Interaction of GDP-4-keto-6-deoxymannose-3,5-epimerase-4-reductase with GDP-mannose-4,6-dehydratase stabilizes the enzyme activity for formation of GDP-fucose from GDP-mannose

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We cloned the GDP-4-keto-6-deoxymannose-3,5-epimerase-4-reductase gene from Arabidopsis thaliana (AtFX/GER1). The yeast Saccharomyces cerevisiae was transfected with the AtFX/GER1 gene coexpressed with GDP-mannose-4,6-dehydratase gene of A. thaliana (MUR1). In vitro GDP-fucose synthesis activity was observed in the cytoplasmic fraction of cells coexpressing the AtFX/GER1 gene and MUR1 gene. However, the cytoplasmic fraction of cells expressing MUR1 alone did not show the GDP-mannose-4,6-dehydratase activity. This result suggests that the AtFX/GER1 protein may contribute to maintenance of the MUR1 protein as the active form. Immunoprecipitation experiments showed that both proteins interact with each other, indicating that this interaction is required to maintain MUR1 protein as the active or stable form. Finally, in vivo GDP-fucose synthesis activity was analyzed by measuring the amount of GDP-fucose produced in the cytoplasm of yeast cells. The amount of GDP-fucose in cells coexpressing MURI and AtFX/GER1 genes was 3.5 times higher than the amount of GDP-mannose in the same cells, indicating that this coexpression system is suitable for production of the valuable sugar nucleotide GDP-fucose in yeast.

Key words: AtFX/GER1 gene/coexpression/GDP-fucose synthesis/MURI gene/Saccharomyces cerevisiae

Introduction

Fucose is a component of sugar chains commonly found in humans in forms such as Lewis a (Leα) and Lewis x (Leβ). Sialyl Leα, which binds to selectin, is an important element of functional sugar chains in endothelial cells (Low et al., 1990; Phillips et al., 1990; Polley et al., 1991; Walz et al., 1990). Fucosylation of these eukaryotic glycans by fucosyltransferases requires GDP-fucose as the donor substrate.

GDP-fucose is synthesized from GDP-mannose by a three-step reaction. The first step, oxidation of C-4 of mannose to keto group and reduction of C-6 of mannose to methyl residue, is catalyzed by GDP-mannose-4,6-dehydratase to yield GDP-4-keto-6-deoxymannose (Bonin et al., 1997). This enzyme requires the cofactor nicotinamide adenine dinucleotide phosphate (NADPH), which is reduced to NADPH during the reaction (Menon et al., 1999). In the second step, epimerization occurs at the C-3 and C-5 position of 4-keto-6-deoxymannose. In the third step, ketone at the C-4 position of 4-keto-6-deoxymannose is reduced, yielding GDP-fucose (Tonetti et al., 1996). The second and third steps are catalyzed by GDP-4-keto-6-deoxymannose-3,5-epimerase-4-reductase, which requires NADPH as a cofactor.

These enzymes have been well characterized, and genes have been cloned in humans, plants, and bacteria. The gene for GDP-mannose-4,6-dehydratase was isolated in Arabidopsis thaliana as MUR1 (Bonin et al., 1997), and in human as GMD (Ohyama et al., 1998; Sullivan et al., 1998). The MUR1 protein and GMD protein were expressed in Escherichia coli, and GDP-mannose dehydratase activity was determined in vitro (Bonin et al., 1997; Sullivan et al., 1998). GDP-mannose-4,6-dehydratase in E. coli was isolated and its three-dimensional structure was determined by X-ray crystallography (Somoza et al., 2000), indicating that the purified enzyme is stable and active. GDP-4-keto-6-deoxymannose-3,5-epimerase-4-reductase, which has enzyme activities for the second and third reaction steps in a single polypeptide, has been cloned in humans and E. coli (Sullivan et al., 1998; Tonetti et al., 1996).

In vitro synthesis of fucosylated sugar chains using fucosyltransferase is still problematic because of the difficulty of economically producing GDP-fucose. We recently developed a system to produce GDP-fucose in vitro by introducing GDP-fucose synthesis genes in yeast cells. Another group reported a similar system using genes of E. coli (Mattila et al., 2000), but GDP-fucose productivity was unclear. Our system allows high production of GDP-fucose because yeast cells contain a large amount of the precursor, GDP-mannose. Furthermore, yeast cells do not consume GDP-fucose, so it is accumulated inside the cells as a final product.

We report the properties of the two enzymes in the yeast system and confirm their physical interactions. Because it is unclear whether these enzymes convert GDP-mannose to GDP-fucose in a cooperative fashion, we also aimed to clarify the interaction of the enzymes with each other, the effect of interaction on enzyme activity, and how the intermediate is transferred to the next enzyme.
Our results reveal that the presence of epimerase-reductase is a prerequisite for functional expression of dehydratase in synthesis of GDP-fucose, suggesting that interaction between the two enzymes plays a significant role in regulation of GDP-fucose synthesis.

Results

Cloning of GDP-mannose-4,6-dehydratase and GDP-4-keto-6-deoxymannose-3,5-epimerase-4-reductase genes from A. thaliana

The MURI gene, which encodes GDP-mannose 4,6-dehydratase, was cloned by polymerase chain reaction (PCR) from the A. thaliana cDNA library using primers having the consensus sequence. The cloned gene was identical to that previously reported by Bonin et al. (1997). The new gene was partially different from GER1, which was deduced as GDP-4-keto-6-deoxymannose-3,5-epimerase-4-reductase gene. Six independent clones were isolated, nucleotide sequences of four clones were analyzed, and these were confirmed to be identical with the new gene shown in Figure 1. The new gene differed from the old gene in 3 amino acid deletions, 1 amino acid insertion, and a new extension of 18 amino acids. Because the amino acid sequences were completely different from that of GER1 gene, we gave the cloned gene a new name, AtFX. However the whole genome sequence data of A. thaliana were subsequently published, and the AtFX sequence was found to be identical to one in the database. The GER1 gene was later revised (Bonin and Reiter, 2000), and the revised sequence was the same as that of AtFX. We therefore used AtFX/GER1 as the gene coding for GDP-4-keto-6-deoxymannose-3,5-epimerase-4-reductase.

Expression of MURI and AtFX/GER1 genes in yeast

MURI was fused to the gene encoding influenza hemagglutinin epitope tag (3HA), whereas AtFX/GER1 was fused to the Myc tag, in frame at the C-terminus of each open reading frame for easy detection of the produced enzymes. Expression vectors in which the two genes were expressed under the geralddehyde phosphate dehydrogenase (GAPDH) promoter were introduced into S. cerevisiae cells either separately or together. Cells harboring one or both expression vectors were cultivated for 12 h. Cytoplasmic fractions were prepared as described in Material and methods, and analyzed for production of the enzymes using monoclonal antibodies against the respective tags. Both gene products were observed clearly (Figure 2). MURI protein was expressed more highly in cells transfected with both MURI and AtFX/GER1 than in cells transfected with either gene alone (Figure 2A, lanes 2, 5). However, no such difference was observed for AtFX/GER1 protein (Figure 2B, lanes 3, 5).

Assay of GDP-mannose-4,6-dehydratase and GDP-4-keto-6-deoxymannose-3,5-epimerase-4-reductase activity

Enzyme activities were measured in vitro using cytoplasmic fractions of the yeast transfectants as enzyme sources. GDP-fucose synthesis assay was conducted using GDP-mannose as substrate and NADPH as cofactor (Bonin et al., 1997). For assay of GDP-mannose-4,6-dehydratase activity alone, the same method was used, except that NADPH was excluded, and the intermediate product was reduced by adding NaBH₄ (Tonetti et al., 1996). The reaction mixture was subjected to high-performance liquid chromatography (HPLC) for measuring formation of GDP-fucose and intermediate (GDP-4-keto-6-deoxymannose) product (Tonetti et al., 1996). Control cells transfected with vector alone (no MURI or AtFX/GER1) showed no conversion of GDP-mannose to GDP-fucose or intermediate product (Figure 3a, 3j). Surprisingly, the cytoplasmic fraction of cells expressing MURI only also showed no activity (Figure 3b, 3k). As expected, enzyme extracts of cells expressing AtFX/GER1 only showed no GDP-fucose peak because the enzyme necessary for the first step of GDP-fucose synthesis was lacking (Figure 3c). The cytoplasmic fraction of MURI and AtFX/GER1 coexpressing

Fig. 1. Alignment of A. thaliana AtFX/GER1 amino acid sequence with human and E. coli GDP-4-keto-6-deoxymannose-3,5-epimerase-4-reductase (hFX, wcaG). Identical residues are shaded in gray.
cells showed a GDP-fucose peak (Figure 3e), indicating that in vitro synthesis of GDP-fucose from GDP-mannose was successful in the yeast system. However, in these assays, the amount of MUR1 protein was not normalized.

Because the amount of MUR1 protein in coexpressing cells was higher than that in MUR1-only expressing cells (Figure 2A, lane 2, 5), it is possible that limited production of MUR1 protein resulted in loss of GDP-mannose dehydratase activity. We therefore assayed GDP-fucose synthesis again after normalizing the MUR1 protein amount. GDP-fucose or intermediate product peaks were not observed in MUR1-only expressing cells even if AtFX/GER1 protein was added (Figure 3g, 3h). In contrast, extract of MUR1 and AtFX/GER1 coexpressing cells that contained the same amount of MUR1 protein as extract of MUR1-only expressing cells showed GDP-fucose synthesis activity (Figure 3i). This result suggests that GDP-mannose dehydratase activity does not depend on amount of MUR1 protein expressed but on MUR1 enzyme stability.

Bonin et al. (1997) reported that MUR1 protein in MUR1-only expressing E. coli showed activity, which is contrary to our result in yeast cells. E. coli cells have inherent GDP-4-keto-6-deoxymannose-3,5-epimerase-4-reductase enzyme encoded by wcaG gene, and the wcaG protein has high homology to AtFX/GER1 protein (Figure 1). Therefore the wcaG protein may function as a substitute for AtFX/GER1 protein. To test this hypothesis, we cloned wcaG gene from E. coli with Myc tag at the C-terminus and coexpressed it with MUR1 gene in yeast. The wcaG protein was expressed well in yeast cells (Figure 2B, lane 4, 6), and GDP-fucose synthesis activity was observed in assay of the cell lysate (Figure 2B).

**Expression of GDP-fucose synthesis genes in yeast**

Because enzyme activity was observed in cytoplasmic fractions of MUR1 and AtFX/GER1 or MUR1 and wcaG coexpressing cells and not in that of MUR1-only expressing cells, it is possible that MUR1 protein had some interaction with AtFX/GER1 protein or wcaG protein. To investigate such interactions, we performed immunoprecipitation analysis using anti-Myc antibody and Protein A Sepharose. Because AtFX/GER1 protein and wcaG protein have Myc tag at the C-terminus, MUR1 protein would be coprecipitated with them if such an interaction took place. As expected, western blotting showed a MUR1 protein band in MUR1 and AtFX/GER1 coexpressing cells and in MUR1 and wcaG coexpressing cells (Figure 4A, lane 4, 5). The amount of MUR1 protein in MUR1 and AtFX/GER1 coexpressing cells was very close to that in MUR1 and wcaG coexpressing cells (Figure 4A, lane 4, 5), even though productivity of wcaG protein was higher than that of AtFX/GER1 protein (Figure 4B, lane 4, 5). This result indicates that interaction between MUR1 protein and AtFX/GER1 protein is stronger than that between MUR1 protein and wcaG protein. MUR1 protein production was not observed in cytoplasmic fractions of cells expressing MUR1, AtFX/GER1, or wcaG only (Figure 4A, lanes 1–3).

Because levels of MUR1 protein in MUR1 and AtFX/GER1 coexpressing or MUR1 and wcaG coexpressing cells were much higher than in MUR1-only expressing cells, it is unlikely that the detected band was due to nonspecific binding to antibody or Protein A Sepharose in coexpressing cells. To confirm that the interaction was specific, we coexpressed HA-tagged MUR1 and native AtFX/GER1, then subjected cytoplasmic fraction from these cells

**Fig. 3. In vitro GDP-mannose-4,6-dehydratase and GDP-4-keto-6-deoxymannose-3,5-epimerase-4-reductase assay.** (a–i) Assay of GDP-fucose synthesis activity. (j–l) Assay of GDP-mannose-4,6-dehydratase activity. Enzyme activities were assayed as described in Materials and methods. These reaction samples were analyzed by HPLC using reverse phase column. (a) W303 harboring only vectors. (b) W303 harboring expression plasmid for MUR1. (c) W303 harboring expression plasmid for AtFX/GER1. (d) W303 harboring expression plasmid for wcaG. (e) W303 harboring expression plasmids for MUR1 and AtFX/GER1. (f) W303 harboring expression plasmids for MUR1 and wcaG. (g) 240 μg of total protein of MUR1 singly expressed cytoplasmic fraction was used for assay. (h) 240 μg of total protein of AtFX/GER1 only expressing cytoplasmic fraction was added to g. (i) 40 μg of total protein of MUR1 and AtFX/GER1 coexpressing cytosolic fraction was used for assay. (j) W303 harboring only vector. (k) W303 harboring expression plasmid for MUR1. (l) W303 harboring expression plasmids for MUR1 and AtFX/GER1. A, B, and C indicate retention time of GDP-mannose, GDP-fucose, and the intermediate product.

**Fig. 4. Immunoprecipitation analysis using anti Myc antibody.** (A) Detection by anti-HA antibody. (B) Detection by anti Myc antibody. Lane 1, W303 harboring expression plasmid for HA-tagged MUR1 gene; Lane 2, W303 harboring expression plasmid for Myc-tagged AtFX/GER1 gene; Lane 3, W303 harboring expression plasmid for Myc-tagged wcaG gene; Lane 4, W303 harboring expression plasmids for HA-tagged MUR1 gene and Myc-tagged AtFX/GER1 gene; Lane 5, W303 harboring expression plasmids for HA-tagged MUR1 gene and Myc-tagged wcaG gene; Lane 6, W303 harboring expression plasmids for HA-tagged MUR1 gene and AtFX/GER1 without tag gene. Arrow on A indicates HA-tagged MUR1 protein. Upper arrow on B indicates Myc-tagged wcaG protein and lower arrow indicates Myc-tagged AtFX/GER1 protein.
to immunoprecipitation analysis using anti-Myc antibody and Protein A Sepharose. The amount of MUR1 protein in cells coexpressing MUR1 and AtFX/GER1 without Myc tag was almost the same as that in cells coexpressing MUR1 and Myc-tagged AtFX/GER1 or wcaG; however, the MUR1 protein band was not detected after immunoprecipitation (Figure 4A, lane 6). This result shows that the observed MUR1 protein bands are specifically coprecipitated by anti-Myc antibody.

The amounts of MUR1 protein, AtFX/GER1 protein and wcaG protein were calculated by densitometric analysis of the western blot results in Figure 4. Based on the amounts of these proteins, the ratios of interacting MUR1 protein/AtFX/GER1 protein and MUR1 protein/wcaG protein were approximately 3:1 and 1:1, respectively. The free MUR1 protein was also calculated by comparing the amounts of MUR1 protein, AtFX/GER1 protein, and wcaG protein observed in western blot (Figure 2 and Figure 4), indicating that no free MUR1 protein is present in MUR1 and AtFX/GER1 or wcaG coexpressing cells.

**Activity of GDP-fucose synthesis enzymes in vivo**

To confirm the activity of GDP-fucose synthesis enzymes in yeast cell cytoplasm, we quantitated GDP-mannose and GDP-fucose in cell extracts (Shimma et al., 1997). Cells were grown in SD-leu and -ura medium until OD 1.0 at 30°C, and then pelleted. Formic acid (1 M) saturated with 1-butanol was added to the pellet, and cytoplasmic GDP-mannose and GDP-fucose were extracted. The extracts were subjected to Mono-Q column chromatography, and GDP-sugar fractions were isolated. These fractions were analyzed by HPLC, and quantities of GDP-mannose and GDP-fucose were determined by peak areas.

Only GDP-mannose was detected in wild-type yeast cells, and in transfectants expressing MUR1, AtFX/GER1, or wcaG gene only (Figure 5a–d). The level of GDP-mannose was about 1.0 nmol per 2×10⁸ cells (Table I). As expected, GDP-fucose and GDP-mannose were detected in MUR1 and AtFX/GER1, or MUR1 and wcaG coexpressing cells (Figure 5e, 5f), with quantitated levels of ~3.5 nmol and 1.5 nmol per 2×10⁸ cells, respectively (Table I). Levels of GDP-fucose and GDP-mannose in these coexpressing transfectants were respectively 3.5 times and 1.5 times higher than corresponding GDP-mannose levels in wild-type cells. In analogy to results of enzyme assay, intermediate peak was not observed and quantity of GDP-mannose remained the same in MUR1-only expressing cells. These results indicate that MUR1 protein in MUR1-only expressing cells showed no enzyme activity in vivo or in vitro, whereas when AtFX/GER1 or wcaG was present in the vicinity of MUR1 in the coexpressing cells, GDP-mannose was effectively converted to GDP-fucose both in vivo and in vitro.

**Discussion**

We cloned the AtFX/GER1 gene that encodes GDP-keto-6-deoxymannose-3,5-epimerase-4-reductase of *A. thaliana*. This gene was coexpressed in yeast *S. cerevisiae* with MUR1 gene that encodes GDP-mannose-4,6-dehydratase of *A. thaliana* (Figure 2) and the GDP-mannose to GDP-fucose converting activity was observed in vivo and in vitro (Figures 3 and 5).

MUR1 protein in MUR1-only expressing yeast cells did not show any dehydratase activity (Figure 3). The amount of MUR1 protein produced by MUR1 and AtFX/GER1 or MUR1 and wcaG coexpressing cells was six times higher than that in MUR1-only expressing cells (Figure 2A). It is plausible that in the MUR1-only expressing cells, degradation of MUR1 protein occurred through denaturation,
resulting in instability and loss of activity (Figure 6). The results presented here suggest that the AtFX/GER1 and wcaG proteins may function to maintain MUR1 protein in active form by stabilizing its conformation. However, once MUR1 protein is in an active and stable form, further stabilization of conformation seems to be unnecessary (Figure 6). AtFX/GER1 protein disappeared when MUR1 and AtFX/GER1 coexpressing cells were cultivated for 60 h, but MUR1 protein still remained active (data not shown). This is consistent with the previously reported activity of purified MUR1 protein (Bonin et al., 1997).

Immunoprecipitation experiments showed that MUR1 protein interacts with AtFX/GER1 protein and wcaG protein (Figure 4). This interaction is necessary to maintain the active and stable form of MUR1 protein. The amount of AtFX/GER1 protein needed to coprecipitate MUR1 protein was less than the amount of wcaG protein needed (Figure 4A, lane 5, 6; 4B, lane 5, 6). This suggests that MUR1 protein interacts more strongly with AtFX/GER1 protein than with wcaG protein. In vitro experiments also support this hypothesis. GDP-fucose synthesis activity of MUR1 and AtFX/GER1 coexpressing cytoplasmic fraction was higher than that of MUR1 and wcaG coexpressing fraction, even though amount of AtFX/GER1 protein was less than that of wcaG protein (Figures 2 and 3).

The interaction between dehydratase and epimerase-reductase may have a significant role in vivo. The intermediate GDP-4-keto-6-deoxymannose is unstable, as reported earlier. If these two GDP-fucose synthesis enzymes existed separately, the intermediate would be broken down. However, if the dehydratase and epimerase-reductase form a complex, the intermediate can be quickly transferred from the former to the latter, and efficient synthesis of GDP-fucose is possible (Figure 6). Thus GDP-fucose synthesis activity of MUR1 and AtFX/GER1 coexpressing cytoplasmic fraction was higher than that of MUR1 and wcaG coexpressing cytoplasmic fraction due to strong interaction between MUR1 protein and AtFX/GER1 protein. In addition, this dehydratase and epimerase-reductase interaction may also have another role in controlling the amount of GDP-mannose and GDP-fucose in vivo. GDP-mannose-4,6-dehydratase activity is inhibited by GDP-fucose (Koizumi et al., 2000). However, GDP-mannose-4,6-dehydratase activity is not inhibited in cells where only GDP-mannose-4,6-dehydratase is active due to the absence of both GDP-4-keto-6-deoxymannose-3,5-epimerase-6-reductase and GDP-fucose, resulting no accumulation of GDP-mannose. The lack of GDP-mannose causes inhibition of N-linked oligosaccharide synthesis, then yielding cell death, because GDP-mannose is essential for the synthesis of N-linked endoplasmic reticulum core oligosaccharide. Therefore the GDP-mannose-4,6-dehydratase activity without GDP-4-keto-6-deoxymannose-3,5-epimerase-4-reductase will be toxic for yeast cells. This may be the other reason for the absence of MUR1 enzyme activity in MUR1-only expressing cells.

The methods for chemical or biological synthesis of GDP-fucose have been reported (Adelhorst and Whitesides, 1993; Ichikawa et al., 1992; Yamamoto et al., 1984). Synthesizing GDP-fucose using these methods was expensive, because it required expensive starting materials or enzymes. Recently, Koizumi et al. (2000) reported a method for GDP-fucose synthesis using bacteria. This method is capable of producing large amounts of GDP-fucose but still has some problems. First, for production of GDP-fucose using their system, three E. coli gene transfectants are needed, E. coli’s GDP-mannose synthesis enzymes, GDP-mannose-4,6-dehydratase (gmd), and GDP-4-keto-6-deoxymannose-3,5-epimerase-4-reductase (wcaG), along with Corynebacterium ammoniagenes for production of GDP-fucose. Furthermore, they used detergent and organic solvents to transfer intermediates from transfectant to transfectant. Therefore new cells are required for each reaction. Second, feeding the starting materials, GMP and mannose, were essential.

Fig. 6. Predicted model for maintaining the MUR1 protein in an active form by the interaction with the AtFX/GER1 protein.
Finally, because *E. coli*’s GDP-mannose-4,6-dehydrase activity is strongly suppressed by low concentration of GDP-fucose, a low yield of GDP-fucose resulted from using the *gmd* transfectant and *wcaG* transfectant simultaneously (Koizumi et al., 2000). Therefore they had to add the *wcaG* transfectants after accumulation of the intermediate GDP-4-keto-6-deoxymannose product. Thus their method is complicated and requires monitoring the accumulation of GDP-4-keto-6-deoxymannose.

Compare to Koizumi et al. (2000) method, our system is simpler and easier. Our method requires the culture of *MURI* and *AtFX/GERI* coexpressing yeast only, without the starting materials or the control of intermediate accumulation. Therefore it is easy to scale up the production level. Mattila et al. (2000) also have reported a similar GDP-fucose production system, but they failed to show the amount of GDP-fucose. They used the *E. coli gmd* gene (the same gene used in the method of Koizumi et al.) instead of *MURI* gene. Because GDP-mannose-4,6-dehydratase encoded by the *gmd* gene is inhibited its activity by low concentration of GDP-fucose, enough GDP-fucose may be not accumulated in their system.

Commercially available GDP-mannose is produced by yeast at a price about 30 times lower than that of GDP-fucose. In this study, we showed that the amount of GDP-fucose was 3.5 times larger than that of GDP-mannose using *MURI* and *AtFX/GERI* coexpressing yeast. The result indicates that the efficient production of GDP-fucose is possible using *MURI* and *AtFX/GERI* coexpressing yeast system. We believe that our GDP-fucose synthesis method using *MURI* and *AtFX/GERI* coexpressing yeast is more suitable than any previous reported GDP-fucose production methods.

In conclusion, we successfully cloned *AtFX/GERI* gene from *A. thaliana* cDNA library and coexpressed it with *A. thaliana MURI* gene in yeast. AtFX/GERI protein in combination with MURI protein not only showed GDP-fucose synthesis ability but also formed a complex with MURI protein and stabilized its activity. These results show that the yeast expression system is useful for analysis of cofactors or interaction with a protein of foreign origin that does not exist in yeast.

Materials and methods

Strains and plasmids

*S. cerevisiae* strain W303-1A (MATa leu2-3,112 his3-11,15 ade2-1 ura3-1 trp1-1 can1-100) was used for expression of *MURI, AtFX/GERI*, and *wcaG* genes. *E. coli* strain DH5α (*deoR endA1 gyrA96 hsdR17(rk- mK-) recA1 relA1 supE44 thi-1 Δ[lacZΔMX-argF]U169 80lacZΔM15 F- λ-) was used for preparation and construction of plasmids. *S. cerevisiae* cells were grown in YPD (2% Bacto peptone, 1% yeast extract, 2% glucose) or SD -leu and -ura (0.67% Bacto yeast nitrogen base without amino acids, 2% glucose, and 20-400 μg/ml amino acid mixture lacking leucine and uracil). *E. coli* cells were grown in Luria Bertani broth (1% Bacto tryptone, 0.5% yeast extract, 0.5% NaCl, 0.2% glucose).

*MURI* gene was amplified by PCR with primers (5'-GTCGAATTCTAGGCGTCAGAAACACC-3' and 5'-GAACCTCGAGGTTGCTGCTTAGC ATC-3') that incorporated EcoRI and XhoI sites. *AtFX/GERI* gene was amplified by PCR with primers (5'-ATTGGTACCATG TCTGCAAATCTGCCAAAATCTCTGC-3' and 5'-TTAGTGCAGCATATCTCGTGGAAACATTCT TCAAATACCAATCATAG-3') that incorporated KpnI and EcoRV sites. For amplification of the genes the cDNA library of *A. thaliana* (Arabidopsis QUICK-Clone cDNA, Clontech, Palo Alto, CA) was used as the template DNA. *WcaG* gene was amplified by PCR with primers (5'-AAGGTACCATGAGTAAACAGGATT TTTATGTCGTCG-TC-3' and 5'-TTGTGACCTTACCCGGGCG AAAGC-GGTCTTGTATTCTCAAGGAAC-3') that incorporated KpnI, Smal, and SalI sites. Genomic DNA of *E. coli* was used for amplification of *wcaG* gene, and the amplified genes were ligated into pCR2.1.

After confirming the nucleotide sequence by sequencing, these genes were introduced into expression vectors. *MURI* gene was cut by EcoRI and XhoI restriction enzymes and introduced into *EcoRI–XhoI* sites of the expression vector YEp352GAP, which contains the GAPDH promoter (Kainuma et al., 1999). A triple influenza hemagglutinin epitope (HA) gene fragment was excised from HAp316 vector with *NraI* and *Eco136I* and inserted into *PvuII* site of YEp352GAP that contains the *MURI* gene; this plasmid was designated YEp-MURIHA. Gene of triple c-Myc epitope tag (Myc) was amplified by PCR with primers (5'-GGTGAA ACAAAAATGTGATTCGAGAAGAT-3' and 5'-CTAGAAGGTCCAAGTCTCTTTCTGAGA TAA-3'). This PCR product was introduced directly to *EcoRV* site of *AtFX/GERI* gene and *SalII* site of *wcaG* gene. *Myc* and *AtFX/GERI* fusion gene (AtFXMyc) was cut by *KpnI* and *XhoI* enzymes and inserted into *KpnI–SalI* site of YEp352GAP-II of which the multicloning site was exchanged for *EcoRI–SalI* oligomer of pUC vector. The gene fragment containing a GDPH promoter, AtFXMyc, and a GAPDH terminator was cut by *BamHI*, and this fragment was inserted to *BamHI* site of pYO325 (Sikorski and Hieter, 1989). This plasmid was designated pYO-AtFXMyc. The same method was followed to construct the plasmid pYO-wcaGMyc. WcaGMyc gene was cut by *KpnI* and *SalI* and inserted into YEp352GAP-II. A GDPH promoter, wcaGMyc, and a GAPDH terminator fragment were obtained by *BamHI* digestion and introduced in pYO325. This plasmid was designated pYO-wcaGMyc. Expression vector of AtFX/GERI protein without Myc tag (pYO-AtFX) was constructed similarly to the construction of pYO-AtFXMyc, except that insertion of Myc tag epitope was not performed.

Preparation of cytoplasmic fractions

The yeast W303 was transformed with expression plasmids YEp352GAP, pYO325, YEp-MURIHA, pYO-AtFXMyc, pYO-wcaGMyc, and pYO-AtFX and grown in 200 ml of SD -leu and -ura medium for 12 h at 30°C. Cells were collected by centrifugation at 3000 × g for 5 min, washed with 1% KCl, and resuspended in 5 ml 100 mM Tris–HCl (pH 7.5), 2 mM dithiothreitol (DTT), protease inhibitor (1 tablet of Complete/50 ml, Roche, Mannheim, Germany; 1% apro tinin, Sigma, St. Louis, MO). Glass beads (0.45–0.5 μm)
were added to half the cell suspension volume and homogenized by vortex mixer for 1 min; this was repeated three times with cooling. Homogenates were filtered by G1 glass filter and centrifuged at 10,000×g for 20 min. Supernatant was collected and centrifuged at 100,000×g for 1 h. The supernatant was collected as cytoplasmic fraction, and (NH₄)₂SO₄ (516 mg/ml) was added. After 1 h at 4°C, the sample was centrifuged at 10,000×g for 20 min, and the pellet was dissolved in water. The resulting sample was desalted by Fast Desalting Column HR 10/10 (Amersham Pharmacia AB, Little Chalfont, UK) with 20 mM Tris–HCl (pH 7.5) containing 1 mM ethylenediaminetetraacetic acid (EDTA).

Western blot analysis

Protein concentration was determined by BCA protein assay reagent. Sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) was performed using 100 μg protein from the cytoplasmic fraction. Proteins were then transferred to polyvinylidene fluoride membrane filter using electrolotter (0.4 A, 1 h). After incubation of the membrane filter for 1 h in 3% skim milk (Difco, Detroit, MI), 100 mM Tris–HCl (pH 7.5), and 500 mM NaCl (blocking buffer), it was transferred to 10 ml of anti-HA monoclonal antibody (16B12, Convance, Berkeley, CA) or anti-Myc monoclonal antibody (9E10, Convance) at a dilution of 1:1000 in blocking buffer with 0.05% Tween 20. The membrane filter was incubated for 1 h at room temperature; washed three times with 100 mM Tris–HCl (pH 7.5), 500 mM NaCl, and 0.5% Tween 20 (wash buffer) for a total of 30 min; and then incubated for 1 h with anti-mouse IgG alkaline phosphatase conjugate (ICN Pharmaceuticals, Inc., Aurora, OH) at a dilution of 1:1000. The membrane filter was then washed three times and incubated with 10 ml of 100 mM Tris–HCl (pH 9.5), 100 mM NaCl, and 50 mM MgCl₂ containing nitro blue tetrazolium chloride and X-phosphate. Coloring reaction was stopped by washing the membrane filter for 5 min with 50 ml of 10 mM Tris–HCl (pH 8.0) containing 1 mM ethylenediaminetetraacetic acid (EDTA).

Assay of GDP-fucose synthesis activity and GDP-mannose-4,6-dehydratase activity

GDP-fucose synthesis activity was assayed in 50 μl of 20 mM Tris–HCl (pH 7.5), 10 mM EDTA, 5 mM NADPH, and 1 mM GDP-mannose for 1 h at 37°C. Each cytoplasmic fraction containing 400 μg protein was used for the standard assay. The reaction was stopped by boiling for 3 min, followed by centrifugation for 1 min at 15,000 rpm. Supernatants were filtered by Ultrafree C3LGC to remove proteins having molecular weight >10 kDa. Samples were analyzed by HPLC using Wakosil 5C18-200 (4.6×250 mm) column with 0.5 M KH₂PO₄ as running buffer at a flow rate of 1.0 ml/min (Tonetti et al., 1996). GDP-mannose and GDP-fucose were detected by absorbance at 254 nm.

GDP-mannose-4,6-dehydratase activity was assayed in 50 μl of 20 mM Tris–HCl, pH 7.5, 10 mM EDTA, 1 mM GDP-mannose for 1 h at 37°C. After the reaction was completed, the intermediate product was reduced by adding 1 μmol of NaBH₄ and incubating for 90 min at 37°C (Tonetti et al., 1996).

Immunoprecipitation

A cytoplasmic fraction containing 500 μg protein was pre-incubated with Protein A Sepharose (Amersham Pharmacia Biotech AB) in 10 μl suspension of water overnight at 4°C, followed by centrifugation for 1 min at 15,000 rpm. Two microliters of anti-Myc monoclonal antibody was added to the supernatant and incubated for 1 h at 4°C, then 10 μl of Protein A Sepharose was added and incubated for 3 h at 4°C. Protein A Sepharose was pelleted by centrifugation for 1 min at 15,000 rpm and washed three times with the wash buffer used in western blot analysis. SDS-PAGE sample buffer (20 μl) was added, and the proteins analyzed by western blot analysis as described.

Extraction of GDP-sugars from yeast cells

Yeast cell culture (10 ml) was cultivated until OD₆₀₀ 1.0 (2×10⁶ cells) and harvested. Two milliliters of ice-cold 1 M formic acid saturated with 1-butanol were added to the cells and incubated for 30 min at 0°C (Shimma et al., 1997). The supernatant was collected and dried by lyophilization. The dried sample was dissolved in 200 μl water, and the nucleotide diphosphate sugar fraction was isolated by Mono-Q column chromatography using SMART System (Amersham Pharmacia Biotech AB). The Mono-Q column was equilibrated with 10 mM KH₂PO₄ at a flow rate of 100 μl/min. After 5 min of sample injection, the ratio of 0.5 M KH₂PO₄ was increased linearly up to 100% for 20 min. Isolated nucleotide diphosphate sugar fractions were analyzed by HPLC as described previously.

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Abbreviations

DTT, dithiothreitol; EDTA, ethylenediaminetetraacetic acid; GAPDH, glyceraldehyde phosphate dehydrogenase; HPLC, high-performance liquid chromatography; Le, Lewis; NADP, nicotinamide adenine dinucleotide phosphate; PCR, polymerase chain reaction; SDS-PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis.

References

