Conserved methionine dictates substrate preference in Nramp-family divalent metal transporters

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Natural resistance-associated macrophage protein (Nramp) family transporters catalyze uptake of essential divalent transition metals like iron and manganese. To discriminate against abundant competitors, the Nramp metal-binding site should favor softer transition metals, which interact either covalently or ionically with coordinating molecules, over hard calcium and magnesium, which interact mainly ionically. The metal-binding site contains an unusual, but conserved, methionine, and its sulfur coordinates transition metal substrates, suggesting a vital role in their transport. Using a bacterial Nramp model system, we show that, surprisingly, this conserved methionine is dispensable for transport of the physiological manganese substrate and similar divalents iron and cobalt, with several small amino acid replacements still enabling robust uptake. Moreover, the methionine sulfur’s presence makes the toxic metal cadmium a preferred substrate. However, a methionine-to-alanine substitution enables transport of calcium and magnesium. Thus, the putative evolutionary pressure to maintain the Nramp metal-binding methionine likely exists because it—more effectively than any other amino acid—increases selectivity for low-abundance transition metal transport in the presence of high-abundance divalents like calcium and magnesium.

results

Conserved Metal-Binding Site Methionine Is Not Required for Transition Metal Transport. To validate *Deinococcus radiodurans* MntH (DraNramp) as a model system, we developed a cell-based cobalt uptake assay adapted from a similar assay in yeast (16). In this colorimetric assay, we quantify the relative Co²⁺ accumulation in Nramp-expressing *E. coli* at various time points by precipitating the transported Co²⁺ to the black solid cobalt (II) sulfide (Fig. S1A). We observed time-dependent Co²⁺ uptake for DraNramp, *Escherichia coli* MntH (EcoNramp), and *Staphylococcus capitis* MntH (ScaNramp) (Fig. S1B), which roughly correlated with expression levels of these His-tagged proteins (Fig. S1C).

In the inward-facing conformational state revealed by the ScaNramp crystal structures, three conserved side chains and a backbone carbonyl group formed the coordination sphere of bound transition metals (9). In DraNramp, these correspond to D56, N59, M230, and the backbone carbonyl of A227 (Fig. 1 A

**Significance**

Transition metals are micronutrients that all organisms use in essential metabolic processes. The ubiquitous Natural resistance-associated macrophage protein (Nramp) family facilitates the acquisition of these metal ions by transporting them across cellular membranes, including dietary iron absorption in mammals. We show that a conserved methionine, an unusual metal-binding residue found in the Nramp metal-binding site, is not essential for the transport of physiological environmentally scarce transition metals like iron and manganese. Instead, it confers selectivity against the abundant alkaline earth metals calcium and magnesium, with the tradeoff of making the toxic metal cadmium a preferred substrate. Using protein structure information, biochemical results, molecular dynamics simulations, and inorganic chemistry theory, we propose a model for how metal discrimination is enforced.


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M-to-A mutants exhibited similar or enhanced Fe$^{2+}$ transport relative to their WT counterpart. In contrast, the S. capitis, Staphylococcus aureus, Streptococcus mutans, and Deinococcus maricopensis M-to-A mutants showed significantly impaired Fe$^{2+}$ transport. The M-to-A mutation phenotype does not cluster in the phylogenetic tree; members of two distinct evolutionary clades of Nrams tolerate an M-to-A substitution for Fe$^{2+}$ transport (Fig. S4) (22, 23). Thus, the observed differences are unlikely due to a broad mechanistic divergence within the Nram family, and instead likely depend on sequence and structural context. We therefore decided to use DraNram and its transport-competent methionine-less mutant to test the hypothesis that a primary role of this methionine is to specifically discriminate against binding or transport of certain metals.

The Methionine Reduces Alkaline Earth Metal Competition. To test our metal-discrimination hypothesis, we compared Co$^{2+}$ transport by WT DraNram and M230A in the presence of various concentrations of competing Mg$^{2+}$, Ca$^{2+}$, Mn$^{2+}$, Zn$^{2+}$, and Cd$^{2+}$, none of which form dark sulfide salts, and determined IC$_{50}$ values for their inhibition of Nram-dependent Co$^{2+}$ transport (Fig. 3). Consistent with our hypothesis, both alkaline earth metals Mg$^{2+}$ and Ca$^{2+}$ inhibited Co$^{2+}$ transport by M230A more effectively relative to WT (Fig. 3 C and D and Fig. S5A). In contrast, the first row transition metals Mn$^{2+}$ (the physiological substrate) and Zn$^{2+}$, as well as the second row transition metal Cd$^{2+}$, competed Co$^{2+}$ transport more effectively in WT relative to M230A (Fig. 3 A, B, and D and Fig. S5A). For most tested metals, this assay cannot discriminate between simple inhibition of cobalt transport or uptake of the competing metal, as their sulfide salts are white (MgS, CaS, ZnS) or light brown (MnS). However, WT DraNram-expressing cells exposed to equal concentrations of Cd$^{2+}$ and Co$^{2+}$ turned yellow on (NH$_4$)$_2$S addition, due to formation of the yellow solid cadmium (II) sulfide, whereas M230A cells remained black (Fig. SSC). Thus, WT DraNram preferentially transports Cd$^{2+}$ over Co$^{2+}$, whereas M230A preferentially transports Co$^{2+}$ over Cd$^{2+}$. In summary, the metal-binding site methionine reduces the competition from the environmentally abundant Mg$^{2+}$ and Ca$^{2+}$ ions while also altering the relative substrate preferences among the rarer transition metals.

The Methionine Is Crucial for Both Promoting Cadmium and Reducing Calcium Transport. To directly test transport of additional metals, we reconstituted purified DraNram into Fura-2-loaded proteoliposomes and monitored DraNram-dependent transport over a range of Cd$^{2+}$, Mn$^{2+}$, or Ca$^{2+}$ concentrations. Mn$^{2+}$ quenches Fura-2 fluorescence at 340 nm, allowing relative influx measurements.

![Fig. 1](image1.png)

**Fig. 1.** The conserved metal-binding site methionine is not required for transport Co$^{2+}$ and Fe$^{2+}$. (A) The SaNram structure identified four residues that directly bind metal substrate as D56, N59, A227, and M230 (DraNram numbering). (B) Sequence logo of metal-binding site from 2,691 related sequences. Alignment was generated using HMMER with the DraNram sequence as the search sequence within the UniprotRef database, with an E-value cutoff of 1 × 10^{-2}. Metal-coordinating residues are identified by gray rectangles. (C) In vivo Co$^{2+}$ transport was greatly reduced for D56A and N59A compared with WT DraNram, but M230A retained full activity. (D) Pheno- types for in vivo Fe$^{2+}$ uptake were similar to Co$^{2+}$. Error bars are SDs (n = 4).

(9) To better understand the roles of the three metal-coordinating side chains, we mutated each residue to alanine and measured Co$^{2+}$ transport by the resulting DraNram variants (Fig. 1C). Loss of a metal-coordinating residue that uses oxygen to bind the metal, D56 or N59, was detrimental to Co$^{2+}$ uptake, consistent with the previously demonstrated importance of these residues to the transport of Co$^{2+}$, Fe$^{2+}$, Mn$^{2+}$, and Cd$^{2+}$ in bacterial and eukaryotic Nram homologs (9, 17–20).

![Fig. 2](image2.png)

**Fig. 2.** The M-to-A mutation has species-specific effects on Nram-dependent Fe$^{2+}$ transport. (A) The M-to-A mutant in human Nramp2 does not transport Fe$^{2+}$. We monitored Fe$^{2+}$ uptake in HEK 293 cells transfected with WT, D86A, or M265A HsNramp2, or an empty vector (EV), using Fura-2 quenching on binding Fe$^{2+}$. Traces are representative of observed metal uptake activity (n = 3). (B and C) The M-to-A mutation in the bacterial homologs EcoNram (B) and SaNram (C) display starkly different phenotypes for Fe$^{2+}$ transport when expressed in E. coli. Error bars are SD (n = 3). Mutants all expressed similarly to their respective WT (Figs. S2E and S3B).
and we determined intraliposome Cd\textsuperscript{2+} or Ca\textsuperscript{2+} concentrations using ratiometric Fura-2 fluorescence (Fig. S6 A–D). Mn\textsuperscript{2+} was transported similarly by WT and M230A, whereas D56A showed little Mn\textsuperscript{2+} transport activity (Fig. 4A). In contrast, WT transported Cd\textsuperscript{2+} much more efficiently than either M230A or D56A (Fig. 4B), which demonstrates the importance of both residues to making Cd\textsuperscript{2+} a good substrate. Importantly, M230A transported Ca\textsuperscript{2+} more efficiently than either WT or D56A (Fig. 4C), showing how this methionine serves to deter Ca\textsuperscript{2+} transport. Our in vitro data suggest that the binding-site methionine is dispensable for transport of the biological substrate Mn\textsuperscript{2+}, consistent with our in vivo observations for the similar metals Co\textsuperscript{2+} and Fe\textsuperscript{2+}. However, the methionine is essential to both promote transport of the toxic metal Cd\textsuperscript{2+} and prevent transport of the alkaline earth metal Ca\textsuperscript{2+}. Of note, the D56A mutation impaired transport of all tested substrates, demonstrating the importance of the conserved aspartate to the general metal transport mechanism. In contrast, the M230A mutation retains transport function, although with altered metal transport preferences, suggesting the methionine functions as a selectivity filter.

To further investigate the underlying cause of the pronounced difference in Cd\textsuperscript{2+} transport by WT and M230A DraNramp, we measured the in vitro Cd\textsuperscript{2+}-binding affinity of these two proteins using microscale thermophoresis (Fig. 4 D and E). We used purified Steap-tagged protein that was Cy5-maleimide–labeled at a lone cysteine replacing R211 in extracellular loop 5–6; this R211/C382S construct shows WT-like Co\textsuperscript{2+} transport activity and similar Cd\textsuperscript{2+} and Mg\textsuperscript{2+} competition phenotypes (Fig. S6E). WT protein bound Cd\textsuperscript{2+} with a K\textsubscript{D} of 10.6 ± 3.6 μM, similar to the 29 ± 10 μM value determined for WT ScaNramp using ITC (9). In contrast, M230A showed no clear Cd\textsuperscript{2+} binding signal. This result demonstrates the importance of M230 for Cd\textsuperscript{2+} binding in WT DraNramp and suggests that reduced binding affinity directly causes Cd\textsuperscript{2+} transport impairment in M230A. In contrast, we observed a similar trend in binding behavior of Mn\textsuperscript{2+} (which WT and M230A both transport) for WT and M230A (Fig. S6 F and G), although we did not calculate K\textsubscript{D} values due to the higher noise in the data.

To extend these observations regarding the differential importance of the methionine to the transport of different metals, we performed a series of molecular dynamics simulations, which we discuss in depth in SI Discussion, using the available ScaNramp structure to compare how different ions equilibrate in and exit the conserved binding site (Fig. S7). These simulations showed that if an ion can interact favorably with the methionine, it experiences greater stabilization in the binding site and a lower energy exit pathway in the intracellular conformation than an ion that cannot interact with the methionine.

Only the Native M230 Allows Transition Metal Transport in High Mg\textsuperscript{2+} Concentrations. To determine how the unique chemical properties of the binding-site methionine affect metal transport and selectivity, we used our in vivo assay to measure Co\textsuperscript{2+} and Fe\textsuperscript{2+} uptake by a panel of 20 DraNramp variants, with each possible amino acid substituted at position 230 (Fig. 5A and Fig. S8). The clear trend is that only small residues—glycine, alanine, serine, threonine, and cysteine—or the native methionine enabled efficient metal transport. This result suggests that other electron-donating functional groups can substitute for the thioether sulfur while preserving transition metal transport. For example, alanine or glycine could provide enough room for one or more water molecules to coordinate the metal (Fig. S8A). Similarly, the threonine or serine hydroxyl group may either directly bind the metal or help align a metal-binding water molecule, but the striking functional difference between the isosteric threonine and valine residues demonstrates the importance of this side chain hydroxyl. Additionally, little metal transport was observed when we replaced M230 with the sterically similar but purely aliphatic isoleucine or leucine side chain, emphasizing the importance of having an electron-pair donor at this position to stabilize the metal substrate. Interestingly, we observed little metal uptake with larger residues that do contain common metal-binding functional groups (asparagine, glutamine, histidine, aspartate, and glutamate). This result may reflect the inability for large polar side chains to optimally orient within the metal-binding site.

Fig. 3. The metal-binding site methionine dictates DraNramp substrate competition against Co\textsuperscript{2+}. Uptake of 200 μM Co\textsuperscript{2+} by E. coli (black) or M230A (red) DraNramp was measured in the presence of varying concentrations of competing divalent metals. The transition metals Mn\textsuperscript{2+} (A), Cd\textsuperscript{2+} (B), and Zn\textsuperscript{2+} (Fig. 5A) compete more effectively for WT than for M230A DraNramp Co\textsuperscript{2+} transport. Conversely, the alkaline earth metals Mg\textsuperscript{2+} (C) and Ca\textsuperscript{2+} (Fig. 5B) compete more effectively for M230A than for WT DraNramp Co\textsuperscript{2+} transport. Error bars are SD (n = 4).

Fig. 4. In vitro transport and binding assays reveal M230’s role in metal selectivity. (A–C) Initial transport rates for Mn\textsuperscript{2+} (A), Cd\textsuperscript{2+} (B), and Ca\textsuperscript{2+} (C) are shown for WT DraNramp (black), M230A (red), D56A (blue), and empty (no protein) liposomes (gray). Error bars are SD from n = 3 or 4. (D and E) Binding isotherms showing WT DraNramp (D) and M230A (E) affinity for Cd\textsuperscript{2+}. Representative data are shown (n = 4) and the error on K\textsubscript{D} is SD.
Substitutions to small amino acids at position 230 retain metal (gold bars). We used competing (black bars, 5-min time point) and Fe (magenta 2+ concentrations and Cd www.pnas.org/cgi/doi/10.1073/pnas.1607734113 in inhibited D56A. (4). Metal toxicity assays demonstrate the importance of M230 in filtering (gray that D56A-expressing cells tolerated. (C 1:1 ratio with Co inhibits all that WT-expressing cells tolerated (Fig. 6 G and) transport in some conditions. In addition, a preferred DraNramp substrate. In transport (25), which agrees with our or Mg inhibited growth of WT- and M230A-expressing strains at (E). While facilitating concentrations, M230A- E. coli). Our study demonstrates the role of the conserved metal-binding site methionine as the selectivity filter in Nramps. In contrast to the presence of various metals us (A) initial Co2+ (black bars, 5-min time point) and Fe2+ (gray bars, 1.5-min time point) transport levels measured for WT DraNramp or variants containing substitutions of M230 to each of the 19 other amino acids expressed in E. coli (Fig. S8B). We observed significant transport (relative to WT) of both metals with the small residues alanine, cysteine, glycine, serine, and threonine replacing M230. (B) Although several mutants transported Co2+ efficiently over the course of 60 min (black bars), only WT retains significant Co2+ transport with a moderate concentration of competing Mg2+ (magenta bars). However, only WT and M230C showed drastic reduction in Co2+ transport with an equal concentration of Cd2+ (gold bars). We used competing Mg2+ and Cd2+ concentrations showing significant differences between WT and M230A (Fig. 3). Error bars are SD (n = 4).

For WT DraNramp and the most active mutants, we measured end point Co2+ uptake in the absence or presence of the competing metals Cd2+ or Mg2+ (Fig. 5B). Strikingly, 10 mM Mg2+ inhibits all mutants, with only WT facilitating significant Co2+ transport. In contrast, 200 μM Cd2+ (1:1 ratio with Co2+) severely reduced WT DraNramp Co2+ uptake, but did not impair the M230A, G, T, or S mutants to the same degree. Interestingly, Co2+ transport by M230C was inhibited by Cd2+ similarly to WT, which suggests the cysteine thiol group can directly coordinate the metal. Thus, the presence of a binding-site sulfur, whether from cysteine or methionine, is essential to making the toxic metal Cd2+ a preferred DraNramp substrate. In conclusion, although five small amino acid side chains can effectively replace the methionine to enable transition metal transport, only the native methionine offers robust selectivity in favor of transition metals in the presence of competing Mg2+.

The Methionine Confers Tolerance to Ca2+ and Mg2+. While Facilitating Cd2+ Toxicity. To explore the biological relevance of metal selectivity, we determined the impact of different metal-binding site configurations on growth of DraNramp-expressing E. coli strains in the presence of various metals using a plate-based toxicity assay. We attribute impaired growth of Nramp-expressing E. coli to toxic intracellular accumulation of metal substrates. M230A- and D56A-expressing strains similarly tolerated Cd2+ at levels which prevented growth of WT-expressing cells (Fig. 6A), confirming the importance of both residues observed for Cd2+ uptake in liposomes. Likewise, Mn2+ inhibited growth of WT- and M230A-expressing strains at high micromolar concentrations that the D56A-expressing strains tolerated (Fig. 6B), which also echoes our liposome assay results. Interestingly, at low micromolar Mn2+ concentrations, M230A-expressing cells grew better than WT-expressing cells, which suggests that M230 is important for Mn2+ uptake at low concentrations and/or among competing alkaline earth metals (Ca2+ and Mg2+ are both present at hundreds of micromolar in LB agar) (24). Additionally, WT- and M230A-expressing cells were more susceptible to Zn2+ and Fe2+ but not equivalent concentrations of Fe3+, than their D56A counterparts (Fig. 6 C and E).

Notably, M230A-expressing cells fared worse at concentrations of Ca2+ or Mg2+ that WT-expressing cells tolerated (Fig. 6 F and G), supporting our Co2+ competition assay data. Interestingly, D56A-expressing cells grew noticeably less than WT-expressing cells at high Mg2+ or Ca2+ concentration, with a similar Ca2+-mediated impairment as M230A. This result suggests that removing either of these two residues may allow nondiscriminatory metal inflow at high concentration gradients, with the intact binding site perhaps functioning as the principal metal transport gate. These metal toxicity observations were not unique to DraNramp, as EcoNramp-expressing cells likewise showed increased Cd2+, decreased Ca2+, and similar Mn2+ toxicity compared with the EcoNramp M-to-A mutant-expressing cells (Fig. S9). Overall, these metal toxicity assays provide indirect evidence of metal transport in a biological setting that largely corroborates our in vitro metal transport findings and emphasizes the importance of the methionine residue in selecting against the environmentally abundant Mg2+ and Ca2+, but also increasing the ability to transport the toxic heavy metal Cd2+.

Discussion
Our study demonstrates the role of the conserved metal-binding site methionine as the selectivity filter in Nramps. In contrast to the other binding site residues, it is not essential for transport of many biologically useful transition metals in DraNramp and several other homologs, although a mutation to alanine does slightly impair Mn2+ transport in some conditions. In addition, while this mutation drastically decreases transport of the toxic metal Cd2+, it increases transport of Mg2+ and Ca2+, which would likely be deleterious to most organisms.

Mutations of the binding site methionine have previously been investigated in a few species. In EcoNramp, an M-to-I or M-to-K mutation eliminated Mn2+ transport (25), which agrees with our lack of observed Fe2+ and Co2+ transport for those mutants in

![Fig. 5. Substitutions to small amino acids at position 230 retain metal transport ability.](Image)

![Fig. 6. Metal toxicity assays demonstrate the importance of M230 in filtering out abundant divalent metals.](Image)
Consistent with our findings regarding the methionine’s importance for Cd\(^{2+}\) binding and transport in DraNramp, an M-to-A mutation in ScaNramp significantly decreased Cd\(^{2+}\) binding affinity, and the analogous mutation in human Nramp2 reduced Cd\(^{2+}\) transport (9), a phenotype we replicated and extended by showing loss of Co\(^{2+}\), Mn\(^{2+}\), and Fe\(^{2+}\) transport. We discovered a range of relative Fe\(^{2+}\) transport abilities for M4o-A mutants in different bacterial homologs (Fig. 2 and Fig. S3) that stretched from no apparent activity for M226A ScaNramp to approximately WT-level transport in M230A DraNramp and M209A EcoNramp to enhanced transport in M326A S. marcescens Nramp.

Our DraNramp results suggest that the main purpose of Nramp’s binding site methionine is to reduce Mg\(^{2+}\) and Ca\(^{2+}\) transport, thus avoiding their overaccumulation and preventing competition with the rarer transition metals that are Nramp’s intended substrates. Inorganic chemistry theory helps explain how many proteins, including Nramp, evolved to discriminate among similar metal ions by tuning their binding-site chemical and structural properties. Interactions between metals and electron-donating ligands are typically strongest when either the ionic or covalent nature of the bond is maximized (26). Thus, highly electronegative ligands such as oxygen form strong bonds with “hard” metal ions like Mg\(^{2+}\), which has a high charge in a small radius. Conversely, less electronegative, more polarizable ligands such as sulfur form as stable bonds with “soft” metal ions like Cd\(^{2+}\), which better share electron density in a covalent manner. In the empirical classification of metal ions as hard (favoring ionic interactions), intermediate (capable of both ionic and covalent interactions), or soft (favoring covalent interactions), Nramp’s biological substrates—the first row transition metals—are considered intermediate, whereas Ca\(^{2+}\) and Mg\(^{2+}\) are hard, and Cd\(^{2+}\), Pb\(^{2+}\), and Hg\(^{2+}\) are soft. In Nramp’s binding site, the soft methionine sulfur is the sole exception; all other metal-binding ligands are hard oxygens better suited to ionic bonds. The methionine therefore selectively stabilizes transition metals capable of covalent and semicovalent interactions.

Our data show that this methionine provides significant stabilization necessary for the binding and transport of Cd\(^{2+}\), which like other soft toxic heavy metals can forge strong covalent-like interactions with sulfur. Additionally, our results suggest that the magnitude of the net methionine stabilization may be minimal for Nramp’s biological substrates (the intermediate first row transition metals) and the most likely reason why the soft-capping hard alkaline earth metals Ca\(^{2+}\) and Mg\(^{2+}\) (which become better substrates upon the methionine’s removal) are effectively destabilized by its presence.

One potential explanation for the M-to-A mutant phenotype is that alanine enables substrate metal ions to retain one or more water ligands that they would otherwise shed upon coordination by the WT binding site. The M-to-A mutation would therefore both improve the binding environment for metals that prefer hard oxygen ligands (Mg\(^{2+}\) and Ca\(^{2+}\)) and impair the ability of a metal like Cd\(^{2+}\) that prefers soft ligands to bind while having only a minor effect on net affinity for intermediate metals that lack a strong preference between a hard and soft ligand. Our M-to-X substitution panel results support this model (Fig. 5): Small residues, which could leave free space for water, all enabled transport of intermediate metals Co\(^{2+}\) and Fe\(^{2+}\) while remaining susceptible to Mg\(^{2+}\) competition and decreasing Cd\(^{2+}\) preference (with the exception of the sulfur-containing cysteine), whereas larger residues such as leucine, which may not provide space for water, severely impaired transport.

Methionine is rarely found in binding sites for most metals, with the notable exception of copper (27, 28). However, the putative metal-binding site in the iron exporter ferroporin contains a methionine (29), suggesting a strategy similar to Nramps for selective transition metal transport. Underscoring the methionine’s key metal selectivity role, a functionally diverged Nramp-related transporter of the hard metal Al\(^{3+}\) in rice has a threonine (providing a hard oxygen ligand) in place of the methionine while retaining the other binding-site residues (30). Similarly the methionine is also replaced with threonine in two bacterial Nramp-related Mg\(^{2+}\) transporters (31). Intriguingly, Nramp homologs from several extremophilic prokaryotes that flourish in highly acidic and heavy metal rich environments (32) have an alanine in place of the methionine, suggesting this substitution is favored under the right selective pressure.

In this evolutionary context, Nramp’s binding site methionine likely represents a tradeoff. We propose that this residue was selected and conserved primarily for its ability to deter competition from and transport of Ca\(^{2+}\) and Mg\(^{2+}\), rather than for it being an optimal ligand for its intended substrates such as Mn\(^{2+}\) and Fe\(^{2+}\). The alkaline earth metals are highly abundant in the environment (the ocean contains 50 mM Mg\(^{2+}\) and 10 mM Ca\(^{2+}\)) (33), and intracellular Ca\(^{2+}\) concentration is tightly regulated, with unchecked import disrupting cell viability, thus providing a strong selective pressure to guard against accidental transport. In addition, as the first row transition metals are approximately six orders of magnitude less abundant than the alkaline earth metals (33), organisms require a robust discriminatory mechanism to find those essential metals within a sea of Ca\(^{2+}\) and Mg\(^{2+}\). However, the binding site methionine also serves as an optimal ligand for the toxic metal Cd\(^{2+}\), enabling it to become an ideal substrate for Nramps: a detrimental property as Nramp activity has been linked to cadmium poisoning in mammals (34, 35). Nevertheless, the normally low environmental abundance of Cd\(^{2+}\) (two orders of magnitude below Mn\(^{2+}\)) likely did not provide significant selective pressure against the methionine on evolutionary time scales. In conclusion, our results explain the ability of Nramps to transport a variety of transition metals while highlighting the role of the conserved methionine in specifically discriminating against the abundant hard alkaline earth metals.

Methods

Additional methods are described in SI Methods.

**DraNramp Microscale Thermophoresis Binding Assay.** Purified streg-tagged fluorescently labeled DraNramps were diluted to 100 nM in 150 mM NaCl, 10 mM Heps, pH 7.5, and 0.03% β-dodecylmaltoside (β-DDM), mixed 1:1 with 14 serially diluted CdCl\(_2\) solutions, and loaded into Premium Coated capillaries (NanoTemper Technologies). Thermophoresis data were obtained with a NanoTemper Monolith NT.115 at 70% light-emitting diode, 80% microscale thermophoresis (MST) power and 30-s MST time, and analyzed using GraphPad prism.

**DraNramp Reconstitution and Proteoliposome Activity Assays.** Lipids (50:35:15 1-stearoyl-2-oleoyl-sn-glycerol, 1-stearoyl-2-oleoyl-sn-glycero-3-phosphoethanolamine (POPE); 1-palmitoyl-2-oleoyl-sn-glycero-3-phospho-rac-glycerol (POPG); Avanti Polar Lipids) and freshly purified DraNramp at a 1:500 mass ratio and 5 mM β-decylmaltoside (β-DDM) were dialyzed against 10 mM Mops, pH 7.0, and 100 mM KCl at 4 °C for 2-3 d and at room temperature for 1 d. After three freeze-thaw cycles to incorporate Fura-2 (100 μM), liposomes were extruded through a 400-nm filter, separated from bulk dye on a PD-10 column (GE Life Sciences), and diluted threefold into 10 mM Mops, pH 7.0, and 100 mM NaCl in a 96-well black clear-bottom assay plate (Greiner). After measuring baseline fluorescence for 5 min, metal substrate was added, along with 50 nM valinomycin to establish a negative internal potential. Fluorescence (F0 = 340 and 380 nm; F∞ = 510 nm) was monitored at room temperature for 70 min, adding iodonycin (0.25 μM) at 60 min to measure maximum signal. The intraliposome concentration of Cd\(^{2+}\) or Ca\(^{2+}\) was determined using [M\(^{2+}\) + I\(^-\)] = ([Fura-2-[Fura-2]^−][K\(_0\) + [M\(^{2+}\)]]) + [M\(^{2+}\) + I\(^-\)] where K\(_0\) is for M\(^{2+}\) and for Ca\(^{2+}\) = 1 μM; Ca\(^{2+}\) = 135 mM (Fig. S7). Transport rates were determined by linear regression of the data for the first 10 min after adding Cd\(^{2+}\) or 60 min after adding Mn\(^{2+}\) or Ca\(^{2+}\).

**In Vivo Metal Uptake Assays in E. coli.** E. coli expressing bacterial Nramps at OD = 5.26 in 190 μL assay buffer (50 mM Heps, pH 7.3, or 2-(N-morpholino)ethanesulfonic acid (Mes), pH 6.4, for fig. 2 and Fig. S3 data, 60 mM NaCl, 10 mM KCl, 0.5 mM MgCl\(_2\), and 0.5 mM MnCl\(_2\)) were distributed into 96-well plates at 37 °C. To initiate uptake, 10 μL 20x metal solution [4 mM Co(NO\(_3\))\(_2\), 4 mM freshly made FeSO\(_4\) in 4 mM ascorbic acid, or a mixture of 4 mM Co(NO\(_3\))\(_2\) with competing metal as chloride salt] was added. To quench uptake,
10 μL 100 mM EDTA (20 μL 200 mM EDTA when Ca²⁺ or Mg²⁺ was included) was added. Cells were pelleted and washed three times before precipitating the metal with 1% (NH₄)₂SO₄. Scanned images of the resulting pellets were converted to black and white, and pellet darkness was determined using ImageJ64 (NIH).

In Vivo Metal Uptake Assays in HEK293 Cells. Fifty microilters of 150 mM NaCl, 4.5 mM KCl, 0.2 mM MgCl₂, 10 mM glucose, and 20 mM Mes, pH 5.5, was added to transfected Fura-2–loaded HEK293 cells. Fluorescence (λex = 340 and 380 nm; λem = 510 nm) was monitored for a 2- to 4-min baseline and 45 min after adding 25 μL 3x metal solution [Co(NO₃)₂, MnCl₂, CdCl₂, CaCl₂, or FeSO₄ + ascorbic acid].

Metal Toxicity Assay. Isopropyl β-D-thiogalactopyranoside (IPTG)-induced E. coli expressing bacterial Nramp (1 μL of dilutions to OD₆₀₀ = 0.01, 0.001, or 0.0001 in LB) were plated onto LB ampicillin agar containing various concentrations of added metal, and the plates were imaged using an Alphalager system after overnight incubation at 37 °C. Gamma values were adjusted the same way for all images to increase contrast.

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