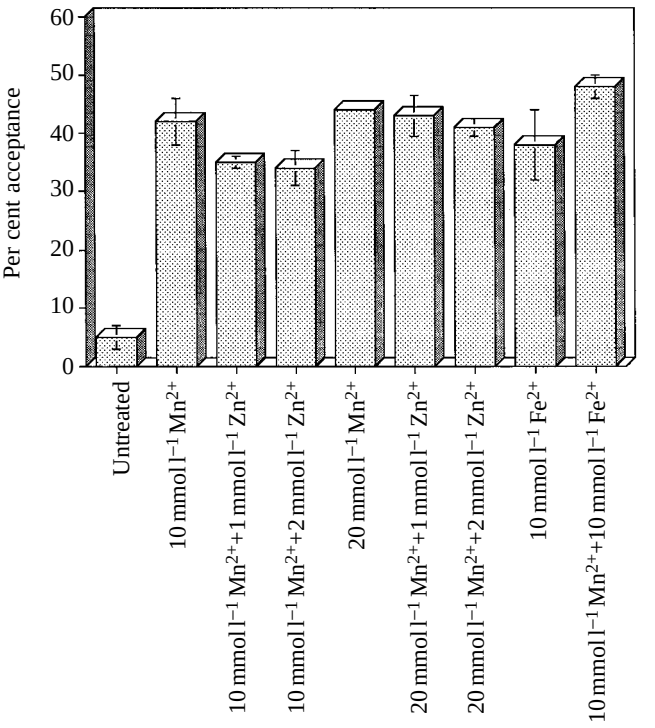

Fig. 5. Effect of metal ions provided during the taste preference test on the behavioral response of the flies. *Mvl*^{97f} mutant flies were reared throughout development on standard medium, aged for 3 days on the same medium, and starved for 18 h. The flies were tested for feeding preference on plates containing the indicated ions in all the wells, those containing the sugar as well as those containing the dye. Values are means ± S.D., $N \geqslant 6$.

Discussion

Our experiments suggest that the yeast Smf1p and the *Drosophila* MLV protein are metal-ion transporters. On the basis of the sequence homology between the MVL protein and the mammalian NRAMP protein, it is tempting to suggest that all the other members of this family, including the mammalian ones, have a similar function. In mice, natural resistance to infection with unrelated intracellular parasites such as *Mycobacteria*, *Salmonella* and *Leishmania* is controlled by a single gene on chromosome 1, designated *Bcg*, *Ity* or *Lsh*. The gene product of *Bcg*, designated natural resistance-associated macrophage protein (NRAMP), has been shown to be altered in susceptible animals (Vidal *et al.* 1993, 1995*a*,*b*; Cellier *et al.* 1995; Govoni *et al.* 1995). The discovery of a yeast homolog of *Nramp* and *malvolio* that functions as a metal-ion transporter raised the possibility that the mammalian protein has a similar function (Supek *et al.* 1996, 1997). Following phagocytosis of a parasite into the phagosome, the macrophage produces reactive oxygen and/or nitrogen intermediates that are toxic for the internalized bacteria. Survival of the pathogen during the burst of macrophage respiratory activity is thought to be partly mediated by microbial metalloproteins such as superoxide dismutase (SOD), which contain Mn^{2+}, Cu^{2+}–Zn^{2+} or Fe^{2+} in their active centers (Chan *et al.* 1992; Fridovich, 1995; Cooper *et al.* 1995; Lah *et al.* 1995). We propose that NRAMP may transport Mn^{2+} and/or other metal ions from the extracellular milieu into the cytoplasm of the macrophage and, following the generation of the phagosome, remove the metal ions from the organelle (Supeck *et al.* 1997). Thus, metal-ion depletion of the microenvironment in the phagosome by NRAMP may limit the rate of production of metalloenzymes by the engulfed bacteria. This limitation will restrict the pathogen's ability to produce metalloenzymes such as SOD and prevent the propagation of the ingested microorganisms. Conversely, an increased concentration of metal ions in the phagosome, caused by a defective NRAMP transporter (*Bcg*^{s}), may promote the growth of the parasites and render the organism sensitive to the pathogen.

Several lines of evidence point to a direct involvement of metal ions in neurotransmission. Zn^{2+} has been implicated in several processes in the nervous system. For example, Zn^{2+} can interact strongly with a variety of ligands including sulfur in cysteine, nitrogen in histidine and oxygen in acidic amino acids (Berg and Shi, 1996). In mammalian brain cells, Zn^{2+} is accumulated in presynaptic vesicles of excitatory neurons and is released during synaptic activity (Assaf and Chung, 1984; Howell *et al.* 1984; Palmiter *et al.* 1996). Zn^{2+} interacts with some ionotropic receptors in the brain, such as the ionotropic ATP receptor (P2x3), which is potentiated by Zn^{2+} (Seguela *et*

al. 1996). Zn^{2+} blocks currents mediated by *N*-methyl-D-aspartate (NMDA) or γ-aminobutyric acid (GABA) as well as voltage-gated Ca^{2+} channels (Westbrook and Mayer, 1987; Peters *et al.* 1987). It also interacts with neurotransmitter uptake systems, such as the dopamine transporter, and inhibits dopamine uptake and cocaine binding (Richfield, 1993). Finally, it has been demonstrated that Zn^{2+} may play a role in neuronal death after transient cerebral ischemia (Koh *et al.* 1996).

The role of Mn^{2+} and Fe^{2+} in neurotransmission and taste perception is not clear. Their possible involvement in neural development has been ruled out since Mn^{2+} and Fe^{2+} suppressed the mutant phenotype even when supplied to flies only at the adult stage, when the nervous system had already been developed. Furthermore, the short period that was sufficient for the mutant flies to recover their taste (see Fig. 5) prompts us to suggest that Mn^{2+} and/or Fe^{2+} participate in a novel signal transduction pathway involved in taste perception. However, it is possible that an appropriate metal-ion concentration in specific nerve cells is required for the optimal performance of these cells in one of the known signal transduction pathways. In *Drosophila*, the inhibition by Zn^{2+} of the restoration of taste behavior by metal ions can be explained by at least two different mechanisms. Zn^{2+} may compete with Mn^{2+} for uptake by metal-ion transporters other than MVL and, by so doing, prevent the suppression of the *malvolio* mutant phenotype by the added high concentrations of Mn^{2+}. Alternatively, Zn^{2+} may interact directly with the site in the signal transduction pathway that requires Mn^{2+} for its activity. The steps in the signal transduction sequence that may involve metal ions include the modulation of receptor activity in the nervous system, the interaction of receptors with second messengers and the expression of signals in the modulation of Ca^{2+} concentration. Recent studies on the effect of metal ions on neuronal receptors (Shuto *et al.* 1997) makes it conceivable that Mn^{2+} and Fe^{2+} may modulate a subset of excitatory receptors involved in taste perception.

Recently, a novel mechanism for regulating ion concentrations in yeast cells has been discovered (Liu *et al.* 1997). It was demonstrated that in *Saccharomyces cerevisiae* a mutation in the *BSD2* gene suppresses oxidative damage in cells lacking superoxide dismutase (Liu and Culotta, 1994). The mechanism for this effect was explained when it was subsequently found that *BDS2* prevents metal hyperaccumulation by exerting negative control over the SMF1 and SMF2 metal transport systems (Liu *et al.* 1997). The gene product of *BSD2* (Bds2p) is situated in the endoplasmic reticulum, while Smf1p and presumably Smf2p function in the plasma membrane (Supek *et al.* 1996, 1997; Liu *et al.* 1997). The most plausible explanation for the function of Bds2p is that it acts as an endoplasmic reticulum receptor that binds several plasma membrane transporters and, by doing so, prevents them from over-accumulating on the plasma membrane. Null mutations in the *BSD2* gene cause an increase in the number of metal-ion transporters in the plasma membrane, resulting in over-accumulation of metal ions, presumably by chemically catalyzing the superoxide dismutation reaction. Bsd2p must also sense, directly or indirectly, the metal-ion concentration inside the cells in order to be able to distribute the specific transporters appropriately between the endoplasmic reticulum and the plasma membrane. When the metal-ion concentration is elevated above a specific threshold, the transporters may be tightly bound and remain in the endoplasmic reticulum. When the metal-ion concentration decreases, the transporters are released and distributed to the plasma membrane. This kind of regulation may be particularly useful for the nervous system, the blood barriers and the digestive tract. We also anticipate the involvement of such transporter receptors in mutants of taste perception in which the MVL protein is intact. Very recently, NRAMP2 (DCT1) was shown to transport Fe^{2+} and other metal ions (Gunshin *et al.* 1997), confirming our findings that the members of this family of membrane proteins are metal-ion transporters (Supek *et al.* 1996, 1997). A genetic disorder that causes hemochromatosis due to elevated rates of uptake of iron from the intestine was localized to a gene that may produce a protein analogous to Bsd2p (Feder *et al.* 1996). We suggest that this mechanism of holding excess and ready-to-use transporters in the endoplasmic reticulum and/or the Golgi body is widespread.

The ramification of this work lies far beyond the fact that the addition of metal ions corrects a defect in *Drosophila* taste behavior. The data not only suggest that *Drosophila* MVL, like yeast Smf1p, is a metal-ion transporter but that all the other family members are also metal-ion transporters. Furthermore, it suggests that these family members function in key metabolic processes that are involved in systems such as signal transduction, neuronal activity and resistance to bacterial infection. We anticipate that the other members of this family will play key roles in metal-ion homeostasis in a variety of processes that are yet to be discovered.

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