Saccharomyces cerevisiae GNA1, an Essential Gene Encoding a Novel Acetyltransferase Involved in UDP-N-acetylglucosamine Synthesis*

(Received for publication, September 21, 1998, and in revised form, October 20, 1998)

Toshiyuki Mio‡, Toshiko Yamada-Okabe§, Mikio Arisawa‡, and Hisafumi Yamada-Okabe‡‡

From the ‡Department of Mycology, Nippon Roche Research Center, 200 Kajiwara, Kamakura, Kanagawa 247-8530 and the §Department of Hygiene, School of Medicine, Yokohama City University, 3-9 Fukaura, Kanazawa, Yokohama 236-0004, Japan

The Saccharomyces cerevisiae gene, YFL017C, for a putative acetyltransferase was characterized. Disruption of YFL017C was lethal, leading to a morphology similar to those caused by the depletion of AGM1 or UAP1, the genes encoding phospho-N-acetylglucosamine mutase and UDP-N-acetylglucosamine pyrophosphorylase, respectively. This implies the involvement of YFL017C in UDP-N-acetylglucosamine synthesis. The recombinant protein for YFL017C displayed phosphoglucomosamine acetyltransferase activities in vitro and utilized glucosamine-6-phosphate as the substrate. When incubated with Agm1p and Uap1p, the Yfl017c protein produced UDP-N-acetylglucosamine from glucosamine-6-phosphate. These results indicate that YFL017C specifies glucosamine-6-phosphate acetyltransferase; therefore, the gene was designated GNA1 (glucosamine-6-phosphate acetyltransferase). In addition, whereas bacterial phosphoglucomosamine acetyltransferase and UDP-N-acetylglucosamine pyrophosphorylase activities are intrinsic in a single polypeptide, they are encoded by distinct essential genes in yeast. When the sequence of ScGna1p was compared with those of other acetyltransferases, Ile97, Glu110, Val112, Gly115, Leu115, Ile116, Phe142, Tyr143, and Gly147 were found to be highly conserved. When alanine was substituted for these amino acids, the enzyme activity for the substituted Phe142 or Tyr143 enzymes was severely diminished. Although the activity of Y143A was too low to perform kinetics, F142A displayed a significantly increased \( K_m \) value for acetyl-CoA, suggesting that the Phe142 and Tyr143 residues are essential for the catalysis.

Acetyltransferases catalyze the acetylation of their specific substrates using the cofactor acetyl-CoA (Ac-CoA). These enzymes play important roles in a wide variety of biological processes, including metabolism, chromatin structure, gene expression, and cell cycle (1–3). At least one acetyltransferase is necessary for the biosynthesis of UDP-N-acetylgalactosamine (UDP-GlcNAc), which is an essential metabolite both in pro-
EXPERIMENTAL PROCEDURES

Yeast Data Base Search and Screening of DNA Libraries—The entire open reading frame (ORF) of ScGNA1 (YFL017C) was amplified by polymerase chain reaction with the S. cerevisiae genomic DNA extracted from strain A451 (MATa can1 ar07 can1 leu2 trp1 ura3) as a template. It was then cloned at the XbaI site of pUC18 or pYEuRa3 (Toyobo), generating pUC-ScGNA1 and pYE-ScGNAI, respectively. Primers used for polymerase chain reaction were 5'-AAAGATCCACCATGATGGATTTTATATAGTTGATTATATA-3' and 5'-AAAGAATTCCTTATTCTTCTATTGGCATTTCC-3'. The Candida albicans homolog of ScGNA1 was cloned by screening a C. albicans genomic DNA library as a probe using the 0.2-kilobase fragment of the C. albicans genomic DNA whose sequence was available in the C. albicans data base and was found to be related to YFL017C. The filters were hybridized and washed under stringent conditions (20 mM sodium phosphate (pH 7.2), 5 × SSC (1 × SSC contains 150 mM NaCl and 15 mM sodium citrate), 5 × Denhardt's solution, 0.1% SDS, 25% formamide at 42 °C for hybridization; 0.1 × SSC and 0.1% SDS at 50 °C for washing). After the third screening, DNA was extracted from bacterial cells that were strongly hybridized with the probe DNA, and the insert DNA was cloned between the BamHI and SalI sites of pUC19 for further plasmid construction. Radiolabeling of the probe DNA was performed by the random priming method using [α-32P]dCTP (23), and DNA sequencing was carried out as described elsewhere (23). Construction of the C. albicans genomic DNA library was already reported (24).

Expression and Purification of the Recombinant Proteins—The coding regions of ScGNA1 and CaGNA1 were cloned between the BamHI and EcoRI sites of pGEX-2T (25), and the resulting plasmids were transformed into E. coli JM109 to express the recombinant proteins as a fusion with glutathione S-transferase (GST). Induction and expression of the recombinant Gna1 protein was carried out with isopropyl-β-D-thiogalactopyranoside as described (24, 25). At 4 h after the addition of isopropyl-β-D-thiogalactopyranoside, the bacterial cells were harvested, suspended in a buffer containing 20 mM Tris-HCl (pH 7.5), 0.5 mM EDTA, 10% (v/v) glycerol, 1 mM phenylmethylsulfonyl fluoride, and 0.1% SDS, and centrifuged at 15,000 × g at 4 °C for 30 min. GST-Gna1 fusion proteins were purified by glutathione-Sepharose CL-4B column chromatography, as described (25), and analyzed by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis. The primers used for amplifying the CaGNA1 ORF were 5'-AAAGATCCATCCACCAAGGGTTATACATTACA-GA-3' and 5'-AAAGAATTCCTTATTCTTCTATTGGCATTTCC-3'. Assays for Phospho-GlcNAc Acetyltransferase—Because CoA reacts with 2-nitrobenzoic acid and releases 4-nitrophenol (26, 27), an assay for phospho-GlcNAc acetyltransferase was performed in 50 μl of a reaction mixture containing 50 mM Tris-HCl (pH 7.5), 5 mM MgCl2, 200 μM GlcNAc-6-P, 200 μM Ac-CoA, 10% (v/v) glycerol, and approximately 0.1 μg of the recombinant GST-Gna1p fusion protein. After incubation at 30 °C for the indicated time, the reaction was terminated by adding 50 μl of a solution containing 50 mM Tris-HCl (pH 7.5) and 6.4 g of NaOH to give pH 12, and the absorbance at 405 nm was measured.

RESULTS

Identification of the Yeast GlcN-6-P Acetyltransferase Gene—

Previously, we demonstrated that unlike the bacterial enzyme, the eukaryotic UDP-GlcNAc pyrophosphorylase is a nonfunctional protein with no phospho-GlcNAc acetyltransferase activity (20). When searching the S. cerevisiae data base, we found YFL017C on chromosome VI encodes a protein with homology to known acetyltransferases. To examine the physiological function of YFL017C, we created an S. cerevisiae strain in which the endogenous YFL017C gene was deleted, but where episomal copies of YFL017C, whose transcription was under the control of the GAL1 promoter, were maintained. The resulting strain grew on galactose plates, but died on glucose plates, confirming that YFL017C is an essential gene. When cultured on glucose plates, most of the YFL017C-deficient cells swelled and often lysed. This morphology was quite similar to that caused by the depletion of AGM1 or UAP1 (Fig. 1), suggesting that YFL017C is involved in the synthesis of UDP-GlcNAc. Although the expected product of YFL017C showed only limited sequence similarity to the C-terminal phospho-GlcNAc acetyltransferase domain of the bacterial GlmU gene protein (15% identity; 64% below), YFL017C was called GNA1 (glucosamine synthesis acetyltransferase 1) in this study.

If GNA1 is really a gene required for an essential metabolic pathway, such as UDP-GlcNAc synthesis, it should be preserved in other organisms. A search of the C. albicans data base revealed a partial nucleotide sequence that shared sequence similarity with S. cerevisiae GNA1 (ScGNA1). By
screening the \textit{C. albicans} genomic DNA library with the DNA fragment of the above nucleotide sequence as a probe, we were able to clone and sequence the \textit{C. albicans} homolog of \textit{GNA1} (\textit{CaGNA1}). The predicted products of \textit{ScGNA1} and \textit{CaGNA1} are closely related to each other (Fig. 2) (44% sequence identity over the entire protein). In addition, when expressed under the control of the \textit{ADH1} promoter, \textit{CaGNA1} rescued an \textit{S. cerevisiae} \textit{gna1} \textit{D} null mutant (data not shown), demonstrating that the \textit{CaGNA1} functionally complements \textit{ScGNA1}. Furthermore, a search of the \textit{Schizosaccharomyces pombe} and \textit{Caenorhabditis elegans} data bases revealed that \textit{S. pombe SPAC16E8} and \textit{C. elegans B0024-12} encode proteins which share a high degree
of sequence identity with ScGna1p but the entire protein (25.2% identity between ScGna1p and the SPAC16E8 product and 28.9% identity between ScGna1p and the B0024-12 product) (Fig. 2). Therefore, we referred to these genes as S. pombe GNA1 (SpGNA1) and C. elegans GNA1 (CeGNA1), respectively. Although the functionality of SpGNA1 and CeGNA1 remains to be established, all the above results strongly support the idea that GNA1 encodes ubiquitous acetyltransferase necessary for UDP-GlcNac synthesis.

In UDP-GlcNac synthesis, there is a process involving acetyltransferase that is the acetylation of phospho-GlcN. Accordingly, we asked whether GNA1 specifies phospho-GlcN acetyltransferase. Both ScGna1p and CaGna1p proteins were expressed in E. coli as a fusion with GST and then purified by affinity column chromatography (Fig. 3A). When incubated with GlcN-6-P and Ac-CoA, GST-ScGna1p and GST-CaGna1p produced CoA, whereas GST alone did not (Fig. 3B). The amounts of CoA released from Ac-CoA reached a plateau within 5 min (Fig. 3B), and GlcN-6-P was preferably utilized as the substrate (Fig. 3C). Although small amounts of CoA were produced when higher concentrations of GlcN-1-P were present (at concentrations higher than 100 μM), the Kᵥ value for GlcN-1-P was about 25 times higher than that for GlcN-6-P (3.0 mM for GlcN-1-P versus 124 μM for GlcN-6-P) (Fig. 3C, Table I). No CoA production was observed when galactosamine 1-phosphate (GalN-1-P) was used as the substrate (Fig. 3C).

Synthesis of UDP-GlcNac from GlcN-6-P in Vitro—Previously, we demonstrated that together with Agm1p, Uap1p produced UDP-GlcNac from GlcN-6-P (20). If Gna1p really catalyzes the acetylation of GlcN-6-P and produces GlcNac-6-P, UDP-GlcNac would be synthesized from GlcN-6-P in the presence of Gna1p, Agm1p, and Uap1p. As expected, GST-ScGna1p and GST-CaGna1p, but not GST alone, produced [32P]UDP-GlcNac from GlcN-6-P, when the yeast Agm1p, Uap1p, and [α-32P]UTP were added to the reaction mixture (Fig. 4). A minor spot corresponding to UDP was also observed in each lane. This is presumably due to a minor contamination of [α-32P]UTP in the [α-32P]UTP preparation, because it appeared even in the absence of the enzyme (Fig. 4). Synthesis of UDP-GlcNac from GlcN-6-P required acetyl-CoA, GlcN-6-P, and Gna1p, confirming that Gna1p generates GlcNac-6-P using Ac-CoA (Fig. 5). Although Gna1p did not efficiently utilize GlcN-1-P as the substrate in the acetyltransferase assay (Fig. 3C), [32P]UDP-GlcNac was efficiently synthesized from GlcN-1-P when Agm1p, Uap1p, and [α-32P]UTP were present (Fig. 5). Moreover, phospho-GlcNac mutase remained an essential factor even for the synthesis of UDP-GlcNac from GlcN-1-P; no [32P]UDP-GlcNac was detected in the absence of Agm1p (Fig. 5). This result suggests that Agm1p recognizes both phospho-GlcN and phospho-GlcNac as the substrates; Agm1p might first convert GlcN-1-P into GlcN-6-P, providing the substrate for the GlcN-6-P acetyltransferase.

Fig. 5. Factors required for the production of UDP-GlcNac from GlcN-1-P. Approximately 0.1 μg of the purified GST-ScUap1p and 0.1 μg [α-32P]UTP in the presence or absence of 0.1 μg of GST-ScAgm1p, 200 μM GlcN-1-P, and 200 μM Ac-CoA. After incubation at 30 °C for 10 min, the reaction products were separated by polyethyleneimine-cellulose TLC and visualized by autoradiography. The positions of UDP-GlcNac, UDP, and UTP visualized under UV light are indicated.

Fig. 6. Comparison of the amino acid sequences of several acetyltransferases. The amino acid sequence of ScGna1p was compared with those of other acetyltransferases using the FASTA and BLAST programs. Amino acids that were replaced by alanine are marked by □.
for Gna1p, and then might attack GlcNac-6-P which was generated from GlcN-6-P by Gna1p. Taken together, we concluded that GNA1 encodes GlcN-6-P acetyltransferase, and defined GNA1 as glucosamine-6-phosphate acetyltransferase.

Possible Active Sites of ScGna1p—A number of acetyltransferases have been identified in various organisms. When the amino acid sequence of ScGna1p was compared with known and putative acetyltransferases, we found that although the bacterial GluM protein is not highly related to ScGna1p, some of the amino acids in the two short regions of ScGna1p are highly conserved among several acetyltransferases. These regions, designated domain I and domain II, encompass the amino acid positions from 97 to 117 and from 142 to 147, respectively. To examine the importance of these domains for catalysis, we mutated the highly conserved amino acids in these regions, Ile97, Glu98, Val102, Gly112, Leu115, Ile116, Phe142, Tyr143, and Gly147 to alanine. As a control, a nonconserved Leu119 was also converted to alanine. After confirming the nucleotide sequences, all the mutant enzymes were expressed as a fusion with GST and purified by affinity chromatography (Fig. 7A). As shown in Fig. 7B, alanine substitutions for Val102, Gly112, Leu115, Ile116, Leu119, and Gly147 only weakly impaired the activity. In contrast, those for Phe142 and Tyr143 severely diminished the activity. Furthermore, although the activity of Y143A was too low to perform kinetics, alanine substitution for the neighboring amino acid Phe142 increased the Km values for Ac-CoA and GlcN-6-P by about 10- and 5-fold, respectively (Table I). The activities of I97A and E98A were also affected to a lesser extent (Fig. 7B). Interestingly, these two mutants displayed different features of the binding affinities to the cofactor and the substrate. Mutation of Ile97 increased the Km value for GlcN-6-P by about 3-fold, whereas that of Glu98 raised the Km value for Ac-CoA by about 2.5-fold (Table I).

In human spermine/spermidine N2-acetyltransferase (HsSSAT), it was demonstrated that the amino acid sequence motif RGFIGIGS beginning at the position of 101 is required for the Ac-CoA binding and that Arg103 and the proximal glycine loops in this motif are essential for the enzyme activity (30). Moreover, the double mutant R101A/E152K acts as a dominant negative (31). In our sequence alignment, Arg101 and Gly106 of HsSSAT correspond to Gln107 and Gly112 of ScGna1p, respectively. To examine the importance of these domains for catalysis, we mutated the highly conserved amino acids in these regions Ile97, Glu98, Val102, Gly112, Leu115, Ile116, Phe142, Tyr143, and Gly147 to alanine. As a control, a nonconserved Leu119 was also converted to alanine. After confirming the nucleotide sequences, all the mutant enzymes were expressed as a fusion with GST and purified by affinity chromatography (Fig. 7A). As shown in Fig. 7B, alanine substitutions for Val102, Gly112, Leu115, Ile116, Leu119, and Gly147 only weakly impaired the activity. In contrast, those for Phe142 and Tyr143 severely diminished the activity. Furthermore, although the activity of Y143A was too low to perform kinetics, alanine substitution for the neighboring amino acid Phe142 increased the Km values for Ac-CoA and GlcN-6-P by about 10- and 5-fold, respectively (Table I). The activities of I97A and E98A were also affected to a lesser extent (Fig. 7B). Interestingly, these two mutants displayed different features of the binding affinities to the cofactor and the substrate. Mutation of Ile97 increased the Km value for GlcN-6-P by about 3-fold, whereas that of Glu98 raised the Km value for Ac-CoA by about 2.5-fold (Table I).

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DISCUSSION

In this paper, we have identified the yeast GNAI as the gene for phospho-GlcN acetyltransferase, and demonstrated that unlike the case in bacteria, yeast UDP-GlcNAc pyrophosphorylase and phospho-GlcN acetyltransferase are encoded by distinct genes. Because UAP1, AGMI, GNAI, and GFAI are all essential genes (18–20), every step of the UDP-GlcNAc synthetic is essential for the viability of yeast cells. In addition, yeast has only one type of acetylated amino sugar, that is GlcNAc; neither GalNAc nor N-acetylmannosamine is present in yeast cells. Thus, Gna1p is expected to be the sole amino sugar acetyltransferase present in yeast cells.
the S. cerevisiae YRL4 cells (33). Depletion of Pat1p function results in multiple phenotypical changes. The PAT1-deficient cells displayed enlarged cell volume, increased sensitivity to the microtubule inhibitor, benomyl, aberrant spindle structure, and incompletion of cytokinesis. Furthermore, by fluorescence-activated cell sorter analysis, it was demonstrated that PAT1 is required for multiple steps in the cell cycle, such as exit from G0, progression of DNA synthesis, and mitosis (33). All these facts suggest a physiological link between UDP-GlcNAc synthesis and cell cycle progression. As UDP-GlcNAc is mainly used as the cell wall precursor in yeast, we wonder whether Gna1p has additional substrates, such as core histones, whose acetylation is essential for gene expression and cell cycle progression; however, no histone acetyltransferase activity was detected in the recombinant ScGna1p.

Lin et al. (33) cloned the Pat1p/GNA1 homolog from S. pombe by the functional complementation of an S. cerevisiae pat1Δ null mutation. Although the amino acid sequence of the C-terminal region of S. pombe Pat1p (SpPat1p) has 245 additional amino acids at the N terminus (33). Because we could not find the DNA sequence corresponding to SpPat1p in the S. pombe data base, the presence of the extra N-terminal amino acids in SpPat1p may be unique to a certain S. pombe strain.

Sequence comparisons of the various acetyltransferases revealed that ScGna1p contains two short domains, where several amino acids are highly conserved among several known and putative acetyltransferases. By the mutation study, we found that Phe142 and Tyr143 of ScGna1p are crucial amino acids for the catalytic reaction and that Ile97, Glu98, and Gln107 are involved in the Ac-CoA binding. In contrast, alanine substitu-

sequence alignment, Y. Miyazaki for assisting with the experiments, and S. B. Miwa for reading the manuscript.

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