Yeast and human glycosylphosphatidylinositol (GPI) precursors differ in the extent to which a fourth mannose is present as a side branch of the third core mannose. A fourth mannose addition to GPIs has scarcely been detected in studies of mammalian GPI synthesis but is an essential step in the Saccharomyces cerevisiae pathway. We report that human SMP3 encodes a functional homolog of the yeast Smp3 GPI fourth mannosyltransferase. Expression of hSMP3 in yeast complements growth and biochemical defects of smp3 mutants and permits in vivo mannosylation of trimannosyl (Man$_3$)-GPIs. Immunolocalization shows that hSmp3p resides in the endoplasmic reticulum in human cells. Northern analysis of mRNA from human tissues and cell lines indicates that hSMP3 is expressed in most tissues, with the highest levels in brain and colon, but its mRNA is nearly absent from cultured human cell lines. Correspondingly, increasing expression of hSMP3 in cultured HeLa cells causes abundant formation of three putative tetramannosyl (Man$_4$)-GPIs. Our data indicate that hSmp3p functions as a mannosyltransferase that adds a fourth mannose to certain Man$_3$-GPIs during biosynthesis of the human GPI precursor, and suggest it may do so in a tissue-specific manner.

Glycosylphosphatidylinositol (GPI)s are essential glycolipids synthesized by all eukaryotes. Many GPIs become covalently attached to the carboxyl termini of various secretory proteins and serve to anchor them to the exterior face of the plasma membrane (1, 2). Others remain protein-free and are distributed in the membranes of major cellular organelles and the plasma membrane (3, 4). GPIs are synthesized in the endoplasmic reticulum (ER) by stepwise addition of components to phosphatidylinositol. The end product of GPI synthesis is a “complete precursor” (5) that is substrate for the GPI transamidase complex that attaches it to proteins. Many of the steps and enzymes of GPI precursor assembly are conserved between humans and Saccharomyces cerevisiae and produce precursors with a common core structure of EthN-PO$_4$-6Man$_1$2Man$_1$6Man$_1$4GlcN$_1$6Ins-PO$_4$-lipid. In both pathways, the glycan portion of the GPI may be modified further with side branching ethanolamine phosphate (EthN-P) moieties on the first and second mannoses (6–19).

A notable difference in GPI structure between yeast and mammals is the extent to which a fourth mannose (Man-4) is present as a α1,2-linked side branch of the third mannose (Man-3). In S. cerevisiae, late stage intermediates in GPI precursor synthesis (17, 19–21), the presumed GPI transamidase substrates (5), and protein-bound GPIs (22) all contain four mannosyl residues. Addition of Man-4 to trimannosyl-GPIs (Man$_3$-GPIs) by the essential Smp3 mannosyltransferase is a mandatory step in yeast GPI precursor assembly which precedes the addition of the terminal EthN-P to Man-3 through which the GPI is ultimately attached to protein (23). Thus, it is probable that all yeast GPI transamidase substrates bear four mannosyl residues. Conversely, studies of the synthesis of mammalian GPI precursors have uncovered little evidence that a similar pathway step occurs in mammals. The largest characterized mammalian GPI precursors and presumed GPI transamidase substrates contain only three mannoses (10, 14, 24). Only trace amounts of GPI intermediates with chromatographic mobilities consistent with the possible presence of Man-4 have been observed in some mammalian cell lines (10, 15, 25). However, Man-4 is present on the GPIs of many purified mammalian GPI-anchored proteins (6, 26–31), raising questions as to how and when transfer of Man-4 to mammalian GPIs occurs.

In this study, we show that the human Smp3 protein (hSmp3p), a sequence homolog of S. cerevisiae Smp3, is a candidate human GPI fourth mannosyltransferase. We demonstrate that hSmp3p is a resident ER protein that adds Man-4 to certain human Man$_3$-GPIs in vitro during synthesis of GPI precursors. Our data suggest that the addition of Man-4 to both human and yeast GPIs occurs via similar mechanisms but that Man$_3$-GPI formation is likely not necessary for transfer of GPIs to proteins in mammals. Our results also suggest that although the addition of Man-4 is rarely detectable in cultured cell lines, this modification may be more common in the cells of many human tissues.

**EXPERIMENTAL PROCEDURES**

Materials—myo-[2-3H]inositol (specific activity 15–20 Ci/mmol) and D-[2-3H]mannose (specific activity 20–30 Ci/mmol) were from American Radiolabeled Chemicals (St. Louis, MO). [α-32P]dATP (specific activity 3,000 Ci/mmol) was from PerkinElmer Life Sciences. Genetin (G418), 5-fluorouracil, Calcofluor white, and tunicamycin were from Sigma.

**Yeast Strains and Media**—S. cerevisiae strains used in this study are listed in Table I. Heterozygous diploids with disrupted alleles of smp3, gpi10, and ypr013w were from Research Genetics (Huntsville, AL). YPD and SD (also referred to as SGlc) medium were as described previously (32). YPDgal medium has the same composition as YPD but with 2% galactose in place of glucose. Inositol-free synthetic medium and synthetic medium containing 0.2% yeast extract and glycerol (SGalYE), galactose (SGalYE) or glucose (SGlcYE) were prepared as described.
were made by transforming the potassium acetate agar containing 5 mM uracil. Strains were constructed as follows. A double mutant was transformed with p425-smp3-2 and pGAL-hGPI13 gpi13::KAN R MAT a/ ade2:: His3-11,15 leu2-3,112 trpl-1 ura3-1 can1-100. Plasmid pGAL-hGPI13 was made by cloning PCR-amplified human PIG-B cDNA was amplified by PCR from a human liver cDNA library using the primers 5'-CCGGAATTCATGCAGATCTGTGGATCCAGC-3' and 5'-GGATCCGGGATGAGGAGGCCCCT-CCAGGAATTC-3' to create a translational fusion reporter with a his3-11,15 leu2-3,112 trpl-1 ura3-1 can1-100, respectively. Expression of hGPI3 and ScSMP3 in S. cerevisiae—The expression vector p416-PGK was made by PCR amplifying a 501-bp fragment of the yeast PGK1 promoter and cloning it into the NotI-EcoRI sites of pRS416 (33). Plasmid p416-PGK-hGPI3-HA, in which hGPI3-HA is expressed from the PGK1 promoter, was made by cloning PCR-amplified hGPI3-HA into the EcoRI-XhoI sites of p416-PGK. Plasmid p416-PGK-hGPI3-HA was then used to transform a Schizosaccharomyces pombe strain, designated by cloning a SacI-XhoI fragment containing the PGK1 promoter and hGPI3-HA from p416-PGK-hGPI3-HA into the SacI-XhoI sites of pRS425. These plasmids for yeast expression of ScSMP3 were constructed as follows. Plasmid p416-PGK ScSMP3 was made by cloning a 1243-bp BglII-HindIII genomic fragment containing ScSMP3 and 513 bp of its native promoter from a YCp50-LEU2 library clone (library obtained from P. Hieter, University of British Columbia) into pRS425. A PCR-amplified fragment containing the ScSMP3 coding region, 513 bp of its native promoter, and 346 bp immediately downstream of its stop codon was cloned into pRS416 to make p416-PGK-ScSMP3. Yeast cells were transformed using lithium acetate (35).

| Strain name | Plasmid(s) | Genotype | Reference or source |
|-------------|------------|----------|---------------------|
| YMW1        | MAT a ade2-1 ade3-322 his3-11,15 leu2-3,112 trpl-1 ura3-1 can1-100 |  |  |
| YJG1        | MAT a his3Δ1 met150 ura3Δ0 ade3Δ22 smp3Δ2 MAT a ade2-1 ade3Δ22 his3-11,15 leu2-3,112 trpl-1 ura3-1 can1-100 | This study |  |
| smp3-2 (a)  | smp3-2 MAT a his3Δ1 ura3Δ0 ade2-1 smp3-2 MAT a ade2-1 ade3Δ22 his3-11,15 leu2-3,112 trpl-1 ura3-1 can1-100 | This study |  |
| smp3-2 (b)  | smp3-2 MAT a his3Δ1 ura3Δ0 ade2-1 smp3-2 MAT a ade2-1 ade3Δ22 his3-11,15 leu2-3,112 trpl-1 ura3-1 can1-100 | This study |  |
| smp3-2 (D4) | smp3-2 MAT a his3Δ1 ura3Δ0 ade2-1 smp3-2 MAT a ade2-1 ade3Δ22 his3-11,15 leu2-3,112 trpl-1 ura3-1 can1-100 | This study |  |
| smp3:KAN #/smp3 | smp3:KAN #/smp3 MAT a his3Δ1 ura3Δ0 ade2-1 ade3Δ22 his3-11,15 leu2-3,112 trpl-1 ura3-1 can1-100 | This study |  |
| smp3:KAN #/YPGal | smp3:KAN #/YPGal MAT a his3Δ1 ura3Δ0 ade2-1 ade3Δ22 his3-11,15 leu2-3,112 trpl-1 ura3-1 can1-100 | This study |  |
| smp3-2 (a)  | smp3-2 MAT a ade2-1 ade3Δ22 his3-11,15 leu2-3,112 trpl-1 ura3-1 can1-100 | This study |  |
| gpi13:KAN #/gpi13 | gpi13:KAN #/gpi13 MAT a his3Δ1 ura3Δ0 ade2-1 ade3Δ22 his3-11,15 leu2-3,112 trpl-1 ura3-1 can1-100 | This study |  |
| smp3-2-gpi13 | smp3-2-gpi13 MAT a his3Δ1 ura3Δ0 ade2-1 ade3Δ22 his3-11,15 leu2-3,112 trpl-1 ura3-1 can1-100 | This study |  |
| smp3-2-gpi13 | smp3-2-gpi13 MAT a his3Δ1 ura3Δ0 ade2-1 ade3Δ22 his3-11,15 leu2-3,112 trpl-1 ura3-1 can1-100 | This study |  |
| smp3-2-gpi13 | smp3-2-gpi13 MAT a his3Δ1 ura3Δ0 ade2-1 ade3Δ22 his3-11,15 leu2-3,112 trpl-1 ura3-1 can1-100 | This study |  |
| gpi10::KAN #/gpi10 | gpi10::KAN #/gpi10 MAT a his3Δ1 ura3Δ0 ade2-1 ade3Δ22 his3-11,15 leu2-3,112 trpl-1 ura3-1 can1-100 | This study |  |
| yjr013c::KAN #/yjr013c | yjr013c::KAN #/yjr013c MAT a his3Δ1 ura3Δ0 ade2-1 ade3Δ22 his3-11,15 leu2-3,112 trpl-1 ura3-1 can1-100 | This study |  |

Mammalian Cell Culture and Transfection Conditions—HeLa cells were obtained from the American Type Culture Collection (ATCC; Manassas, VA) and cultivated in minimum Eagle’s medium (ATCC) or RPMI 1640 medium (Invitrogen) supplemented with 10% fetal bovine serum. Cells were cultured at 37 °C in a humidified atmosphere containing 5% CO2. For transient transfection of cells for metabolic labeling experiments, 1.5–3 × 10^6 cells were plated at 15–21 cm^2 dishes and transfected with FuGENE 6 Transfection Reagent (Roche Applied Science) the next day. The transfected cells were incubated at 37 °C in 5% CO2 for 24–48 h prior to labeling (see below). Stable hSMP3-expressing HeLa cell lines were made by transfecting HeLa cells in 60-mm dishes with 2 μg of pRS416-hGPI3-HA using FuGENE 6. Stable transfectants were selected in minimum Eagle’s medium containing 500 μg of G418/ml.

Expression of hGPI3, ScSMP3, and Pig-B in HeLa Cells—A human SMP3 cDNA was amplified by PCR from a human brain cDNA library using the primers 5’-CCGGAATTCATGCAGATCTGTGGATCCAGC-3’ and 5’-GGATCCGGGATGAGGAGGCCCCT-CCAGGAATTC-3’ to create a translational fusion reporter with a carboxyl-terminal HA epitope, was cloned into the EcoRI-Xhol sites of the mammalian expression vector pCDNA3.1 (Invitrogen). A human Pig-B cDNA was amplified by PCR from a human liver cDNA library using the primers 5’-GCTGATCGCTGAGAGGAGCCCT-ACAGC-3’ and 5’-TTCCCTGCTCACCTCCGACCTGATCCTGCTCTGATCCTAGGAATTC-3’. The product, encoding hSmp3p with a carboxyl-terminal HA epitope, was cloned into the BamHI-Xhol sites of pCDNA3.1. ScSMP3 was amplified from p416-Sc- SMP3 by PCR using the primers 5’-GTAGGATCCACCCCATGATGATGATCATTGAGGAGGAGGAGCCCT-ACAGC-3’ and the product, encoding ScSmp3p with a carboxyl-terminal HA epitope, was cloned into the BamHI-Xhol sites of pCDNA3.1.
maintained at 25\(/{^\circ}C\) before labeling with \([3H]\)inositol for 2 h as described previously (19). Viable spores grew into colonies after 3 days at 30\(/{^\circ}C\). Four viable haploids from a tetrad of sporulated smp3::KanR/SMP3 cells harboring pGAL-hSMP3-3HA, pGAL-ScSMP3-3HA, or an empty vector control were sporulated. Haploid ascospores were dissected from asci onto YPGal agar medium. Viable spores were grown for 16 h at 25\(/{^\circ}C\) on YPGal agar medium containing 200 \(\mu\)g of G418/ml or SGal agar medium with 1 \(\mu\)g of 5-fluoroorotic acid (5-FOA)/ml for 3 days at 30\(/{^\circ}C\). 10-fold serial dilutions of smp3-2 cells harboring pPGK-hSMP3-3HA, p416-ScSMP3, or an empty vector were spotted onto YPD agar medium with or without 16 \(\mu\)g of Calcofluor white (CFW)/ml and grown for 4 days at 25\(/{^\circ}C\).

**Enzyme Treatment of Human GPI Intermediates**—[\(^3H\)]Mannose-labeled GPI mannolipids were treated with PI-phospholipase C as described previously (19), with 10 units of jack bean \(\alpha\)-mannosidase (JBeM; Sigma) for 20 h at 57\(/{^\circ}C\) in 3 \(M\) \(\left(NH_4\right)_2SO_4, 0.1 \text{ mM zine acetate, pH 7.5, 0.5\% Nonidet P-40 Substitute (Sigma), or in 10\% fetal bovine serum as a source of GPI-phospholipase D. After labeling, the cells were washed twice with 5 ml of PBS and released from culture plates by incubation in 1 mM EDTA containing 0.25\% trypsin at room temperature for 10 min. Cells were pelleted and washed with 10 ml of PBS.

Radiolabeled lipids were extracted from both yeast and mammalian cells in chloroform:methanol:water (10:3:3, v/v/v) and partitioned in 1-butanol as described previously (19). Radiolabeled lipids were analyzed by separation on silica 60 TLC plates (EM Science, Darmstadt, Germany). TLC plates were pre-run in solvent A (chloroform:methanol:water, 65:25:4, v/v/v), after which lipids were separated in solvent B (chloroform:methanol:water, 10:10:2.5, v/v/v), solvent C (chloroform:methanol:water, 10:10:2.75, v/v/v), or solvent D (chloroform:methanol:water, 10:10:3, v/v/v). TLC-separated lipids were visualized by fluorography using a BioMax Transcreen LE intensifier screen and BioMax MS film (Eastman Kodak) for 4–6 days.

**Northern Analysis**—PCR was used to amplify a 428-bp probe spanning bases 74–503 of the human hSMP3 cDNA. To radiolabel the probe, 100 ng of the product was used as template in a second PCR containing a 1 \(\mu\)M concentration of each primer, 50 \(\mu\)M each dCTP, dGTP, and dTTP, 0.5 \(\mu\)M dATP, 10 pmol \((\alpha\text{-}32\text{P})\)dATP, and 1 unit of platinum \(P_fX\) DNA polymerase. Thermocycling consisted of incubation at 94\(/{^\circ}C\) for 30 s followed by 15 rounds of successive incubations at 94\(/{^\circ}C\) for 30 s and 68\(/{^\circ}C\) for 2 min, then a final incubation at 68\(/{^\circ}C\) for 5 min. Radiolabeled probe was purified by passage through a ChromaSpin column (Clontech, Palo Alto, CA). A multiple human tissue mRNA blot (BD Biosciences) was prehybridized at 65\(/{^\circ}C\) for 1 h in 10 ml of ExpressHyb.

** FIG. 1.** hSMP3 restores viability and full cell wall synthesis to Sc smp3 mutants. A, heterozygous smp3::Kan\(^{-}/\)SMP3 diploid S. cerevisiae cells harboring pGAL-hSMP3-3HA, pGAL-ScSMP3-3HA, or an empty vector control were sporulated. Haploid ascospores were dissected from asci onto YPGal agar medium. Viable spores grew into colonies after 3 days at 30\(/{^\circ}C\). B, four viable haploids from a tetrad of sporulated smp3::KanR/SMP3 cells harboring pGAL-hSMP3-3HA were grown on YPGal agar medium containing 200 \(\mu\)g of G418/ml or SGal agar medium with 1 \(\mu\)g of 5-fluoroorotic acid (5-FOA)/ml for 3 days at 30\(/{^\circ}C\). C, 10-fold serial dilutions of smp3-2 cells harboring pPGK-hSMP3-3HA, p416-ScSMP3, or an empty vector were spotted onto YPD agar medium with or without 16 \(\mu\)g of Calcofluor white (CFW)/ml and grown for 4 days at 25\(/{^\circ}C\).
**RESULTS**

Identification and Cloning Human SMP3—We reasoned that the protein responsible for Man-4 addition to human GPIs might resemble the fourth GPI mannosyltransferase of *S. cerevisiae* Smp3p. Locus 80235 of human chromosome 3 encodes a predicted 579-amino acid protein (FLJ12769) that has been grouped into a family of dolichol phosphate mannose-utilizing mannosyltransferase sequences by its homology with the asparagine glycosylation pathway mannosyltransferases Alg9p and Alg12p and the GPI mannosyltransferases PIG-Bp and *S. cerevisiae* Smp3p (23, 36). Human FLJ12769 contains a HQE/RF amino acid motif that is characteristic of the SMP3 group within this sequence family. Additionally, it exhibits 30% identity and 56% similarity to *S. cerevisiae* Smp3p and 26% identity and 50% similarity to *Schizosaccharomyces pombe* Smp3p, thus making it a plausible candidate human GPI fourth mannosyltransferase. We refer to this protein as hSmp3p in this study. Because Man-4 has been identified on the GPIs of two purified brain proteins (6, 26), we used a human brain cDNA library as template for PCR amplification of a hSMP3 cDNA for cloning. The deduced protein sequence of the cloned hSMP3 cDNA was identical to that of FLJ12769.

**Human SMP3 Complements the Growth Defects of Yeast smp3 Mutants—**Haploid *S. cerevisiae* cells harboring a deleted *smp3* locus are inviable (23). Therefore, to determine whether hSMP3 functions similarly to *ScSMP3*, we tested its ability to restore viability to a yeast *smp3* null mutant. A hSMP3 cDNA was expressed from the *S. cerevisiae GAL10* promoter (pGAL-hSMP3) in a heterozygous *SMP3/smp3::Kan* strain diploid. Diploid cells were sporulated, and complementation was assessed via tetrad analysis. Asci from diploids harboring pGAL-hSMP3 gave rise to four viable haploid progeny (Fig. 1A). Additionally, two haploids from each tetrad were resistant to G418 and sensitive to 5-fluoroorotic acid, indicating that they harbored the *smp3::Kan* allele and that their viability was dependent upon the complementing *URA3*-containing pGAL-hSMP3 plasmid (Fig. 1B). Expression of hSMP3 also restored viability to a temperature-sensitive (t-s) *smp3* mutant. *S. cerevisiae* strain at nonpermissive temperature (data not shown) and a haploid strain of the fission yeast *S. pombe* harboring a lethal *smp3* null mutation.

**Fourth Mannose Addition to Human GPI Precursors**

*Fig. 2. Human SMP3 complements Man₄-GPI accumulation in yeast smp3 mutants.* A, expression of hSMP3 suppresses accumulation of Man₃-GPIs. *S. cerevisiae* Δsmp3 cells harboring the pGAL-ScSMP3 or pGAL-hSMP3 were grown and [³H]inositol labeled in medium containing galactose (lanes 3 and 5) or glucose (lanes 2 and 4) to induce or repress SMP3 expression, respectively. Radiolabeled lipids were extracted from cells and separated by TLC in solvent B. Lane 1 consists of lipids from smp3-2 cells radiolabeled with [³H]inositol at 37 °C. The Man₃-GPI that accumulates in smp3-2 cells is a mixture of isoforms bearing EthN-P on either Man-1 or Man-2 (23) as shown. B, in vivo mannosylation of Man₄-GPIs in a *S. cerevisiae* smp3Δgpi13 Δsmp3-2 mutant. The smp3-2gpi13Δ-KAN*-pGAL-GPI13 strain was [³H]inositol labeled in glucose-containing medium at 37 °C to repress GPI13 expression and arrest the t-s smp3-2 allele. Lipids were extracted and separated by TLC in solvent B (lane 1). The same labeling conditions were applied to smp3-2gpi13Δ-KAN*-pGAL-GPI13 cells harboring p425-PGK-hSMP3-HA (lane 3) or a pScSMP3 control plasmid (lane 2). Control lanes 4, 5, and 6 represent [³H]inositol-labeled lipids isolated from smp3-2 cells, gpi13Δ:KAN*-pGAL-GPI13 cells, or wild-type (WT) cells, respectively. The schematic depicts the pathway steps surrounding the addition of Man-4 to yeast GPIs and the structures of GPIs that accumulate in smp3 and gpi13 cells (19, 23). M₁, M₂, M₃, and M₄ represent GPI mannoses in the order of their addition to GPIs; G, glucosamine; PE, ethanolamine phosphate. 

(Contech) containing 1 mg of denatured salmon testes DNA (Sigma). Labeled probe was mixed with 30 μg of denatured salmon testes DNA, and 50 μl of 20× SSC (3 M NaCl, 0.3 M sodium citrate, pH 7.0). This mixture was incubated at 95 °C for 5 min, at 68 °C for 30 min, and then added to the blot. Hybridization was performed overnight at 65 °C followed by five 20-min washes at 65 °C with 200 ml of 2× SSC containing 1.0% SDS and two 20-min washes at 55 °C with 200 ml of 0.1× SSC, containing 0.5% SDS. Hybridized probe was quantitated using a Storm 860 PhosphorImager and ImageQuant software (Amersham Biosciences).
Vivo in GPI synthesis which results in accumulation of a Man3-GPI transferase activity. does not encode a protein with promiscuous GPI mannosyl-

is grown in medium containing glucose, h

expression is regulated by the

expression complemented the cell wall synthesis defect of smp3-2 cells by restoring their ability to grow in the presence of Calcofluor white (Fig. 1C). Considered together, these data show that hSMP3 can specifically replace the ScSMP3 GPI Man-4 transferase in vivo in yeast and suggest that hSMP3 does not encode a protein with promiscuous GPI mannosyl-

intermediates. To expand on this notion, we examined whether hSMP3 expression facilitated conversion of a Man3-GPI to a Man4-GPI in an in vivo mannosyltransferase assay. In this experiment, we utilized a haploid S. cerevisiae strain with conditional defects in two GPI biosynthetic genes: (i) a t-s smp3-2 allele that causes accumulation of a Man3-GPI and (ii) a glucose-repressible wild type allele of GPI13, a downstream gene involved in EthN-P transfer to Man-3, which accumulates a Man4-GPI when grown in medium containing glucose. Cells grown and

labeled with [3H]inositol (23). Therefore, we tested the ability of hSMP3 expression to abolish Man3-GPI accumulation in a smp3 mutant strain. We constructed a haploid S. cerevisiaeΔsmp3 strain harboring pGAL-hSMP3 from which hSMP3 expression is regulated by the G Aly10 promoter. When this strain is grown in medium containing glucose, hSMP3 expression is repressed, cells become depleted of hSmp3p, and Man3-GPIs accumulate (Fig. 2A, lane 4). However, only traces of Man3-GPIs accumulate in cells grown in galactose where hSMP3 expression is induced (Fig. 2A, lane 5). Thus, production of hSmp3p abolishes accumulation of Man3-GPIs in smp3 cells, presumably by facilitating their conversion to downstream GPI intermediates.

Immunolocalization of hSmp3p in the Mammalian ER—The ability of hSmp3p to function in place of ScSmp3p in yeast suggests that the addition of Man-4 to human GPIs may occur during GPI synthesis in the ER. Therefore, we used immunolocalization microscopy to determine the native subcellular location of hSmp3p in human HeLa cells. We first compared localization of hSmp3p to PIG-Bp, a mannosyltransferase involved in the addition of Man-3 to human GPIs during their synthesis in the ER (38). In transiently transfected HeLa cells producing both FLAG-tagged PIG-Bp (PIG-Bp-FLAG) and HA-tagged hSmp3p (hSmp3p-HA), immunostaining revealed colocalization of the two proteins (Fig. 3A). Additionally, we compared the localization of hSmp3p-HA with the endogenous ER and Golgi marker proteins calretilcumin and golgin-97, respectively. The immunostaining pattern of hSmp3p-HA was iden-

tical to that of ER-localized calretilcin (Fig. 3B) and distinct from Golgi-localized golgin-97 (Fig. 3C). We conclude that hSmp3p is targeted to the ER in human HeLa cells.

Distribution of hSMP3 mRNA in Human Tissues and Cell Lines—The distribution of hSMP3 mRNA in various human tissues and cell types was determined by Northern analysis. A commercial RNA expression array containing human mRNA from 58 adult tissues, 7 fetal tissues, and 8 cell lines was probed with a [32P]labeled hSMP3 cDNA fragment. Expression of hSMP3 was detected in most adult tissues and all fetal tissues (Fig. 4, A and B). In adult tissues, hSMP3 mRNA was most abundant in brain tissues with the highest levels in the cerebellum (Fig. 4A, 17 and 18). This pattern was also seen in fetal samples, where hSMP3 mRNA was most abundant in the brain (Fig. 4B, 67). Expression of hSMP3 was notably high in transverse colon, descending colon, and rectal tissues (Fig. 4, 36–38). No hSMP3 expression was detected in spleen tissue (Fig. 4B, 49) or peripheral blood leukocytes (Fig. 4B, 51). Interestingly, hSMP3 mRNA was only weakly detected in each cultured cell line sample represented in the array (Fig. 4A, 1–8). The finding that hSMP3 mRNA levels are higher in some tissues (e.g. brain and colon) leads to the prediction that levels of hSmp3 protein are also elevated in these tissues. However, Western blotting of standardized lysates from colon, brain, thymus, and spleen cells using a polyclonal antiserum raised to amino acids 443–579 of hSmp3 failed to detect hSmp3p (data not shown). Therefore, native levels of hSmp3p appear to be very low.

Human Smp3p Expression Causes Man4-GPI Formation in Vivo in HeLa Cells—Because endogenous hSMP3 mRNA was nearly undetectable in many cultured human cell lines by Northern analysis, we reasoned that increasing hSMP3 expression might result in Man4-GPI formation. We looked for Man4-GPIs in HeLa cells either transiently or stably transfected with a vector expressing HA-tagged hSmp3p by metabolic labeling of GPIs with [3H]mannose. In experiments with transiently transfected cells, typically 50–60% of cells immunostained

3 A. Fabre, B. A. Taron, and C. H. Taron, unpublished data.
FIG. 4. Distribution of hSMP3 mRNA in human tissues and cell lines. A and B, a commercial human multiple tissue mRNA array was probed with a 32P-labeled hSMP3 cDNA. The amount of mRNA in each sample has been adjusted by the vendor to produce normalized signals for various housekeeping genes. Thus, hSMP3 mRNA abundance can be compared between samples. The hybridized array was exposed for 69 h and then quantitated using a PhosphorImager. The numbered samples represent mRNA from the following tissues and cell lines: 1, leukemia (undifferentiated HL-60); 2, HeLa S3; 3, leukemia (K-562); 4, leukemia (MOLT-4); 5, Burkitt’s lymphoma (Raji); 6, Burkitt’s lymphoma (Daudi); 7, colorectal adenocarcinoma (SW480); 8, lung carcinoma (A549); 9, whole brain; 10, cerebral cortex; 11, parietal lobe; 12, occipital lobe; 13, temporal lobe; 14, paracentral gyrus of cerebral cortex; 16, pons; 17, left cerebellum; 18, right cerebellum; 19, corpus callosum; 20, amygdala; 21, caudate nucleus; 22, hippocampus; 23, medulla oblongata; 24, putamen; 25, accumbens nucleus; 26, thalamus; 27, spinal cord; 28, esophagus; 29, stomach; 30, duodenum; 31, jejunum; 32, ileum; 33, iliocecum; 34, appendix; 35, ascending colon; 36, transverse colon; 37, descending colon; 38, rectum; 39, heart; 40, aorta; 41, left atrium; 42, right atrium; 43, left ventricle; 44, right ventricle; 45, interventricular septum; 46, apex of the heart; 47, kidney; 48, skeletal muscle; 49, spleen; 50, thymus; 51, peripheral blood leukocyte; 52, lymph node; 53, bone marrow; 54, trachea; 55, lung; 56, placenta; 57, bladder; 58, uterus; 59, prostate; 60, testis; 61, ovary; 62, liver; 63, pancreas; 64, adrenal gland; 65, thyroid gland; 66, salivary gland; 67, fetal brain; 68, fetal heart; 69, fetal kidney; 70, fetal liver; 71, fetal spleen; 72, fetal thymus; 73, fetal lung; 74, 100 ng of genomic DNA.
Fig. 5. Overexpression of hSMP3 or ScSMP3 in HeLa cells results in formation of novel GPI mannolipids. A, expression vectors harboring hSMP3 or ScSMP3 were introduced into HeLa cells by either transient or stable transfection. Transfected cells were incubated with [3H]mannose to label GPI mannolipids, after which lipids were extracted from cells and separated by TLC in solvent C. [3H]Mannose-labeled GPI mannolipids from HeLa cells transiently transfected with an empty vector (V, lane 1), a hSMP3 expression vector (Hs, lane 2), or a ScSMP3 expression vector (Sc, lane 3) are shown, along with those from untransformed HeLa cells (minus sign, lane 4) and from a stable hSMP3-expressing HeLa cell line (B7T cells, lane 5). B, [3H]mannose-labeled lipids isolated from HeLa cells transiently expressing hSMP3 were incubated without or with JBaM (lanes 2 and 3), PI-phospholipase C (PLC) (lanes 4 and 5), and GPI-phospholipase D (PLD) (lanes 6 and 7), then separated by TLC in solvent C. C, TLC-purified H8 and M4A were incubated without or with JBaM and then separated by TLC in solvent D. Lane 1 of B and C are lipids from HeLa cells transiently expressing hSMP3. In all panels, the positions of M4A, M4B, and M4C, of known human GPI intermediates (H2–H8), and of dolichol phosphomannose (DPM) are indicated. In A, the mannose portion of glycans from characterized Man3-GPIs are shown. M1, M2, and M3 represent GPI mannoses in the order of their addition to GPIs; E, ethanolamine phosphate; O, origin.

Fourth Mannose Addition to Human GPI Precursors

Yeast and human GPI precursors differ in the extent to which a fourth mannose is present as a side branch on the third core mannose. In yeast, all GPI precursors likely receive four mannoses because the addition of Man-4 is a mandatory step in GPI biosynthesis which precedes GPI attachment to proteins (23). However, to date, studies of mammalian GPI synthesis have uncovered little evidence that an analogous pathway step occurs in mammals. In the present study, we report the characterization of hSmp3p, a member of a mannosyltransferase family that includes the S. cerevisiae Smp3p GPI fourth mannosyltransferase. First, we demonstrated that hSMP3 could replace ScSMP3 in vivo in yeast complementation experiments, and that expression of hSMP3 in yeast led to in vivo mannosylation of a Man2-GPI. Second, we showed that constitutive expression of hSMP3 in HeLa cells results in a dramatic increase in formation of human Man2-GPIs. Third, we showed that hSmp3p immunolocalizes to the human ER. Together, these studies indicate that hSmp3p functions as a mannosyltransferase that is capable of adding a fourth α-linked mannose to human Man2-GPI intermediates during GPI precursor synthesis in the ER. In addition, we demonstrated that hSMP3 is expressed in most human tissues but is only weakly expressed in many cultured mammalian cell lines. Our findings lead us to modify current models for the mammalian GPI assembly pathway. They also raise the possibility that Man2- and Man3-GPIs may be expressed in a tissue-specific manner and have impli-
Specificity of Man-4 Addition to Human GPIs—Five mammalian Man₃-GPI intermediates (H₄, H₆, H₇, H₇', and H₈) that differ in the number and position of EthN-P residues have been characterized structurally (10, 12–15, 24, 39, 40). In principle, each could be an acceptor lipid for the transfer of Man-4. However, our observations of the lipids that form in BT7 cells, a HeLa cell line that stably expresses hSmp3p, suggest that only certain Man₃-GPIs receive a fourth mannose. In BT7 cells, the most abundant late stage GPI intermediates are the putative Man₃-GPIs M₄A, M₄B, and M₄C, suggesting that the presence of hSmp3p has nearly completely biased the GPI synthetic pathway toward production of Man₃-GPIs (Fig. 5A, lane 5). These cells form normal levels of only two Man₃-GPIs (H₄ and H₇') and are nearly devoid of lipids H₆, H₇, and H₈. Thus, it is likely that pools of H₆, H₇, and H₈ have become depleted because they are converted efficiently to Man₄-GPIs, whereas H₄ and H₇' are either not acceptors for Man-4 transfer or are not accessible to hSmp3p.

The most recent scheme for mammalian GPI anchor synthesis suggests that the pathway is split into two branches (15). We have amended this model to reflect our current data (Fig. 6). Because there is no evidence from metabolic labeling studies that Man-4 is added to H₄ or H₇', we propose that Man-4 transfer to human GPIs likely occurs only within Pathway I of the branched model. In one model, hSmp3p transfers Man-4 to H₆ to form M₄C, which is then converted to M₄B and M₄A (solid arrows). Alternatively, hSmp3p adds Man-4 directly to the Man₃-GPIs H₈, H₇, and H₆ (dotted arrows). GlcNAc-PI, N-acetylglucosaminyl phosphatidylinositol; GlcN-PI, glucosaminyl phosphatidylinositol; GlcN(acyl)-PI, glucosaminyl acyl phosphatidylinositol; hexagons, inositols; G, glucosamine; M, mannose; E, ethanolamine phosphate. The structures of M₄B and M₄C are predicted.
expressed (see next section). Additionally, because M4A and M4B likely have EthN-P on Man-3 on Man-3, both could be potential Man$_4$-GPI substrates for GPI transamidase.

Formation of the putative Man$_4$-GPIs M4A, M4B, and M4C could occur two ways: (i) by direct addition of Man-4 to lipids H8, H7, and H6, respectively; or (ii) by addition of Man-4 to H6 to form M4C, after which M4C is converted to M4B and then to M4A by the sequential addition of two EthN-P groups. Several observations support the latter model. First, hSmp3p can replace ScSmp3p in vivo, and expression of either ScSMP3 or hSMP3 in HeLa cells causes formation of the same three Man$_4$-GPIs, suggesting that these proteins have similar, if not identical specificities for their acceptor GIPs. Because ScSmp3p likely transfers Man-4 to yeast Man$_4$-GPIs bearing a single EthN-P (23), GPI lipids with similar head groups, including human H6, may be preferred substrates for Smp3p mannosyltransferases. Second, if M4C is a Man$_4$ form of H6, then it does not bear EthN-P on Man-3 and is not able to be transferred to proteins. Thus, M4C is likely a precursor of M4A and M4B. Finally, B7 HeLa cells form predominantly M4A and much lower quantities of M4B and M4C, suggesting that M4A is an end product of Man$_4$-GPI synthesis. Our current efforts to address the specificity of hSmp3p in vitro will help further determine which Man$_4$-GPIs can serve as lipid acceptors for Man-4 transfer.

Abundance of Man$_4$-GPIs in Human Cells—To date, the importance of Man-4 addition to mammalian GPI intermediates has been unclear because Man$_4$-GPI formation has scarcely been observed in studies of GPI biosynthesis in cultured mammalian cell lines. However, our data suggest that cultured cell lines may not provide an accurate picture of the abundance of Man$_4$-GPIs. Using cultured HeLa cells, we showed that: (i) expression of hSMP3 is barely detectable by Northern analysis of HeLa cell mRNA, (ii) HeLa cells form predominantly Man$_3$-GPIs, and (iii) increasing hSMP3 expression in HeLa cells causes abundant formation of Man$_4$-GPIs. Thus, the ability of a cell to form Man$_4$-GPIs likely depends on the level of hSMP3 expression. Our finding that hSMP3 is barely expressed in many cell lines may account for the absence of Man$_4$-GPI formation in studies of GPI synthesis. However, formation of Man$_4$-GPIs may be far more common and perhaps predominant in most human tissues where hSMP3 mRNA levels are significantly higher (Fig. 4). In support of this notion, a fourth mannose is present on 7 of 10 characterized mammalian protein-bound GIPs (6, 7, 26–31, 41, 42). Six of the 7 Man$_4$-GPIs were isolated from proteins purified directly from primary sources like homogenized organ tissue (6, 26, 28, 29, 31) or urine (27). Only 1 was from a protein purified from a cultured mouse cell line (30). In all cases, however, mixtures of both Man$_3$- and Man$_4$-GPIs were observed.

We would predict Man$_4$-GPIs to be most prevalent in brain and colorectal tissues where hSMP3 expression is greatest, and this notion is supported by structural data indicating that Man$_4$-GPIs are present on various GPI-anchored brain proteins (6, 26). Additionally, hSMP3 expression is barely detectable in the spleen and peripheral blood leukocytes, suggesting that Man$_4$-GPIs may predominate in these tissues. Thus, it is possible that the presence or absence of Man-4 on GPI precursors is regulated according to cell type, and we speculate that these differences in GPI glycan structure affect the function or localization of specific GPI-anchored proteins.

Implications for human GPI Transamidase Specificity—Our demonstration that human cells produce both Man$_3$- and Man$_4$-GPI precursors raises the possibility that the human GPI transamidase complex recognizes both forms as substrates for transfer to proteins. Various observations support this notion. First, mammalian cells form both Man$_3$ and Man$_4$ forms of GPI intermediates that appear structurally competent for transfer to proteins because of the presence of EthN-P on Man-3 (e.g., H7, H7’, M4A, and M4B). Second, a mammalian cell line defective in GPI transamidase accumulates both Man$_3$-GPIs and trace amounts of lipids with chromatographic mobilities consistent with their being the Man$_3$-GPIs M4A and M4B (15). Third, the Man$_4$-GPI H8 and a lipid whose chromatographic mobility is consistent with its being the Man$_4$-GPI M4A physically associate with human GPI transamidase in immunoprecipitation experiments (43). Finally, mammalian GPIs having either three or four mannoses have been isolated from purified GPI-anchored proteins (6, 7, 26–31, 41, 42). Thus, in mammalian cells, it is likely that transfer of GPIs to proteins occurs irrespective of the presence or absence of Man-4 on GPI precursors.

In contrast, the addition of Man-4 to S. cerevisiae GPIs is a mandatory step in GPI precursor synthesis which precedes EthN-P addition to Man-3 and subsequent attachment of complete GPI precursors to proteins (23). To date, there is no evidence that yeast can form Man$_3$-GPIs that bear EthN-P on Man-3. Additionally, yeast GPI transamidase mutants accumulate exclusively Man$_4$-GPI intermediates (20, 21, 44). Therefore, it is likely that yeast GPI transamidase exclusively requires Man$_4$-GPIs as substrates for transfer to proteins. Thus, fungal and mammalian GPI transamidases may have important differences in specificity which could potentially be exploited in the development of novel anti-fungal strategies.

In summary, we have shown (i) that the human genome encodes a fourth GPI mannosyltransferase that is functional and ER-localized when expressed in human cells and (ii) that although the activity of a fourth GPI mannosyltransferase is scarcely detectable in cultured HeLa cells, hSMP3 is expressed widely in human tissues. Our findings also suggest that Man$_4$-GPIs may be formed abundantly in certain human tissues, whereas Man$_3$-GPIs may predominate in others, raising the possibility that GPI glycans may differentially influence localization and function of GPI-anchored proteins. Further, our results support the notion that human and fungal GPI transamidases differ in their specificity for GPI precursors, with the human transamidase recognizing both Man$_3$- and Man$_4$-GPIs and the fungal transamidase requiring exclusively Man$_4$-GPIs.

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