Nic1p, a Relative of Bacterial Transition Metal Permeases in Schizosaccharomyces pombe, Provides Nickel Ion for Urease Biosynthesis*

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The Schizosaccharomyces pombe genome sequencing project identified an open reading frame (O74869 and O74912, named Nic1p in the present study) with significant similarity to members of a family of bacterial transition metal permeases. These uptake systems transport Ni$^{2+}$ ion with extremely high affinity across the bacterial cytoplasmic membrane, but they differ in selectivity toward divalent transition metal cations. An S. pombe mutant harboring an interrupted nic1 allele (nic1-1) was strongly impaired in $^{63}$Ni$^{2+}$ uptake in the presence of a high molar ratio of Mg$^{2+}$ relative to Ni$^{2+}$, conditions that reflect the natural situation. Under these conditions, the nic1-1 mutant contained only background activities of the nickel-dependent cytoplasmic enzyme urease and could not catabolize urea. Among a series of divergent transition metal cations tested (Cd$^{2+}$, Co$^{2+}$, Cu$^{2+}$, Mn$^{2+}$, and Zn$^{2+}$), only Co$^{2+}$ caused considerable inhibition of Nic1p-mediated Ni$^{2+}$ uptake. On the other hand, experiments with $^{59}$Co$^{2+}$ (at nM concentrations) did not show significant differences in Co$^{2+}$ uptake between the nic1-1 mutant and the parental strain. Our data suggest that Nic1p acts as a plasma-membrane nickel transporter in fission yeast, a finding that invites searches for isologous counterparts in higher eukaryotes.

Nickel-dependent enzymes catalyze key reactions in energy and nitrogen metabolism in both prokaryotes and eukaryotes (for reviews, see Refs. 1 and 2). The most widespread nickel metalloenzyme is urease (EC 3.5.1.5), which has been identified in prokaryotes, fungi, algae, plants, and invertebrates. Ureases allow their hosts to utilize urea as a source of nitrogen. They are also important virulence factors in bacteria and fungi (reviewed in Refs. 3 and 4). Urease-mediated hydrolysis of urea yielding ammonium ion and carbamater is the common mechanism of biological urea degradation, although an alternative mechanism is known. In baker's yeast Saccharomyces cerevisiae, other yeasts, and certain green algae, the breakdown of urea is mediated by a biotin-dependent ATP-dependent carboxylation to give 1-carboxyurea (allopbanate) and subsequent hydrolysis to ammonia and carbon dioxide (5).

The fission yeast Schizosaccharomyces pombe contains a urease and is able to grow on urea as the sole source of nitrogen. The soluble, cytoplasmic urease has been purified to homogeneity, and its kinetic properties have been determined (6). Its structural gene (ure1) has been cloned and sequenced, and a knockout mutation was shown to inhibit the growth of S. pombe on agar plates containing 10 mM urea as the nitrogen source (7). S. pombe urease is neither controlled by nitrogen repression nor by urea induction (6).

High affinity nickel transport, a prerequisite for the biosynthesis of nickel-containing metalloenzymes, and the underlying uptake mechanisms have been investigated in a number of prokaryotes (reviewed in Refs. 8 and 9). Comparable transporters, however, have not yet been reported for eukaryotes. Trace amounts of Ni$^{2+}$ ion are sufficient for maximal urease activity of S. pombe, and this activity was not stimulated by the addition of Ni$^{2+}$ to the medium (6). This result suggested that an uptake system with a very high affinity for Ni$^{2+}$ operates in fission yeast. The S. pombe genome sequencing project identified an open reading frame (Nic1p) that displays similarity to a family of bacterial transition metal permeases (9). In the present report we show that interruption of the respective gene strongly affected urease activity under conditions of nickel limitation and prevented the growth of S. pombe on urea. We demonstrate that Nic1p is a high affinity nickel permease. This is the first example of this kind of transporter in an eukaryotic organism. We also present evidence that nonspecific metal uptake systems allow S. pombe to transport Ni$^{2+}$ ion under certain conditions.

MATERIALS AND METHODS

Organisms, Media, and Growth Conditions—S. pombe var. pombe 972 h−1 (Lindner, wild type, DSM 70576) was obtained from the Deutsche Sammlung für Mikroorganismen und Zellkulturen GmbH (Braunschweig, Germany). S. pombe strain FY254 (ATCC 201402) (h− can1-1 leu1-32 ade6-M210 ura4-D18) was a gift of Susan Forsburg (The Salk Institute, La Jolla, CA) and was used as the parental strain for gene interruption. S. pombe strains were grown in YES (0.5% w/v yeast extract, 3% w/v D-glucose) medium or in Edinburgh minimal medium (EMM) (10) at 30 °C. Under standard conditions, adenine, l-leucine, and uracil were added as both media to give final concentrations of 100 μg/ml. Cell densities of cultures in YES medium were calculated upon measuring the optical density (A$^{595}$) in a spectrophotometer and establishing the ratio between A$^{595}$ and the cell concentration. One A$^{595}$ unit corresponded to 2.2 × 10$^{7}$ colony-forming units/ml. Growth on urea as the nitrogen source was monitored on agar plates containing a modified EMM medium. The aforementioned supplements were added to final concentrations of 10 μg/ml, and urea (10 mM) was supplied in place of ammonium salt as the nitrogen source. Plasmids containing S. pombe DNA were propagated in Escherichia coli strains DH5α F′ (Life Technologies, Inc.) and XL1-Blue (Stratagene, Amsterdam) and the Dam− strain GM2163 (New England Biolabs, Urbana, IL).

The abbreviations used are: EMM, Edinburgh minimal medium; PCR, polymerase chain reaction; MES, 4-morpholinoethane sulfonic acid; Nramp, natural resistance-associated macrophage protein; NTA, nitrilotriacetate.

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Schwalbach, Germany). Recombinant E. coli strains were grown in LB medium supplemented with appropriate antibiotics.

Gene Interruption—The nic1- gene was cloned following amplification using total DNA of S. pombe wild-type strain 972 h as the template. Cells (10 ml) grown overnight in YES medium were harvested, washed in SCE buffer (900 mM sorbitol, 50 mM sodium citrate, 10 mM EDTA, pH 7.5), and resuspended in 350 μl of SCE buffer containing 2-mercaptoethanol (0.8% v/v) and lytically (Sigma) (1 mg/ml). After 30 min at 37 °C, the cells were pelleted and lysed by vigorous shaking after the addition of 250 μl of lysis buffer (10 mM Tris hydrochloride, pH 8.0, 100 mM NaCl, 2% w/v Triton X-100, 1% w/v SDS), 300 mg of acid-washed glass beads (Sigma), and 250 μl of phenolchloroform/2-pentanol (25:24:1 v/v). After the addition of 200 μl of TE buffer (10 mM Tris hydrochloride, pH 8.0, 1 mM EDTA), and centrifugation, nucleic acids in the aqueous phase were precipitated with ethanol. The pellet was washed with 70% (w/v) ethanol, dried under vacuum, resuspended in 50 μl of distilled water containing 5 μg of RNase A, and stored at 4 °C. 1 μl of this sample was used for PCR (30 cycles of 20 s at 94 °C, 30 s at 50 °C, 2 min at 72 °C) in the presence of primer A (5′-GGGCTCTGAGATATTTAAACGCCTTCG-3′) (Fig. 1), and 2.5 units of Taq DNA polymerase. Approximately 20 μg of the purified PCR product was used to transform S. pombe FY254 by the method of Bähler et al. (11). A 20-ml culture of FY254 in YES was grown to a density of approximately 10^8 cells/ml. The cells were washed twice with distilled water and once with LiAc/TE (100 mM lithium acetate, 10 mM EDTA, pH 7.5), and resuspended in 100 μl of LiAc/TE. 20 μg each of carrier DNA (sheared salmon sperm DNA, Stratagene), and the PCR product was added. After 10 min at room temperature, 260 μl of polyethylene glycol 4000 (40% w/v in LiAc/TE) was added, and the mixture was incubated at 30 °C for 1 h. Finally, 43 μl of dimethyl sulfoxide was added followed by heating to 42 °C for 5 min. The cells were washed and resuspended in 500 μl of distilled water. 250 μl was plated on uracil-free EMM agar. Colonies of ura4+ containing transformants appeared after 4 days and were purified by streaking on uracil-free agar plates. The disruption of nic1+ in recombinants was verified by PCR analysis (primer combinations P1 (5′-GGGGATCCGAGATATTTAAACGCCTTCG-3′)/P4 and P1/B) and Southern blotting (Fig. 1). For the latter purpose, a PCR product obtained with primers P1 and P4 and the nic1+ allele as the template was labeled with digoxigenin-11-dUTP (Roche Molecular Biochemicals) and used as the probe.

Nickel Accumulation in Growing Cells—Nickel accumulation is expressed as pmol/10^9 cells.

Nickel Transport—S. pombe strains were grown in 1 ml of YES medium to an optical density (A_595) of approximately 1.5 in YES medium containing 65NiCl₂ (24.4 TBq/mol) and the indicated supplements. Cells were harvested, washed twice with 50 mM Tris hydrochloride, pH 7.5, and concentrated 10-fold. The radioactivity of 50 μl of the cell suspension was quantitated by liquid scintillation counting in a Canberra-Packard 1600 TR counter using Zinsser Aquasafe 300 Plus as scintillation mixture. Nickel accumulation is expressed as pmol/10^9 cells.

Urease Assay—Cells were grown in 50 ml of YES medium overnight in the presence of the indicated supplements, harvested, washed, and resuspended in 2 ml potassium phosphate buffer (20 mM, pH 7.5). After two passages through a French pressure cell, the crude extracts were separated by ultracentrifugation (50,000 × g, 30 min, 4 °C). 20 μl of the supernatant (containing approximately 300 μg of protein) was added to 880 μl of potassium phosphate buffer (20 mM, pH 7.5), and the mixture was equilibrated at 37 °C. The reaction was started by the addition of 100 μl of a freshly prepared urea solution (100 mM). Urease activity was determined spectrophotometrically by quantitating the rate of ammonium ion release from urea. For this purpose, ammonium ion was converted into indophenol (15). Protein was estimated by a modification
of the Lowry method (16). Urease activity is expressed in milliunits/mg of protein. One milliunit corresponds to 1 nmol of urea hydrolyzed/min.

RESULTS AND DISCUSSION

Nic1p Is a Novel Member of a Family of Transition Metal Permeases—The amino acid sequence alignment shown in Fig. 2 identified Nic1p of S. pombe as the first eukaryotic member of a family of transporters found in Gram-negative and Gram-positive bacteria (for a review, see Ref. 9). The bacterial counterparts consist of 337 to 381 amino acid residues and contain 8 transmembrane segments. Four characteristic amino acid signatures are conserved in these permeases, as follows. The motifs (R/K)HA\_{X}DADH(I/L) and F\_{XX}GHS(T/S)(V/I)V are located within transmembrane segments II and III, respectively, and have been shown to be critical for transport activity. Likewise, the motifs LG\_{X}(D/E)T(A/S)(T/S)E and GM\_{XXX}D(T/S)XD located in transmembrane segments V and VI, respectively, are conserved and important for activity. A common feature of this family of membrane proteins is a highly charged hydrophilic loop connecting transmembrane segments IV and V. Deletions in this loop abolish activity (reviewed in Ref. 9).

Hydropathy profile (9) and amino acid sequence alignments (Fig. 2) revealed that Nic1p is closely related to the bacterial counterparts. The aforementioned sequence motifs are fully conserved. The putative transmembrane helices IV and V of Nic1p are linked by a hydrophilic loop (residues 164 to 208) containing 12 potentially charged residues.

Nickel Uptake—Based on the similarity to the bacterial nickel permeases, we suspected that Nic1p plays a role in nickel transport into S. pombe cells. To investigate this hypothesis, nickel accumulation of growing cells of the S. pombe nic1-1 mutant was compared with metal uptake by the parental strain under various conditions (Table I). In the presence of 5 mM 63NiCl\_2 in complex medium, both strains accumulated high amounts of Ni\^{2+} ion. The addition of magnesium salt to the medium resulted in a 20-fold decrease in nickel accumulation. At a low Ni\^{2+} concentration (100 nM), the nic1-1 mutation caused a moderately reduced Ni\^{2+} accumulation when the medium was not supplemented with Mg\^{2+} ion. In the presence of 10 mM Mg\^{2+}, however, a strong effect was observed. Although 10^9 cells of the parental strain accumulated 48 pmol of nickel, metal accumulation of the nic1-1 mutant decreased to 4 pmol of nickel/10^9 cells (Table I). A series of nickel accumulation experiments performed at substrate concentrations between 25 and 150 nM in the presence of 10 mM MgCl\_2 (Fig. 3) revealed that Nic1p is closely related to the bacterial counterparts. The aforementioned sequence motifs are fully conserved. The putative transmembrane helices IV and V of Nic1p are linked by a hydrophilic loop (residues 164 to 208) containing 12 potentially charged residues.

**Interruption of the nic1** gene—nic1 is located on chromosome III of S. pombe between the long terminal repeat of a Tf2-type retrotransposon and an open reading frame of unknown function. The strategy for gene interruption is illustrated in Fig. 1. 246 base pairs of nic1 were deleted and replaced by an approximately 1.8-kilobase ura4 marker gene (17). Using 20 mg of the amplified construct for transformation into S. pombe FY254, approximately 1,000 transformants were obtained on uracil-free EMM agar plates. Two transformants were chosen and shown by PCR (Fig. 1B) and Southern blotting (Fig. 1C) to contain the disrupted nic1 allele. Both mutants grew normally in mineral medium in the presence of ammonium salt as the nitrogen source as well as in complex medium, indicating that nic1 is dispensable under both conditions.
confirmed the assumption that Nic1p acts as a high affinity nickel transporter in *S. pombe*. This conclusion was further substantiated by uptake assays with resting cells in buffer. While the nic1-1 mutant was unable to transport $^{63}\text{Ni}^{2+}$ over the 50-min test period, significant transport was observed for the parental strain (Fig. 4).

Our data are compatible with the view that *S. pombe* is able to transport Ni$^{2+}$ ion by nonspecific Mg$^{2+}$ uptake systems. A similar situation has been reported for *S. cerevisiae* (18). Lesions in the two *S. cerevisiae* genes ALR1 and ALR2 were found to produce a magnesium-deficient phenotype while conferring increased resistance to certain metal ions including the divalent ions of the transition metals copper, manganese, nickel, and zinc. Alr1p and Alr2p belong to the CorA family of membrane transporters, the most widespread type of nonspecific Mg$^{2+}$ uptake system in bacteria and archaea (19). Alr1p- and Alr2p-like proteins are also encoded in the genome of *S. pombe*.

The respective open reading frames (O13779 and O13657) together with Alr1p and Alr2p contain large N-terminal extensions compared with their prokaryotic counterparts and represent a CorA subfamily (19).

At very low Ni$^{2+}$ concentrations and high molar ratios of Mg$^{2+}$ to Ni$^{2+}$, Ni$^{2+}$ uptake of *S. pombe* was dependent on Nic1p. This result indicated that nonspecific systems contribute little to Ni$^{2+}$ uptake under conditions that reflect the situation in the natural environment.

**Selectivity of Nic1p**—To test the specificity of Nic1p, the effect of cadmium, cobalt, copper, manganese, and zinc ions on

**Table I**

Nickel accumulation of the *S. pombe* nic1-1 mutant during growth in complex medium

| Strain   | $5 \mu\text{M}^{63}\text{Ni}^{2+}$ | $5 \mu\text{M}^{60}\text{Ni}^{2+}$ | $10 \text{mM Mg}^{2+}$ | $100 \text{nm}^{63}\text{Ni}^{2+}$ | $100 \text{nm}^{60}\text{Ni}^{2+}$ |
|----------|-----------------------------------|-----------------------------------|------------------------|-----------------------------------|-----------------------------------|
| Parent   | 11,635                            | 476                               | 164                    | 48                                |
| nic1-1   | 10,245                            | 540                               | 103                    | 4                                 |

**Table II**

Effect of growth conditions on urease activity of the *S. pombe* nic1-1 mutant

| Strain | Supplement | Urease activity* |
|--------|------------|------------------|
|        | None       | Ni$^{2+}$ | Mg$^{2+}$ | NTA | Mg$^{2+}$ + NTA |
| Parent | 210        | 272       | 210      | 205 | 216             |
| nic1-1 | 257        | 241       | 242      | 166 | <10             |

*Expressed as nmol of urea hydrolyzed/mg of protein/min. The values represent the means of triple determinations using extracts from independent cultures.
nickel accumulation of *S. pombe* FY254 was investigated. For this purpose, the cells were grown in YES medium containing 100 nM 63NiCl2 and 10 mM MgCl2. Under these conditions, high level nickel accumulation is dependent on Nic1p. The competing metal ions were added to final concentrations of 1 μM. With the exception of Co2+, none of the metal ions caused significant inhibition of nickel accumulation (data not shown). The effect of Co2+ was analyzed in more detail. Fig. 5 illustrates that Co2+ was an inhibitor. At a 50-fold excess, Co2+ ion abolished 63Ni2+ accumulation in *S. pombe*. We then addressed the question of whether Nic1p is capable of transporting Co2+ ion. The nic1-1 mutant and its parental strain FY254 were grown in YES medium supplemented with 5 mM CoCl2 at concentrations between 100 and 500 nM in the presence of 10 mM MgCl2. Both strains were able to accumulate cobalt, and no obvious difference was found under any conditions tested (data not shown). This result suggested that Nic1p is not the main mediator of Co2+ uptake. The observed Co2+ accumulation could be due to the activity of a nonspecific transition metal transporter of the Nramp (natural resistance-associated macrophage protein) family. Three Nramp-like proteins (Sm1p, Sm2p, and Sm3p) have been identified in *S. cerevisiae*, and a homologous open reading frame (Q10177) is also encoded in the genome of *S. pombe* (see Refs. 20 and 21 for recent reviews).

**Physiological Role of Nic1p**—To elucidate the physiological consequences of the nic1-1 mutation, we first monitored growth on mineral agar plates containing 10 mM urea as the nitrogen source (not shown). Although *S. pombe* FY254 formed colonies similar to those observed on ammonium-containing plates after 3 to 4 days, the nic1-1 mutant failed to grow on urea under standard conditions. Growth of the mutant was partially restored by adding nickel salt at μM concentrations to the medium. At Ni2+ concentrations above 500 μM, growth of both strains as well as the wild-type strain 972 h−1 was completely inhibited. *S. pombe* harbors a putative metallothionein and has recently been shown to produce a phytochelatin synthase (22).Metallothioneins and phytochelatins mainly mediate resistance toward cadmium, copper, and zinc and apparently do not allow *S. pombe* to escape nickel toxicity under the conditions tested.

We next investigated the role of Nic1p in urea metabolism by quantitative urease assays (Table II). Upon growth of the cells in standard YES medium, soluble extracts of the nic1-1 mutant and its parental strain contained urea-specific activities of approximately 200 to 250 milliunits/mg of protein. These values were in good agreement with those published previously (6). The addition of NiCl2 (5 μM) to the growth medium resulted in a slightly increased urease activity of strain FY254. Surprisingly, high concentrations of MgCl2 (20 mM) had almost no effect on urease activity of both strains. Under comparable conditions, efficient Ni2+ uptake was dependent on Nic1p (Table I, Figs. 3 and 4). We hypothesized that low level Ni2+ uptake, which could be due to a nonspecific Nramp-like transporter, is sufficient for maximal urease activity under these conditions. Therefore, a nickel-complexing agent (NTA), which had proved to efficiently inhibit nonspecific nickel uptake in bacteria (13), was added to the growth medium. 50 μM NTA led to a small but significant decrease of urease activity in the absence of Nic1p. The addition of a combination of NTA and Mg2+, however, gave a strong response. Although the parental strain was almost unaffected, urease activity in the mutant was below the threshold of the assay. Since the natural habitats of *S. pombe* contain nutrients with strong metal-complexing capacity and since the molar ratio of Mg2+ to Ni2+ is generally very high, the latter growth conditions reflect the situation in the environment.

Our results identified Nic1p as an important auxiliary factor for urease activity in *S. pombe*, a finding that may be of general significance for the analysis of urease biosynthesis in eukaryotes. Additional urease accessory genes in fission yeast have been tentatively mapped (23). The genome sequencing project revealed homologs of the bacterial urease operon proteins UreD and UreF, which are essential for urease metallo-center assembly (4). An isolog with high similarity to the bacterial UreG, a GTPase that is important for nickel incorporation into urease apoprotein (24), is not obvious from the *S. pombe* genome sequence.

Although at present we have only indirect data on the localization of Nic1p, our results strongly suggest that it represents a plasma-membrane transporter. Protein-sorting signals are not obvious from the primary structure, and recent work on the Nrrmp homolog Sm1p of *S. cerevisiae* has demonstrated that sorting is a mechanism of post-translational activity control of plasma-membrane transporters in yeast (25, 26).

Compared with its bacterial relatives, Nic1p has a unique specificity. Ni2+ transport by HoxN of *Ralstonia eutropha*, for instance, is not inhibited by Co2+, and HoxN does not transport Co2+ ion (14). On the other hand, Ni2+ uptake by NhlP of *Rhodococcus rhodochrous* is specifically inhibited by Co2+, and this permease is able to transport Co2+ ion (14). Nic1p seems to be a third type of nickel permease, since Co2+ was an inhibitor but, if at all, only a weak substrate for transport. Understanding the molecular basis of the differences in ion selectivity is a challenging problem.

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Page 18031: The values for nickel accumulation of the parental strain and the mutant should be 389 pmol/10^9 cells and 32 pmol/10^9 cells, respectively, rather than 48 pmol/10^9 cells and 4 pmol/10^9 cells.

Page 18032: All nickel accumulation values in Table I, Fig. 3, and Fig. 5 should be multiplied by a factor of 8.1.

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Comparison of the two murine terminal deoxynucleotidyltransferase isoforms. A 20-amino acid insertion in the highly conserved carboxyl-terminal region modifies the thermosensitivity but not the catalytic activity.

Jean-Baptiste Boulé, François Rougeon, and Catherine Papanicolaou

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