Acetyl-CoA hydrolase (Ach1p), catalyzing the hydrolysis of acetyl-CoA, is presumably involved in regulating intracellular acetyl-CoA or CoA-SH pools; however, its intracellular functions and distribution remain to be established. Using site-directed mutagenesis analysis, we demonstrated that the enzymatic activity of Ach1p is dependent upon its putative acetyl-CoA binding sites. The ach1 mutant causes a growth defect in acetate but not in other non-fermentable carbon sources, suggesting that Ach1p is not involved in mitochondrial biogenesis. Overexpression of Ach1p, but not constructs containing acetyl-CoA binding site mutations, in ach1-1 complemented the defect of acetate utilization. By subcellular fractionation, most of the Ach1p in yeast was distributed with mitochondria and little Ach1p in the cytoplasm. By immunofluorescence microscopy, we show that Ach1p and acetyl-CoA binding site-mutated constructs, but not its N-terminal deleted construct, are localized in mitochondria. Moreover, the onset of pseudo-hyphal development in homozygote ach1-1 diploids was abolished. We infer that Ach1p may be involved in a novel acetyl-CoA biogenesis and/or acetate utilization in mitochondria and thereby indirectly affect pseudo-hyphal development in yeast.

The concentration of acetyl-CoA in cells is primarily regulated by its rate of synthesis and its utilization in various metabolic pathways. In the yeast Saccharomyces cerevisiae, biosynthesis of acetyl-CoA is mainly achieved by the acetyl-CoA synthetase reaction, whereas oxidative decarboxylation by the mitochondrial pyruvate dehydrogenase complex appears to be of minor importance (reviewed in Ref. 1). Even under glycolytic growth conditions, S. cerevisiae converts pyruvate into acetate, catalyzed by the subsequent action of pyruvate dehydrogenase, acetaldehyde dehydrogenase, and acetyl-CoA synthetase (2). In the presence of a fermentable carbon source, acetyl-CoA may be mainly used as a precursor of fatty acid and sterol biosynthesis. On the other hand, an additional pool of acetyl-CoA is required for the glyoxylate cycle (citrate synthase and malate synthase reactions) when cells grow with a non-fermentable substrate such as ethanol or acetate.

Acetyl-CoA hydrolase, catalyzing the hydrolysis of acetyl-CoA, was first identified in the pig heart (3), and subsequently the enzyme has been found in many mammalian tissues (4–10). During the purification of yeast Naa-acetyltransferase, an endogenous “inhibitor” of acetyltransferase was purified and shown to be acetyl-CoA hydrolase (11, 12). Acetyl-CoA hydrolase also inhibits purified rat brain pyruvate carboxylase (13) and [acyl-carrier-protein]acyltransferase (14).

It has been shown that the expression of acetyl-CoA hydrolase (ACH1) from S. cerevisiae is glucose-repressible (15) and subjected to cAMP-dependent repression (16). The function of Ach1p in vivo is still speculative. Previously, we have shown that the ability of ach1 mutants to grow on acetate is impaired (17). ACH1 is highly homologous to the aarC gene of Acetobacter acetii (18) and the Neurospora crassa gene acu8 (19, 20).

An acu-8 mutant strain, characterized as acetate non-utilizing, shows strong growth inhibition by acetate but will use it as a carbon source at low concentrations (20). The acu-8 mutant was also shown to be deficient in acetyl-CoA hydrolase and to accumulate acetyl-CoA when supplied with acetate. As in Saccharomyces, the Neurospora enzyme is acetate-inducible. The arrC-defective mutant also showed an inability to assimilate acetic acid (18). However, in all three organisms, disruption of these genes yields strains that grow normally on ethanol (17, 18, 20). Possibly, the acetyl-CoA balance during growth on acetate is disturbed in such mutants. Whether or not acetyl-CoA hydrolase is involved in regulating the endogenous pool(s) of acetyl-CoA remains to be established. In this study, we took an initial step to characterize the biochemical property of Ach1p in vivo and determine its subcellular localization. We demonstrate that the enzymatic activity of Ach1p is dependent upon its putative nucleotide (CoA) binding sites and show that Ach1p is a mitochondrial enzyme. In addition, we provide initial evidence that Ach1p is involved in development of pseudohyphae but not in mitochondrial biogenesis.

**MATERIALS AND METHODS**

**Strains, Media, and Microbiological Techniques—**Yeast culture media were prepared as described by Sherman et al. (21). YPD contained 1% Bacto-yeast extract, 2% Bacto-peptone, and 2% glucose. SD contained 0.7% Difco yeast nitrogen base (without amino acids) and 2% glucose. Nutrients essential for auxotrophic strains were supplied at specified concentrations. For comparison of Ach1p expression in different carbon sources, synthetic media containing 5% glucose, 2% galactose, 2% glycerol, and 2% potassium acetate were used. Yeast cells were transformed by the lithium acetate method (22). Plasmids were constructed by standard protocols as described by Sambrook et al. (23). Yeast strains YPH550 (MATa ade2 his3 leu2 lys2 trp1 ura3–52), YPH252 (MATa ade2 his3 leu2 lys2 trp1 ura3–52), and INVSc1 (MATa his3–1 leu2 trp1–289, ura3–52) were used in this study. For creating the yeast expression vector encoding Ach1p, the sequence encoding yeast ACH1 was amplified by PCR and inserted into the
that hydrolyzes 1 nmol of [1-14C]acetyl-CoA in 1 min. As shown previously (15). One unit of activity is defined as the amount of enzyme that hydrolyzes 1 nmol of [1-14C]acetyl-CoA in 1 min.

Expression and Purification of Recombinant Proteins and Polyclonal Antibody Production—The open reading frame of ACH1 was obtained by PCR, by the use of primers that incorporated unique NcoI and BamHI sites at the initiating methionine and 6 bp downstream of the stop codon, respectively. For the His-tagged Ach1p, a DNA fragment containing the ACH1 coding region was generated by amplifying yeast genomic DNA with sequence-specific primers. The PCR product was purified and ligated to the expression vector pET15b (Novagen), yielding pET15b-ACH1. The His-tagged fusion protein was synthesized in Escherichia coli and purified on nickel-nitriiotriacetic acid resin (Qiagen, Chatsworth, CA) as described (25). Denatured, purified recombinant Ach1p isolated from an SDS-PAGE gel was used as antigen for raising polyclonal antibodies in rabbits essentially as described (25).

Preparation of Crude Yeast Lysates and Assay of Acetyl-CoA Hydrolyase Activity—Crude yeast lysates were prepared, and acetyl-CoA hydrolase activity was determined by radioassay, as described previously (15). One unit of activity is defined as the amount of enzyme that hydrolyzes 1 nmol of [1-14C]acetyl-CoA in 1 min.

Western Blot Analysis and Immunofluorescence Microscopy—Yeast total proteins were prepared and subjected to Western blot analysis as described previously (26). Cells were prepared for immunofluorescence staining as described by Huang et al. (25). Alexa 594- or Alexa 488-conjugated anti-IgG antibodies (Molecular Probes, Eugene, OR) were used as secondary antibodies. H33258 was diluted in mounting solution for nucleic acid staining. Fluorescence microscopy was performed with a Nikon Microphot SA microscope.

Subcellular Fractionation—Yeast grown in selective minimal medium or YPD medium were harvested by centrifugation and washed once with 10 mM NaNO₃, before Lyticase digestion of cell walls in a solution containing 12.2 M sorbitol and 100 mM potassium phosphate (pH 6.2). Spheroplasts were suspended in buffer containing 0.1 M sorbitol, 20 mM HEPES (pH 7.4), 50 mM potassium acetate, and 1 mM EDTA with protease inhibitors and disrupted on ice with 20 strokes in a Dounce homogenizer. The lysate was centrifuged (450 × g) to remove debris and unbroken cells. Cleared lysate (0.8 ml) was loaded on top of a manually generated six-step sucrose gradient (0.7 ml each of 60, 50, 40, 30, 20, and 10% sucrose in lysis buffer), which was then centrifuged at 170,000 × g for 3 h in a Beckman SW55 rotor at 4 °C. Proteins in samples (100 μl) of fractions, collected manually from the top, were precipitated with 10% trichloroacetic acid, separated by SDS-PAGE, and analyzed by immunoblotting. Diluted antibodies against mitochondria (1:1,500) (Molecular Probes), Rpl3p (1:1,000), Emp47 (1:5,000), and Arf1p (1:5,000) (26) were used to identify organelles.

Yeast was grown in YPGal to early stationary phase. Mitochondria were isolated as described previously (31). Mitochondria were resuspended in lysis buffer to give an approximate final concentration of 10 mg of protein/ml. To isolate the mitochondrial intermembrane space, a suspension of mitochondria was gently resuspended in lysis buffer and centrifuged at low speed (600 × g) to remove larger aggregates. The organelle suspension was loaded on a 14–36% Nycodenz gradient to further fractionate mitochondria and peroxisomes. The gradient was centrifuged at 32,500 rpm, 4 °C, for 3.5 h. After centrifugation, the sample was divided into 14 fractions from 14 to 36% Nycodenz gradients; then proteins were precipitated with 10% trichloroacetic acid and analyzed by Western blotting.

Isolation and Fractionation of Mitochondria—Yeast was grown in YPGal to early stationary phase. Mitochondria were isolated as described previously (31). Mitochondria were resuspended in lysis buffer to give an approximate final concentration of 10 mg of protein/ml. To isolate the mitochondrial intermembrane space, a suspension of mitochondria (10–20 mg of protein/ml in 0.6 M sorbitol, 10 mM Tris, pH 7.4) was diluted with 5 volumes of 10 mM Tris, pH 7.4, to a final sorbitol concentration of 0.1 M. The suspension was incubated at 4 °C with gentle rocking for 20 min. The “shocked” mitochondria were sedimented at 20,000 rpm in a Beckman SW55 Ti rotor for 20 min. The supernatant contains the contents of the intermembrane space; then proteins were precipitated with 10% trichloroacetic acid and analyzed by Western blotting. To isolate mitochondrial membrane and matrix, shocked mitochondria were resuspended in 10 mM Tris, pH 7.4, to a protein concentration of about 2 mg/ml with five strokes in a Dounce homogenizer and left on ice to allow further swelling of the mitochondrial matrix space. After 5 min, “shrinking buffer” (one-third of the suspension volume) containing 1.8 M sucrose, 8 mM ATP, 8 mM MgCl₂, adjusted to pH 7.4 with KOH, was added. The suspension was mixed carefully by three strokes in the Dounce homogenizer and left on ice. After 5 min, the suspension was exposed to ultrasonic irradiation for 3 × 5 s on ice. Total mitochondrial membranes were sedimented for 60 min at 35,000 rpm in a Beckman SW55 Ti rotor at 4 °C. The supernatant represents the matrix fraction; then proteins were precipitated by 10% trichloroacetic acid and analyzed by Western blotting.

FIG. 1. Western blot analysis and enzyme activity of Ach1p and its mutants. As shown in A, wild-type (WT) yeast, ach1l mutant, and ach1l mutant with expressed Ach1p, Ach1lΔN, Ach1lΔEs, and Ach1lΔSS were cultured with synthetic medium containing 2% glucose. Acetyl-CoA hydrolase activity was determined by radioassay, as described previously (15). One unit of activity is defined as the amount of enzyme that hydrolyzes 1 nmol of [1-14C]acetyl-CoA in 1 min. In B, wild-type yeast was cultured with synthetic medium containing 5% glucose, 2% galactose, 2% glycerol, or 2% potassium acetate. Total proteins (~20 μg/lane) were separated by SDS-PAGE, stained with Coomassie Blue (upper panels), or subjected to Western blot analysis (lower panels). Ach1p was identified with specific antibody (1:5,000). The yeast Arf1p was used as internal control. Positions of protein standards are indicated on the left.
Identification of Endogenous Ach1p—To characterize the Ach1p gene product, we prepared a rabbit antiserum against an E. coli/H11011 fusion protein. Among total cellular proteins, antibodies prepared against Ach1p reacted only with a protein of ~64 kDa, the expected size for Ach1p (Fig. 1). This protein was not detected in an ach1 mutant (Fig. 1) or by the preimmune serum (not shown). Immunoblotting with this antiserum detected nongradient amounts of Ach1p (data not shown) as well as various mutant forms of Ach1p (Fig. 1). As in previous RNA blot analysis (15), Ach1p was subjected to glucose-dependent regulation (Fig. 1B). As in previous RNA blot analysis (15), Ach1p was subjected to glucose-dependent regulation (Fig. 1B).

RESULTS

Subcellular Localization of Endogenous Ach1p—To study the subcellular distribution of Ach1p, the total yeast spheroplast-homogenized lysate was fractionated by 30–60% discontinuous sucrose gradient centrifugation. As shown in Fig. 3A, distribution of most of Ach1p was similar to that of the mitochondrial protein porin, although little Ach1p was found in the cytoplasmic fractions. To determine further whether Ach1p may also be present in peroxisomes, a homogenate of oleate-grown cells was first subjected to differential centrifugation to obtain an organellar pellet. This material was further fractionated by density gradient centrifugation on Nycodenz. Fig. 3B shows good resolution between mitochondria (porin marker) and peroxisomes (thiolase marker), and the distribution of Ach1p was similar to that of the mitochondrial protein porin. By immunofluorescence microscopy, endogenous Ach1p, similar to porin, appeared to be localized to mitochondria (Fig. 3C). Yeast mitochondria can form branched networks distributed evenly around the circumference of the cell in the peripheral cytoplasm. Abnormal mitochondrial morphology was not seen in ach1 mutant yeast. To further localize the Ach1p within the purified mitochondria, we analyzed the intermembrane space, matrix, and membrane fractions (Fig. 4). Ach1p cofractionated with Mge1p but not with cytochrome oxidase subunit IV and porin, indicating that it is localized to the mitochondrial matrix.

N terminus but Not Putative Nucleotide (CoA) Binding Sites of Ach1p Are Required for Ach1p Mitochondrial Localization—

Fig. 2. Alignment of the homologous regions of the Ach1p and other related Co-A transferases. ScAch1, S. cerevisiae Ach1p; SpAch1, S. pombe Ach1; NaAcu8, N. crassa Acu8; EcCat1, E. coli Cat1; CkCat1, C. kluyveri CAT1 (succinyl-CoA:CoA transferase); CkCat2, C. kluyveri CAT2 (butyryl-CoA:CoA transferase). Arrows indicate the site-directed mutagenesis of the Co-A binding sites to generate Ach1p mutant forms of Ach1p (Fig. 1). As in previous RNA blot analysis (15), Ach1p was subjected to glucose-dependent regulation (Fig. 1B). As in previous RNA blot analysis (15), Ach1p was subjected to glucose-dependent regulation (Fig. 1B).
Most proteins targeted to the mitochondrial matrix contain a cleavable N-terminal presequence with basic and hydroxylated amino acids interspersed throughout their length (35, 36). Although the N terminus of Ach1p contains no typical matrix-targeting sequence, we suspected that deletion of the N-terminal domain from Ach1p might interfere with its mitochondrial localization. After expression in the \textit{ach1} mutant, Ach1pdN, lacking 64 amino acids at the N terminus, was recovered in the least dense fractions of the lysate (data not shown). In cells, most of the Ach1pdN mutant was in the cytoplasmic region with a punctate distribution (Fig. 5), and the mitochondrial morphology was similar to that in wild-type cells (Fig. 5). In addition, fusion between the N terminus (64 amino acids) of Ach1p and GFP protein failed to be imported into mitochondria (data not shown).

We further tested whether overexpression of Ach1p or its mutant constructs (Ach1pES and Ach1pSS) in yeast might cause dominant-negative effects on mitochondrial morphology. By immunofluorescence microscopy, Ach1pES and Ach1pSS, like overexpressed Ach1p, were present in some tubular or spherical structures that also stained with anti-porin antibody (Fig. 5). Thus, N terminus, but not putative Co-A binding sites of Ach1p, was required for Ach1p mitochondrial localization. Ach1p Is Not Required for Mitochondrial Biogenesis—Because most of Ach1p is localized in the mitochondria, we next examined whether Ach1p can affect mitochondrial function. Yeast, when cultured in glycerol medium, requires mature active mitochondria for oxidative metabolism and growth. Yeast grown in glucose initially does not need active, mature mitochondria, and the mitochondria are not well developed before being switched from anaerobic glucose fermentation to aerobic ethanol oxidation. The wild-type strain, \textit{ach1} strain, and \textit{ach1} strains overexpressing Ach1p and its deleted or mutated constructs (Ach1pES, Ach1pSS, and Ach1pdN) were cultured overnight in synthetic medium containing glucose. Cells were harvested, suspended in double distilled H₂O, subjected to serial dilution, and dropped onto glucose, acetate, glycerol, succinate, and ethanol media plates. Fig. 6 shows that all strains grew on the glucose, glycerol, succinate, and ethanol media plates; however, the \textit{ach1} mutant and the Ach1pES, Ach1pSS, and Ach1pdN expression strains did not grow in acetate medium plates. Moreover, the Ach1p-overexpressing cells grew as well as the wild-type yeast on acetate plates. These results suggested that Ach1p is not involved in cellular events in mitochondrial biogenesis, which is required for cells to grow in non-fermentable carbon sources.

\textbf{Ach1p Is Required for Pseudohyphal Formation}—Diploid cells of the yeast \textit{S. cerevisiae} undergo pseudohyphal differen...
Ach1p is localized to mitochondrial matrix. The mitochondria of yeast were subfractionated as described under “Materials and Methods.” Protein samples of submitochondrial fractions were subjected to SDS-PAGE, transferred to polyvinylidene difluoride membrane, and probed with specific antisera to detect the following proteins: membrane markers (Porin and cytochrome oxidase IV), matrix marker (Mge1p), and Ach1p protein. Mem., mitochondrial membrane proteins; IMS, mitochondrial intermembrane space; Mtx, mitochondrial matrix; MW, molecular weight markers.

The N terminus, but not putative CoA binding sites of Ach1p, is required for localization to mitochondria. The yeast ach1 mutant cells were transformed with pVTl0lU-ACH1, pVTl0lU-ACH1dN, pVTl0lU-ACH1ES, and pVTl0lU-ACH1SS. Cells were fixed with formaldehyde; spheroplasts were prepared and reacted with anti-Ach1p antibody (1:1000) and anti-mitochondrial porin antibody (1:50) followed by secondary antibodies. Nucleic acids were stained with H33258.

**FIG. 5.** The N terminus, but not putative CoA binding sites of Ach1p, is required for localization to mitochondria. The yeast ach1 mutant cells were transformed with pVTl0lU-ACH1, pVTl0lU-ACH1dN, pVTl0lU-ACH1ES, and pVTl0lU-ACH1SS. Cells were fixed with formaldehyde; spheroplasts were prepared and reacted with anti-Ach1p antibody (1:1000) and anti-mitochondrial porin antibody (1:50) followed by secondary antibodies. Nucleic acids were stained with H33258.

**FIG. 4.** Ach1p is localized to mitochondrial matrix. The mitochondria of yeast were subfractionated as described under “Materials and Methods.” Protein samples of submitochondrial fractions were subjected to SDS-PAGE, transferred to polyvinylidene difluoride membrane, and probed with specific antisera to detect the following proteins: membrane markers (Porin and cytochrome oxidase IV), matrix marker (Mge1p), and Ach1p protein. Mem., mitochondrial membrane proteins; IMS, mitochondrial intermembrane space; Mtx, mitochondrial matrix; MW, molecular weight markers.

**DISCUSSION**

In this study, we show that putative conserved nucleotide (CoA) binding sites and N terminus of Ach1p require its enzyme activity. Our data also show that Ach1p is localized to the mitochondria, and the N terminus of Ach1p is required for its localization. Finally, we show that Ach1p is not involved in mitochondrial biogenesis but may be involved in pseudohyphal differentiation.

The amino acid sequences of Ach1p and homologous CoA-transferases contain two conserved CoA (ADP) binding sites (GXXGXX(G/A)) from heterodimeric CoA transferases (Fig. 2) (9, 33). We determined whether these putative nucleotide (CoA) binding sites are required for Ach1p activity. Enzyme assays confirmed that Ach1pSS and Ach1pES expressed in the ach1 mutant contained little detectable acetyl-CoA hydrolizing activity. We also showed that utilization of acetate as carbon source by the ach1 mutant is impaired, and overexpression of Ach1pES, or Ach1pSS, cannot restore this activity. Our data indicate that one or more putative nucleotide (CoA) binding sites are required for Ach1p enzymatic activity.

The subcellular localization of a protein is an important characteristic with functional implications. Our data show that most of Ach1p is localized to the mitochondria, although a little Ach1p was also found in small punctate form distributed in the cytoplasm. The majority of mitochondrial matrix-targeting signals are cleaved upon import into the mitochondria (36). The matrix-located MTF1 protein in yeast, which is a transcription-stimulating factor (38), is an exceptional case. This protein lacks a recognizable matrix-targeting sequence, and its import is reported to be independent of outer membrane receptors. How specificity of targeting is achieved in this case and whether there is an entirely separate pathway for importing this protein remain to be clarified. We demonstrate that N-terminal but not putative CoA binding sites of Ach1p are required for localization to mitochondria. Thus, translocation of Ach1p from the cytoplasm to the mitochondrial matrix may, like that of MTF1, require that the N-terminal sequence lacks a recognizable matrix-targeting signal. A recent report described that Ach1p has two different protein spots by two-dimensional gel electrophoresis (39). These two Ach1ps have the same relative molecular weight but differ in their pI, suggesting that Ach1p might be modified post-translationally. However, we attempted, but failed, to confirm that there are two different protein spots of Ach1p by two-dimensional gel electrophoresis. Carnitine acetyltransferase (CAT) is known to be present in mitochondria and peroxisomes of oleate-grown *S. cerevisiae*, and both proteins are encoded by the same gene, YCAT (27). We also speculated whether Ach1p in oleate-grown cells might have a different subcellular localization. Our data showed that the majority of Ach1p is present in mitochondria but not in peroxisomes. Thus, we concluded that Ach1p is a mitochondrial enzyme and may execute its physiologic function in the matrix space.

Contemporary knowledge of the structure and function of acetyl-CoA hydrolases (i.e. cytosolic (8, 40) and mitochondrial (5)) is incomplete. Ach1p resembles the rat mitochondrial acetyl-CoA hydrolase, is not affected by ADP or ATP, and is inhibited by βNADH (9, 12). The *Km* of the Ach1p is similar to lack the Ach1p were completely defective in pseudohyphal differentiation, whereas ach1/ach1-overexpressing Ach1p restores pseudohyphal growth (Fig. 7). However, expression of Ach1p deleted or mutated constructs (Ach1pES, Ach1pSS, and Ach1pN) failed to restore pseudohyphal growth (data not shown).

L.-M. Buu, Y.-C. Chen, and F.-J. S. Lee, unpublished data.
A Role of Ach1p in Mitochondria

In conclusion, this study has confirmed that putative conserved nucleotide (CoA) binding sites of Ach1p are required for its enzyme activity in vivo. We also demonstrated that Ach1p is a mitochondrial enzyme, although its potential function in the glyoxylate cycle needs to be investigated further.
We also showed that Ach1p is not involved in mitochondrial biogenesis, and our data suggest that the metabolism of acetyl-CoA by Ach1p is involved indirectly in pseudohyphal differentiation. Although yeast can use acetate or ethanol as carbon source by converting them to acetyl-CoA in the metabolic pathway, it will be interesting to know how ach1 mutants could impair acetate but not ethanol utilization. The exact physiologic role of this mitochondrial Ach1p needs to be investigated further.

Acknowledgments—We thank Drs. T. Langer and M. Rose for providing us with antibodies. We thank Chih-Hsin Chen for preparing the anti-Ach1p antibody.

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A Role of Ach1p in Mitochondria

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Functional Characterization and Localization of Acetyl-CoA Hydrolase, Ach1p, in Saccharomyces cerevisiae
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J. Biol. Chem. 2003, 278:17203-17209.
doi: 10.1074/jbc.M213268200 originally published online February 26, 2003

Access the most updated version of this article at doi: 10.1074/jbc.M213268200

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