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MINIREVIEW

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The many highways for intracellular trafficking of metals

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Abstract Metal ions such as copper and manganese represent a unique problem to living cells in that these ions are not only essential co-factors for metalloproteins, but are also potentially toxic. To aid in the homeostatic balance of essential but toxic metals, cells have evolved with a complex network of metal trafficking pathways. The object of such pathways is twofold: to prevent accumulation of the metal in the freely reactive form (metal detoxification pathways) and to ensure proper delivery of the ion to target metalloproteins (metal utilization pathways). Much of what we currently know regarding these complex pathways of metal trafficking has emerged from molecular genetic studies in baker's yeast, *Saccharomyces cerevisiae*. In this review, we shall briefly highlight the current understanding of factors that function in the trafficking and handling of copper, including copper detoxification factors, copper transporters and copper chaperones. In addition, very recent findings on the players involved in manganese trafficking will be presented. The goal is to provide a paradigm for the intracellular handling of metals that may be applied in a more general sense to metals that serve essential functions in biology.

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Abbreviations *CTR*: cell surface transporter · *GSH*: glutathione · *MCF*: mitochondrial carrier family · *mito*: mitochondria · *MT*: metallothionein · *SOD*: superoxide dismutase

Introduction

Proteins that rely on metal ions for activity can be found at virtually every location within a cell, ranging from the cell surface, to the soluble cytosol, to the heart of membranous organelles such as the nucleus, Golgi and mitochondria. With so many enzymes needing metals in such diverse locations, how is the co-factor delivered to the right site and also at the right time? This seems even more problematic when one considers that many metal-binding proteins appear indiscriminate in their choice of metals. In a test tube setting, a copper-requiring enzyme may also bind zinc or cobalt but exhibit activity only when copper is bound. It is therefore crucial that, among the many different metals accumulated by cells, only the correct ion is presented to the metalloproteins. This problem seems paramount when one considers the fact that all essential metals are also potentially toxic. The cell must somehow deliver the right metals to partner proteins without permitting toxic side reactions of metal ion chemistry. Overall, the task seems formidable.

Although the precise mechanism by which metals are faithfully combined with proteins in vivo is still unclear, bits and pieces of information are emerging from studies on copper and more recently on manganese. The current paradigm is that metal ions are not free agents. Rather, these ions are under careful surveillance by systems designed to detoxify and sequester the metal or to escort the ion to its cognate site in a metalloprotein. Once a metal enters a cell, it is subjected to one of several fates

or pathways, depending on physiological needs. For the purpose of this review, we shall refer to these routes as "metal trafficking pathways".

Results and discussion

As mentioned above, much of what we have learned regarding metal trafficking systems has emerged from studies on copper, and more recently on manganese. The pathways for intracellular trafficking of copper have been discussed in numerous reviews [1, 2, 3, 4, 5, 6, 7, 8]. In this report, we will just briefly provide an overview on copper as a reference point for a more detailed discussion on recent findings of manganese.

Intracellular pathways for trafficking copper

The vast majority of what is known regarding copper trafficking pathways in eukaryotic cells originated from studies on baker's yeast, *Saccharomyces cerevisiae*. Copper largely enters the cell through the action of one or more high-affinity cell surface transporters (CTRs) (Fig. 1) [9, 10, 11], and yeast cells lacking the high-affinity transport systems show deficiencies in copper-requiring enzymes under normal growth conditions [12, 13]. The CTRs of yeast aided in the identification of human and murine CTR1 [9, 14], and a deletion of the CTR1 gene in mice results in profound copper deficiency and embryonic lethality [15]. Yet as critical as the high-affinity transport systems seem, all organisms have back-up mechanisms for obtaining copper [16]. For example, in yeast, copper can also enter the cell via one or more low-affinity transporters when the extracellular medium is supplemented with elevated, but non-toxic, concentrations of copper. Often the low-affinity transporters are not specific for copper, but can also transport other divalent metals (e.g., the FET4 transporter of *S. cerevisiae*) [17, 18] (Fig. 1).

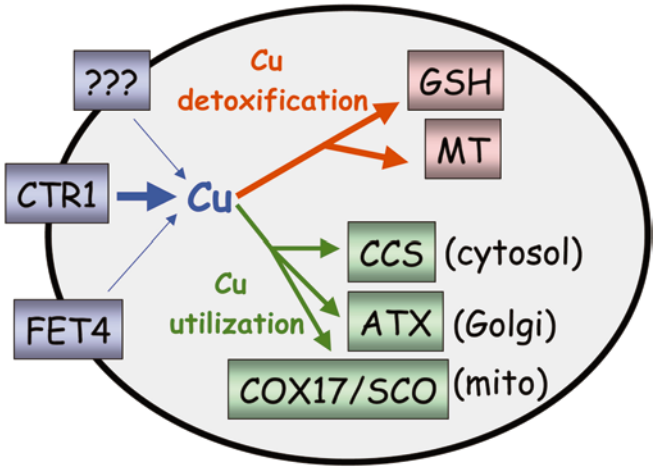

Fig. 1 Trafficking pathways for copper. The cell surface transporters (*blue boxes*), copper chaperone-like molecules (*green boxes*) and detoxification factors (*pink boxes*) for copper are shown, based on pathways established for baker's yeast. CTR1, being a major high-affinity transporter, is the predominant source of environmental copper under physiological conditions (depicted by *heavy arrow*). When environmental copper becomes more available, low-affinity transporters such as FET4 and other unknown molecules ("???") can also contribute to the pool of intracellular copper. Once the metal enters the cell, a substantial fraction is subject to detoxification (*red arrows*) by factors such as metal-binding metallothioneins (MT) or glutathione (GSH). Another pool of copper is reserved by copper chaperone molecules for copper utilization pathways (*green arrows*), i.e. delivery of the metal to copper-requiring targets in the cytosol, mitochondria (*mito*) or Golgi

Once copper enters the cell, the metal is subject to one of two major fates: either copper detoxification or copper utilization. A large fraction of intracellular copper undergoes detoxification and can be sequestered by metal-binding factors such as metallothioneins or glutathionine (Fig. 1) [19, 20, 21, 22, 23]. These detoxification factors effectively prevent copper from accumulating in the toxic and free ionic form [21]. However, a fraction of copper bypasses these detoxification systems and is reserved for copper utilization pathways that separately deliver the ion to at least three classes of copper-containing enzymes: (1) copper enzymes in the secretory pathway destined for the cell surface or extracellular milieu; (2) cytochrome *c* oxidase in the mitochondria; and (3) a copper- and zinc-containing superoxide dismutase (SOD1) in the cytoplasm.

Copper delivery to the Golgi

All copper-requiring enzymes that transit through the secretory pathway are activated with copper in a very specific compartment of the Golgi. Copper is made available to this compartment through the action of copper-transporting P-type ATPase molecules that translocate cytosolic copper into the lumen of the Golgi. In humans, these ATPase molecules are known as the Wilson or Menkes disease proteins and, as the names imply, mutations in the corresponding genes result in inherited disorders of copper metabolism [24, 25]. These transporters do not obtain cytosolic copper from a free ionic pool. Instead, the metal is delivered by a small soluble copper binding protein we have named yeast ATX1, also known as ATOX1 or HAH1 in humans [26, 27, 28, 29, 30, 31, 32, 33]. For the purpose of this review, we shall refer to the protein as ATX1.

ATX1 represents the prototype of a family of molecules known as copper chaperones. O'Halloran coined the phrase "copper chaperone" [33] to describe proteins that spare copper from the detoxification factors and escort the metal to specific copper-requiring targets in the cell. The copper chaperones also directly insert the metal into the cognate target.

Copper delivery to the mitochondria

The delivery of copper to cytochrome *c* oxidase in the mitochondria requires at least two factors that were

originally identified through genetic studies in yeast (by Glerum, Tzagoloff and colleagues at Columbia University [34, 35, 36]). The first, known as COX17, is a small soluble copper-binding protein found in both the cytoplasm and intermembrane space of mitochondria [34, 37, 38, 39]. COX17 has been proposed to deliver copper to a second set of copper-binding proteins, SCO1 and SCO2, that lie in the inner membrane of mitochondria and make copper available to the Cu_A site of cytochrome oxidase [40, 41, 42]. In this regard, the COX17–SCO partnership for cytochrome oxidase is somewhat akin to the ATX1–P-type ATPase partnership for delivering copper to secretory pathway enzymes. The COX17–SCO pathway for copper trafficking indeed plays a vital role in cell physiology, as mutations in the SCO proteins have been associated with neonatal lethality in humans [43, 44, 45].

Copper delivery to the cytosol

A major copper protein in the cytosol is a copper- and zinc-containing superoxide dismutase (SOD1). Copper is known to be inserted into SOD1 through the action of a molecule we have named CCS, for copper chaperone for SOD1 [46]. CCS is able to form a transient heterodimer with SOD1 as the prerequisite to copper transfer [47, 48, 49]. SOD1 also binds one zinc atom per monomer, although the mechanism by which the enzyme acquires zinc is unknown.

It is noteworthy that, unlike the aforementioned pathways for delivering copper to cytochrome oxidase and to enzymes in the secretory pathway, activation of SOD1 appears to require just one protein (CCS) that directly inserts the metal into the enzyme. This may reflect the fact that the SOD1 target largely resides in the cytoplasm, with no intracellular membrane barriers for the metal to cross. However, we noted that a small fraction of yeast SOD1 and its copper chaperone also reside in the intermembrane space of mitochondria [50, 51]. It is quite conceivable that, in the special case of mitochondrial SOD1, an additional factor becomes important for delivering copper to the enzyme.

By using yeast SOD1 as an example, some basic premises of copper trafficking emerge. First, the action of the copper chaperones appears highly specific. Each chaperone molecule interacts with one and only one copper-requiring target. CCS delivers copper to SOD1, but not to the Golgi copper transporter or to cytochrome oxidase in the mitochondria. Secondly, there appears to be very limited backup for the copper chaperones. When CCS is absent, yeast SOD1 generally remains apo for copper. By comparison, there appears to be multiple backup systems for the membrane transporters for copper. High-affinity transporters largely function when copper is limiting, but when extracellular copper is elevated even slightly, low-affinity transporters can also contribute to metal accumulation (Fig. 1) [16, 17, 18]. Yet regardless of the upstream copper transporter used, the metal must go through the copper chaperone CCS to reach yeast SOD1 (Fig. 1) [18]. The only condition where yeast SOD1 can acquire copper independent of CCS is when the cell is challenged with a toxic dose of copper. This saturates the detoxification systems and a fraction of copper now becomes freely available to interact with SOD1 in a CCS-independent manner [21]. In essence, the copper detoxification and copper utilization pathways are in a constant ''tug of war'' over the metal. Such homeostasis is crucial for a metal ion that is both toxic and essential for life.

Trafficking pathways for manganese

Employing a genetic approach in baker's yeast, we have just begun to unravel some of the intracellular trafficking pathways for manganese. Like copper, manganese is essential for the activity of a number of enzymes, but high levels of manganese can also be toxic to the cell. In this section, we shall highlight what is currently known regarding the uptake of manganese from the extracellular environment and then the trafficking pathways involved in the detoxification of the ion or the delivery of this metal to manganese-requiring enzymes. Once again, baker's yeast has proven to be a powerful model system for understanding these processes.

Cell surface transport of manganese

As with copper, there appears to be a number of ports of entry by which extracellular manganese can be taken into the cell (Fig. 2). One such transporter for manganese is yeast SMF1, a member of the Nramp family of divalent metal transporters [52]. SMF1 was originally discovered by Nelson and co-workers as a protein which, when produced to high levels, would overcome a manganese-deficient step in mitochondrial protein processing [53]. Biochemical analysis revealed a high-affinity manganese transport activity for SMF1 and the transporter was indeed found at the cell surface [53, 54]. However, under physiological conditions, SMF1 does not appear to contribute much to cellular manganese. Yeast cells lacking SMF1 show no major deficiencies in manganese accumulation or in the activity of manganese-requiring enzymes [55]. This might reflect the fact that under normal growth conditions there is very little accumulation of SMF1 in the cell [54]. We noted that unless cells are starved for manganese, the bulk of SMF1 synthesized by cells is degraded by proteases in the yeast vacuole [54, 56]. Like other Nramp transporters, SMF1 is relatively non-specific for divalent metals and can also transport toxic metals such as cadmium [57, 58]. To minimize metal toxicity, SMF1 is only present at the cell surface when the demand for manganese is great, i.e. under manganese starvation conditions [54, 56]. We therefore conclude that SMF1 is an important high-affinity transporter for manganese under states of

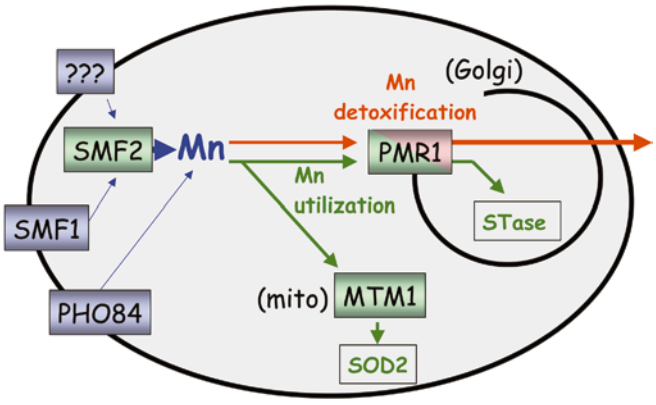

Fig. 2 Trafficking pathways for manganese. Factors involved in the cell surface transport (*blue boxes*), detoxification (*pink boxes/red arrows*) and utilization (*green boxes and green arrows*) of manganese in yeast are shown. SMF1 is believed to only function under extreme manganese starvation conditions and other, as of yet unknown, high-affinity transporters ("???") are thought to contribute the metal under physiological conditions. When cells are exposed to toxic manganese, the metal is largely taken up in the form of manganese phosphate complexes by the PHO84 phosphate transporter. SMF2, localized to intracellular vesicles, plays an important role in the manganese utilization pathways for both Golgi sugar transferases (STase) and mitochondrial SOD2. By pumping manganese into the Golgi, PMR1 plays a dual role in the detoxification of manganese and in delivery of manganese to the Golgi sugar tranferases. MTM1 in the mitochondrial inner membrane is essential for delivering manganese to SOD2

manganese starvation; however, under normal physiological conditions, other high-affinity transporter(s) must be operable. The identity of such transporter(s) remains elusive at this time (Fig. 2).

Under conditions of manganese surplus or manganese toxicity, manganese can enter the cell through a different route [59]. *S. cerevisiae* PHO84 is a well-known transporter for the high-affinity uptake of phosphate and we have recently uncovered a role for this protein in manganese transport (Fig. 2). Yeast cells lacking PHO84 exhibit a resistance to manganese toxicity and accumulate low levels of the metal when cells are exposed to toxic manganese. However, these same mutant cells show no aberration in manganese accumulation under normal conditions when cells are exposed to physiological levels of manganese. As such, PHO84 is behaving like a low-affinity transporter for manganese, contributing to manganese accumulation only when cells are exposed to high levels of the metal [60]. We hypothesized that, in yeast, PHO84 can transport phosphate in the form of $MnHPO_4$; in fact, recombinant PHO84 is seen to exhibit this exact activity in reconstituted proteoliposomes [61]. It is quite possible that phosphate transporters also contribute to manganese uptake in humans, particularly under conditions of manganese toxicity.

Manganese detoxification pathways

Unlike copper, manganese detoxification does not appear to use factors that directly bind and sequester the metal, such as metallothioneins. Instead, *S. cerevisiae* keeps the total intracellular concentration low by minimizing metal uptake and effectively exporting excess manganese. To minimize high-affinity uptake of manganese, the SMF1 transporter is degraded by proteases in the vacuole, as described above. To facilitate manganese efflux from the cell, the metal is pumped into the Golgi and ultimately exported from the cell via secretory pathway vesicles that carry the metal to the cell surface. Such pumping of manganese into the secretory pathway is accomplished by another P-type ATPase, known as PMR1 (Fig. 2), that is a transporter for both calcium and manganese [62, 63, 64]. Yeast cells lacking the PMR1 transporter are exquisitely sensitive to manganese toxicity and accumulate high concentrations of the metal, presumably in the cytosol [65]. Another possible pathway for manganese detoxification in yeast involves trapping the metal in the lumen of the vacuole. As evidence for this, mutants of the vacuolar ATPase also exhibit manganese sensitivity, although not to the degree seen with cells lacking PMR1 [66].

Manganese utilization pathways

Using yeast as a model system, we have focused on two enzymatic targets that require the metal for activity. The first is a manganese-requiring superoxide dismutase (SOD2) that resides in the matrix of the mitochondria [67]. Like Cu/Zn SOD1 in the cytosol, mitochondrial SOD2 protects against oxidative damage by scavenging superoxide radicals [67]. SOD2 needs to acquire its manganese in the matrix of the mitochondria and, therefore, SOD2 activity is a good marker for mitochondrial manganese. The second target of manganese trafficking is represented by a class of sugar transferases in the Golgi ("STase"; see Fig. 2) that transfer mannose, glucose and galactose moieties to proteins in the secretory pathway [68, 69]. In *S. cerevisiae*, manganese-requiring mannose transferases are responsible for glycosylation of invertase [70, 71, 72], and the degree of invertase glycosylation has been shown to be a good marker for manganese availability in the Golgi [55, 63].

The intracellular trafficking of manganese in yeast is highly dependent on another member of the Nramp family of transporters, SMF2 [73]. Yeast cells that are devoid of the SMF2 transporter exhibit deficiencies in both invertase glycosylation and in manganese SOD2 activity, indicative of a cell wide disturbance in manganese trafficking [55]. Yet SMF2 is never found at the cell surface, but rather operates at intracellular vesicles [55, 74]. The nature of these SMF2-containing vesicles is not known, but they may function as storage depots or transient passage stations for manganese. Manganese influx may converge at these vesicles and SMF2 then serves to redistribute the metal by transporting it out of the vesicles into the cytosol for utilization by the cell (Fig. 2) [55].

Following release of manganese by SMF2, the metal may be trafficked to the Golgi, where it serves as the

substrate for transport by PMR1 (Fig. 2). As described above, the PMR1 P-type ATPase pumps manganese into the Golgi, where the metal may exit the cell via the secretory pathway [62, 63, 64, 75, 76]. This not only provides a means for manganese detoxification, but is also crucial for supplying the yeast Golgi with the manganese needed to activate mannose transferases (Fig. 2) [62, 64]. Cells lacking the PMR1 ATPase exhibit defects in invertase glycosylation by mannose transferases and this defect is corrected by high manganese supplements [55, 62]. A similar situation appears to exist in mammals, where the PMR1 homologue hSPCA1 functions in manganese and calcium homeostasis [77]. Patients with mutations in hSPCA1 are associated with Hailey–Hailey disease, a disorder of kertatinocytes consistent with defects in protein glycosylation [78].

How does manganese reach the mitochondrial matrix for incorporation into SOD2? We have very recently undertaken a genetic approach in yeast for identifying potential mitochondrial transporters for manganese. As likely candidates, we screened through a collection of mitochondrial transporters known as the mitochondrial carrier family (MCF). This family of transporters, which lie in the inner membrane of mitochondria, function to regulate the exchange of small molecules between the mitochondria and cytosol. *S. cerevisiae* has ~35 distinct MCFs and humans are estimated to express nearly 40 such transporters. Although the substrates for a subset have been identified (e.g., compounds of the TCA cycle, ADP/ATP [79, 80, 81]), the bulk of MCF molecules transport substrates of unknown identity. We screened through nearly 35 individual MCFs of *S. cerevisiae* and identified a single transporter which, when mutated, rendered SOD2 largely inactive. Activity of SOD2 could be rescued by supplementing the cells with high concentrations of manganese, but not with other metals, indicating that the SOD2 polypeptide is largely devoid of manganese in this MCF mutant. We have named the corresponding protein MTM1 for manganese trafficking factor for mitochondrial SOD2 (Fig. 2) [82].

If MTM1 were indeed the manganese transporter for the mitochondria, then one would expect mutations in the corresponding gene to result in low mitochondrial manganese. However, this was not the case. If anything, yeast cells lacking MTM1 were associated with *elevations* in mitochondrial manganese [82]. Apparently, the cell makes a futile attempt to rescue SOD2 activity by increasing mitochondrial manganese. In spite of this elevated manganese, SOD2 remains apo for manganese. We therefore conclude that MTM1 does not globally supply the mitochondria with manganese, but rather helps traffic manganese directly to SOD2.

To demonstrate that MTM1 is specific for a mitochondrial form of manganese SOD, we expressed in the cytosol of *S. cerevisiae* a manganese-containing SOD from the pathogenic yeast *Candida albicans*. This manganese SOD is highly homologous to mitochondrial SOD2 from *S. cerevisiae*, but localizes to the cytosol, not mitochondria. Our studies demonstrate that manganese acquisition by this cytosolic form of manganese SOD2 does not require MTM1 [82], although it does rely on the global manganese trafficking factor SMF2. Therefore, MTM1 is specifically required for the activation of manganese-containing SOD that resides in mitochondria.

When MTM1 is compared with known metal transporters and metal chaperones, MTM1 does not appear to behave as a classical membrane transporter for manganese (see Table S1, Supplementary material). First of all, MTM1 is not required for intracellular metal accumulation, as there is no loss in mitochondrial manganese associated with loss of MTM1. By comparison, cells lacking the SMF2 transporter (also needed for SOD2 activity) are associated with very low mitochondrial manganese [55]. Secondly, unlike other metal transporters, there appears to be very little backup for MTM1. The metal deficiencies associated with loss of the yeast SMF2 and CTR1 transporters are easily bypassed by supplementing the growth medium with low, non-toxic doses of manganese or copper, respectively [11, 18, 55, 63]. Under these conditions the metal is transported by alternative low-affinity transport systems and metal trafficking is restored. However, one cannot so easily dispense with MTM1. Supplying the growth medium with non-toxic supplements of manganese fails to rescue the SOD2 defect associated with loss of MTM1. Rather, it takes toxic levels of manganese to restore SOD2 activity in yeast cells lacking MTM1 [82]. This is highly reminiscent of what is seen with mutants of the copper chaperones in yeast (Table S1, Supplementary material). These molecules only become dispensable under conditions of copper toxicity, or when the metal escapes detoxification and becomes freely available [21]. We envision that a similar scenario may exist with MTM1, i.e. this factor is somehow necessary for directly supplying SOD2 with manganese and it is not until the metal accumulates in the free ionic or toxic form that SOD2 can be activated in lieu of MTM1.

Concluding remarks

Overall, we have just begun to scratch the surface in understanding how living organisms effectively handle metals that are both toxic ions and essential nutrients. For each metal ion that enters a cell, a complex network of trafficking pathways are available, and the pathway chosen is sure to be dictated by physiological need. The findings of copper and manganese trafficking described herein represent only a fraction of the complete picture. There are likely to be analogous routes of metal trafficking that operate for other essential ions such as zinc and iron. The upcoming years promise to bring new exciting insight into the area of metal ion trafficking.

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