Identification of the Plasma Membrane H⁺-Biotin Symporter of Saccharomyces cerevisiae by Rescue of a Fatty Acid-auxotrophic Mutant*

(Received for publication, March 22, 1999, and in revised form, April 14, 1999)

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Bakers’ yeast is auxotrophic for biotin (vitamin H) and depends on the efficient uptake of this compound from the environment. A mutant strain with strongly reduced biotin uptake and with reduced levels of protein biotinylation was identified. The strain was auxotrophic for long-chain fatty acids, and this auxotrophy could be suppressed with high levels of biotin in the medium. After transformation of this mutant with a yeast genomic library, the unassigned open reading frame YGR065C was identified to complement this mutation. This gene codes for a protein with 593 amino acids and 12 putative transmembrane helices. Northern blot analysis revealed that, in wild-type cells, the corresponding mRNA levels were increased at low biotin concentrations. Likewise, cellular biotin uptake was increased with decreasing biotin availability. Expression of YGR065C under the control of the constitutive ADH1 promoter resulted in very high biotin transport rates across the plasma membrane that were no longer regulated by the biotin concentration in the growth medium. We conclude that YGR065C encodes the first biotin transporter identified for a non-mammalian organism and designate this gene VHT1 for vitamin H transporter 1.

Enzymes depending on enzyme-bound biotin (vitamin H) as a carrier of CO₂ in carboxylation, decarboxylation, and transcarboxylation reactions are found throughout the biological world (1, 2). Some organisms, such as Escherichia coli and all higher plants, are able to synthesize biotin de novo (3). However, many other organisms including mammals have lost this capability and are auxotrophic for biotin. The biotin requirement of yeast is well documented for many industrial and laboratory strains of Saccharomyces cerevisiae (4) including strain S288C (5), whose genome has been sequenced. Due to this deficiency, S. cerevisiae has been used in growth assays for the microbiological determination of biotin (6).

In bakers’ yeast, the covalent attachment of biotin to biotin-dependent enzymes is catalyzed by a biotin-protein ligase, which is encoded by the essential BPL1 gene (7). Among the best studied substrates for Bpl1p are acetyl-CoA carboxylase (Acc1p) and the two isoforms of pyruvate carboxylase (Pyc1p and Pyc2p). Acc1p catalyzes the first step of fatty acid biosynthesis, i.e. the carboxylation of acetyl-CoA to malonyl-CoA. A loss of functionally active Acc1p produces a lethal phenotype that cannot be rescued by the supply of fatty acids from the growth medium (8, 9). This is caused by an impairment of the malonyl-CoA-dependent elongation of long-chain fatty acids to very-long-chain fatty acids, which is assumed to be essential for the nuclear pore structure in yeast (9).

Pyc1p and Pyc2p generate oxaloacetate from pyruvate. This reaction is required for the anaerobic synthesis of tricarboxylic acid cycle intermediates during gluconeogenesis or amino acids biosynthesis. A pyc1/pyc2 double mutant is no longer able to grow on glucose with ammonia as the sole nitrogen source. Growth of the double mutant is restored when L-aspartate is added to regenerate the oxaloacetate pool (10). A similar phenotype was described for another yeast, Pichia pastoris, after deletion of its single pyruvate carboxylase gene (11).

Pioneering work on biotin transport in S. cerevisiae has been done by Rogers and Lichstein (12, 13), who described a carrier-mediated, high affinity (apparent $K_m = 0.3 \mu M$), and energy-requiring uptake mechanism. They showed that intracellular concentrations of biotin could exceed biotin concentration in the medium >1000-fold and that this uptake was stimulated by glucose. Rogers and Lichstein also discovered that uptake of biotin is controlled by the biotin level in the growth medium. High levels of biotin reduce the rate of catalyzed biotin uptake. Further support for a specific biotin uptake mechanism in yeast came from the finding that biotin uptake was irreversibly inhibited by the $p$-nitrophenyl ester of biotin, presumably due to covalent modification of the transport system (14, 15).

Despite the extensive biochemical studies on many aspects of biotin transport in yeast, neither the biotin transporter nor its gene has been identified to date. In this paper, we report on the characterization of a yeast mutant (A7-9) that was isolated in a screen for fatty acid auxotrophy. It is demonstrated that the fatty acid auxotrophy in A7-9 results from a defect in biotin uptake from the environment. Complementation of A7-9 with a genomic library led to the identification of the open reading frame YGR065C. The encoded protein exhibits significant structural and sequence homologies to the large family of 12-transmembrane helix transporters. We provide evidence that this gene encodes the biotin transporter of S. cerevisiae and named the gene VHT1 for vitamin H transporter 1.

EXPERIMENTAL PROCEDURES

Yeast Strains, Media, and Plasmids—The following yeast strains were used in this work: JS 91.15-5 (MATa, ura3, his3, leu2, Δinod::LEU2), JS 91.15-23 (MATa, ura3, leu2, trp1, his3, can1), and BF 89.436 (MATa, Δfas1::HIS3, his3, ura3, trp1). Strains 74.2-12C (MATa, ilv1, ade2, suc2) and JS 89.27-3 (MATa, ura3, leu2, trp1) were used for backcrosses. The fatty acid-auxotrophic mutant A7-9 was isolated upon methanesulfonic acid ethyl ester mutagenesis of JS 91.15-5 and subsequent selection of fatty acid-requiring mutants by replica
plating on synthetic complete dextrose (SCD) agar with and without fatty acid supplementation. Details were as described previously (16).

The YEp24-based genomic library used for complementation was described before (17). The library plasmid pA7-T8, which complemented the mutation of A7-9, was isolated as described in the text. The single copy of the vHT1c fragment in YEp was ligated into the SmaI site of pUC19, excised with SacI and HindIII, and cloned into the corresponding sites of YCP133 (18). For construction of the constitutively VHT1-overexpressing plasmid pVHT1oe, the PCR-amplified open reading frame of VHT1 was cloned into pUC19 (5'-AGTCGAGCTCATAGAAC-3' and SM2-SacI (5'-AGTCGAGCTCATAGAAC-GTAAAAAGTGGACG-3') was cloned into the PstI and SacI sites of the multicopy plasmid pVT100-U (19). The sequence of the PCR product was verified. Plasmid YEp24 (20) was used as a vector control.

Media for S. cerevisiae were prepared as described (21). Synthetic dextrose (SD) medium contained 0.67% Bacto-yeast nitrogen base without amino acids (Difco, Bacto-yeast nitrogen base without amino acids, Difco, Augsburg, Germany) and 2% glucose and was supplemented with the necessary amino acids to meet the growth requirements of the strains. SCD medium was prepared from SD medium by adding a complete mixture of L-amino acids and the nucleobases adenine and uracil. Fatty acids were added as described previously (16).

The fatty acid supplementation to the fatty acid requirements was confirmed by PCR for correct integration of the VHT1 gene into the HIS3 gene with no extra biotin added, A7-9 showed almost wild-type growth. As evident from the degree of protein biotinylation in strains A7-9 and BF83, the fatty acid-auxotrophic phenotype of one of the unassigned mutations, strain A7-9, was determined in Fig. 1. Virtually no growth was observed when A7-9 cells were supplemented with saturated medium-chain fatty acids (caprylate (C9) or caprate (C10)). However, this growth defect was suppressed in the presence of long-chain fatty acids (laurate (C12), myristate (C14), or palmitate (C16)). However, this growth defect was suppressed in the presence of long-chain fatty acids (laurate (C12), myristate (C14), or palmitate (C16)).

Analysis of [14C]biotin transport across the plasma membrane revealed that A7-9 is completely defective in biotin uptake (Fig. 2A). The transport rates in A7-9 were less than half of those in wild-type controls. Although the intracellular concentrations of biotin in wild-type cells clearly exceeded the extracellular concentration, the intracellular concentration in A7-9 cells was far below the concentration equilibrium. Increased extracellular fatty acid concentration suppressed the fatty acid-auxotrophic phenotype in A7-9 and restored normal growth (Fig. 2B), probably due to increased passive diffusion of biotin into the cells. Thus, A7-9 is a conditionally fatty acid-auxotrophic mutant that requires long-chain fatty acids or biotin for growth. Compared with wild-type cells, A7-9 needs an additional 100-fold higher vitamin H concentrations for optimal growth. As evident from Fig. 2B, 2-4 μg of biotin/liter allowed full growth of the wild type, whereas growth of the mutant was barely supported. On YPD complete medium with no extra biotin added, A7-9 showed almost wild-type growth rates (data not shown).

Streptavidin-conjugated peroxidase was used to determine the degree of protein biotinylation in strains A7-9 and BF83. A7-9 cells were grown on SD medium supplemented with both long-chain fatty acids and l-aspartate to suppress deficiencies possibly caused by a reduced biotinylation of Acc1p, Pyc1p, and Pyc2p carboxylases. As a control, the mutant was also grown on SCD medium supplemented with excess biotin (1 mg/liter). After separation of cellular proteins by SDS-polyacrylamide gel electrophoresis and subsequent transfer to

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Fig. 1. Fatty acid-dependent growth of the yeast mutant A7-9. Cells were grown in SCD medium supplemented with 0.5% aspartic acid, 0.5% Brij 58, and 0.02% of the indicated fatty acid. Cell densities were determined after 3 days of growth at 30 °C.

RESULTS

Characterization of a Yeast Mutant Defective in High Affinity Biotin Uptake—Fatty acid-auxotrophic mutants have been assigned to four distinct chromosomal loci, i.e. the fatty acid synthase genes FAS1 and FAS2 and the ACC1 and BPL1 genes encoding the acetyl-CoA carboxylase and the biotin-protein ligase, respectively (16, 28, 27). In addition, several fatty acid-auxotrophic yeast strains that are non-allelic to any of the above-mentioned loci were available to us. We speculated that a defective biotin transport system in a biotin-dependent organism such as S. cerevisiae should cause fatty acid auxotrophy, i.e. a phenotype similar to that observed in bpl1 mutants. The fatty acid-auxotrophic phenotype of one of the unassigned mutants, strain A7-9, was determined in Fig. 1. Virtually no growth was observed when A7-9 cells were supplemented with saturated medium-chain fatty acids (caprylate (C9) or caprate (C10)). However, this growth defect was suppressed in the presence of long-chain fatty acids (laurate (C12), myristate (C14), or palmitate (C16)).

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1 The abbreviations used are: SCD, synthetic complete dextrose; SD, synthetic dextrose; PCR, polymerase chain reaction; bp, base pair(s).

2 E. Schweizer, unpublished data.
DNA fragment containing only the reading frame with one potential transmembrane domain. It overlaps the frames pA7-T8 and pA7-T10 had identical inserts of 8 kilobases, and the third harbored a larger, overlapping DNA fragment. According to the prediction, Ygr065p starts with a long hydrophilic domain of 120 amino acids, and both the N and C termini are on the cytoplasmic side of the membrane. Six asparagine residues are part of consensus sequences for N-glycosylation. Two of these residues are located on the extracellular surface (Asn146, between transmembrane domains 1 and 2, and Asn406, between transmembrane domains 7 and 8) and might thus be accessible for glycosylation during protein secretion.

Ygr065p belongs to a family of eight putative yeast transporter proteins (30) that is part of the major facilitator superfamily (34). So far, a function has been attributed to only two members of this group: Dal5p, the plasma membrane-localized permease for allantoin and ureidosuccinate (35, 36), and Fen2p, the plasma membrane-localized pantothenate transporter (37). Ygr065p shares 21 and 28% amino acid identities with Dal5p and Fen2p, respectively. We named the gene represented by this open reading frame (GenBank accession number Z72850) VHT1 for vitamin H transporter 1.

Disruption of VHT1 Abolishes High Affinity Biotin Uptake—

Biotin Transport in Saccharomyces cerevisiae

FIG. 2. Biotin transport and biotin requirement of wild-type (□) and A7-9 mutant (●) cells. A, comparison of biotin transport rates. Cells were grown in YPD medium, and uptake of [14C]biotin was determined as described under “Experimental Procedures.” B, cells were grown in SCD medium supplemented with 0.5% aspartic acid and the indicated concentrations of biotin. The lowest concentration of biotin in this experiment was 2 μg/liter. Cell densities were monitored after 2 days of growth at 30 °C. JS 9115-23 served as a wild-type reference.

FIG. 3. Protein biotinylation in the biotin transport-deficient mutant A7-9 and in the wild-type strain BF 89.4-36. Extracts from the mutant and the control strain BF 89.4-36 were separated by SDS-polyacrylamide gel electrophoresis and blotted onto nitrocellulose. Experimental conditions were as described previously (27). A7-9 was grown in SCD medium containing 0.5% aspartic acid and 1 mg of biotin/liter (+bio) or 0.5% Brij 58 and 0.03% fatty acids (1:1 mixture of myristate and palmitate, +FA). BF 89.4-36 cells were grown in the same fatty acid-containing SCD medium with (+bio) or without (+FA) biotin supplementation (1 mg/liter). As confirmed by Coomassie Blue staining of a reference gel, identical amounts of protein were applied to each lane (data not shown). Biotinylated reference proteins were purchased from Bio-Rad.

FIG. 4. Complementation of the biotin transport-deficient mutant A7-9 by YGR065C. Strain A7-9 was transformed with plasmid pA7-T8 (carrying the initially obtained genomic fragment), pVHT1sc (a single copy plasmid carrying VHT1 under control of the VHT1 promoter), or pVHT1oe (a multicopy plasmid with VHT1 under control of the ADH1 promoter) or with the empty control vector YEp24 (20). Transformants were streaked on SD plates containing 2 μg or 2 mg of biotin/liter. Growth of the strains was scored after 3 days at 29 °C.

Biotin Transport in Saccharomyces cerevisiae

YGR065C, which has no functional assignment in public data bases, codes for a protein of 593 amino acids (Fig. 5A). A hydrophobic analysis of this sequence (Fig. 5B) (31) suggested 12 transmembrane domains, a number that has been found in numerous plasma membrane transporters (32). According to the prediction, Ygr065p starts with a long hydrophilic domain of 120 amino acids, and both the N and C termini are on the cytoplasmic side of the membrane. Six asparagine residues are part of consensus sequences for N-glycosylation. Two of these residues are located on the extracellular surface (Asn146, between transmembrane domains 1 and 2, and Asn406, between transmembrane domains 7 and 8) and might thus be accessible for glycosylation during protein secretion.

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The VHT1 gene was disrupted in strain JS 91.15-23; transformants were selected on SD plates containing 2 mg of biotin/liter; and disruptants were identified on plates containing 2 μg of biotin/liter. These cells had the HIS3 marker gene correctly integrated at the VHT1 locus and are referred to as JSYΔvht1. By genetic complementation analysis, JSYΔvht1 proved to be allelic to the A7-9 mutant. Moreover, all biochemical parameters investigated, such as biotin- or fatty acid-dependent growth, biotin uptake, and protein biotinylation, were the same in both mutants (data not shown).

Biotin Uptake by Vht1p and VHT1 mRNA Levels Are Modulated by the Biotin Content of the Medium—Biotin transport activity in S. cerevisiae is modulated by the biotin content of the media. The activity increases with decreasing biotin concentrations, and protein biotinylation is necessary for this increase in activity (13). We analyzed the biotin transport activities of A7-9, A7-9 cells expressing VHT1oe and grown in SD medium containing 0.2 μg (●), 2 μg (○), or 20 μg (△) of biotin/liter. Uptake of [14C]biotin was determined as described under “Experimental Procedures.”

I. Regulation of biotin transport by extracellular biotin concentrations. A, A7-9 cells were transformed with the VHT1 gene on a single copy plasmid (pVHT1sc) and grown in SD medium containing 0.2 μg (●), 2 μg (○), or 20 μg (△) of biotin/liter. Uptake of [14C]biotin was determined as described under “Experimental Procedures.”

For further analyses, RNA was prepared from JS 91.15-23 wild-type cells grown on 0.2, 2, or 20 μg of biotin/liter and probed with a radiolabeled VHT1 fragment on a Northern blot. A single hybridization signal corresponding to a mRNA of ~1900 bp was observed in each of the three samples (Fig. 7). VHT1 mRNA was most abundant at 0.2 μg of biotin/liter. VHT1 mRNA was least abundant at 2 μg/liter, and the level was even further reduced at 20 μg/liter. These results are in good agreement with the biotin transport activities determined above (Fig. 6A) and show that extracellular biotin concentrations modulate VHT1 mRNA levels and thereby Vht1p transport activity.

Transport by Vht1p Is Specific for Biotin and Sensitive to Protonophores—A recently identified mammalian vitamin transporter, SMVT, transports the three vitamins biotin, pantothenate, and lipoate with similar affinities by means of a Na⁺ symport mechanism (38, 39). Transport of [14C]biotin by Vht1p was not inhibited when pantothenate was added at 10 times the concentration of biotin (data not shown). Also other compounds with structural similarity to biotin, such as allantoin, allantoate, xanthine, uric acid, and urea, had no effect on biotin transport (data not shown), suggesting that Vht1p is specific for biotin.

Vht1p-dependent [14C]biotin transport was further characterized by measuring its activity in the presence of protonophores (such as 2,4-dinitrophenol and carbamyl cyanide m-chlorophenylhydrazone), the membrane-impermeable SH-modifying agent p-chloromercuribenzenesulfonic acid, or the above-mentioned inhibitor of biotin transport (biotinyl-p-nitrophenyl ester) (Fig. 8). In accordance with published data (14), biotinyl-p-nitrophenyl ester strongly reduced biotin transport to values <4% of the uninhibited activity. The protonophores 2,4-dinitrophenol and carbamyl cyanide m-chlorophenylhydrazone inhibited transport rates by 81 and 86%, respectively, suggesting a proton symport mechanism for biotin uptake.

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After growth in SD medium with 0.2, 2, or 20 μg of biotin/liter, biotin uptake was determined (Fig. 6). In agreement with previous data (13), we observed a pronounced effect of the extracellular biotin concentration on biotin uptake in pVHT1sc transformants expressing VHT1 from its genuine promoter. The biotin transport rate was maximal at 0.2 μg of biotin/liter and became very low at 2 and 20 μg of biotin/liter (Fig. 6A). As expected, biotin transport rates in pVHT1oe transformants were increased and independent of extracellular biotin concentrations (Fig. 6B), suggesting that regulatory sequences in the VHT1 promoter are responsible for the biotin responsiveness seen in Fig. 6A.

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affinity biotin transporter encoded in the yeast genome. (iii) Overexpression of VHT1 under the control of the ADH1 promoter causes a dramatic increase in biotin transport activity irrespective of the biotin content of the medium (Fig. 6B). (iv) VHT1 mRNA levels and biotin uptake rates are modulated by extracellular biotin and increase as a consequence of biotin limitation (Figs. 6A and 7). (v) Biotinyl-p-nitrophenyl ester, a strong inhibitor of biotin transport in S. cerevisiae, inhibits Vht1p-dependent biotin transport by >90%. The last two findings confirm results previously obtained from biochemical and kinetic analyses of yeast biotin transport (12–14).

Vht1p Shares No Homology with a Mammalian Na+–dependent Vitamin Transporter—The sensitivity of biotin uptake to uncouplers of transmembrane proton gradients (such as 2,4-dinitrophenol and carbonyl cyanide m-chlorophenylhydrazon) suggests that Vht1p mediates the H+-coupled symport of biotin. Consistent with this interpretation, biotin transport leads to intracellular accumulation of biotin and is stimulated by glucose (12), a sugar that directly activates the proton-extruding ATPase of the yeast plasma membrane (41).

Biotin uptake studies performed with intact mammalian cells (42) or with tissue-derived membrane vesicles (43) clearly indicated a Na+-symport mechanism for biotin. Most remarkably, uptake of biotin was competitively inhibited by pantothentic acid and lipoate, suggesting a single protein for the uptake of all three vitamins (38, 44). This was confirmed after the recent cloning and heterologous expression of the rat SMVT gene in Xenopus laevis oocytes (39). The Na+-dependent SMVT protein, which belongs to the Na+-dependent glucose transporter family, exhibits no structural homology to the H+-dependent Vht1p protein characterized in this study.

E. coli, like most bacteria, is able to synthesize biotin, and indeed, most of the biotin required by humans is not derived from the diet, but rather from the intestinal microflora (45). Although being prototrophic for biotin, E. coli also takes up this vitamin by a carrier-mediated, saturable, high affinity (kd = 0.14 μM), and energy-dependent process (46). As in S. cerevisiae, biotin transport in E. coli is suppressed by the biotin concentration in the media (46, 47). To date, no information is available on bacterial biotin transporters. A BLAST search (48) in publicly available data banks using Vht1p as query sequence yielded no match.

In conclusion, plants and most bacteria are able to synthesize biotin and do not depend on biotin uptake. In contrast, mammals and yeast are unable to synthesize biotin, but efficiently accumulate biotin from their environment. In yeast, the biotin transporter is only synthesized when required, i.e. at very low extracellular biotin concentrations. The biotin transporters of mammals and yeast are clearly different, both structurally and functionally. Thus, the yeast biotin transporter may represent a suitable target for new antifungal drugs with applications in agriculture and medicine.

Acknowledgments—We thank H.-J. Schuller (Universität Greifswald, Greifswald, Germany) for providing yeast strains and for fruitful discussions and M. Weider, P. Rothe, and M. Ramsperger for experimental help.

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