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FUNCTION OF METAL-ION HOMEOSTASIS IN THE CELL DIVISION CYCLE, MITOCHONDRIAL PROTEIN PROCESSING, SENSITIVITY TO MYCOBACTERIAL INFECTION AND BRAIN FUNCTION

FRANTISEK SUPEK*, LUBICA SUPEKOVA†, HANNAH NELSON AND NATHAN NELSON‡
Department of Biochemistry, Tel Aviv University, Ramat Aviv, 69978 Tel Aviv, Israel

Summary

A novel *Saccharomyces cerevisiae* mutant, unable to grow in the presence of 12.5 mmol l^{-1} EGTA, was isolated. The phenotype of the mutant is caused by a single amino acid change (Gly149 to Arg) in the essential yeast cell division cycle gene *CDC1*. The mutant could be suppressed by overexpression of the *SMF1* gene, which codes for a plasma membrane Mn^{2+} transporter. We observed that the yeast *SMF1* gene shares homology with the mouse *Nramp* gene. *Nramp* (*Bcg*) was cloned as a gene responsible for mouse resistance to infection with mycobacteria and is identical with the *Ity* and the *Lsh* genes conferring resistance to infection by *Salmonella typhimurium* and *Leishmania donovani*, respectively. Although the cloning of *Nramp* identified the gene responsible for the resistance of mice to mycobacteria, its function is unknown. We propose that the mammalian protein, like the yeast transporter, is a Mn^{2+} and/or Zn^{2+} transporter. Following the phagocytosis of a parasite into the phagosome, the macrophage produces reactive oxygen and/or nitrogen intermediates that are toxic for the internalized bacteria. The survival of the pathogen during the burst of macrophage respiratory activity is thought to be partly mediated by microbial superoxide dismutase (SOD), which contains Mn^{2+} or Fe^{2+} in its active centre. Nramp may transport Mn^{2+} from the extracellular milieu into the cytoplasm of a macrophage and, after the generation of the phagosome, remove Mn^{2+} from the organelle. Thus, the Mn^{2+}-depletion of the phagosome microenvironment by the *Nramp* gene product may be a rate-limiting step in the metalloenzyme's production by the engulfed bacteria. This limitation will restrict the mycobacterial ability to produce active enzymes such as SOD and prevent the propagation of the ingested microorganisms. Conversely, an increased concentration of Mn^{2+} in the phagosome caused by a defective Nramp transporter (Bcg^s) may promote the growth of the mycobacteria and render the organism sensitive to the pathogen.

We use a similar approach to identify, clone and study other metal-ion transporters.

Key words: metal ions, transport, homeostasis, manganese, mycobacteria, yeast, brain.

Introduction

Mineral nutrients are continuously cycled through organisms and their environment, yet cellular nutrient concentrations remain more or less constant. This homeostasis is maintained by a delicate balance of transport activities across the plasma and organelle membranes. In addition, several metabolic effects, together with modulation of chelating agent concentrations, help to maintain the required metal-ion concentrations in the various cellular compartments in different tissues. Modulation of the metal-ion concentrations may result from signal transduction and may participate in the signalling processes. Though most attention has been drawn to the involvement of Ca^{2+} as a second messenger in signal transduction, it is likely that other cations are also involved in signalling pathways. The cytoplasm, endoplasmic reticulum, nucleus, mitochondria and/or chloroplasts and the vacuolar system are the five major compartments that maintain metal-ion homeostasis in eukaryotic cells. Although metal ions in the cytoplasm are generally maintained at low concentrations and are modulated according to the metabolic state of the cell, the organelles frequently serve as storage compartments for some of the ions. Specific signals cause the movement of ions into and out of the storage organelles or the cell milieu. Metal-ion transporters play a major role in this process. There are different pathways for entry into and exit out of the cytoplasm for the various ions. For example, iron uptake into mammalian cells involves receptor-mediated endocytosis, whereas in *S. cerevisiae* an uptake system containing a reducing step functions in iron uptake across the plasma membrane (Lesuisse

*Present address: Department of Molecular and Cellular Biology, University of California – Berkeley, Berkeley, CA 94720, USA.
†Present address: Gladstone Institute of Cardiovascular Disease, University of California – San Francisco, San Francisco, CA 94141-9100, USA.
‡Author for correspondence (e-mail: nelson@post.tau.ac.il).

et al. 1987). Calcium homeostasis is also maintained by the coordination of several transport systems, including Ca^{2+}-ATPases (Rudolph *et al.* 1989; Cunningham and Fink, 1994; Ghislain *et al.* 1990), Na^{+}/Ca^{2+} (Herchuelz *et al.* 1980; Crompton *et al.* 1978) and H^{+}/Ca^{2+} exchangers (Ohsumi and Anraku, 1983; Rooney and Gross, 1992) as well as several other carriers and channels. Very little is known about the transport systems for other metal ions, such as Mn^{2+} and Zn^{2+}, that are essential for the life cycle of eukaryotic cells (White and Gadd, 1987). *S. cerevisiae* cells are quite resilient to the stress of low divalent cation concentrations (Askwith *et al.* 1994; Dancis *et al.* 1994). Wild-type cells can readily grow in the presence of 12.5 mmol l^{-1} EGTA in a medium buffered at pH 6.0. However, mutants in which the V-ATPase is inactive cannot grow under these conditions (Noumi *et al.* 1991). To explore the transport systems of metal ions, we selected mutants that cannot grow in the presence of 12.5 mmol l^{-1} EGTA but contain an intact V-ATPase (Supek *et al.* 1996).

The cell division cycle *CDC1* gene requires manganese to function

S. cerevisiae has been shown to accumulate many transition metals, which are present in trace amounts in the natural environment. It has been demonstrated that high-affinity uptake of these metals is mediated by specific uptake systems (White and Gadd, 1987; Dancis *et al.* 1994). To identify possible candidates for these transporters, yeast cells were mutagenized using ethyl-methansulphonate (EMS). Colonies that were not able to grow on YPD (1 % yeast extract, 2 % bactopeptone, 2 % glucose and 50 mmol l^{-1} Mes) medium (pH 6.0) supplemented with 12.5 mmol l^{-1} EGTA were collected. Screening yielded 16 *csp* mutants (*c*helator-sensitive *p*henotype), and the mutant strain *csp2* was characterized in greater detail (Supek *et al.* 1996). To identify a growth-limiting metal, plates containing EGTA were supplemented with the different metals. Only $MnCl_2$ at 0.05 mmol l^{-1} or higher concentrations rescued the growth of the mutant on plates containing EGTA. Of all the metals studied, only 1 mmol l^{-1} $ZnCl_2$ showed specific growth inhibition of the *csp2* mutant. This inhibition could be fully reversed by supplementing the zinc-containing medium with 0.2 mmol l^{-1} $MnCl_2$. Supplementation with $CaCl_2$, $CoCl_2$, $CuCl_2$, $MgCl_2$ or $NiCl_2$ had no effect.

The *csp2* mutant was transformed using a yeast genomic library, and two different plasmids able to complement *csp2* mutant were isolated (Supek *et al.* 1996). One of them was identified as the gene *CDC1* encoding a soluble protein involved in the cell division cycle of yeast (Halbrook and Hoekstra, 1994; Loukin and Kung, 1995). The second was the *SMF1* gene encoding a membrane protein reported to be a multicopy suppressor of a temperature-sensitive mutant defective in the function of mitochondrial processing peptidase (Hawlitschek *et al.* 1988; West *et al.* 1992). Null mutation in the *CDC1* gene was lethal, and null mutation in the *SMF1* gene was reported to have no detectable effect on cell viability (West *et al.* 1992). We observed that null mutants in the *SMF1* gene failed to grow in the presence of 12.5 mmol l^{-1} EGTA (Supek *et al.* 1996). It is noteworthy that, when cloned into a low-copy-number plasmid (YPN2), *SMF1* failed to complement the *csp2* mutant. Using the polymerase chain reaction (PCR), the *CDC1* and *SMF1* genes were cloned from the *csp2* mutant (Supek *et al.* 1996). Whereas the *SMF1* gene was intact, the *CDC1* gene exhibited one Gly to Arg mutation replacing Gly149 with Arg. It was demonstrated that the *CDC1* gene codes for an essential yeast protein and that the mutant allele of this gene determines the observed phenotype of the *csp2* strain. The mutant allele of the *CDC1* gene bearing the Gly149 to Arg change was denoted *cdc1-200*. The data suggest that *CDC1* is a Mn^{2+}-dependent protein.

The complementation of the *csp2* phenotype by Mn^{2+} suggested that Cdc1p is a metalloprotein with Mn^{2+} as a prosthetic group. Recently, using biophysical and genetic means, it has been demonstrated that Cdc1p may indeed be a Mn^{2+}-dependent cell division cycle factor (Halbrook and Hoekstra, 1994; Loukin and Kung, 1995).

Identification of a Mn^{2+} transporter in yeast plasma membrane

Because our experiments indicated the possible involvement of the *SMF1* gene in metal-ion homeostasis, we examined the phenotype of *smf1* null mutant cells under the same conditions as described for the *csp2* mutant. The strain bearing the null allele of the *SMF1* gene could not grow on YPD plates containing 12.5 mmol l^{-1} EGTA (Supek *et al.* 1996), but it was no more sensitive than the wild-type strain to other chelators (e.g. BAPTA, bathophenanthrolinedisulphonic acid). The deletion mutant was tested for sensitivity to high concentrations of metals in the medium. The only observed difference between the wild-type and Δ*smf1* strains was increased sensitivity of the latter to $NiCl_2$. While the growth of the wild-type strain on plates was completely inhibited by 4 mmol l^{-1} $NiCl_2$, the growth of the deletion mutant was abolished by 2 mmol l^{-1} $NiCl_2$.

West *et al.* (1992) reported experiments with epitope-tagged Smf1p, introduced into the yeast cells on a multicopy plasmid, that indicated the presence of the tagged protein in purified mitochondria. Because overexpression of the protein may lead, in some cases, to its mislocalization and/or because Smf1p could have been present in the mitochondrial preparation as a result of contamination by some other organelle, we decided to re-examine the subcellular localization of Smf1p. To identify the correct location of Smf1p, we applied the cell fractionation approach that is commonly used in yeast cell biology studies (Goud *et al.* 1988). The presence of Smf1p in the endoplasmic reticulum or Golgi complex was ruled out by cell fractionation on sucrose gradients (Supek *et al.* 1996). To identify the location of Smf1p more precisely, purified preparations of mitochondria, plasma membrane and vacuolar membranes were subjected to western analysis using antibodies against marker proteins and Smf1p. Neither mitochondria nor vacuolar membranes contained significant

amounts of Smf1p, but it was localized to the plasma membrane (Supek *et al.* 1996).

The *SMF1* gene mediates high-affinity Mn^{2+} uptake

The sensitivity of the Δ*smf1* strain to EGTA may indicate that the *SMF1* gene codes for a plasma membrane metal transporter. Therefore, we measured Mn^{2+} uptake by yeast strains in which the *SMF1* gene was disrupted, in wild-type yeast and in transformed cells containing single or multiple copies of the gene (Supek *et al.* 1996). The *smf1* null mutant exhibited reduced Mn^{2+} uptake activity in comparison with the wild-type strain. Moreover, the strain containing *SMF1* on a multicopy plasmid had a Mn^{2+} uptake activity approximately fivefold higher than that of the wild-type strain. These results suggest that Smf1p functions in high-affinity Mn^{2+} uptake by the yeast cells. They also indicate the presence of a low-affinity system operating at Mn^{2+} concentrations of approximately $5\,\mu mol\,l^{-1}$ and higher. Recently, we identified Smf2p as a possible low-affinity Mn^{2+} transporter and we analyzed an additional yeast gene product, Smf3p, as a potential manganese and/or other metal-ion transporter (A. Kahn and N. Nelson, unpublished results).

A *mas1-ts* mutant can be rescued by Mn^{2+}

The *SMF1* gene was previously cloned as a multicopy suppressor of a temperature-sensitive mutation in the *MIF1* (*MAS1*) gene (Hawlitschek *et al.* 1988; West *et al.* 1992). The *MAS1* and *MAS2* (*MIF2*) genes encode proteins in which a heterodimeric form constitutes the yeast mitochondrial 'signal peptidase' (Pollock *et al.* 1988; Yang *et al.* 1988; Witte *et al.* 1988). *In vitro* experiments demonstrated that the purified peptidase activity is dependent on Mn^{2+}, Co^{2+} or Zn^{2+} (Hawlitschek *et al.* 1988). However, the identity of the *in vivo* cofactor(s) remains unknown. On the basis of our finding that Smf1p transports Mn^{2+}, we proposed that overexpression of Smf1p might lead to an increased intracellular Mn^{2+} concentration which would stabilize the mutant metallopeptidase at 37 °C and thus enable growth at this temperature. To test this hypothesis, different yeast strains bearing a *mas1-ts* mutation were streaked on YPD plates supplemented with $1\,mmol\,l^{-1}$ $MnCl_2$, $0.5\,mmol\,l^{-1}$ $CoCl_2$ or $1\,mmol\,l^{-1}$ $ZnCl_2$ and incubated at either 23 °C or 37 °C. The *ts*-sensitive E20 strain, in which a *mas1-ts* mutation could be suppressed by overexpression of *SMF1*, could also grow at 37 °C in the presence of $1\,mmol\,l^{-1}$ $MnCl_2$ (Supek *et al.* 1996). In contrast, $0.5\,mmol\,l^{-1}$ $CoCl_2$ or $1\,mmol\,l^{-1}$ $ZnCl_2$ failed to support growth at 37 °C. These experiments strongly suggest that Mn^{2+} is the *in vivo* cofactor of the mitochondrial processing peptidase.

Smf1p is related to Nramp-1, which is involved in the resistance of macrophages to mycobacteria

Natural resistance to infection with unrelated intracellular parasites such as *Mycobacterium*, *Salmonella* and *Leishmania* is controlled in the mouse by a single gene on chromosome 1, designated *Bcg*, *Ity* or *Lsh*. A candidate gene for *Bcg*, designated natural resistance-associated macrophage protein (*Nramp*), has been isolated and shown to encode a macrophage-specific membrane protein, which is altered in susceptible animals (Cellier *et al.* 1995; Govoni *et al.* 1995; Vidal *et al.* 1995*a*). *Nramp* is part of a small family of at least two genes, *Nramp1* and *Nramp2*. A mouse mutant bearing a null allele at *Nramp1* was generated and found to have pleiotropic effects on natural resistance to infection with intracellular parasites (Vidal *et al.* 1995*b*). It eliminated resistance to *Mycobacterium bovis*, *Leishmania donovani* and lethal *Salmonella typhimurium* infection, establishing that *Nramp1*, *Bcg*, *Lsh* and *Ity* are the same locus. *Nramp1* plays a key role only in the early part of the macrophage–parasite interaction and may function through an initial cytocidal or cytostatic mechanism.

SMF1 encodes a highly hydrophobic protein containing 575 amino acids with a molecular mass of 63 271 Da. The amino acid sequence of the yeast protein is 30 % identical with the corresponding sequence of the human and mouse *Bcg* gene product (Vidal *et al.* 1993; Kishi, 1994; Cellier *et al.* 1994; Barton, 1994). Similar homology was observed for the recently discovered *Drosophila malvolio* (*MVL*) gene involved in taste behaviour (Rodrigues *et al.* 1995). Their relatively high degrees of identity and structure suggest similar functions for these membrane proteins. In addition, a search in GenBank revealed homologous proteins in *Caenorhabditis elegans* and plants (Supek *et al.* 1996). Moreover, a highly homologous gene was recently identified in *Mycobacterium leprae* (about 32 % identity over 200 amino acids), suggesting that this family of transporters is widespread from bacteria to humans. We suggested that the members of this gene family function in divalent cation transport (Supek *et al.* 1996).

Although the cloning of *Nramp* identified the gene responsible for the resistance of mice to mycobacteria, its function is unknown. We propose that the mammalian and insect membrane proteins, like the yeast transporter, are Mn^{2+} and/or Zn^{2+} transporters. Fig. 1 depicts a proposed model for the role of Nramp in macrophage defence against microbial invasion. Following the phagocytosis of a parasite into the phagosome, the macrophage produces reactive oxygen and/or nitrogen intermediates that are toxic for the internalized bacteria (Segal and Abo, 1993). Phagosomes contain several plasma membrane proteins, of which one may be the Nramp protein. The survival of the pathogen during the burst of macrophage respiratory activity is thought to be partly mediated by microbial superoxide dismutase (SOD) and other enzymes (Chan *et al.* 1992) that contain Mn^{2+}, Cu^{2+}/Zn^{2+} or Fe^{2+} in their active centre. We propose that Nramp, like its yeast homologue, transports Mn^{2+} and/or Zn^{2+} from the extracellular milieu into the cytoplasm of a macrophage and, after the generation of the phagosome, removes metal ions from the organelle. Thus, the depletion of the metal ion from the phagosome microenvironment by the *Nramp* gene product

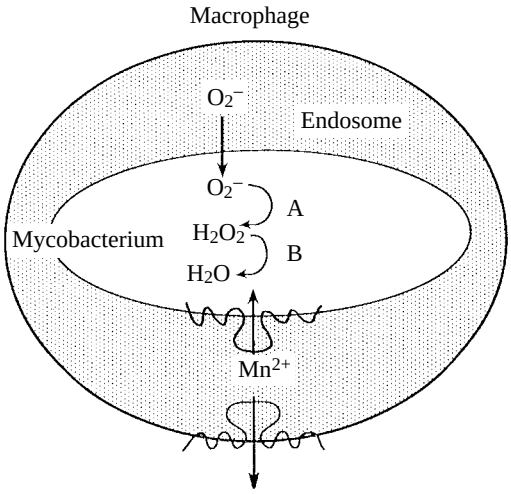

Fig. 1. A proposed role of *Nramp* in macrophage–pathogen interactions. Nramp is proposed to function in Mn^{2+} and/or other metal-ion uptake by the macrophage. After the formation of the phagosome, it is present in the phagosomal membrane and active in the transport of Mn^{2+} or other metal ions from the lumen to the cytoplasm. The mycobacterium contains a similar transporter that may transport Mn^{2+} or other metal ions from the lumen of the phagosome into its cytoplasm. A, superoxide dismutase; B, catalase; O_2^-, reactive oxygen intermediate.

may be a rate-limiting step in the production of the metalloenzyme by the engulfed bacteria. This will restrict the mycobacterial ability to produce active enzymes 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 mycobacteria and render the organism sensitive to the pathogen. The discovery of a homologous gene in *M. leprae* supports our suggestion that Nramp might be a Mn^{2+} or other metal-ion transporter (Supek *et al.* 1996). Our suggestion for the possible function of the *Nramp* and *malvolio* proteins as metal-ion transporters can be tested in several experimental systems. (1) The *malvolio* and *Nramp* proteins can be expressed in *Xenopus laevis* oocytes and assayed for their transport activity. (2) The substrate of the *malvolio* protein can be identified by growing the *Drosophila malvolio* mutant on medium containing different metal ions and looking for the ion(s) that cures the taste behaviour phenotype. Preliminary experiments indicate that only Mn^{2+} reverses the taste behaviour phenotype (S. Orgad, H. Nelson and N. Nelson, unpublished results). (3) The *malvolio* and *Nramp* proteins can be expressed in yeast mutants that are unable to grow on medium containing limited amounts of metal ions. Suppression of this phenotype may identify the substrate of the insect and mammalian transporters and may also help in the development of new drugs against mycobacterial infections.

We have demonstrated that Smf1p mediates a high-affinity Mn^{2+} uptake by yeast cells (Supek *et al.* 1996). Similar experiments with Smf2p indicate that this membrane protein may also function as a metal-ion transporter. A recent search in the GenBank revealed the presence of a third gene in this family, denoted as *SMF3* (A. Kahn and N. Nelson, unpublished results). Since the entire sequence of the yeast genome has been deposited in the GenBank, we assume that this family of transporters includes only three homologous genes in the yeast genome. The experiments presented so far demonstrate the transport of Mn^{2+} and Zn^{2+} by these transporters. Even though Smf2p and Smf3p may add some other metal ions to this list, it is likely that this family of transporters will not cover the transport of all the metal ions whose transporters have not yet been identified. We have therefore utilized the same genetic selection system to identify the missing metal ion transporters. The selection utilizes several chelating agents to cover several metal ions. So far, we have isolated 20 additional *csp* mutants denoted as *csp3–csp23* (A. Cohen, L. Bourvine and N. Nelson, unpublished results).

Function of the vacuolar system of eukaryotic cells in metal-ion homeostasis

Mammalian cells accumulate iron by binding transferrin to its high-affinity receptor or through a transferrin-independent pathway (Jordan and Kaplan, 1994). A defect in the transferrin pathway results in iron deficiency, suggesting the predominance of this pathway. This pathway involves the adsorption of iron onto transferrin, followed by the binding of the Fe–transferrin to its receptor, internalization of transferrin-bound receptors into endosomes, the bound iron being released by the low pH generated by V-ATPase, and finally iron transport across the endosomal membrane into the cytoplasm. The kinetics of iron transport through the transferrin pathway has been studied in rabbit reticulocytes (Watkins *et al.* 1991). It was demonstrated that the dissociation of iron from transferrin was the rate-limiting step in this process. The existence of a transferrin-independent transport system situated in the plasma membrane of mammalian and most other eukaryotic cells raises questions about the advantage of the transferrin pathway in mammals. Apparently, mammalian cells experience an iron stress that can be overcome by the tight binding of iron onto transferrin at neutral pH and its release at low pH. This would provide an iron uptake system that can compete with the natural iron-chelating materials that are present outside the cell. Since the affinity of most iron chelators decreases at low pH, a similar mechanism of iron uptake, operating through the endocytotic pathway, may exist in every eukaryotic cell. This mechanism may operate with any other metal ion that has its affinity for natural chelators decreased at low pH. V-ATPase may play a crucial role in this process (Nelson, 1991, 1992).

It has been shown that *S. cerevisiae* accumulates iron by two distinct transport systems, one of low and one of high affinity. The high-affinity system operates *via* a process requiring ferric reductase activity (Dancis *et al.* 1990). The reduction of Fe^{3+} takes place outside the cell. Fe^{2+} is then transported across the plasma membrane through an iron transporter that requires the

oxidation of Fe^{2+} to Fe^{3+} by a copper-dependent ferro-oxidase (Askwith *et al.* 1994; Dancis *et al.* 1994). Consequently, iron transport across the yeast plasma membrane is dependent on copper transport. Two gene products were shown to be necessary for iron transport. One (Fet3p) is a multicopper oxidase anchored into the outer surface of the plasma membrane by a single transmembrane domain. The second (Ftr1p) is a Fe^{2+} transporter that transports the reduced iron across the membrane. The low-affinity iron uptake system prefers Fe^{2+} over Fe^{3+} as substrate and it contains the *FET4* gene product with six predicted transmembrane domains (Dix *et al.* 1994).

Does iron uptake in yeast cells follow a pathway similar to that of mammalian transferrin? Yeast cells in which genes encoding V-ATPase subunits were inactivated were unable to grow on medium containing 50 mmol l^{-1} Ca^{2+} or 15 mmol l^{-1} EGTA (Nelson, 1991, 1992). The explanation for the sensitivity of V-ATPase null mutants to EGTA is shown in Fig. 2. It was proposed that a high-affinity Ca^{2+} uptake pathway operates in yeast through endocytosis and through the vacuolar system (Ohsumi and Anraku, 1983; Nelson, 1991, 1992; Nelson *et al.* 1992; Supek *et al.* 1996). Accordingly, Ca^{2+}-EGTA is taken up by endocytosis to the vacuole and is dissociated into Ca^{2+} and EGTA by the low pH inside the vacuole. The Ca^{2+} can then be transported into the cytoplasm by the available transporters and channels. V-ATPase null mutants fail to acidify the vacuole and are therefore sensitive to EGTA. Experiments utilizing V-ATPase null mutants indicated that these iron uptake systems are not exclusive for iron uptake by yeast cells. As shown in Fig. 3, V-ATPase null mutants are sensitive to 0.1 mmol l^{-1} bathophenantroline sulphonate, a condition under which wild-type cells grow well. The growth arrest by bathophenantroline sulphonate can be relieved by the inclusion of either 10 μmol l^{-1} Cu^{2+} of Fe^{2+} in the growth medium. We propose that, similar to the effect of EGTA on Ca^{2+} homeostasis in V-ATPase null mutants, bathopenantroline sulphonate reduces the external iron concentration to a level at which the plasma membrane uptake system is not efficient enough to sustain growth. In wild-type yeast, the iron–bathophenantroline complex is taken up into the vacuolar system through the endocytotic pathway. Acidification of the interior of the vacuolar system by the V-ATPase causes dissociation of the iron from the chelating agent and allows iron uptake through the organelle membrane (Noumi *et al.* 1991; Nelson, 1991, 1992; Supek *et al.* 1996). The absence of proper acidification of the vacuolar system in V-ATPase null mutants prevents the dissociation of iron from the chelating agent, resulting in growth arrest of the mutants. This pathway is analogous to the iron uptake system of mammalian cells.

Fig. 4 depicts a schematic proposal for the main factors

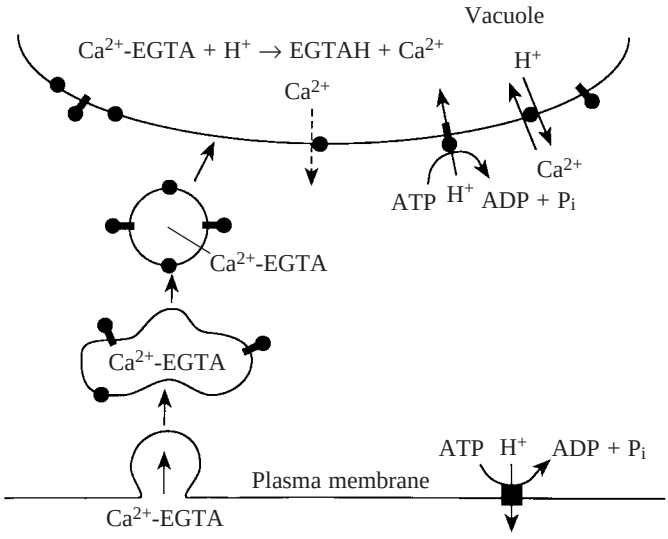

Fig. 2. Schematic explanation of the sensitivity of V-ATPase mutants to EGTA. Ca^{2+} entry through the endocytotic pathway is depicted as representative of a divalent cation. Similar mechanisms may exist for other ions and their chelating agents. (🏆) V-ATPase; (■) plasma membrane H^+-ATPase; (●) vacuolar Ca^{2+} transporters.

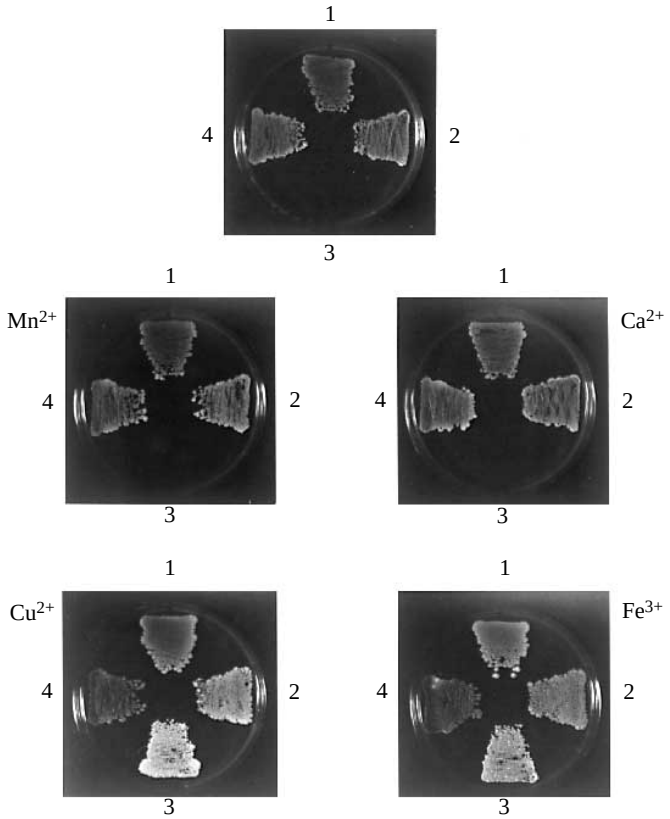

Fig. 3. Effect of bathophenantroline disulphonic acid on growth of various yeast strains. *Saccharomyces cerevisiae* strains W303-1a (*MATa, leu2, his3, ade2, trp1,ura3*) were grown on YPD plates containing 2 % bactopeptone, 1 % yeast extract, 2 % dextrose and 2 % agar, buffered with 50 mmol l^{-1} Mes and adjusted to pH 6.0 with NaOH. The medium was supplemented with 0.1 mmol l^{-1} bathophenantroline sulphonate and, where indicated, 10 μmol l^{-1} divalent cations were added as chloride salts. (1) Wild-type W303-1a cells. (2) *csp2-1* mutant. (3) Δ*vma3::URA3* mutant in which V-ATPase is not active. (4) Δ*smf1::URA3* mutant in which the gene *SMF1* is deleted.

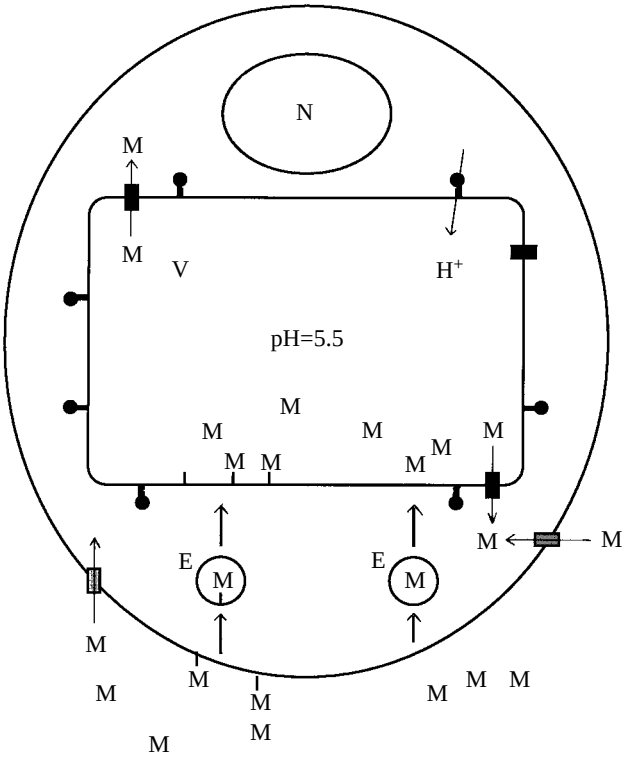

Fig. 4. Schematic proposal for the function of the vacuolar system in metal-ion homeostasis. Two distinct pathways for metal import into the cell by the vacuolar system are proposed. One involves metal receptors and the other fluid-phase endocytosis. The metal-ion transporters on the vacuolar and plasma membrane are also depicted. (🌹) V-ATPase; (▬) vacuolar metal-ion transporter; (▭) plasma membrane metal-ion transporter; (▮) metal-ion receptor; N, nucleus; V, vacuole; E, endosome; M, metal ion.

involved in metal-ion transport in eukaryotic cells. Reversible metal-ion transporters operate in both the plasma and vacuolar membranes. During metal-ion stress, two similar pathways can overcome the metal-ion shortage. One involves metal-ion receptors that operate in a similar fashion to transferrin-mediated iron transport. The second system utilizes the endocytotic pathway for fluid-phase endocytosis of free metals or metal ions bound to chelating agents.

The involvement of V-ATPase in metal-ion homeostasis has been studied biochemically and genetically (Eide *et al.* 1993; Li *et al.* 1994). It was shown that purified V-ATPase from reticulocyte endosomes reconstituted into liposomes was active in ATP-dependent Fe^{2+} transport into the liposomes (Li *et al.* 1994). This experiment suggests that the transport of iron across the endosomal membrane does not require a special transporter. The genetic approach stemmed from the phenotype of V-ATPase null mutants (Nelson and Nelson, 1990). It was demonstrated that, under certain conditions, the V-ATPase null mutants exhibited a pet^- phenotype that is unable to grow on a non-fermentable carbon source (Umemoto *et al.* 1990; Supek *et al.* 1994). Li *et al.* (1994) demonstrated that the inclusion of elevated iron concentrations in the growth medium can suppress this phenotype. It was suggested that the low rate of iron transport from the vacuoles of the null mutants into the cytoplasm caused an iron shortage in the mitochondria and rendered them respiration-incompetent. Recently, we employed the genetic selection system used to identify the yeast manganese transporter (Supek *et al.* 1996) to obtain yeast mutants that are sensitive to bathophenantroline sulphonate (A. Cohen, L. Bourvine and N. Nelson, unpublished results). The resulting *csp3* mutant grows well on medium buffered at pH 7.5 and on 12.5 mmol l^{-1} EGTA, indicating that its V-ATPase is intact (Noumi *et al.* 1991; Nelson, 1991, 1992; Nelson *et al.* 1992). We anticipate that the mutation is located on a gene encoding an essential metalloprotein (iron?) or an iron transporter. This approach may yield the missing information on the mechanism of metal-ion transport across the membranes of the vacuolar system.

The vacuolar system may play a critical role not only in the uptake of metal ions from the environment but also in their storage and secretion. Several organelles of eukaryotic cells contain high concentrations of Ca^{2+}, Mg^{2+}, Fe^{2+} and other metal ions. Specific transporters must be present in the membranes of these organelles to maintain the ionic balance across their membranes. In addition, there are numerous transcription factors with 'zinc fingers' (Berg and Shi, 1996). If, indeed, zinc is present in some of these structures, its concentration should influence their *in vivo* activities. Mammalian cDNA encoding a zinc transporter (ZnT-1) was recently cloned from a rat kidney library (Palmiter and Findley, 1995). The gene product was predicted to be a membrane protein with six membrane-spanning domains and a large intracellular loop. It was localized to the plasma membrane by the use of the myc epitope-tag. It was suggested that ZnT-1 transports zinc out of the cells and that its absence causes an increased sensitivity to zinc toxicity. Recent studies in our laboratory indicate that ATP-dependent transporters may also play a role in metal-ion homeostasis of eukaryotic cells. A null mutation in the *S. cerevisiae DRS2* gene caused an increased tolerance to high manganese concentrations in the growth medium (H. Nelson, A. Cohen and N. Nelson, unpublished results). This gene was discovered as a yeast gene required for ribosome assembly (Ripmaster *et al.* 1993). We identified some sequence homology between Drs2p and amino acid sequences derived from a vanadate-sensitive ATPase from bovine chromaffin granules (Moriyama and Nelson, 1988). The latter was suggested to function as a phospholipid flipase (Zachowski *et al.* 1989). The connection among these three apparently unrelated processes may turn out to be metal-ion homeostasis.

Several metal ions are poisonous at moderate or high concentrations, and there exist specific transport systems to clear excess amounts from the cytoplasm. The structurally related *COT1* and *ZRC1* genes of *S. cerevisiae* encode membrane proteins that are dosage-dependent suppressors of metal toxicity (Conklin *et al.* 1992, 1994). Cot1p confers increased tolerance to high levels of cobalt and Zrc1p confers increased tolerance to high levels of zinc. Strains that carry null alleles at both genes are viable but are metal-

hypersensitive (Conklin *et al.* 1994). Remarkably, Cot1p was localized to the yeast mitochondria (Conklin *et al.* 1994). Even though mitochondria may accumulate cobalt, the mechanism of cobalt tolerance through mitochondrial function is not apparent. The site of action of Smf1p may eventually be shown to be the vacuolar system or the plasma membrane, as was observed for Smf1p (Supek *et al.* 1996). It was demonstrated that the function of the *COT1* or *ZRC1* gene products is more important for metal-ion homeostasis than that of the *GRR1* gene product, which is also involved in metal metabolism (Flick and Johnston, 1991; Conklin *et al.* 1993). The latter may represent a connection between metal-ion homeostasis and gene regulation in the glucose repression system. The interplay between metal-ion transport, metal-ion homeostasis and gene regulation is likely to attract more attention in the near future.

Recently, it was shown that Ca^{2+} homeostasis in *S. cerevisiae* is controlled by three genes, *VCX1*, *PMC1* and *PMR1*, encoding intracellular Ca^{2+} transporters (Cunningham and Fink, 1996). Mutants lacking all three Ca^{2+} transporters are inviable, but mutants containing only a single functional transporter are viable. *VCX1* encodes a vacuolar H^+/Ca^{2+} exchanger and its function requires V-ATPase. Pmc1p also functions in the vacuole and Pmr1p functions primarily in the Golgi complex. The Ca^{2+} homeostasis provided by these transporters is controlled by calmodulin and calcineurin (Cunningham and Fink, 1996). In addition to its function in Ca^{2+} homeostasis, Pmr1p also functions in Mn^{2+} tolerance. In contrast to Vcx1p, which derives its energy from the proton gradient generated by V-ATPase, Pmc1p and Pmr1p are Ca^{2+}/Mn^{2+}-ATPase and Ca^{2+}-ATPase, respectively (Cunningham and Fink, 1994; Lapinskas *et al.* 1995). These and other observations stress the crucial function of the vacuolar system in metal-ion homeostasis (Lin and Culotta, 1995).

Metal-ion homeostasis and brain function

Although metal-ion homeostasis is vital for every eukaryotic cell, it may serve a special function in certain organs and cell types, e.g. iron metabolism in red blood cells. Here, the iron in haemoglobin functions as an oxygen ligand and thereby plays a crucial role in respiration. The function of Ca^{2+} homeostasis in the brain has been discussed frequently because of the importance of Ca^{2+} in signal transduction through many receptors. In addition, brain damage during hypoxia or ischaemia has been attributed to an imbalance in Ca^{2+} concentrations. Similarly, the presence of unusual concentrations of other metal ions can cause impaired brain function or cell death. The different ions may be distinct redox-active ions, such as Fe^{2+}, Cu^{2+}, Co^{2+} and to a lesser extent Mn^{2+}, or non-redox-active ions, such as Ca^{2+} and Zn^{2+}. Zn^{2+} and Ca^{2+} may be targeted to transcription factors and other enzymes involved in DNA metabolism, because targeting redox-active metal ions to these places can lead to the promotion of radical reactions that result in nucleic acid damage. The redox-active ions normally function in enzymes that participate in redox reactions and in the conversion of active oxygen-containing components. All of these processes require defined amounts of specific metal ions at the right position and at the correct time. Very little is known about the mechanisms of metal-ion homeostasis, about the maintenance of appropriate levels of the metal ions under a variety of environmental conditions, or about the transporters involved in these processes.

The transport of metal ions from the blood to the brain involves the crossing of the blood–brain barrier. Studies of iron transport in the brain using ^{59}Fe (Morris *et al.* 1992; Ueda *et al.* 1993) have shown that the permeability of the blood–brain barrier to Fe–transferrin is similar to that of albumin. The experiments suggested that the uptake of iron into the brain involves the transport of iron from iron-loaded blood transferrin to a brain-derived transferrin for extracellular iron transport within the brain (Morris *et al.* 1992). Subsequently, the iron is taken up *via* transferrin receptors into the brain cells. The introduction of monoclonal antibodies against transferrin receptors into the brain fluid reduced the iron transport by 35–85 %. The experiments suggested that, together with transferrin-dependent iron uptake, there exists a non-transferrin iron transport that readily crosses the blood–brain barrier (Ueda *et al.* 1993). Perturbation of iron homeostasis was observed in response to chronic treatment of rats with chlorpromazine, a medication for schizophrenic patients (Ben-Shachar *et al.* 1994). This observation suggests that some of the side effects caused by psychotic drugs may be due to perturbation of metal-ion homeostasis.

Zinc can interact strongly with a variety of ligands including sulphur in cysteine, nitrogen in histidine and oxygen in acidic amino acids. Therefore, it is likely to be bound to serum proteins and it may cross the blood–brain barrier as a ligand of amino acids or other components that bind zinc. Indeed, it was found that histidine facilitates the transport of zinc across the blood–brain barrier, but this was attributed to the diffusion of a zinc–histidine complex across unstirred layers, making the zinc ion accessible to its transport sites (Buxani-Rice *et al.* 1994). In brain cells, zinc is accumulated in the presynaptic vesicles of excitatory neurones and is released during synaptic activity (Assaf and Chung, 1984; Howell *et al.* 1984). Zinc interacts with some ionotropic receptors in the brain. For example, the ionotropic ATP receptor (P2x3) 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). Since Zn^{2+} is secreted from synaptic vesicles, interacts with certain receptors and should have a specific transport system, it may be considered as a neurotransmitter. It also interacts with neurotransmitter uptake systems such as the dopamine transporter. It inhibits dopamine uptake and cocaine binding (Richfield, 1993). Finally, it has been demonstrated that it may play a role in neuronal death after transient cerebral ischaemia (Koh *et al.* 1996).

Manganese is also readily taken up into the central nervous system, probably as a free ion (Rabin *et al.* 1993; Murphy *et al.* 1991). However, the transport is affected by plasma proteins such as albumin and transferrin that bind Mn^{2+}. Mn^{2+} uptake into rat astrocytes is not inhibited by Co^{2+}, Zn^{2+} or Pb^{2+}, indicating the presence of a specific Mn^{2+} uptake system (Aschner *et al.* 1992). Except for its function in metalloproteins, there is no indication of specific neuronal modulation by Mn^{2+}. However, if the *malvolio* protein in *Drosophila* is indeed a Mn^{2+} transporter (Supek *et al.* 1996; Rodrigues *et al.* 1995), its neuronal function may become apparent. Our working hypothesis is that the Mn^{2+} functions in a novel signal transduction pathway(s).

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