Photoaffinity labelling with P^3-(4-azidoanilido)uridine 5′-triphosphate identifies Gpi3p as the UDP-GlcNAc-binding subunit of the enzyme that catalyses formation of GlcNAc-phosphatidylinositol, the first glycolipid intermediate in glycosylphosphatidylinositol synthesis

Zlatka KOSTOVA*[1], David M. RANCOUR†[1], Anant K. MENON† and Peter ORLEAN*[2].

*Department of Biochemistry, University of Illinois at Urbana-Champaign, Roger Adams Laboratory, 600 South Mathews Avenue, Urbana, IL 61801, U.S.A., and †Department of Biochemistry, University of Wisconsin-Madison, 433 Babcock Drive, Madison, WI 53706, U.S.A.

Glycosylphosphatidylinositols (GPIs) are made by all eukaryotes. The first step in their synthesis is the transfer of GlcNAc from UDP-GlcNAc to phosphatidylinositol (PI). Four proteins in mammals and at least three in yeast make up a complex that carries out this reaction. Three of the proteins are highly conserved between yeast and mammals: the Gpi1 protein, the Pig-C/Gpi2 protein and the Pig-A/Gpi3 protein. The function of the individual subunits is not known, but of the three, the Pig-A/Gpi3 proteins resemble members of a large family of nucleotide-sugar-utilizing glycosyltransferases. To establish whether Gpi3p is the UDP-GlcNAc-binding subunit of the yeast GlcNAc-PI synthetic complex, we tested its ability to become cross-linked to the photoactivatable substrate analogue P^3-(4-azidoanilido)-uridine 5′-triphosphate (AAUTP). We report that Gpi3p bearing the FLAG epitope at its C-terminus becomes cross-linked to AAUTP[α-^{32}P], but that Gpi2p-FLAG does not. Furthermore, Gpi3p-FLAG expressed in *Escherichia coli* is also cross-linked. These results indicate that Gpi3p is the UDP-GlcNAc-binding and probable catalytic subunit of the GlcNAc-PI synthetic complex.

Key words: endoplasmic reticulum, glycosyltransferase, GPI anchor.

INTRODUCTION

Glycosylphosphatidylinositols (GPIs), which contain the structural motif Manα1-4GlcNα1-6Ins-PO_4-lipid, are found in all eukaryotic cells. GPIs can be extended by mannosylation and phosphoethanolamine addition to form a structure (NH_2-CH_2-CH_2-PO_4-6Manα1-2Manα1-6Manα1-4GlcNα1-6Ins-PO_4-lipid) that can be covalently linked to the C-terminus of certain secretory glycoproteins to act as a membrane anchor [1–4]. However, not all GPIs are transferred to protein, and some remain instead as 'free' GPIs. In certain protozoa, the Manα1-4GlcN-α1-6Ins-PO_4-lipid core is converted into cell-surface glycoinositol phospholipids and lipophosphoglycans.

GPIs are assembled in a stepwise pathway whose enzymes are localized in subcompartments of the endoplasmic reticulum, as well as other intracellular membranes. Biosynthesis of all GPIs is initiated by the transfer of GlcNAc from UDP-GlcNAc to an acceptor phosphatidylinositol (PI) ([5]; also reviewed in [1,3,4,6,7]). This first step is mediated by a complex of four proteins in mammals and at least three proteins in yeast. The mammalian proteins, Gpi1p, Pig-Cp and Pig-Ap [8–11], have their sequence and functional homologues in the *Saccharomyces cerevisiae* Gpi1, Gpi2 and Gpi3 proteins respectively [12–16]. However, GlcNAc-PI synthesis in mammals also requires the Pig-H protein [17], which, so far, has no obvious homologue in yeast. The genes for these proteins were cloned by complementation of mammalian GPI anchoring-defective cell lines and temperature-sensitive yeast *gpi* mutants.

The mammalian Gpi1, Pig-C, Pig-A, and Pig-H proteins form a complex [8,18]. The subunit stoichiometry of the complex and the exact biochemical function of the subunits is less clear. In only one case does the deduced amino acid sequence of the protein suggest a function: Gpi3p/Pig-Ap resembles members of a superfamily of nucleoside diphosphate sugar-utilizing α-glycosyltransferases [16,19,20], which includes the *rfaK* protein, a putative UDP-GlcNAc-utilizing transferase involved in lipopolysaccharide biosynthesis in *Salmonella typhimurium* [21]. This raises the possibility that Gpi3p is the catalytic subunit of the GlcNAc-PI synthetic complex. Of the other subunits, Gpi1p is least likely to be catalytic: deletion of the yeast *GPI1* gene yields viable, though temperature-sensitive cells, suggesting a role for Gpi1p in stabilizing the complex [8,14]. Gpi1p indeed has such a role in mammalian cells: it is required for association of Pig-Cp with a complex of Pig-Ap and Pig-Hp [22]. The hydrophobic Gpi2/Pig-Cp protein has been suggested to be involved in recognition of the acyl chains of the GlcNAc-accepting PI species [8], and as such, this member of the GlcNAc-PI synthetic complex may present the lipid to the glycosyltransferase subunit.

To determine which of the essential proteins of the yeast GlcNAc-PI synthetic complex binds UDP-GlcNAc, we tested Gpi2p and Gpi3p for their ability to become cross-linked to the photoactivatable UDP-GlcNAc analogue P^3-(4-azidoanilido)-uridine 5′-triphosphate (AAUTP) [23]. We report that the photoprobe becomes cross-linked to Gpi3p, but not Gpi2p, and that Gpi3p is therefore the UDP-GlcNAc-binding subunit.

EXPERIMENTAL

Materials

UDP-[U-^{14}C]GlcNAc (specific radioactivity of 283 mCi/mmol) was obtained from NEN Life Science Products (Boston, MA,

Abbreviations used: AAUTP, P^3-(4-azidoanilido)-uridine 5′-triphosphate; GalT, galactosyltransferase; GPI, glycosylphosphatidylinositol; PI, phosphatidylinositol; RT-PCR, reverse transcriptase PCR.

[1] These authors contributed equally to this work.

[2] To whom correspondence should be addressed (e-mail p-orlean@uiuc.edu).

U.S.A.). Lyticase, palmitoyl-CoA and tunicamycin were obtained from Sigma, and Nikkomycin-Z was purchased from Calbiochem. Silica Gel 60 TLC plastic-backed sheets were purchased from Merck. X-Ray Films, X-OMAT AR and BIOMAX-MS, and Transcreen-LE intensifying screens were from Eastman Kodak Company (Rochester, NY, U.S.A.). Restriction endonucleases, T4 DNA ligase, and T4 DNA polymerase were purchased from Gibco BRL. GeneClean was obtained from Bio 101 (La Jolla, CA, U.S.A.). *Pwo* polymerase and EDTA-free protease inhibitor tablets were obtained from Boehringer Mannheim. Anti-FLAG M2 monoclonal antibody (where FLAG corresponds to Asp-Tyr-Lys-Asp-Asp-Asp-Asp-Lys) was obtained from Eastman Kodak and Sigma, and horseradish peroxidase-labelled goat-anti-mouse IgG was from Kirkegaard and Perry Laboratories (Gaithersburg, MD, U.S.A.). Hybond nitrocellulose membranes and the ECL® Western blotting detection reagent were from Amersham Life Science. Oligonucleotides were synthesized at the University of Illinois Genetic Engineering and Sequencing Facility. The vectors pET-11d and p6HisF-11d, and the *Escherichia coli* BL21(DE3) strain were gifts from C.-M. Chiang (University of Illinois), and pET-13a was provided by M. Churchill (University of Illinois).

Yeast strains and culture media

The temperature-sensitive *gpi3* allele was described previously [13]. Other yeast strains used in this study were derived from YMW3, a diploid obtained by crossing strains YMW1 and YMW2 [24] (genotype: *MATa/α*; *ade2-1*; *ade3Δ22*; *his3-11,15*; *leu2-3,112*; *trp1-1*; *ura3-1*; *can1-100*).

Yeast strains were routinely grown on YPD [yeast extract/peptone/2% (w/v) dextrose] medium or on selective SD (synthetic minimal) medium [25]. Strains harbouring plasmids on which *GPI2* or *GPI3* were expressed behind the *GAL10* promoter were maintained on SGly medium [0.67% Yeast nitrogen base, 0.2% yeast extract and 3% (v/v) glycerol]. Diploid strains were sporulated on 2% (w/v) Bacto-agar plates containing 1% (w/v) potassium acetate.

The *GPI2* and *GPI3* genes were disrupted by replacing 88 and 97% of their respective coding regions with the *kanMX4* module using the procedure of Wach [26]. High-fidelity *Pwo* polymerase was used for all amplifications by PCR. Linear *gpi2::kanMX4* and *gpi3::kanMX4* DNA fragments were used to transform diploid strain YMW3 to be resistant to 200 μg of geneticin/ml. Integration of the disrupting DNA cassettes at the correct chromosomal loci was verified by whole-cell PCR using an oligonucleotide primer complementary to the chromosomal region upstream of the disrupted gene, and a primer complementary to the internal *kanMX4* sequence. Heterozygous diploids were sporulated and submitted to tetrad analysis to confirm the lethal disruption of the *GPI2* and *GPI3* genes

Expression of FLAG-tagged Gpi2p and Gpi3p in yeast

An intronless copy of *GPI3* was obtained by reverse transcriptase-PCR (RT-PCR) using yeast mRNA as the template. Total yeast mRNA (140 μg) was extracted from wild-type strain XM37-10c using the Qiagen RNEasy Kit, and mRNA was isolated from this material using the Promega mRNA Kit. The product was used for the amplification of the target *GPI3* cDNA using the Gibco BRL Superscript preamplification system for first strand cDNA synthesis. The first strand cDNA obtained was then used as the template in a PCR amplification reaction using the oligonucleotide primers 5′USNde (5′-CATTATTATATAAACATATGGGCTTCAATATAGC-3′) and 3′BSBam (5′-CTATCTTATCCCTACTTTGTTTTCTC-3′). The former introduces an *Nde*I site at the ATG codon at the 5′ end of the cDNA, and the latter introduces a 3′ *Bam*HI site, which is present in the *GPI3* coding region. The sequence of the resulting 570 bp DNA fragment was obtained to verify the presence of spliced 5′ *GPI3* cDNA, and *Nde*I and *Bam*HI sites at its 5′ and 3′ ends respectively. The complete, intronless *GPI3* gene was assembled by cloning the 570 bp *Nde*I–*Bam*HI fragment and a 1.3 *Bam*HI–*Eco*RI fragment that included the remaining 3′ *GPI3* coding sequence into p6HisF-11d [27].

For expression of epitope-tagged Gpi3p in yeast, primers 5′*GPI3*pMW20 (5′-CTACAGCTGAGCTCACTCTAAC-3′) and 3′*GPI3FLAG*Sma (5′-TCCCCCGGGTGATCATTAGGGGGTGGTTTTATCGTCATCGTCTTTGTAGTCAGTTTCTCTTGCTTCCTTCGTCTC-3′), and *Pwo* polymerase were used to amplify a *Sac*I–*Sma*I fragment encoding C-terminally FLAG-tagged Gpi3p. The resulting 1.5 kb fragment was cloned into the *Sac*I and *Sma*I sites behind the GAL10 promoter of plasmid pMW20 [24] to give pMW20-*GPI3-FLAG*. Primer 3′*GPI3FLAG*Sma introduces DNA encoding the C-terminal FLAG epitope. A pMW20-*GPI3* control plasmid was made analogously, but using the 3′ primer 5′-TCCCCCGGGTTAGACGAGCTTCCAGCTG-3′.

For expression of epitope-tagged Gpi2p in yeast behind its native promoter, a DNA fragment encoding C-terminally FLAG-tagged Gpi2p was amplified from genomic DNA using the oligonucleotide primers 5′Sac*GPI2FLAG* (5′-GATAGAGCTCCTGAATTTCTAATATAG-3′; *Sac*I site underlined) and 3′*GPI2FLAG*Bam (5′-CGCGGATCCTATTAGGGGGTGGTTTTATCGTCATCGTCTTTGTAGTCATCCAATATTGGTGTTCTTGCATC-3′). The resulting 1.3 kb fragment was cloned into the 2 μm plasmid pRS425. For cloning into pMW20, a 930 bp DNA fragment was amplified using oligonucleotide primers 5′BamEco*GPI2* (5′CGCGGATCCGGAATTCAATAGGTACAATGAC3′; *Bam*HI and *Eco*RI sites underlined) and 3′*GPI2FLAG*Bam, and cloned into the *Eco*RI–*Bam*HI sites of pMW20. Similarly, pMW20-*GPI2* was made by amplifying a 900 bp DNA fragment using primers 5′BamEco*GPI2* and 3′*GPI2*Sma (5′-TCCCCCGGGACAGGCAATAAGCC-3′).

The pMW20-based expression plasmids were introduced into *GPI/gpi::kanMX4* diploids, heterozygous at the corresponding *GPI* locus. The transformants were sporulated and the resulting asci were dissected on to YPGal [yeast extract/peptone/2% (w/v) galactose] medium. Tetrads yielded four viable segregants and these were tested for the presence and correct segregation of the appropriate *gpi::kanMX4* allele by whole-cell PCR and by plating on to geneticin-containing YPGal medium. Segregants from representative tetrads were maintained on glycerol-containing medium.

For galactose-induced expression of tagged and untagged Gpi2p and Gpi3p, 50–100 ml pre-cultures were grown at 30 °C for 2 days in SGly medium, after which the cells were collected, resuspended in YP medium containing 2% galactose, and grown for a further 5–6 h in this inducing carbon source. Cells were then harvested and washed. Mixed membranes [28] or crude lysates [29] were immediately prepared from them for enzyme assays, photoaffinity labelling and Western blotting.

Expression of FLAG-tagged Gpi3p in *E. coli*

For expression of C-terminally FLAG-tagged Gpi3p in *E. coli*, intronless *GPI3* was amplified with *Pwo* polymerase using the oligonucleotides 5′USNde and 3′*GPI3FLAG*Sma as forward and reverse primers respectively. The former primer was designed to allow the ATG codon of *GPI3* to be ligated behind the T7

promoter of vector pET13a, whereas the latter added DNA encoding the FLAG epitope immediately before the stop codon of the gene. The resulting 1.4 kb DNA fragment was digested with *Nde*I and *Bcl*I and ligated into the *Nde*I and *Bam*HI sites of pET13a, and the plasmid, pET13a-*GPI3-FLAG*, was introduced into *E. coli* BL 21(DE3) [30].

Pre-cultures (5–10 ml) of *E. coli* BL21(DE3) cells harbouring pET13a-*GPI3-FLAG* were grown overnight at 30 °C in Luria–Bertani medium containing 50 μg of kanamycin/ml, and were subsequently diluted (1:100) into 250–500 ml of fresh medium. The culture was allowed to grow to a D_{600} of 0.4–0.6, at which point expression of Gpi3p-FLAG was induced for 2.5–3 h. Cells were harvested and washed once with cold water. Cells were resuspended in cold bacterial lysis buffer [20 mM Tris/HCl (pH 7.9), 20 % (v/v) glycerol, 0.2 mM EDTA and 0.5 M NaCl] [27] if only protein gels were run and Western blots were performed, or in cold TM buffer [50 mM Tris/HCl (pH 7.5) and 2 mM $MgCl_2$] if *in vitro* assays were performed. Both buffers were supplemented with protease inhibitors. In some experiments, the detergents Nonidet P40 or Triton X-100 were added to give a final concentration of 0.1 % (v/v). Cells were kept on ice and lysed by sonication for 5 × 20–25 s, with 30 s intervals on ice in between sonications. Unbroken cells and cell debris were removed by centrifugation, and the crude lysates were used in subsequent Western blots and in *in vitro* assays. Alternatively, the cell slurry was centrifuged at 25000 ***g*** for 20 min to sediment inclusion bodies, and the supernatant was then centrifuged at 100000 ***g*** to recover membranes.

GlcNAc-PI synthesis assay

Washed mixed membranes were prepared and assayed for *in vitro* synthesis of GlcNAc-PI, GlcN-PI and GlcN-(acyl-Ins)PI essentially as described previously [28].

Western blotting

Protein samples were separated by SDS/PAGE, using either 12 % or 9 % polyacrylamide gels, for Gpi2p and Gpi3p, respectively. After electrophoresis, gels were equilibrated in transfer buffer [48 mM Tris, 39 mM glycine, 0.037 % (w/v) SDS and 20 % (v/v) methanol, pH 9.2], then transferred on to pre-wetted nitrocellulose membranes using BioRad's SemiDry transfer apparatus. Membranes were then incubated at room temperature for 1 h in blocking solution consisting of 1 × TBS-T [10 mM Tris/HCl (pH 8.0), 150 mM NaCl and 0.05 % (v/v) Tween-20] containing 5 % (w/v) dry milk. After blocking, the membrane was rinsed with distilled water and incubated for 30–60 min at room temperature with a 1:1000 dilution of the primary anti-FLAG M2 antibody (approximately 4.2 μg/ml) in 15 ml of blocking solution. The membrane was rinsed 3 times with distilled water and washed three times, for 5–10 min each, with 1 × TBS-T, then incubated for 30–40 min with a 1:2000 dilution of horse radish peroxidase-coupled goat anti-mouse secondary antibody in blocking solution. The membrane was then rinsed three times with distilled water and washed four times for 10–15 min with 1 × TBS-T. Detection of epitope-tagged Gpi3p was performed using Amersham's ECL® kit, and exposing membranes to X-OMAT AR film.

Synthesis of AAUTP[α-^{32}P]

AAUTP[α-^{32}P] was synthesized and purified as described previously [23].

Photoaffinity labelling, immunoprecipitation, and detection of Gpi3p-FLAG and Gpi2p-FLAG

Cells from galactose-induced cultures were harvested by centrifugation, washed once with cold water and resuspended in 5 ml of buffer [50 mM Tris/HCl (pH 7.40, 5 mM $MgCl_2$, 150 mM NaCl, 20 % (v/v) glycerol and 1 mM dithiothreitol] containing protease inhibitors (Boehringer Mannheim). A mixed membrane fraction was prepared in this buffer. The 100000 ***g*** pellets were resuspended in 250 μl of buffer [50 mM Hepes/NaOH (pH 7.5)/250 mM sucrose]/100 D_{600} units of cells. Photolabelling reactions were then performed in a dark room under a filtered safe-light. A volume of membranes corresponding to approx. 200 μg of protein was diluted into buffer [50 mM Hepes/NaOH (pH 7.5), 250 mM sucrose and 10 mM EDTA] and reactions were initiated by the addition of 1 μM AAUTP[α-^{32}P] (total volume of 100 μl). The competition experiments also contained a 10–1000-fold molar excess of one of the following: UDP; UDP-GlcNAc; GDP; GDP-Man; ADP. Mixtures were incubated at room temperature for 5 min, then for 1 min on ice, then photolysed for 2 min by irradiation with 254 nm UV light, after which they were quenched with 100 μl of 10 mM 2-mercaptoethanol and incubated on ice for 5 min. Protease inhibitors were added and the samples were diluted with 0.9 ml of IP buffer [15 mM Hepes/NaOH (pH 7.5), 150 mM NaCl and 1 % (v/v) Triton X-100]. Anti-FLAG M2 affinity resin (40 μl; 50 % slurry in IP buffer) was added, and samples were gently shaken overnight at 4 °C. The anti-FLAG resin was then washed three times with 1 ml volumes of IP buffer, then twice with 10 mM Hepes/NaOH (pH 7.5) containing 50 mM NaCl and 0.1 % Triton X-100. All the liquid was subsequently removed using a syringe. To elute the FLAG-tagged Gpi2 proteins from the resin, 2 × SDS-loading buffer was added directly to the beads, and these were incubated at 45 °C for 30 min. Eluted proteins were separated by SDS/PAGE, and Western blotting was performed using monoclonal anti-FLAG M2 antibodies as described above. Following detection, the nitrocellulose membrane was rinsed several times with TBS and allowed to incubate in that buffer, with gentle shaking, at room temperature for 15–20 min. Excess liquid was removed and the membrane was allowed to dry overnight under normal laboratory lights, after which ^{32}P-labelled proteins were revealed by exposing the nitrocellulose membrane to a PhosphorImager screen (Molecular Dynamics) for approx. 3 days.

Photoaffinity labelling of bovine milk galactosyltransferase

Nucleotide titration

Reaction mixtures were set up containing buffer [50 mM Hepes/NaOH (pH 7.5), 5 mM $MnCl_2$ and 5 mM $CaCl_2$], nucleotide protectors, and 1 μM AAUTP[α-^{32}P], in a final reaction volume of 20 μl. Bovine milk galactosyltransferase (GalT) (0.5 μg; from −20 °C frozen stock in 50 mM Hepes/NaOH, pH 7.5) was added last to the ice-cold buffer. Samples were incubated for 15 min in a water bath maintained at room temperature, followed by 1 min on ice and 2 min of UV irradiation (also on ice). Reactions were quenched by the addition of 5 mM dithiothreitol (2.5 mM final concentration), and samples were then incubated for 5 min at room temperature. SDS [10 % (w/v) stock] was added to a final concentration of 1.1 % (w/v) and the samples were heated at 100 °C for 4 min, and then cooled. The samples were subsequently diluted to 100 μl with the addition of BSA (8 μg), water and NaCl (5 M stock; 500 mM final concentration), and the protein was precipitated with organic solvent as previously described [31]. Dried protein pellets were dissolved

in 25 μl of 2 × SDS/PAGE sample buffer by heating at 100 °C for 5 min. A 10 μl sample was taken for liquid scintillation counting and another 10 μl sample was resolved by SDS/PAGE (10 % polyacrylamide). The polyacrylamide gel was fixed in a mixture of 50 % (v/v) methanol and 100 μl of 37 % formaldehyde/100 ml, then dried. An autoradiogram was obtained on a PhosphorImager screen (Molecular Dynamics).

Photolabelling of GalT in a background of Gpi3p-FLAG-containing microsomes

Reaction mixtures were set up containing buffer [50 mM Hepes/NaOH (pH 7.5), 5 mM $MnCl_2$, 5 mM $CaCl_2$, 250 mM sucrose], UDP (either 10 or 100 μM, as indicated) and 1 μM of AAUTP[α-^{32}P] in a final reaction volume of 50 μl. The background protein (50 μg) used was a microsomal fraction from the *gpi3::kanMX4*-pMW20-*GPI3-FLAG* yeast strain. GalT (2 μg; from −20 °C frozen stock in 50 mM Hepes/NaOH, pH 7.5) was added last to the ice-cold reaction mixtures. Reactions were irradiated and quenched as above. Reactions that did not contain background protein during the labelling had the equivalent amount of protein added at the time of quenching to insure precipitation efficiency. SDS [10 % (w/v) stock; 0.5 % (w/v) final concentration] and NaCl (5 M stock; 500 mM final concentration) were added to give a final volume of 100 μl. The protein was precipitated with organic solvent [31], and the dried protein pellets were solubilized and resolved by SDS/PAGE. The polyacrylamide gels were either fixed directly or Coomassie-Blue-stained and de-stained [in 10 % (v/v) acetic acid/40 % (v/v) methanol] and subsequently dried. Autoradiograms were obtained as above. Coomassie Brilliant Blue staining of the gels indicated that the protein bands in each lane were of equal intensity. In addition, no apparent differences were observed between autoradiogram intensities of gels that had been directly fixed in acid-free fixative and those that had been Coomassie-Blue-stained. This indicated that dilute weak acid did not significantly promote the hydrolysis of the AANTP photoaffinity reagents under the conditions used.

RESULTS

Our strategy to test Gpi2p and Gpi3p for their ability to bind UDP-GlcNAc was to introduce the FLAG epitope [27] into each protein, to express the tagged proteins in *GPI2*- or *GPI3*-deleted haploid *S. cerevisiae* strains, to incubate membranes from such strains with AAUTP[α-^{32}P], to purify the tagged proteins using an immuno-affinity resin, and to determine whether the tagged protein had become [^{32}P]-labelled. We also investigated whether Gpi3p could bind AAUTP[α-^{32}P] in the absence of its partner proteins by expressing tagged-Gpi3p in *E. coli* and testing whether the bacterially expressed protein could become cross-linked to the photoprobe.

C-terminally FLAG-tagged Gpi3p and Gpi2p restore *in vivo* and *in vitro* GlcNAc-PI synthetic activity to GPI3 and GPI2-disruptants

The *GPI3/SPT14* gene has been predicted to be spliced [15], which might interfere with bacterial expression of Gpi3p. We isolated *GPI3* mRNA, generated the corresponding cDNA sequence by RT-PCR, and showed that the *GPI3* message indeed lacks the predicted intron, and the amino acid sequence at the start of Gpi3p is the same as that proposed [15].

cDNA constructs encoding both N- and C-terminally FLAG-tagged Gpi3p restored viability to *gpi3::kanMX4* disruptants; however, only membranes from haploid cells expressing C-terminally tagged Gpi3p had *in vitro* GlcNAc-PI synthetic activity

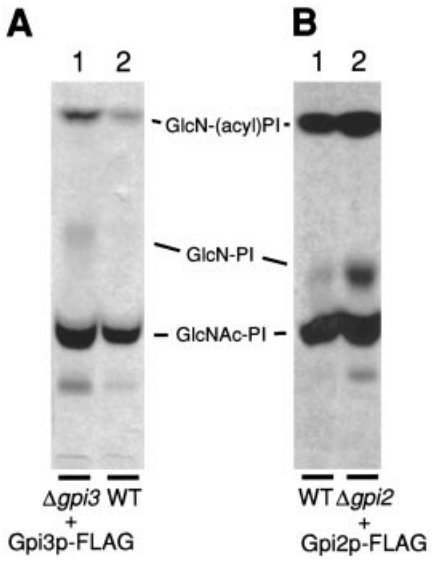

Figure 1 ***In vitro*** **GlcNAc-PI synthetic activity of C-terminally FLAG-tagged Gpi3p and Gpi2p.**

Mixed membranes were prepared from wild-type haploids and from *gpi3::kanMX4* and *gpi2::kanMX4* haploids harbouring pMW20-*GPI3-FLAG* and pRS425-*GPI2-FLAG*, respectively, and assayed for GlcNAc-PI synthetic activity. ^{14}C-labelled GlcNAc-PI, GlcN-PI and GlcN-(acyl-Ins)PI [GlcN-(acyl)PI] were extracted, separated by TLC, and detected by fluorography. (**A**) Wild-type haploid (WT, lane 2) and a *gpi3::kanMX4* haploid harbouring pMW20-*GPI3-FLAG* (*Δgpi3* + Gpi3p-FLAG, lane 1). (**B**) Wild-type haploid (WT, lane 1) and a *gpi2::kanMX4* haploid harbouring pRS425-*GPI2-FLAG* (*Δgpi2* + Gpi2p-FLAG, lane 2).

(Figure 1A), and such cells were therefore used in subsequent analyses. Plasmids encoding C-terminally FLAG-tagged Gpi2p both complemented the *gpi2::kanMX4* null mutation and conferred *in vitro* GlcNAc-PI synthetic activity on membranes from the rescued strains (Figure 1B). Membranes from strains expressing Gpi3p-FLAG and Gpi2p-FLAG under the control of the *GAL10* promoter exhibited slightly higher levels of synthesis of [^{14}C]GlcNAc-PI and of the GlcN-PI and GlcN-(acyl-Ins) species that are generated from it *in situ*. One explanation for this is that a large excess of one essential subunit ensures that more, fully assembled, GlcNAc-PI synthetic complexes are present in these cells.

To detect FLAG-tagged Gpi2p and Gpi3p by Western blotting, using a monoclonal anti-FLAG antibody, it was necessary to express *GPI2-FLAG* and *GPI3-FLAG* under the control of the galactose-inducible *GAL10* promoter. Western blotting of proteins in detergent extracts of crude lysates [29] or membranes [28] of galactose-induced *gpi3::kanMX4*-pMW20-*GPI3-FLAG* cells revealed an anti-FLAG-reactive protein of approximately 53 kDa, the predicted mass of Gpi3p-FLAG (Figure 2A, lanes 4 and 5), whereas lanes containing extracts of cells expressing untagged Gpi3p contained no anti-FLAG-reactive material (Figure 2A, lanes 2 and 3). Bacterially expressed Gpi3p (see below) was also loaded as a control (lane 1). The sizes of the yeast and bacterially expressed Gpi3p were very similar, indicating that Gpi3p-FLAG was not extensively modified after it had been translated. The same procedures were used to detect Gpi2p-FLAG in extracts of galactose-induced *gpi2::kanMX4*/pMW20-*GPI2-FLAG* cells. Cross-reactive material was detected that migrated with apparent molecular masses of 29, 55 and 84 kDa (Figure 2B, lanes 1 and 2). The Gpi2-FLAG protein is predicted to have a molecular mass of 29 kDa, and the 55 and

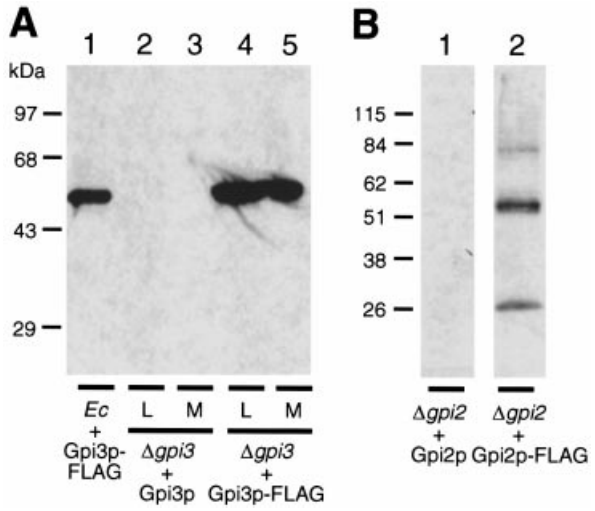

Figure 2 Immunodetection of C-terminally FLAG-tagged Gpi3p and Gpi2p expressed in yeast.

(**A**) Lysates (L) or membranes (M) from cells from galactose-induced cultures of *gpi3::kanMX4*/pMW20-*GPI3* and *gpi3::kanMX4*/pMW20-*GPI3-FLAG* were solubilized in detergent, the extracts separated by SDS/PAGE, and FLAG-tagged material detected by Western blotting using the M2 anti-FLAG antibody. Lanes 2 and 3, extracts from cells harbouring pMW20-*GPI3* (*Δgpi3* + Gpi3p); lanes 4 and 5, extracts from cells expressing pMW20-*GPI3-FLAG* (*Δgpi3* + Gpi3p-FLAG). Lane 1 contains a sample of protein from *E. coli* cells in which expression of pET13a-*GPI3-FLAG* had been induced (*Ec* + Gpi3p-FLAG). (**B**) Gpi2p-FLAG was immunoprecipitated from extracts of membranes from *gpi2::kanMX4*/pMW20-*GPI2* cells (lane 1) and *gpi2::kanMX4*/pMW20-*GPI2-FLAG* cells (lane 2) in 15 mM Hepes/NaOH (pH 7.5), 150 mM NaCl and 1% (v/v) Triton X-100 using anti-FLAG M2 affinity resin. Proteins were extracted from the resin, separated by SDS/PAGE, and FLAG-tagged material was detected by Western blotting using the M2 anti-FLAG antibody. Positions of molecular-mass markers are indicated to the left.

84 kDa proteins probably correspond to dimers and trimers of Gpi2p, which may be formed upon aggregation of this very hydrophobic protein. A very similar electrophoretic pattern has been observed with the human Gpi2p homologue, Pig-Cp [10]. Taken together, these results indicate that Gpi2p and Gpi3p bearing the FLAG epitope at their respective C-termini are functional *in vivo*, have *in vitro* GlcNAc-PI synthetic activity, and can be expressed at levels sufficient for immunodetection and immunoaffinity purification.

Photolabelling of yeast membranes containing Gpi3p-FLAG

We tested whether Gpi3p-FLAG expressed in the *gpi3::kanMX4*-pMW20-*GPI3-FLAG* strain could become cross-linked to AAUTP[α-^{32}P]. Unlabelled AAUTP is a competitive inhibitor of murine GlcNAc-PI synthase [23] and is therefore expected to be a good probe of the UDP-GlcNAc-binding component of the yeast enzyme. As a control to identify material that cross-reacted with AAUTP[α-^{32}P], but whose presence in anti-FLAG resin-treated extracts did not dependent on pMW20-*GPI3-FLAG*, we used the *gpi3-6A* strain, whose membranes have undetectable *in vitro* GlcNAc-PI synthetic activity. Because *GPI3*-deleted strains are not viable, the *gpi3-6A* mutant is the best candidate for a control strain whose Gpi3p may be present at lower levels than in wild-type cells, or bind UDP-GlcNAc less well, or both.

Mixed membranes were isolated from both strains, and portions containing 200 μg of protein were used for photolabelling. Samples were incubated with the photoprobe in the presence or absence of excess unlabelled UDP-GlcNAc, then photolysed for 2 min. One sample of membranes from each strain was not treated with UV, for comparison. Following photolabelling, proteins were immunoprecipitated using anti-FLAG resin, and eluted from the resin by gently warming the sample in SDS/PAGE loading buffer. Samples were then separated by SDS/PAGE and transferred on to nitrocellulose membranes, after which ^{32}P-labelled material was detected using a PhosphorImager, and Gpi3p-FLAG then detected by Western blotting.

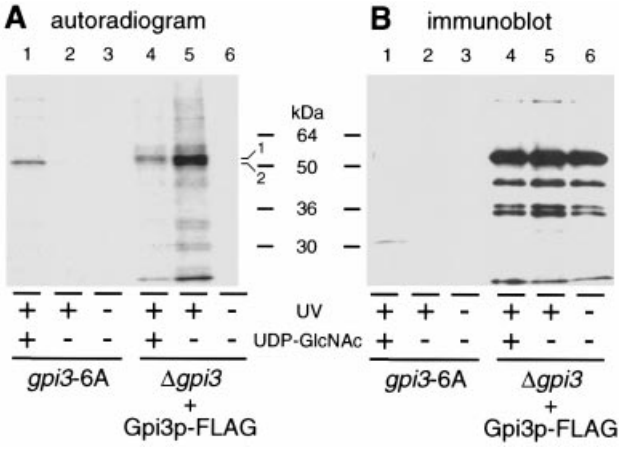

Figure 3 Photoaffinity labelling of Gpi3p-FLAG expressed in *S. cerevisiae*

Mixed membranes were isolated from *gpi3*-6A cells or from *gpi3::kanMX4* cells harbouring pMW20-*GPI3-FLAG*, and incubated with AAUTP[α-^{32}P] with or without UV irradiation, and with or without UDP-GlcNAc. Gpi3p-FLAG was detected following separation of the proteins in the incubation mixtures by SDS/PAGE, transfer of the proteins on to nitrocellulose membranes and Western blotting with M2 anti-FLAG antibody. (**A**) ^{32}P-labelled proteins from *gpi3*-6A (lanes 1–3) and *gpi3::kanMX4*/pMW20-*GPI3-FLAG* (*Δgpi3* + Gpi3p-FLAG) membranes (lanes 4–6) incubated with AAUTP[α-^{32}P]. Incubation mixtures were either exposed to UV light (lanes 1, 2, 4 and 5) or were not irradiated (lanes 3 and 6), and UDP-GlcNAc was included in the reaction mixtures displayed in lanes 1 and 4). Band 1 (lanes 4 and 5) is the material selectively photolabelled in the Gpi3p-FLAG sample, and Band 2 (lanes 1 and 5) is non-selectively-labelled material. (**B**) Western blot of the membrane in (**A**) with M2 anti-FLAG antibody.

The results of the Gpi3p photolabelling are shown in Figure 3. Gpi3p became cross-linked to the photoprobe in a light-dependent and competitive manner (Figure 3A, lanes 4 and 5). There was no signal in the non-UV-irradiated control sample (lane 6). All but one of the multiple bands present in the *gpi3::kanMX4*/pMW20-*GPI3-FLAG* strains + UV, and + UV + UDP-GlcNAc lanes were also present in samples from the *gpi3-6A* control (Figure 3A, lanes 1 and 2), and correspond to non-specifically labelled proteins. The size of the band selectively photolabelled in the Gpi3p-FLAG sample (Figure 3A, lane 5, band 1) corresponded to that predicted for Gpi3p-FLAG. The intensity of cross-linked-Gpi3p was decreased by about 60% in the presence of 1000-fold molar excess non-radioactive UDP-GlcNAc (Figure 3A, lanes 4 and 5). The corresponding Western blot confirmed that the labelled and immunoprecipitated protein was Gpi3p (Figure 3B, lanes 4 and 5); the blot also showed that, under the incubation conditions used, some degradation of Gpi3p occurred leading to non-photolabelled albeit anti-FLAG-immunoreactive material.

Photolabelling of Gpi3p expressed in *E. coli*

To establish whether binding of AAUTP[α-^{32}P] to Gpi3p required the presence of Gpi1p and Gpi2p, we expressed Gpi3p-FLAG in *E. coli*, which does not contain these GlcNAc-PI synthase

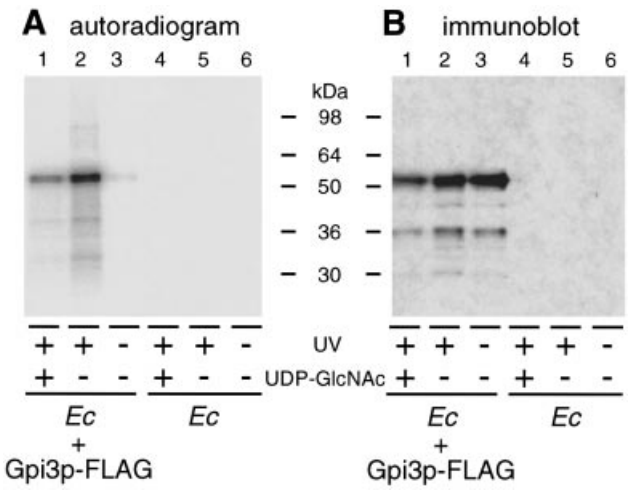

Figure 4 Photoaffinity labelling of Gpi3p-FLAG expressed in *E. coli*

Lysates of *E. coli* BL21 (DE3) cells in which expression of pET13a-*GPI3-FLAG* had been induced (lanes 1–3; *Ec*+Gpi3p-FLAG) were incubated with AAUTP[α-^{32}P] with or without UV irradiation, and with or without UDP-GlcNAc, and Gpi3p was immunoprecipitated using anti-FLAG affinity gel. *E. coli* BL21 (DE3) cultures harbouring pET13a were grown and processed in parallel as negative controls for photolabelling and Western blotting (lanes 4–6; *Ec*). (**A**) ^{32}P-labelled proteins immunoprecipitated by anti-FLAG resin, separated by SDS/PAGE, and transferred on to a nitrocellulose membrane. (**B**) Western blot of the membrane in (**A**) with M2 anti-FLAG antibody.

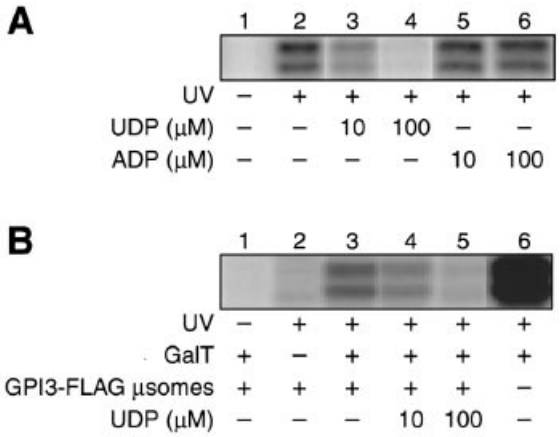

Figure 5 Specificity of photolabelling via AAUTP[α-^{32}P] demonstrated with a purified, soluble protein

(**A**) Bovine milk GalT (0.5 μg) was photolabelled with AAUTP[α-^{32}P] (1 μM) in buffer [50 mM Hepes/NaOH (pH 7.5), 5 mM $MnCl_2$ and 5 mM $CaCl_2$] in the absence or presence of the indicated nucleotides. The total labelling reaction volume was 20 μl. (**B**) GalT (2 μg; lanes 1 and 3–6) was photoaffinity labelled with 1 μM AAUTP[α-^{32}P] in buffer [50 mM Hepes/NaOH (pH 7.5), 250 mM sucrose, 5 mM $MnCl_2$ and 5 mM $CaCl_2$] alone (lane 6), or in buffer containing 50 μg of protein from microsomes of the *gpi3::kanMX4*-pMW20-*GPI3-FLAG* strain (lanes 1–5). UDP was added as a competitor as indicated (lanes 4 and 5). The final labelling reaction volumes were 50 μl. The signal in the absence of UV irradiation is shown in lane 1, and the background of photolabelled microsomal proteins in the molecular-mass range of GalT is shown in lane 2.

subunits. Photolabelling, immunoprecipitation and Western blotting were carried out with samples of bacterial membranes containing 100 μg of protein, using the procedure used for yeast-expressed Gpi3p-FLAG. *E. coli* harbouring the empty pET13a vector was used as a control strain. Bacterially expressed Gpi3p (Figure 4B, lanes 1–3) was also specifically labelled by the photoprobe in a UV light-dependent manner (Figure 4A, lanes 1–3). Inclusion of a 1000-fold molar excess of UDP-GlcNAc (over AAUTP[α-^{32}P]) in the reaction mixture reduced photolabelling of Gpi3p (Figure 4A, lanes 1 and 2). Although bacterially expressed Gpi3p bound the photoprobe, extracts of the cells had no detectable GlcNAc-PI synthetic activity under any of the conditions tested. This suggests that auxiliary proteins are needed for GlcNAc transfer to PI.

Further analysis of the specificity of AAUTP[α-^{32}P]-mediated photolabelling of Gpi3p

The finding that Gpi3p could be protected from photolabelling by including UDP-GlcNAc in the reaction mixture argues strongly that labelling is specific. In order to test the specificity of photo cross-linking further, we carried out similar photolabelling reactions in the presence of uridine diphosphate at concentrations ranging from 10–1000-fold molar excess over AAUTP[α-^{32}P], and compared the results obtained with UDP to those obtained with other nucleoside diphosphates (ADP and GDP). Although UDP reduced photolabelling of Gpi3p at a 1000-fold excess over AAUTP[α-^{32}P] (similar to the results with UDP-GlcNAc described above), no reduction in photolabelling was seen when lower molar ratios of UDP to AAUTP[α-^{32}P] were tested (results not shown). Also, surprisingly, similar data were obtained with the irrelevant nucleotides GDP and ADP, i.e. there was no effect on photolabelling at low molar excess over AAUTP[α-^{32}P], but labelling was reduced when the nucleotides were included at 1000-fold excess.

In order to understand these results, we carried out identical photolabelling analyses of bovine milk GalT, an enzyme that we previously showed could be specifically labelled with AAUTP[α-^{32}P] [23]. For the purposes of these experiments, GalT represents a simple model because it is water-soluble and can be readily obtained as a pure protein. Figure 5(A) shows that GalT can be photolabelled with AAUTP[α-^{32}P] in a UV-light-dependent fashion, and that the extent of labelling can be progressively diminished by including a 10–100-fold molar excess of UDP, but not ADP, in the reaction mixture. These results indicate that labelling of GalT by AAUTP is binding-site specific and that AAUTP is interacting with GalT solely as a uridine-nucleotide analogue.

As an additional test, we photolabelled GalT in a background of microsomal protein obtained from Gpi3p-FLAG-expressing yeast. The purpose of this experiment was to test whether the complexity of the reaction mixture could significantly alter the ability of a protein to be specifically photolabelled. As shown in Figure 5(B), the principal effect of including the microsomal protein background was to decrease the intensity of GalT labelling, most probably because of the increase in the number of potential binding targets for AAUTP. However, GalT labelling retained its sensitivity to the presence of uridine nucleotides (Figure 5B, lanes 3–5): when UDP was titrated into the reaction mix, the extent of labelling decreased in a manner similar to that shown in Figure 5(A). We conclude that GalT recognizes AAUTP solely as a uridine-nucleotide analogue and that the specificity of the interaction holds up regardless of whether GalT is pure or in a heterogeneous biomolecule mixture.

These results indicate that AAUTP[α-^{32}P]-mediated photolabelling of Gpi3p is more complicated than labelling of GalT. It is clear that the inability of Gpi3p-FLAG to be protected from labelling is not due to diffusible components of the heterogeneous protein mixture that may alter the properties of the target,

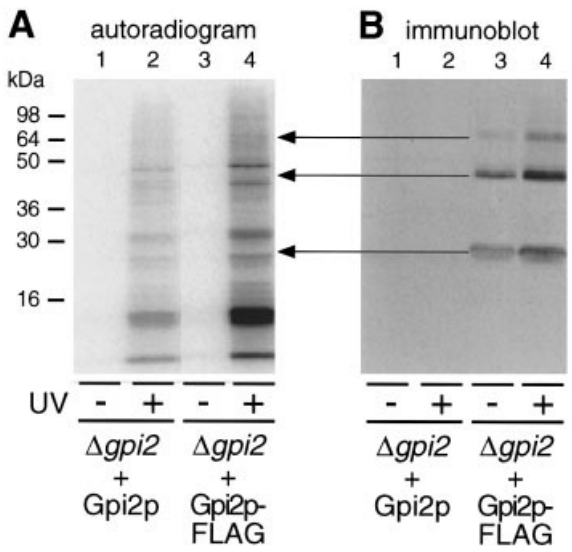

Figure 6 Incubation of *S. cerevisiae* membranes containing Gpi2p-FLAG with AAUTP[α-^{32}P]

Mixed membranes from *gpi2::kanMX4*/pMW20-*GPI2* and *gpi2::kanMX4*/pMW20-*GPI2-FLAG* cells were incubated with AAUTP[α-^{32}P] with or without UV irradiation. Gpi2p-FLAG was immunoprecipitated using anti-FLAG resin and proteins were eluted from the resin, separated by SDS/PAGE and transferred on to nitrocellulose membranes. Gpi2p-FLAG was detected by Western blotting with M2 anti-FLAG antibody and ^{32}P-labelled proteins were then detected by exposing the nitrocellulose filter to X-ray film. (**A**) Autoradiogram of blot. ^{32}P-labelled proteins from *gpi2::kanMX4*/pMW20-*GPI2* (*Δgpi2*+Gpi2p) (lanes 1 and 2) and *gpi2::kanMX4*/pMW20-*GPI2-FLAG* (*Δgpi2*+Gpi2p-FLAG) (lanes 3 and 4) were incubated with AAUTP[α-^{32}P]. Incubation mixtures were either exposed to UV light (lanes 2 and 4) or were not irradiated (lanes 1 and 3). (**B**) Western blot of the membrane in (**A**) with M2 anti-FLAG antibody. The autoradiogram was superimposed on the immunoblot that had been exposed to X-ray film to assess whether any ^{32}P-labelled bands corresponded to those detected with the anti-FLAG antibody. The arrows indicate the positions on the PhosphorImager scan that correspond to the positions of the anti-FLAG antibody-reactive material.

because the specificity of GalT labelling was not affected under the same conditions (Figure 5B). The protective effect on Gpi3p photolabelling observed with a 1000-fold excess of UDP-GlcNAc (but not with lower levels of nucleotide competitors) is more likely to be due to a general perturbation of the experimental system rather than the result of competition with AAUTP[α-^{32}P] for a binding site on Gpi3p. The hydrophobicity of AAUTP[α-^{32}P] (contributed by the arylazide moiety) is likely to be a factor in our inability to protect Gpi3p from photolabelling in the presence of hydrophilic nucleotide competitors. Gpi3p contains a hydrophobic membrane anchoring sequence that may serve to retain AAUTP[α-^{32}P] preferentially over soluble nucleotide competitors, accounting for our inability to protect the protein from photolabelling in the presence of low levels of UDP (see the Discussion).

AAUTP[α-^{32}P] does not label Gpi2p

To discover whether AAUTP[α-^{32}P] labelling of Gpi3p was solely determined by hydrophobic interactions rather than its recognition as a uridine nucleotide analogue, and also to determine whether the other essential component of the yeast GlcNAc synthase complex, Gpi2p, was involved in nucleotide sugar binding, we tested whether Gpi2p could be cross-linked to AAUTP[α-^{32}P]. Because Gpi2p is a membrane protein with a high complement of hydrophobic amino acids [10,13], it should provide an excellent way to test whether the AAUTP[α-^{32}P]-labelling of Gpi3p that we observed occurred solely via a nucleotide-independent hydrophobic interaction.

Mixed membranes were prepared from the *gpi2::kanMX4* strain harbouring pMW20-*GPI2-FLAG*, and photolabelling experiments were carried out under the same conditions as used for Gpi3p-FLAG. Gpi2p-FLAG was immunoprecipitated from the photolabelling mixture using anti-FLAG resin, although, owing to a different commercial source of the resin, the immunoprecipitation was not as clean as seen with Gpi3p-FLAG. Nevertheless, a direct overlay of the autoradiogram (obtained after transfer on to nitrocellulose) on the immunoblot profile (obtained by probing the same piece of nitrocellulose) clearly showed that, although a number of photolabelled bands were evident, none of these corresponded to Gpi2p (or its multimers). The same results were obtained in four independent experiments.

The results of a representative experiment are shown in Figure 6. Neither the Gpi2p-FLAG monomer, nor the dimer, both of which were prominent on the Western blot, corresponded to any of the ^{32}P-labelled bands that became cross-linked to the probe (Figure 6A and B, lanes 4). These data argue strongly that AAUTP[α-^{32}P] is not recognized by Gpi2p and that AAUTP[α-^{32}P]-mediated labelling of Gpi3p involves nucleotide recognition and does not occur solely through an interaction between the arylazide moiety and a hydrophobic sequence in the protein. Photolabelling is therefore selective for Gpi3p: the photoprobe does not bind to another component of the GlcNAc-PI synthetic complex.

DISCUSSION

The first step in the assembly of GPIs is the transfer of GlcNAc from UDP-GlcNAc to PI. This seemingly straightforward glycosyl transfer reaction is carried out by a protein complex, three of whose members are highly conserved between yeast and mammals. We show here that of the conserved proteins that are essential for GlcNAc-PI synthesis in yeast, i.e. Gpi2p and Gpi3p, only Gpi3p binds the photoreactive UDP-GlcNAc analogue AAUTP[α-^{32}P] and can do so in the absence of the other subunits of the GlcNAc-PI synthetic complex. This strongly implicates Gpi3p as the substrate-binding and likely catalytic subunit of the first enzyme in GPI synthesis.

Our results, together with previous findings, further indicate that transfer of GlcNAc to PI requires one or more additional proteins. Thus membranes from Gpi1p- and Gpi2p-defective yeast mutants grown at 25 °C lack detectable GlcNAc-PI synthetic activity [12,13], as do membranes from mammalian mutants defective in the Gpi1, Pig-C, and Pig-H proteins [22, 32–34], even though Gpi3p/Pig-Ap is present. Our finding that *E. coli* cells expressing Gpi3p have no detectable *in vitro* GlcNAc-PI synthetic activity is also consistent with the notion that Gpi3p does not function autonomously.

Our inability to protect Gpi3p from AAUTP[α-^{32}P]-mediated photolabelling in the presence of a low molar excess of UDP suggests that AAUTP[α-^{32}P] interacts with Gpi3p in a complex way. The ability of unlabelled AAUTP to act as a competitive inhibitor of GlcNAc-PI synthase [23] originally suggested a simple recognition event in which AAUTP, acting as a nucleotide sugar analogue, competed with UDP-GlcNAc for the nucleotide (product) or nucleotide sugar (substrate) binding site in the enzyme. It is clear that some of the specificity of this interaction is lost in the photolabelling studies reported in the present study, possibly because of protein overexpression (necessary for immunodetection; Figure 2). Thus it is likely that only a portion of the Gpi3p-FLAG molecules expressed in yeast are integrated into functional GlcNAc-PI synthase complexes. Although the

remainder of the expressed Gpi3p-FLAG molecules would still bind AAUTP[α-^{32}P] [which is supported by our studies of the bacterially expressed protein (Figure 4)], these molecules will have lost the potential influence of other subunits of the GlcNAc-PI synthase complex. Thus it is possible that a hidden hydrophobic interface between Gpi3p and Gpi2p (or Gpi1p) in the GlcNAc-PI synthase complex is exposed in unintegrated Gpi3p and is available for secondary interactions with the arylazide moiety of AAUTP[α-^{32}P]. However, this putative hydrophobic interaction cannot be the sole means by which Gpi3p recognizes AAUTP[α-^{32}P], because labelling is selective: Gpi2p, a significantly more hydrophobic protein and the component of the GlcNAc-PI synthase complex implicated in binding the acyl chains of the PI substrate of the enzyme [8], cannot be labelled by AAUTP[α-^{32}P].

We believe, on the basis of our studies of bovine milk GalT and other UDP-sugar-recognizing enzymes (Figure 5 and reference [23]), that Gpi3p most probably binds AAUTP[α-^{32}P] as a uridine nucleotide analogue, and that once recognition and binding are achieved, the interaction between Gpi3p and AAUTP[α-^{32}P] becomes potentially complicated by interactions between the arylazide moiety in AAUTP[α-^{32}P] and hydrophobic sequences in the protein. These results, together with the sequence similarity between Gpi3p and members of a superfamily of prokaryotic nucleoside diphosphate sugar-utilizing α-glycosyltransferases, indicate that that Gpi3p is the nucleotide sugar binding component of the GPI GlcNAc-PI synthase complex.

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