Identification of the tRNA-binding Protein Arc1p as a Novel Target of *in Vivo* Biotinylation in *Saccharomyces cerevisiae* *

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Received for publication, June 25, 2004, and in revised form, July 21, 2004
Published, JBC Papers in Press, July 22, 2004, DOI 10.1074/jbc.M407137200

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Biotin is an essential cofactor of cell metabolism serving as a protein-bound coenzyme in ATP-dependent carboxylation, in transcarboxylation, and certain decarboxylation reactions. The involvement of biotinylated proteins in other cellular functions has been suggested occasionally, but available data on this are limited. In the present study, a *Saccharomyces cerevisiae* protein was identified that reacts with streptavidin on Western blots and is not identical to one of the known biotinylated yeast proteins. After affinity purification on monomeric avidin, the biotinylated protein was identified as Arc1p. Using 14C-labeled biotin, the cofactor was shown to be incorporated into Arc1p by covalent and alkali-labile linkages. Similar to the known carboxylases, Arc1p biotinylation is mediated by the yeast biotin:protein ligase, Bpl1p. Mutational studies revealed that biotinylation occurs at lysine 86 within the N-terminal domain of Arc1p. In contrast to the known carboxylases, however, *in vitro* biotinylation of Arc1p is incomplete and increases with BPL1 overexpression. In accordance to this fact, Arc1p lacks the canonical consensus sequence of known biotin binding domains, and the bacterial biotin:protein ligase, BirA, is unable to use Arc1p as a substrate. Arc1p was shown previously to organize the association of MetRS and GluRS tRNA synthetases with their cognate tRNAs thereby increasing the substrate affinity and catalytic efficiency of these enzymes. Remarkably, not only biotinylated but also the biotin-free Arc1p obtained by replacement of lysine 86 with arginine were capable of restoring Arc1p function in both *arc1Δ* and *arc1Δlos1Δ* mutants, indicating that biotinylation of Arc1p is not essential for activity.

Biotin was first identified in 1934 by Kögl and Tönns (1) when they were studying the growth requirements of yeast cells in synthetic media. Even though its synthesis is restricted to plants, most bacteria, and certain fungi, the vitamin is now recognized as a general metabolic cofactor required by all forms of life. Like most water-soluble vitamins, biotin serves, in common with the other dietary B vitamins, in the synthesis of various metabolic products. Biotin was first identified in 1934 by Kögl and Tönns (1) when they were studying the growth requirements of yeast cells in synthetic media. Even though its synthesis is restricted to plants, most bacteria, and certain fungi, the vitamin is now recognized as a general metabolic cofactor required by all forms of life. Like most water-soluble vitamins, biotin serves, in common with the other dietary B vitamins, in the synthesis of various metabolic products.
of 45-kDa apparent molecular mass, exhibiting a strong interaction with streptavidin-peroxidase conjugate. Here, we show that this protein is encoded by \textit{ARC1}, a gene encoding a yeast aminoaoyl tRNA synthetase cofactor (13). The \textit{Arc1} protein has no detectable sequence similarity to other known biotin proteins. Despite this fact, biotin is covalently linked to a specific lysine residue within the N-terminal domain of Arc1p. Biotin attachment requires the yeast biotin:protein ligase BirA.

The targeted replacement of a 1.33-kb KanMX-encoding PCR fragment prepared from pFA6KanMX4. Plasmid pHSK4 was thereby obtained. Using the one-step gene disruption procedure of Rothstein (21), the 2-kb Sail/BamHI restriction fragment of pHSK4 was thereby used for disruption of chromosomal \textit{ARC1} DNA in the haploid \textit{S. cerevisiae} strain JS89.27-3. The \textit{los1/His5} deletion was generated in strain JS91.15-23 by the "short flanking homology PCR" method. For homologous recombination with chromosomal yeast DNA, the \textit{Schizosaccharomyces pombe his5} gene was amplified from plasmid pFA6-HISSMX6. In the \textit{los1/His5} deletion construct, 1,563 bp (from position 28 to + 1,535 relative to the start codon) of \textit{LoS1} were replaced by the HIS5 marker.

**Affinity Purification of Biotinylated Yeast Proteins—**\textit{S. cerevisiae} cells were grown to mid-logarithmic phase in yeast extract peptone dextrose medium. The harvested cells (20 g, fresh weight) were broken with glass beads, and the cleared lysate was dialyzed against PBS to remove free biotin. 30 ml of protein extract was loaded onto a 5-ml monomeric avidin-Sepharose column (Pierce), which was subsequently washed with PBS until the fractions were free of protein. Biotinylated proteins were then eluted from the column with PBS containing 2 mM biotin. 1-ml fractions were collected and assayed by SDS-PAGE and Western blotting with streptavidin-horseradish peroxidase conjugate (Amersham Biosciences) as described below (22).

**EXPERIMENTAL PROCEDURES**

**Yeast Strains, Vectors, and Media—**The \textit{S. cerevisiae} strains used in this study are listed in Table I. For recombinant plasmid constructions, the \textit{S. cerevisiae}/\textit{E. coli} shuttle vectors YCplac33 (14), p414MET25 (15), pVT-100U (16), pTRC-HISA (Invitrogen), and the \textit{E. coli} plasmids pQE70 (Qiagen) served as recipients. Plasmid pFA6-HISSMX6 (17) and pFA6KanMX (18) were used for PCR amplification of \textit{HisS} and \textit{KanMX} insertion cassettes, respectively. Yeast cells were routinely grown in complex yeast extract peptone dextrose medium (19). Cells with the \textit{KanMX} insertion cassette were selected on yeast extract peptone dextrose medium that contained 200 mg/ml G418 (Roche Applied Science).

Semisynthetic omission medium lacking either uracil (SS-U) or histidine (SS-H) contained 2% glucose, 0.7% yeast nitrogen base, and an otherwise complete set of amino acids and nucleic acid bases (19). Biotin-free semisynthetic media (SC-Bio) were prepared according to the formula indications of Invitrogen.

**Plasmid Constructs—**The \textit{ARC1} gene together with 465-bp upstream and 167-bp downstream flanking sequences was isolated by whole cell PCR from chromosomal \textit{S. cerevisiae} DNA and subsequently integrated, as a 1.76-kb Sail/BamHI restriction fragment, into the yeast centromere and expression plasmids YCplac33 and p414MET25, respectively. Plasmids pHSK1 (from YCplac33) and pHSK2 (from p414MET25) were thus obtained. Using His\textsubscript{s}-encoding C-terminal and appropriate N-terminal primers, the \textit{ARC1} reading frame was PCR amplified from pHSK1 and, integrated, as Sail/BamHI and PstI/BamHI restriction fragment, into the yeast centromere and expression plasmids YCplac33 and p414MET25, respectively. Correspondingly, the \textit{ARC1} deletion constructs pARC1-A, pARC1-B, pARC1-C, pARC1-D, and pARC1-E were all amplified by PCR using primers prepared from plasmid pFA6-HISSMX6. In the \textit{los1/His5} deletion construct, 1,563 bp (from position 28 to + 1,535 relative to the start codon) of \textit{LoS1} were replaced by the HIS5 marker.

**Heterologous Expression of Intact and C-Terminally Truncated Arc1p in \textit{E. coli}—**For expression of intact Arc1p, plasmid pHSK15 was transformed to \textit{E. coli} strain M15 (pREP4) (Qiagen). Transformants were grown overnight at 30 °C in 50 ml of LB medium containing 50 μg/ml kanamycin and 80 μg/ml ampicillin. Cells were harvested by centrifugation and, after washing, resuspended in 200 ml of kanamycin-free LB medium. After a 1-h incubation \textit{ARC1} expression was induced with 5 mM isopropyl-1-thio-β-D-galactopyranoside, and the cells were incubated for another 5 h at 30 °C. Then, cells were harvested, resuspended in 20 ml of PBS containing 2 mg/ml phenylmethanesulfonyl fluoride, and subsequently disrupted by sonication. After a 20-min centrifugation at 16,000 × g, the cleared lysate was analyzed by SDS-PAGE and Western blotting. The \textit{N-term} deletion was generated in strain JS91.15-23 by the "short flanking homology PCR" method. For homologous recombination with chromosomal yeast DNA, the \textit{Schizosaccharomyces pombe his5} gene was amplified from plasmid pFA6-HISSMX6. In the \textit{los1/His5} deletion construct, 1,563 bp (from position 28 to + 1,535 relative to the start codon) of \textit{LoS1} were replaced by the HIS5 marker.

**In Vitro Mutagenesis and Gene Disruptions—**The targeted replacement of specific lysine residues in Arc1p by arginine was performed using a one- or two-step PCR procedure as described by Landt et al. (20). In the two-step procedure used for plasmids pK79R, pK86R, pK89R, pK185R, and pK300R the internal primer contained the arginine triple AGA at the appropriate position, whereas the primers determining the N and C termini of Arc1p were the same as those used for pAB1 construction. Plasmids pK132R, pK133R, pK134R, and pK135R were constructed by a one-step PCR method using a His tag encoding an appropriately mutated C-terminal primer in combination with the N-terminal primer used for pARC1-A construction. Again, the resulting PCR products were integrated, as PstI/BamHI restriction fragments, into the yeast expression vector pVT-100U. For chromosomal \textit{ARC1} disruption, the N-terminal 812 bp of the \textit{ARC1} reading frame were removed, as a 1,087-bp Nsil/Hpal restriction fragment, from pHSK3 and replaced by an appropriate 1.33-kb KanMX-encoding PCR fragment prepared from pFA6KanMX4. Plasmid pHSK4 was thereby obtained. Using the one-step gene disruption procedure of Rothstein (21), the 2-kb Sail/BamHI restriction fragment of pHSK4 was thereby used for disruption of chromosomal \textit{ARC1} DNA in the haploid \textit{S. cerevisiae} strain JS89.27-3. The \textit{los1/His5} deletion was generated in strain JS91.15-23 by the "short flanking homology PCR" method. For homologous recombination with chromosomal yeast DNA, the \textit{Schizosaccharomyces pombe his5} gene was amplified from plasmid pFA6-HISSMX6. In the \textit{los1/His5} deletion construct, 1,563 bp (from position 28 to + 1,535 relative to the start codon) of \textit{LoS1} were replaced by the HIS5 marker.

**In Vitro Biotinylation of the Purified Arc1p Fragment, Arc1p—**For \textit{in vitro} biotinylation, about 0.5 μg of purified Arc1p was incubated with 40 μg of cell extract from JS89.27-3, the \textit{bpi1} mutant C25/17, or from JS89.27-3 transformed with plasmid pVT100U-BPL1-2Z. After incubation at 30 °C for 3.5 h in the presence of 50 mM trisec-HCl, pH 7.5,
5 mM MgCl₂, 50 mM NaCl, 10 μM biotin, and 0.5 mM ATP the reaction was stopped by boiling in SDS-sample buffer. Protein biotinylation was analyzed by Western blotting with streptavidin-horseradish peroxidase conjugate (22).

MALDI-TOF Analyses—A Coomassie-stained protein band of Arc1p was in-gel digested with trypsin (Promega) in principle as reported by Shevchenko et al. (23) or by the endopeptidases Arg-C, Lys-C, or Glu-C (Roche Applied Science), respectively, according to the manufacturer’s recommendations. For MALDI-TOF analysis a 0.5-μl aliquot of peptide digest was co-crystallized with α-cyano,4-hydroxy-cinnamic acid (24) or with a 9:1 mixture of 2,5-dihydroxybenzoic acid with 2-hydroxy-5-methoxybenzoic acid (25) on AnchorChip targets (Bruker Daltonics, Bremen) (26). Peptide fingerprints were acquired with a Reflex III (Bruker Daltonics) mass spectrometer in reflector mode. The resulting peak lists were searched against the NCBI nonredundant database using Mascot (www.matrixscience.com) allowing a 100-ppm tolerance for peptide mass values.

RESULTS

Identification of Arc1p as a Novel Streptavidin-reactive Yeast Protein—Three different types of biotin-containing carboxylases are known in yeast, i.e. acetyl-CoA carboxylase, pyruvate carboxylase, and urea amidase. Acetyl-CoA carboxylase is encoded by two different genes, ACC1 and HFA1 (27, 28). Both encode high molecular mass (250 kDa for Acc1p, 242 kDa for Hfa1p) multienzymes catalyzing cytoplasmic and mitochondrial acetyl-CoA carboxylation, respectively. Similarly, two independent pyruvate carboxylases are encoded by the genes PYC1 and PYC2 (29). Urea amidase, the third known biotin-containing yeast enzyme, comprises two activities, urea carboxylase and allophanate hydrolase, which are fused within a single polypeptide of 202-kDa molecular mass (30). Because of the similar masses of the respective multifunctional proteins, Acc1p and Hfa1p on the one side and Pyc1p and Pyc2p on the other do not separate upon SDS-PAGE but superimpose at the ACC- and PYC-specific positions, respectively. As shown in Fig. 1A, the Acc1p/Hfa1p and Pyc1p/Pyc2p carboxylase proteins present in the yeast cell homogenate are readily detected by SDS-PAGE and subsequent Western blotting with streptavidin-horseradish peroxidase conjugate. In some of our experiments, Dur1,2p has also been seen as a faint band migrating slightly slower than Acc1p (cf. Fig. 7A). Besides these known carboxylases, however, an additional and so far unassigned streptavidin-reactive protein (p45 in Fig. 1) was uncovered by this assay. This protein with an apparent molecular mass of 45 kDa has occasionally been observed before (8, 31–33), but it has, to our knowledge, never been investigated in more detail. Homology searches in the yeast genome with biotin binding domains of known carboxylases revealed no unexpected hits.

Therefore, identification of the 45-kDa protein seemed to us of particular interest.

In a first step toward identification, the unknown protein was enriched from the yeast cell extracts by affinity chromatography on monomeric avidin. Bound proteins were released by adding biotin to the wash buffer and analyzed by SDS-PAGE (Fig. 1B). The streptavidin-reactive 45-kDa protein eluting slightly in front of the yeast carboxylases, ACC and PYC, could be highly enriched by this procedure (Fig. 1B). The purified 45-kDa protein was subjected to endoprotease digestion with subsequent Edman sequencing. Thereby, the two peptide sequences, LEINHDLPLHEVI and APEKPKPSAIDFRVG, were obtained. These sequences were assigned to the S. cerevisiae open reading frame YGL105w encoding the Arc1p/44p1 protein (Swiss Protein Database P46672). Arc1p has been described both as a tRNA-binding protein and as an activator of MetRS and GlnRS tRNA synthetases (13). Besides this, it was reported, as 44p1, to bind specifically to quadruplex DNA (34). None of these characteristics relates to the canonical functions of known biotin-containing enzymes, nor is a typical biotin binding consensus motif evident from the Arc1p sequence. Therefore, a more detailed verification of Arc1p as a biotin-containing protein seemed necessary. For this, we disrupted the ARC1 gene by integration of the Geneticin resistance marker, KanMX. Details of the disruption protocol are described under “Experimental Procedures.” Probing a protein extract of the arc1Δ-null mutant, SC1478, with streptavidin-horseradish peroxidase revealed that the 45 kDa signal observed with wild type cells had indeed been abolished (Fig. 2A). On the other hand, the 45 kDa signal reappeared when ARC1 DNA was reintroduced into the arc1Δ mutant by transformation with the multicopy plasmid pAB1 (Fig. 2A). Correct production of pAB1-encoded Arc1p in the transformants is evident from the immune response of its His₆-tagged recombiant protein with PentaHis monoclonal antibodies (Fig. 2B). Together, these experiments confirmed that Arc1p in fact represents the novel streptavidin-reactive yeast protein, even though its calculated molecular mass of 42.1 kDa is somewhat lower than the experimentally observed value.

Biotin Is Covalently Linked to Arc1p—Streptavidin is well documented to bind to a variety of biotin-free peptides collectively referred to as strep tags (35). These peptide tags can be added to a protein of interest and are useful for purification as well as for detection in Western blots. To exclude the possibility that Arc1p interacted with streptavidin in a biotin-independent way, we investigated whether Arc1p was capable of incorporating ¹⁴C-labeled biotin. Yeast cells are unable to produce biotin de novo and possess a high affinity biotin transporter (Vht1p) in their plasma membrane (36). This facilitates effi
A Novel Biotinylated Protein in Yeast

Localization of the Biotin Binding Site within Arc1p—The data in Table II suggested an alkali-resistant amide linkage of biotin to one of the lysine residues of Arc1p. To identify this lysine residue, pAB1-encoded Arc1p was purified, in successive steps, by affinity chromatography on monomeric avidin-Sepharose and on Ni²⁺-NTA-agarose. Final purification was achieved by preparative SDS-PAGE. Purified Arc1p was subsequently digested with a variety of different endopeptidases such as trypsin, V8, Lys-C, and Arg-C. The resulting fragments were analyzed by MALDI-TOF mass spectrometry. Together, these analyses allowed us to exclude 335 of the 376 amino acids of Arc1p as possible sites of biotinylation (data not shown). The data indicated that amino acids 80–100 and/or 133–147 likely represented the site(s) of modification. In an independent series of experiments, five different segments of Arc1p comprising amino acids 1–130 (A), 1–140 (B), 1–270 (C), 123–376 (D), and 199–376 (E) were combined with a C-terminal His₉ tag and expressed in yeast. SDS-PAGE and Western blot analysis of the resulting cell extracts revealed that only the three N-terminal fragments A (not shown), B, and C were biotinylated, whereas fragments D and E covering amino acids 123–376 contained no biotin (Fig. 4). According to these data and consistent with the above-mentioned MALDI-TOF data, the site of Arc1p biotinylation is located within the first 130 amino acids of the protein. Finally, to identify the biotinylated lysine residue within this sequence, we systematically mutated selected lysine codons to codons for arginine, using a two-step PCR-based protocol. By this technique 9 lysine residues in Arc1p were replaced. The replacements were performed either with the full-length ARC1 gene (Lys at positions 79, 86, 89, 185, and 300) or with a 420-bp fragment encoding the N-terminal domain (Lys at positions 132, 133, 134, and 135). All constructs contained, in addition to the lysine replacement, a C-terminal His₉ tag. This enabled us to ensure, by immunoblotting with PentaHis antibodies, that the proteins were produced correctly and, at the same time, incorporation of biotin could be analyzed by means of their reactivity with streptavidin. The results of these mutagenesis studies are summarized in Figs. 5 and 6. It turned out that most of the lysine replacements examined were without effect on Arc1p biotinylation. As the only exception, the K98R mutated Arc1 protein did not react with streptavidin peroxidase. From these results it is evident that Arc1p is biotinylated within its N-terminal 130 amino acids and, second,
that a single amino acid, lysine 86, represents the site of biotinylation.

**Arc1p Is Specifically Modified by the Unique Yeast Biotin: Protein Ligase, Bpl1p**—The five known biotin-containing carboxylases of yeast are biotinylated by a specific biotin:protein ligase encoded by the essential *BPL1* gene. Because Arc1p lacks the canonical biotin binding domain of known carboxylases, we wanted to investigate whether Arc1p is also modified by Bpl1p. In contrast to the lethality of *bpl1Δ*-null mutants, certain *bpl1*-missense mutants retaining a critical level of biotin:protein ligase activity are viable and grow upon supplementation with long chain fatty acids. It is presumed that the marginal level of malonyl-CoA biosynthesis which is retained in these mutants allows the formation of very long chain fatty acids to an extent that is necessary for cell survival. Because of this leakiness, viable *bpl1* mutants retain detectable though drastically reduced levels of acetyl-CoA and pyruvate carboxylase holoenzymes. This is evident from Fig. 7, which compares the abundance of streptavidin-reactive proteins in extracts of wild type and *bpl1* mutant cells. Even though comparable amounts of total cell proteins were applied to the gel, the streptavidin reactivity of ACC and PYC was very faint in the mutant but was strong in the wild type. As is also evident from Fig. 7, the effect of Bpl1p inactivation on Arc1p biotinylation is even more pronounced than that on ACC and PYC. The Arc1p signal, which was very prominent in the wild type, was abolished completely in the *bpl1* mutant. From these results it is concluded that in yeast, Bpl1p is involved not only in the biotinylation of pyruvate and acetyl-CoA carboxylases but also in the modification of Arc1p.

This conclusion was corroborated further by *in vitro* experiments where a N-terminal fragment of Arc1p was added to extracts of wild type and *bpl1* mutant cells. To investigate whether the N-terminal domain of Arc1p functions as an independent acceptor of biotin we fused amino acids 7–140 of Arc1p to an N-terminal His6 tag for biosynthesis by a bacterial expression system. The recombinant protein fragment Arc1-N thus produced was purified from *E. coli* by nickel-chelate chromatography and analyzed by SDS-PAGE and Western blotting. It was found that a protein of the expected molecular mass had indeed been produced in the heterologous system, but this protein did not react with streptavidin (Fig. 7). This finding is in contrast to the ability of other biotin binding domains to be recognized and modified by heterologous biotin:protein ligases even when they are transferred between different kingdoms of life (8, 9). Because the three-dimensional structure rather than a specific protein sequence of the biotin binding domain appears to be essential for recognition by biotin:protein ligases (37), it had to be excluded that the Arc1-N fragment was misfolded and therefore not modified in *E. coli*. Therefore, we analyzed whether the purified fragment was modified by yeast Bpl1p *in vitro*. To this end, protein extracts from wild type yeast cells, from the *bpl1* mutant and from yeast cells overexpressing an extrachromosomal version of *BPL1*, were incubated with the Arc1-N fragment. Although only a faint Arc1-N signal was observed when the wild type cell extract was used as
biotinylating agent, biotinylation was increased drastically with protein extracts from the BPL1-overexpressing strain as an enzyme source (Fig. 7). Under the same conditions, extracts from bpl1 mutants were unable to effect Arc1-N biotinylation. Because of the use of total yeast cell extracts as an enzyme source, the known biotin-containing yeast proteins were also detected on the protein blot. Furthermore, it is evident from Fig. 7 that not only biotinylation of Arc1-N but also that of full-length Arc1p increases when BPL1 is overexpressed. This demonstrates that Arc1p is only partially biotinylated, even in wild type yeast cells. In contrast, the streptavidin reactivity of Arc1p/Hfa1p, Dru1.2p, and Pyc1p/Pyc2p was not increased upon BPL1 overexpression, indicating that these proteins were exhaustively biotinylated in wild type yeast. As is also evident from Fig. 7, the extent of biotinylation of all biotinylated proteins was reduced in the bpl1 mutant cell extract used for in vitro biotinylation of Arc1-N. As expected, no Arc1-N biotinylation was observed with an E. coli cell extract or when full-length Arc1p was expressed in E. coli (data not shown). Moreover, in vitro biotinylation of Arc1-N by yeast biotin:protein ligase failed when the fragment contained the K86R mutation (data not shown). Taken together, these results indicate that Arc1p is biotinylated by the same enzyme, Bpl1p, which also modifies the known biotin-containing yeast carboxylases. Because of the unique molecular structure of its biotin binding site, however, Arc1p modification appears to be inefficient in yeast and becomes even impossible with the heterologous E. coli biotin:protein ligase.

Functional Relevance of Arc1p Biotinylation—As an activator of aminoacyl-tRNA synthetases, Arc1p is involved in the nuclear export of aminoacyl-tRNA. Because of the existence of both an aminoacylation-dependent and an aminoacylation-independent export pathway, however, deletion of ARC1 is not lethal but simply reduces cellular growth. This characteristic, which has been first reported by Simos et al. (13), was confirmed again by the experiment shown in Fig. 8 demonstrating the meiotic segregation of the arc1Δ-null allele in the presence and absence of multiple extrachromosomal copies of ARC1. As expected, plasmid-encoded Arc1p effectively complements the growth defect of the arc1Δ-null mutants. Surprisingly, however, the same effect was observed if the biotin-free K86R mutant allele instead of wild type ARC1 was used in the complementation assay (Fig. 8). According to these data, protein-bound biotin appears to be irrelevant for the growth-supporting function of Arc1p.

The functional importance of Arc1p biotinylation was also investigated from a different point of view. In yeast, LOS1 encodes the nuclear export factor exportin-t, which is presumed to mediate nuclear export of nonacylated tRNAs. Los1p and Arc1p mediate two parallel pathways of nuclear tRNA export, and simultaneous deletion of both genes is lethal. To address the question of whether biotinylation is essential for Arc1p function, we transformed, in separate experiments, a los1Δ/los1Δ, arc1Δ/arc1Δ heterozygous diploid with the wild type and mutant (K86R) ARC1 DNA, respectively. The transformants were sporulated, and the viability of resulting meiotic segregants was analyzed. As is shown in Table III, no viable arc1Δarc1Δ double mutants were obtained from transformants harboring an ARC1-free control plasmid. In contrast, vital spores carrying both chromosomal mutations were recovered in the presence of extrachromosomal ARC1 DNA indicating that the chromosomal arc1Δ lesion was complemented. Again, no difference was observed between wild type ARC1 and the ARC1 K86R allele because both were capable of restoring the viability of arc1Δlos1Δ double mutants. Together, these experiments demonstrate that the Arc1 protein is biotinylated at a specific lysine residue within the N-terminal domain of the protein. This modification requires the activity of yeast biotin:protein ligase, Bpl1p. No evidence was obtained that protein biotinylation is required for the physiological activity of Arc1p.

DISCUSSION

In the present work, a novel biotinylated yeast protein of 45 kDa apparent molecular mass was identified which reacts with streptavidin in Western blots. Affinity purification with avidin-Sepharose and N-terminal sequencing of several proteolytic peptides revealed the novel protein as Arc1p. Recombinant Arc1p was shown to incorporate 14C-labeled biotin by covalent and alkali-stable linkage. Biotinylation of Arc1p required the presence of extrachromosomal DNA indicating that Arc1p mediate two parallel pathways of nuclear tRNA export, to mediate nuclear export of nonacylated tRNAs. Los1p and

FIG. 7. In vitro biotinylation of the purified Arc1p fragment, Arc1-N (amino acids 7–140). For biotinylation, Arc1-N was incubated with cell extracts of JS89.27-3 (wt), bpl1-C2S/17, or JS89.27-3 transformants overexpressing BPL1 from the multicopy plasmid pVT100-U-BPL1-ZZ (mc). In a control experiment, Arc1-N was incubated with buffer instead of yeast cell extracts (–). A, Western blot of the reaction products with streptavidin-horseradish peroxidase. In addition to Arc1p, other proteins were reduced in the wild type yeast cells. In contrast, the streptavidin reactivity of Arc1p/Hfa1p, Dur1.2p, and Pyc1p/Pyc2p was not increased upon BPL1 overexpression, indicating that these proteins were exhaustively biotinylated in wild type yeast. B, the same samples are shown after staining with Coomassie Brilliant Blue.

FIG. 8. Complementation of arc1Δ mutant by biotinylated and nonbiotinylated Arc1p. The heterozygous ARC1/arc1Δ diploid, SC1477, was transformed with the indicated plasmids. After sporulation, individual spores were analyzed for their growth characteristics on yeast extract peptone dextrose medium at 30 °C. Plasmid pK86R was as indicated in Fig. 6. WT, wild type.

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In vitro biotinylation of the purified Arc1p fragment, Arc1-N (amino acids 7–140). For biotinylation, Arc1-N was incubated with cell extracts of JS89.27-3 (wt), bpl1-C2S/17, or JS89.27-3 transformants overexpressing BPL1 from the multicopy plasmid pVT100-U-BPL1-ZZ (mc). In a control experiment, Arc1-N was incubated with buffer instead of yeast cell extracts (–). A, Western blot of the reaction products with streptavidin-horseradish peroxidase. In addition to Arc1p, other proteins were reduced in the wild type yeast cells. In contrast, the streptavidin reactivity of Arc1p/Hfa1p, Dur1.2p, and Pyc1p/Pyc2p was not increased upon BPL1 overexpression, indicating that these proteins were exhaustively biotinylated in wild type yeast. B, the same samples are shown after staining with Coomassie Brilliant Blue.
A Novel Biotinylated Protein in Yeast

The heterozygous diploid SC1468 containing the arc1Δ/ARC1, los1Δ/LOS1 allele pairs was sporulated after transformation with the indicated plasmids. Viable spores were isolated on YPD medium. By replicating onto appropriate omission media, uracil-prototrophic cated plasmids. Viable spores were isolated on YPD medium. By repli-LOS1 allele pairs was sporulated after transformation with the indi-

| Plasmid | Total | Wild type | arc1Δ | los1Δ | arc1Δ los1Δ |
|---------|-------|-----------|-------|-------|-------------|
| pVT-100U | 47    | 11        | 17    | 19    | 0           |
| pAB1    | 69    | 15        | 18    | 25    | 11          |
| pK36R   | 56    | 16        | 11    | 16    | 13          |

boxylases. According to the extensive studies of Hurt and co-workers (13, 38, 39), Arc1p is involved in the aminocarboxylation-dependent pathway of tRNA export from the yeast nucleus. Although the C-terminal and the central portion of Arc1p are required for tRNA binding, its N-terminal domain specifically binds the two aminocarboxyl-tRNA synthetases, MetRS and GluRS (40, 41). This interaction is mediated by the noncatalytic, N-terminally appended domains of both synthetases. Association of Arc1p with MetRS and GluRS appears to be required for effective recruitment and acylation of the cognate tRNAs. Arc1p-mediated aminocarboxylation of tRNA is supposed to occur within the nucleus, thereby relieving the nuclear retention of uncharged tRNA. Transport of aminocarboxyl-tRNA through the nuclear pore is facilitated further by its release, on the cytoplasmic face of the pore, from the synthetases to eEF-1A (42). Despite this vital function of Arc1p, arc1Δ mutants are not lethal. This is because of the operation of a second, aminocarboxylation-independent nuclear tRNA export pathway in yeast which requires the exportin Los1p (43). Only simultaneous inactivation of both ARC1 and LOS1 results in lethality, indicating that no additional tRNA export pathway exists in yeast (41). Despite this redundancy of tRNA export mechanisms from the nucleus, arc1Δ mutants are characterized by a slow growth phenotype (13). We used both criteria, i.e., the synthetic lethality of arc1Δ los1Δ double mutants and the growth retardation of arc1Δ mutants, for investigating the effect of protein-bound biotin on Arc1p function. It turned out that biotin-free Arc1p was indistinguishable from biotinylated Arc1p in its ability to complement the above mentioned phenotypes of arc1Δ and arc1Δ los1Δ mutants. According to these results, biotinylation appears to be dispensable for the general functioning of Arc1p. Nevertheless, it cannot be excluded, at present, that biotinylation modulates the interaction of Arc1p with GluRS, MetRS, or tRNA and thereby affects the efficiency of its function.

The biotin binding site of Arc1p has no resemblance to the consensus sequence present in the known biotin-containing carboxylases. Biotinylation of Arc1p is nevertheless performed by the same enzyme, Bpl1p, which also modifies the various yeast carboxylases. In contrast to holocarboxylase formation, however, biotinylation of Arc1p is inefficient with only a minor fraction of the cellular Arc1 protein being modified, in vivo. In contrast to yeast biotin:protein ligase, the respective

biotinylated (pAB1) and nonbiotinylated (pK86R) Arc1p were indistinguishable from biotinylated Arc1p in its ability to modify Arc1p, the 13-amino acid consensus sequence derived from the biotinylated peptides is absent from Arc1p. In analogy to the activity of BirA, it is concluded that the folded structure of the Arc1 protein around lysine 86 resembles that around biocytin in ACC and Fyc, making it a substrate for Bpl1p. Functionally competent biotin binding domains have a minimal size of 63–66 amino acids (2, 36, 48) and fold into a flattened beta-barrel that exposes the modified lysine residue (49–51). From this it is expected that a major portion of the N-terminal domain of Arc1p should be involved in constituting its biotin binding domain.

There exist a few examples of biotin-associated proteins that are involved in functions other than carboxylation reactions. Among these are, apart from Arc1p, yeast acetyl-CoA carboxylase (52–54), mammalian histones (55, 56), and the bacterial biotin:protein ligase, BirA (10, 57). Kadowaki et al. (52) reported on a temperature-sensitive yeast acetyl-CoA carboxylase mutant being defective in mRNA nuclear export. Even though Schneider and co-workers (53, 58) attributed this defect to an altered lipid composition of the nuclear envelope rather than to a carboxylation-independent function of ACC, molecular details of this mutation remain to be elucidated. As was demonstrated recently by Hymes and co-workers (55, 56), human histones may be specifically biotinylated, in vivo, by the action of biotinidase functioning as a biotin transferase rather than as hydrolase. The packaging and regulatory properties of rat liver chromatin were shown to be affected by a similar histone modification (59, 60). Similarly, noncovalently associated biotinyl-AMP facilitates binding of the BirA aporepressor to the E. coli biotin operator (10, 57, 61). Remarkably, specific protein-nucleic acid interactions as observed with Arc1p (13, 34) represent a common feature in all of the above listed processes. Nevertheless, neither our own data nor those reported earlier by Simos et al. (13) support an effect of biotin on Arc1p functioning and, in particular, on its tRNA binding characteristics. In vitro, both the biotin-free Arc1p synthesized in E. coli and an Arc1p fragment lacking the biotinylated N-terminal domain exhibited unimpaired tRNA binding capacities (41). Furthermore, comparing the S. cerevisiae Arc1p sequence with the homologous proteins of other fungi reveals significant sequence conservation in their C-terminal parts but, in most cases, a drastically less conserved N-terminal protein sequence. Obviously, only N-terminal domains with distinct similarity to S. cerevisiae Arc1p, like those of the Eremothecium gossypii or Candida albicans homolog, contain a lysine corresponding to lysine 86 of the yeast protein. Therefore, more detailed molecular studies will be necessary to demonstrate whether biotinylation in fact participates, in a more subtle way, in the biochemical functioning of Arc1p rather than being only an accidental protein modification that does not interfere with Arc1p function.

Acknowledgment—We acknowledge the skilful technical assistance of Sabine Laberer.

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