THE JOURNAL OF BIOLOGICAL CHEMISTRY
Vol. 270, No. 34, Issue of August 25, pp. 19709–19715, 1995
Printed in U.S.A.

Biosynthesis of the Side Chain of Yeast Glycosylphosphatidylinositol Anchors Is Operated by Novel Mannosyltransferases Located in the Endoplasmic Reticulum and the Golgi Apparatus*

(Received for publication, March 27, 1995, and in revised form, May 31, 1995)

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Glycosylphosphatidylinositol (GPI) anchors of the yeast *Saccharomyces cerevisiae* have been reported to contain three different types of side chains attached to the α1,2-linked mannose of the conserved protein-ethanolamine-PO_4-Manα1,2Manα1,6Manα1,4GlcNH_2-inositol glycan core. The possible side chains are Manα1,2- or Manα1,2Manα1,2- or Manα1,3Manα1,2- (Fankhauser, C., Homan, S. W., Thomas Oates, J. E., McConville, M. J., Desponds, C., Conzelmann, A., and Ferguson, M. A. (1993) *J. Biol. Chem.* 268, 26365–26374). To determine in what subcellular compartment these side chains are made, we metabolically labeled GPI-anchored membrane proteins with *myo*-[2-^{3}H]inositol in secretion mutants blocked at various stages of the secretory pathway and analyzed the anchor structure of the labeled glycoproteins. When the exit of vesicles from the endoplasmic reticulum or entry into the *cis*-Golgi were blocked in *sec12* or *sec18* cells, all anchors contained a side chain consisting of a single α1,2-linked mannose. GPI proteins trapped in the *cis*-Golgi of *sec7* contained Manα1,3Manα1,2- but no Manα1,2Manα1,2- side chains. Mutants blocked at later stages of the secretory pathway made increased amounts of side chains containing two mannoses. Manα1,2Manα1,2- and Manα1,3Manα1,2- side chains were preferentially associated with ceramide- and diacylglycerol-containing GPI anchors, respectively. *Mnn1, mnn2, mnn3, mnn5,* and *mnt1(=kre2), i.e.* mutants which lack or down-regulate 1,2- and 1,3-mannosyltransferases used in the elongation of *N*- and *O*-glycans in the Golgi, add the fifth mannose to GPI anchors normally. The same conclusion was reached through the analysis of deletion mutants in *KTR1, KTR2, KTR3, KTR4,* and *YUR1* which all are open reading frames with high homology to *MNT1*. Mutants deficient in the Golgi elongation of *N*-glycans such as *anp1, van1, mnn9* are deficient in the maturation of the *N*-glycans of GPI-anchored glycoproteins, but process the GPI anchor side chain normally. Data are consistent with the idea that the fourth mannose is added to proteins as part of the anchor precursor glycolipid in the endoplasmic reticulum, whereas the fifth mannose is added by not yet identified α1,3- and α1,2-mannosyltransferases located in the Golgi apparatus.

The addition of glycosylphosphatidylinositol (GPI)[1] anchors to the carboxyl terminus of newly synthesized polypeptides occurs as an early post-translational modification of proteins entering the secretory pathway (1, 2). While the core carbohydrate structure linking the protein to the lipid moiety (Fig. 1) is conserved throughout eukaryotic evolution, many organisms attach additional sugars and/or other groups to this core. All GPI anchors of *Saccharomyces cerevisiae* contain a fourth mannose residue (*M4*, Fig. 1) attached to the α1,2-linked mannose of the glycan core and part of yeast GPI anchors also contain a fifth mannose (*M5*) which is linked either α1,2 or α1,3 to M4 (3). It is unknown if a given protein is made with several different kinds of side chains or if each protein is made with only one kind. The presence of a fourth, α1,2-linked mannose has also been found as a species- and tissue-specific modification in mammalian GPI anchors (4, 5). Recently, the same α1,2-linked mannose has also been found in *Dictyostelium discoideum* (6) and on a GPI glycolipid made by merozoites of *Plasmodium falciparum* (7). Here we undertook to exploit the well defined secretion and glycosylation mutants of *S. cerevisiae* in order to investigate the subcellular localization and identity of the mannosyltransferases involved in the biosynthesis of the mannose side chains of yeast GPI anchors.

EXPERIMENTAL PROCEDURES

Yeast Strains and Materials—The secretion mutants developed by Peter Novick and Randy Schekman (8, 9) were: HMSF1 *MATa sec1–1*, SF294–2B *MATa sec7–1*, SF226–1C *MATa sec12–4*, HMSF169 *MATa sec14–3*, HMSF176 *MATa sec18–1*, with all of them being derived from X2180–1A *MATa SUC2 mal gal2 CUP1*. Glycosylation mutants were the ones described by C. Ballou (10) and P. Robbins *et al.* (11): LB1–22D *MATα mnn1 SUC2 mal gal2 CUP1*, LB1–16A *MATα mnn2 SUC2 mal gal2 CUP1*, LB54–3A *MATa mnn3* and LB-65–5D *MATa mnn5* are derived from X2180. YAH 116 *ura3–52 lys 2–801 ade2–101 trp1-Δ1 his3-Δ200 mnt1::TRP1* is derived from YAH-115 which is identical except that it has an intact *MNT1* gene. Strains containing disruptions in open reading frames which are highly homologous to *MNT1(=KRE2)* were developed by M. Jaquet and M. Lussier in SEY6210 *leu2–3,112 ura3–52 his3Δ200 lys2–801 trp1Δ901 suc2Δ9*. A quadruple deletion contained *leu2–3,112 ura3–52 his3Δ200 lys2–801 trp1Δ901 suc2Δ9 kre2::TRP1 yur1::HIS3 ktr1::LYS2 ktr2::URA3*; strains containing single deletions were YBR1445 *leu2–3,112 ura3–52 his3Δ200 lys2–801 trp1Δ901 suc2Δ9 ktr3::HIS3* and YBR1411 *leu2–3,112 ura3–52 his3-Δ200 lys2–801 trp1Δ901 ktr4::HIS3* (12). Disruptants of *ANP1* (RCY1 *MATa leu2–3, 112 ura3–52 his3Δ200 lys2–801 trp1Δ901 suc2Δ9 anp1::LEU2*) and *VAN1* (RCY2 *MATa leu2–3, 112 his3Δ200 lys2–801 trp1Δ901 suc2Δ9 van1::TRP1*) constructed in SEY6210 were obtained from R. Chapman and S. Munro (13). ZY100 (*MATa ade2–101 leu2–3,*

* This work was supported by Swiss National Foundation Grant 3100–032515. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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[1] The abbreviations used are: GPI, glycosylphosphatidylinositol; ASAM, *Aspergillus saitoï* α-mannosidase; CP, complete precursor; ConA, concanavalin A; HF, hydrofluoric acid; JBAM, jack bean α-mannosidase; PI, phosphatidylinositol; PI-PLC, phosphatidylinositol-specific phospholipase C; ER, endoplasmic reticulum.

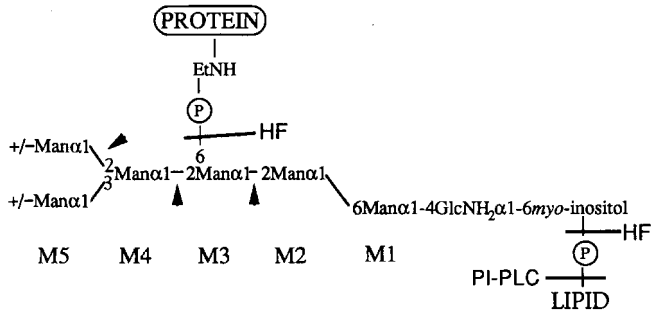

FIG. 1. **Glycosylphosphatidylinositol anchor structures of yeast proteins.** The scheme outlines the structural variants found in *S. cerevisiae* (3). The mannoses are annotated by *M1-M5*. The majority of GPI anchors of wild type cells contain only M1 to M4. A fifth mannose (*M5*) is present only on part of the anchors and is linked either α1,2 or α1,3 to M4 (3). The sites of cleavage obtained by HF dephosphorylation and phosphatidylinositol-specific phospholipase C (*PI-PLC*) are indicated. *Arrows* point toward the linkages which can be hydrolyzed by the α1,2-linkage-specific exomannosidase from ASAM. *EtNH*, ethanolamine; *R*, alkyl chain.

112 ura3–52 suc2Δ9 gal2 pep4::CAT) and ZY400 (*MATa ade2–101 leu2–3, 112 ura3–52 suc2Δ9 gal2 pep4::CAT mnn9::URA3*)(14) were obtained from Vivian MacKay, ZYMOGENETICS. SEY2102 (*MATα suc2Δ9, ura3–52 leu2–3, 112 his4*) and a strain with a deletion in *ERD1* in this same background were obtained from H. Pelham (15). Cells were kept on YPD agar plates and cultured on minimal SDC media containing salts, vitamins, and trace elements but no *myo*-inositol (16), 2% glucose as a carbon source, 1% of casein hydrolysate, adenin and uracil (40 μg/ml). SDC medium of the same composition was used for radiolabeling of cells. *myo*-[2-^{3}H]Inositol (20Ci/mmol) was purchased from Amersham Corp. (Buckinghamshire, United Kingdom). All glycosidases used (α-mannosidases from jack bean and *Aspergillus saitoï*) were purchased from the Oxford GlycoSystems, (Oxford, UK). ConA-Sepharose and octyl-Sepharose CL-4B were from Pharmacia (Uppsala, Sweden).

Preparation and Analysis of Radiolabeled GPI Anchors—Unless indicated otherwise, all radiolabelings were done under identical conditions at 24 °C except for temperature sensitive secretion mutants which were concomitantly labeled at 37 °C. Exponentially growing cells were resuspended to give an OD_{600} of 10 and labeled with *myo*-[2-^{3}H]inositol (30 μCi/ml) for 40 min. At this stage the labeling medium was diluted with 4 volumes of fresh SDC medium, and incubation was continued for another 90 min. Cells were lysed and the glycoproteins were delipidated and adsorbed onto ConA-Sepharose as described (17) (procedure C followed by A). Control experiments showed that all labeled GPI proteins were quantitatively adsorbed onto ConA-Sepharose. The Sepharose beads were incubated with Pronase and resulting anchor peptides were purified over octyl-Sepharose as described (18). Anchor glycopeptides from up to 150 OD_{600} units of cells were taken up in 100 μl of 10 mM Tris-HCl, pH 7.5, 0.2 mM EDTA, 20% propanol, and treated with 0.1 units of PI-PLC for 3 h at 37 °C. Lipids were removed by extraction with *n*-butanol. The lipid-free anchor head groups were dephosphorylated by HF, treated with α-mannosidases, and analyzed by paper chromatography in methylketone/pyridine/H_2O (20:12:11) as described (19). Before chromatography samples were *N*-acetylated and desalted (20). Mild base-sensitive lipid moieties were removed from anchor peptides by a 5-h incubation in 8 M NH_3 in H_2O/CH_3OH (1:1) at 37 °C.

RESULTS

GPI Anchors of the Endoplasmic Reticulum Contain Four Mannoses—Recently two very polar GPIs named CP1 and CP2 have been identified in *S. cerevisiae*. CP1 and CP2 are present in only very low amounts and are rapidly turning over; they contain a single α1,2-linked mannose (M4) added onto the conserved core plus a phosphoethanolamine group on M3 (18) (Fig. 1). The presence of phosphoethanolamine suggests that they potentially might represent the complete precursor GPIs used for GPI anchoring of newly made proteins, a process which takes place in the ER (21, 22). If CP1 and CP2 are the donor lipids for GPI anchoring, we predict that GPI proteins residing in the ER contain four mannoses. To show this, temperature-sensitive secretion mutants deficient in the vesicular

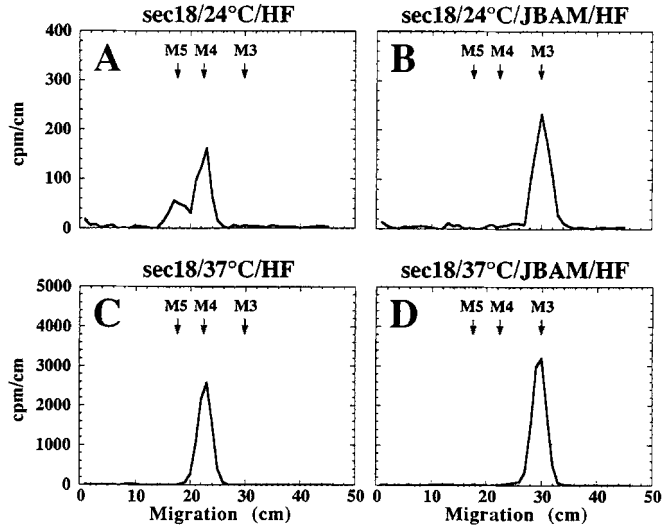

FIG. 2. **Sec18 cells were radiolabeled with *myo*-[2-^{3}H]inositol at 24 °C (*panels A* and *B*) or 37 °C (*panels C* and *D*).** Glycopeptides were generated and either left untreated (*panels A* and *C*) or treated with jack bean α-mannosidase (*JBAM*) (*panels B* and *D*). Subsequently all glycopeptides were dephosphorylated with hydrofluoric acid (HF), resulting fragments were *N*-acetylated and sized by paper chromatography and scintillation counting of 1-cm wide paper strips. *M3-M5* indicate the migrations of radiolabeled Man_3-GlcNAc-Ins to Man_5-GlcNAc-Ins standard oligosaccharides (18) run in parallel on the same paper. Labeling temperatures and the sequential order of treatments are summarized at the top of each panel.

transport from the ER to the Golgi were metabolically labeled with *myo*-[^{3}H]inositol, the anchor peptides of GPI proteins were isolated, treated with HF, and sized by paper chromatography. As shown in Fig. 2, when the transport of vesicles from ER to Golgi was blocked at 37 °C, the anchor peptides of *sec18* indeed contained four mannoses of which only one could be removed by JBAM (Fig. 2, *panels C* and *D*). However, when *sec18* cells were labeled at 24 °C, an additional, slower migrating peak was observed (Fig. 2, *panel A*). The additional, slower migrating peak was also found in wild type cells, both at 24 °C as well as 37 °C Fig. 3, *panel B*). Results similar to the ones shown for *sec18* were also obtained in other mutants blocking between ER and Golgi, namely *sec12* (Table I) and *sec16*.

The slower migrating peak was assumed to represent a fragment with a fifth mannose (*M5*, Fig. 1) which is predicted by the previous analysis of yeast anchors (3). Its identity was confirmed by the following observations: (i) when isolated from a preparative paper chromatogram and treated with jack bean α-mannosidase (JBAM), the slower migrating peak yielded a fragment comigrating in thin layer chromatography with GlcNAc-Ins, not Ins (not shown). (ii) When JBAM treatment of total anchor peptides was done *before* HF treatment, counts were quantitatively recovered in a peak comigrating with the Man_3-GlcNAc-Ins standard indicating that three mannoses were protected by an HF-sensitive group also in this slower migrating peak (Fig. 3, *panel C*). (iii) The HF fragment migrated much slower if *N*-acetylation was omitted, thus indicating the presence of an amino group which is typically found on the glucosamine of GPIs (Fig. 3, *panel A*). (iv) Treatment of the total of anchor peptides with α-mannosidase from *A. saitoi* (ASAM) produced a fragment comigrating with Man_2-GlcNAc-Ins whereby part of the material proved to be resistant (Fig. 3, *panel D*). These findings strongly argue that this additional HF fragment represents a GPI structure. On the basis of the previous analysis of the GPI anchor of mature GPI proteins from the same strain (X2180) (3), it seems safe to assume that the slower migrating peak represents a mixture of

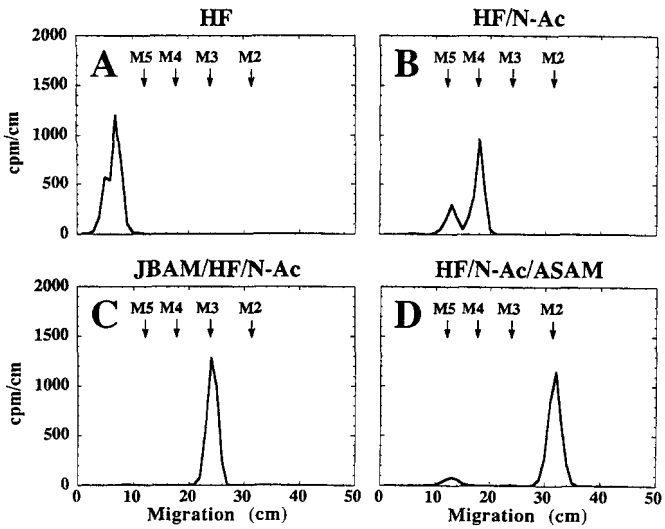

FIG. 3. **Anchor glycopeptides were prepared from radiolabeled X2180 cells, were divided into 4 equal aliquots, and were subjected to sequential treatments in the order indicated on top of each panel.** *N-Ac*, *N*-acetylation. Resulting fragments were sized by paper chromatography. Recoveries of counts/min were close to 100% in all treatments.

TABLE I

Different side chains are found in GPI proteins trapped in various compartments of the secretory pathway

Each cell line was labeled with *myo*-[2-^{3}H]inositol at 24 or 37 °C. Proteins were extracted, anchor peptides were prepared, and untreated or ASAM-treated HF fragments thereof were run on paper chromatography. Quantitation of results allowed calculation of the relative amounts of Manα1,3Manα1,2Manα1,2Manα1,6Manα1,4GlcNH$_2$-Ins (% M5α1,3) and Manα1,2Manα1,2Manα1,2Manα1,6Manα1,4GlcNH$_2$-Ins (% M5α1,2). Where experiments have been done twice with the same mutant, both results are shown.

Strain/labeling temperature	% M5	% M5α1,3	% M5α1,2
X2180 24 °C	18.0	11.1	6.9
X2180 37 °C	11.0	8.0	3.0
sec1 24 °C	34.7	27.8	6.9
sec1 37 °C	48.0	36.7	11.3
sec7 24 °C	25.0 25.0	7.9 7.3	17.1 17.7
sec7 37 °C	11.0 7.7	11.0 7.7	0.0 0.0
sec14 24 °C	30.0	17.2	12.8
sec14 37 °C	37.8	30.2	7.6
sec12 24 °C	16.8	10.4	6.4
sec12 37 °C	0.0	0.0	0.0
sec18 24 °C	30.9	16.0	14.9
sec18 37 °C	0.0	0.0	0.0

Manα1,2Manα1,2Manα1,2Manα1,6Manα1,4GlcNAcα1,6Ins and Manα1,3Manα1,2Manα1,2Manα1,6Manα1,4GlcNAc-α1,6Ins, the latter being resistant to ASAM. There is some variability among different wild type strains with regard to the fraction of GPI anchors containing a fifth mannose (*M5*, Fig. 1, Tables II and IV). It should be noted that the percentages obtained for wild type cells represent the status of GPI anchors in the Golgi and/or in post-Golgi compartments. This can be stated because, when analyzed by SDS-polyacrylamide gel electrophoresis, ER forms of GPI proteins are no more detectable after a 2-h pulse labeling with *myo*-[2-^{3}H]inositol as was done in the experiments described in here (23). (ER forms of GPI proteins have much lower molecular masses than more mature GPI proteins.)

In summary, ER forms of GPI-anchored proteins contain the same four mannoses as the candidate precursor lipids CP1 and CP2. Moreover, the absence of M5 in secretion mutants which block vesicular traffic between the ER and Golgi strongly suggests that the addition of M5 occurs in the Golgi.

TABLE II

None of the known α1,2- and α1,3-mannosyltransferases is essential for side chain addition

Percentages of various types of anchors were determined through comparison of ASAM-treated and untreated HF fragments as in Table I. All labelings were carried out at 24 °C.

strain	% M5	% M5α1,3	% M5α1,2
YAH115[a]	20.5	16.4	4.1
mnt1	20.3	13.4	6.9
SEY6210[a]	31.5	9.1	22.4
kre2 yur1 ktr1 ktr2 quadruple knockout	39.4	16.2	23.2
ktr3	32.1	5.9	26.2
ktr4	33.8	6.8	27.0
X2180[a]	18.0	11.1	6.9
mnn1	27.6	17.6	10.0
mnn2	19.0	13.0	6.0
mnn3	11.5	4.3	7.2
mnn5	35.8	22.6	13.2

[a] Mutant strains are listed below their corresponding wild type strains (YAH115, SEY6210, X2180) with which they ought to be compared.

Addition of M5 Occurs in Early and Late Golgi Compartments—The ASAM treatments of the type shown in Fig. 3 appear to be exhaustive since they quantitatively eliminated the Man$_4$-GlcNAc-Ins peak which can be considered as an internal control. Therefore, performing an experiment as described in Fig. 3, *panels B* and *D*, we could obtain the percentage of α1,3-linked M5 from the percentage of ASAM-resistant Man$_5$-GlcNAc-Ins and could calculate the percentage of α1,2-linked M5 by subtracting the percentage of ASAM-resistant Man$_5$-GlcNAc-Ins from the percentage of total Man$_5$-GlcNAc-Ins present before ASAM treatment (Tables I-IV). In a control experiment we preparatively isolated the Man$_5$-GlcNAc-Ins peak from a paper chromatogram, treated it with ASAM and reanalyzed the products by a further paper chromatography. By this procedure we obtained the same ratio of ASAM-resistant to ASAM-sensitive Man$_5$-GlcNAc-Ins as was calculated from the ASAM digest of the *total* anchor peptide fraction (containing Man$_4$-GlcNAc-Ins and Man$_5$-GlcNAc-Ins as in Fig. 3, *panels B* and *D*). The relative contributions of α1,2- and α1,3-linked M5 seem to be quite variable among different wild type strains, with most of the variability stemming from variations in the amount of α1,2 linkages (Table II, IV).

To probe the distribution of transferases involved in the addition of M5, we used *sec7*, a secretion mutant which blocks between early and mid Golgi compartments (24) (Table I). The disappearance of α1,2-linked but not α1,3-linked M5 in *sec7* upon shift to 37 °C strongly suggests that an α1,3-mannosyltransferase is encountered by newly made GPI proteins already in the earliest Golgi compartment lying proximal to the *sec7* block whereas the α1,2-mannosyltransferase is localized in later Golgi compartments lying beyond the *sec7* block. While interpreting this result one should keep in mind that significant amounts of enzymes destined for a distal compartment will accumulate proximally to a secretory block that is maintained for some time. This implies that during a prolonged secretory arrest in *sec7*, the *cis*-Golgi compartment might actually take on characteristics of a mid or *trans*-Golgi compartment and carry out distal modifications. Thus, the absence of α1,2-linked M5 in *sec7* allows to formally conclude that in wild

type cells, α1,2-linked M5 is added in the mid or *trans*-Golgi. On the other hand, in spite of the persistence of α1,3-linked M5 in *sec7*, the corresponding α1,3-mannosyltransferase cannot be assigned unequivocally to the *cis*-Golgi since α1,3-mannosyltransferase might be a *trans*-Golgi enzyme which, if artificially retained proximal to the *sec7* block, assumes an active conformation. The *trans*-Golgi localization of the α1,3-mannosyltransferase may appear less likely since the enzyme is not active if retained in the ER proximal to a *sec18* block and because the related α1,2-mannosyltransferase is not active if retained in the *cis*-Golgi.

Late Sec Mutants Accumulate GPI Anchors Containing Five Mannoses—Two alternative possibilities may be considered to explain the fact that not all GPI anchors receive M5 residues in wild type strains. (i) Only part of the GPI proteins might be substrates for the M5-transferases, and thus the limiting factor might be some steric or topological problem which restricts access of proteins to these enzymes. (ii) Alternatively, the rapid transit of GPI proteins through the Golgi might render the time for interaction with processing enzymes limiting. In this latter case, the prolonged retention of proteins proximal to a secretion block might increase the frequency of M5 addition.

In this context it should be noted that there is no increase in α1,3-linked M5 in *sec7* as compared to wild type. Assuming that the corresponding α1,3-transferase is located in the *cis*-Golgi also in wild type cells, this result suggests that in wild type the exposure time of GPI proteins to α1,3-mannosyltransferase in the *cis*-Golgi is not limiting or else that Sec7p is required to maintain the α1,3-mannosyltransferase of the *cis*-Golgi in a functional state. Later secretion mutants (*sec14* and *sec1*) seem to enhance addition of M5 (Table I), possibly because GPI proteins remain for prolonged periods in contact with α1,3- and the α1,2-mannosyltransferases present in the later Golgi. It is noteworthy that a block beyond the Golgi (*sec1*) induces an increase in both α1,2- and α1,3-linked M5, whereas the intra-Golgi block of *sec14* results in a decrease of α1,2- but a compensatory increase in α1,3-linked M5 (Table I). Since the *sec14* block is supposed to be distal to the *sec7* block, this might indicate that the *sec14* block renders the access of GPI proteins to the α1,2-mannosyltransferase difficult and that part of the α1,2-transferase is located in a very late Golgi compartment. Alternatively, the *sec14* block might delay transition of GPI proteins through the early and middle Golgi, thus increasing the time of exposure of the anchors to α1,3-mannosyltransferase(s) so that less substrate would be left for the α1,2-mannosyltransferase.

Involvement of Known Mannosyltransferases in Side Chain Addition—MNN1 encodes for a Golgi α1,3-mannosyltransferase which adds terminal mannoses onto *O*- and *N*-glycans (14, 28) whereas *MNN2*, *MNN3*, and *MNN5* control the addition of α1,2-linked mannoses onto the outer chains of *N*-glycans in the Golgi (29–31). *MNT1* (=*KRE2*) encodes for a Golgi α1,2-mannosyltransferase which adds the third mannose of *O*-glycans. *YUR1, KTR1, KTR2, KTR3*, and *KTR4* were identified as open reading frames with considerable homology to *MNT1* (12, 32, 33). As shown in Table II, normal amounts of α1,3-linked M5 are added in *mnn1* and similarly, no reduction in α1,2-linked M5 was observed in *mnn2, mnn3, mnn5*, and *mnt1* relative to the corresponding wild types. Also, single or combined deletions of *YUR* and *KTR* sequences produced at most a moderate (1.5-fold) reduction in α1,3-linked M5 (*ktr3*). Thus, none of these genes seems to be essential for addition of either α1,2-linked or α1,3-linked M5 residues. *YUR* and *KTR* deletion mutants also made normal amounts of mannosylinositolphosphoceramide (34), thus indicating that these open reading frames are not essential for the GDP-Man-dependent mannosylation of inositolphosphoceramide in the Golgi (data not shown) (35). The α1,3-linked M5 is significantly reduced in *mnn3*, a mutant with a general shortening of *N*- and *O*-linked glycans (10). The fact that only addition of α1,3- but not α1,2-linked M5 is reduced may indicate that the pleiotropic mutation of this strain affects mainly the early Golgi compartments.

TABLE III
Correlation of lipid moieties with type of side chain

Exponentially growing X2180 or SEY6210 cells were labeled with *myo*-[2-^{3}H]inositol and anchor peptides were purified by chromatography on octyl-Sepharose (= starting material). Anchor peptides were then treated with mild base (8 M NH_3, 37 °C, for 5 h) and the hydrolysate was passed once more over an octyl-Sepharose column. Head groups of mild base-sensitive anchors were recovered in the run-through fraction whereas mild base-resistant anchor peptides had to be eluted with propanol. Counts were recovered quantitatively from this second octyl-Sepharose column and were set as 100%. Both of these fractions were worked up separately to determine the proportion of the various kinds of side chains as described in Table I. Percentages obtained by summing up experimental data are in italic. A control experiment showed that no anchor peptides came through a second octyl-Sepharose column when the mild base treatment was replaced by a mock incubation without NH_3. To evaluate the accuracy of measurements, the starting material, not treated by mild base, was worked up in parallel: results (fourth row) agreed reasonably with the sum of values obtained for base-sensitive and base-resistant anchors (third row).

Strain/type of anchors	% M4	% M5α1,3	% M5α1,2	Total
X2180, mild base-sensitive anchors	12.1	5.4	1.9	*19.4*
X2180, mild base-resistant anchors	69.8	3.7	7.1	*80.6*
X2180, mild base-sensitive plus base-resistant anchors	*81.9*	*9.1*	*9.0*	100
X2180, starting material	81.8	8.9	9.3	100
SEY6210, mild base-sensitive anchors	13.9	11.1	2.2	*27.2*
SEY6210, mild base-resistant anchors	53.5	3.6	15.7	*72.8*
SEY6210, mild base-sensitive plus base-resistant anchors	*67.4*	*14.7*	*17.9*	100
SEY6210, starting material	69.6	14.3	16.1	100

Correlation between the Type of Side Chain and the Lipid Moiety of GPI-anchored Proteins—GPI-anchored proteins are made with two different lipid moieties, mild base-resistant ceramides, and mild base-sensitive diacylglycerols (3, 17). To see whether both types of anchors were associated with all types of side chains, mild base-sensitive and mild base-resistant anchor peptides were analyzed separately as described in Table III. The results indicate that all types of side chains can be associated with both lipid moieties. Yet, the ratio of α1,3-linked M5 over α1,2-linked M5 anchors is different in base-sensitive and base-resistant anchors, namely 2.8 (X2180), 5.1 (SEY6210), or infinite (ZY100) in base-sensitive but only 0.5 (X2180), 0.23 (SEY6210), or 0.07 (ZY100) in base-resistant anchors (Tables III and IV). Thus, base-sensitive anchors have a tendency to receive α1,3-linked M5 whereas base-resistant anchors preferentially are provided with an α1,2-linked M5 in all strains.

Mnn9 Abolishes N-Glycan Elongation but Not Addition of M5 on GPI-anchored Proteins—There exist several mutants (*mnn9, anp1, van1, erd1*, and *pmr1*) which appear to be unable to elongate *N*-glycans in the Golgi because they do not add

TABLE IV
Mutants deficient in the elongation of N-glycans are still able to add α1,2- and α1,3-linked M5 to GPI anchors
Data were obtained as described in Table III.

Strain/type of anchors	% M4	% M5α1,3	% M5α1,2	Total
ZY100[a], mild base-sensitive-anchors	21.8	8.5	0.0	*30.3*
ZY100, mild base-resistant anchors	52.4	1.2	16.1	*69.7*
ZY100, mild base-sensitive plus base-resistant anchors	*74.2*	*9.7*	*16.1*	100
ZY100, starting material	73.2	9.4	17.4	100
mnn9, mild base-sensitive anchors	6.6	9.7	0.0	*16.3*
mnn9, mild base-resistant anchors	58.9	13.0	11.8	*83.7*
mnn9, mild base-sensitive plus base-resistant anchors	*65.5*	*22.7*	*11.8*	100
mnn9, starting material	65.4	22.8	11.8	100
SEY6210, mild base-sensitive anchors	13.9	11.1	2.2	*27.2*
SEY6210, mild base-resistant anchors	53.5	3.6	15.7	*72.8*
SEY6210, mild base-sensitive plus base-resistant anchors	*67.4*	*14.7*	*17.9*	100
SEY6210, starting material	69.6	14.3	16.1	100
van1, mild base-sensitive anchors	9.5	9.0	0.6	*19.1*
van1, mild base-sensitive anchors	65.6	4.9	10.4	*80.9*
van1, mild base-sensitive plus base-resistant anchors	*75.1*	*13.9*	*11.0*	100
van1, starting material	72.2	15.5	12.3	100
anp1, mild base-sensitive anchors	10.0	2.3	0.5	*12.8*
anp1, mild base-resistant anchors	76.7	2.5	8.0	*87.2*
anp1, mild base-sensitive plus base-resistant anchors	*86.7*	*4.8*	*8.5*	100
anp1, starting material	87.2	4.6	8.2	100
SEY2102, starting material	71.9	7.2	20.9	100
erd1, starting material	84.2	4.7	11.1	100

[a] Mutant strains are listed below their corresponding wild type strains with which they ought to be compared.

α1,6-linked mannose onto the *N*-glycans of secretory proteins such as invertase and mannoproteins (10, 13, 15, 36). It is unlikely that these mutants are deficient in some glycosyltransferase, but it is rather suspected that their problem might originate from some abnormality of protein trafficking, from an anomalous Golgi organization, from an inability to retain glycosyltransferases in the Golgi, or from a disturbed ionic environment in the Golgi (14, 37, 38). *MNN9, VAN1*, and *ANP1* are homologous, and Anp1p has been shown to reside in the ER (13). In studies concerning the functional organization of the Golgi of such mutants, it obviously is not possible to rely on the *N*-glycan maturation as a criterion for the normal progression

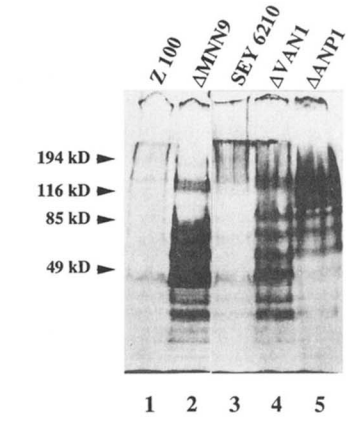

FIG. 4. **1.5×10^7 cells from different insertional mutants in *N*-glycan elongation and corresponding parental cells were labeled for 2 h with 20 μCi of *myo*-[2-^{3}H]inositol at 24 °C.** Proteins were extracted and analyzed by SDS-polyacrylamide gel electrophoresis/fluorography. Exposure was for 1 week. The GPI anchor maturation of these mutants is summarized in Table IV.

of proteins through the Golgi apparatus. We thus exploited the above established Golgi localization of M5-transferases as a means to assay the traffic of GPI proteins through the Golgi in these mutants. To demonstrate that their defects affect not only soluble but also GPI proteins, *mnn9, anp1*, and *van1* were labeled with *myo*-[2-^{3}H]inositol and GPI proteins were visualized by SDS-polyacrylamide gel electrophoresis/fluorography. As shown in Fig. 4, these mutants make large amounts of GPI proteins which are very much smaller than in wild type cells. This is consistent with the view that the *N*-glycans of GPI proteins, as the ones of invertase (13), are not elongated in *mnn9* and *van1* and are only partially elongated in *anp1*. It also is evident that the labeling of GPI proteins in mutant cells is significantly enhanced, possibly because the transit of GPI proteins through the Golgi and the ensuing removal of the anchor at the cell surface is slowed down in these mutants (39). (Invertase secretion by *mnn9* has been reported to be slowed down (37).) Analysis of the GPI anchors shows that all mutants can add α1,2-linked as well as α1,3-linked M5 (Table IV). In all elongation mutants the proportion of base-sensitive anchors is diminished, and the percentage of α1,2-linked M5 is reduced. Beyond these similarities two mutants show quite specific changes which are not to be found in others: *Mnn9* shows a drastic increase in α1,3-linked M5 in the base-resistant anchor fraction, thus accumulating a normally quite uncommon kind of anchor; *anp1* has a severely reduced amount of M5 with losses occurring in both the mild base-sensitive α1,3-linked M5 as well as the mild base-resistant α1,2-linked M5; in *van1* and *erd1* the changes are less extensive. While the alterations in *mnn9* are difficult to interpret it is likely that the drastic reduction of M5 in *anp1* results from an inability to efficiently retain M5-transferases in the Golgi apparatus (13). Whatever the cause of the deficiency in *N*-glycan elongation of *mnn9, van1, anp1*, and *erd1* may be, the GPI proteins do get into contact with the M5-transferases normally found in the Golgi and their GPI anchor can be matured, although the *N*-glycans on the same GPI proteins remain immature. Data however cannot tell whether these M5-transferases really reside in their normal location in these mutants.

DISCUSSION

As for many other glycan structures, the exact role of the mannose side chain on GPI anchors is presently not well understood. Nevertheless, single mannose side chains (M4) have been found in mammals, *D. discoideum, P. falciparum*, and *S. cerevisiae* and therefore, the possibility to add M4 seems to

have been maintained during evolution in several phyla. On the other hand, the GPI biosynthesis machinery, at least of mammals and trypanosomes, does not require the presence of M4, since most mammalian and trypanosomal cells do not contain this residue either on the complete precursor lipids or on the GPI proteins. In contrast to M4, M5 residues have only been described in *S. cerevisiae*. In various wild type strains, M5 residues are present in 18–32% of anchors, and the ratio of α1,2- *versus* α1,3-linked mannose varies from 1:4 to 2.5:1, depending on the strain (Table II).

The identity of the mannosyltransferases involved in the addition of M4 and M5 are unclear at the moment. The general experience in glycobiosynthesis is that each kind of linkage is achieved by a different glycosyltransferase. Since M4, as M3, is α1,2-linked, it is conceivable that the same mannosyltransferase is responsible for the addition of both M3 and M4. On the other hand, the M5 adding mannosyltransferases are definitely different from the ones that add M4 since, according to our data with secretion mutants, they reside in the Golgi whereas the M4 addition must occur in the ER. Our data show that none of a panel of cloned Golgi mannosyltransferases or genes regulating such transferases is essential for the addition of α1,2-linked or α1,3-linked M5. The slight reduction in α1,3-linked M5 observed in *mnn3, ktr3,* and *ktr4* mutants might be taken as an indication that all of these enzymes are involved in the addition of α1,3-linked M5, but this would be against the general "one linkage-one enzyme" rule mentioned before. Also, this seems unlikely in view of the fact that *KTR3* shows much less homology with *KTR4* than with *KTR1* which latter is without influence on the addition of M5 (Table II) (12). Thus, although we cannot exclude that redundant enzymes are responsible for the addition of M5, it seems more likely that M5 addition is due to the presence of some other, yet unknown Golgi mannosyltransferases.

The GPI anchor peptides analyzed here were purified over octyl-Sepharose and hence contain a lipid moiety. We conclude that the M5-transferases get access to M4 without any need for previous removal of the lipid moiety. This is in agreement with the idea that the glycan part of GPIs can assume a relatively extended configuration and form a broad platform between protein and lipid (40). Yet, we routinely find that 10–20% of the anchor peptides eluted from ConA-Sepharose do not bind to octyl-Sepharose. Indeed it has recently been reported that for some proteins the GPI anchor represents a necessary and sufficient signal for their incorporation into the cell wall and that upon arrival at the plasma membrane part of the GPI anchor including the lipid moiety is removed (39, 41, 42). Having restricted our analysis to lipid-containing anchors, it is obvious that the possible further additions of glycans onto the GPI core structure or onto the GPI side chain during this incorporation process would have escaped detection.

Through analysis of *N*-glycan structures of glycoproteins accumulating in *sec7, sec14, sec18,* and *sec23*, of α-factor maturation events in these secretion mutants and through subcellular fractionation studies, the yeast Golgi could be divided into two distinct early (cis) compartments containing α1,6-mannosyltransferases for the elongation of *N*-glycans, a later (mid) compartment containing the *N*- and *O*-glycan elongating α1,3-mannosyltransferase coded for by *MNN1,* and an even later (trans) compartment containing the processing protease coded for by *KEX2* (24–27). The presence of α1,3-linked or α1,2-linked M5 residues on GPI anchors can now serve as an alternative means for tracking GPI proteins and their vesicular flow through the Golgi. The presence of α1,2-linked M5 indicates that a GPI protein has reached or passed the later Golgi compartments whereas the addition of α1,3-linked M5 in *sec7* cells indicates that a GPI protein has reached the *cis*-Golgi. Subcellular fractionation studies will be required to decide whether the presence of an α1,3-mannosyltransferase is a distinguishing feature of the *cis*-Golgi also in wild type cells. It should be noted that early and late Golgi modifications of GPI anchors are independent or even mutually exclusive events, whereas in the *N*-glycan elongation, addition of α1,6-mannose in the early Golgi is a prerequisite for the addition of α1,3-linked mannose in the later Golgi.

Base-sensitive anchors have a tendency to receive α1,3-linked M5 whereas base-resistant anchors preferentially are provided with an α1,2-linked M5. The bias of α1,3-linked M5 for base-sensitive anchors can be interpreted in several alternative ways which at present are not easy to distinguish experimentally. (i) α1,3-Mannosyltransferases might prefer diacylglycerol-based anchors over ceramide-based ones, and the inverse might be true for the α1,2-mannosyltransferase. (ii) Since lipid moieties of GPI anchors might still get exchanged in the Golgi (17), the Golgi lipid exchange enzyme might introduce ceramides preferentially on anchors with α1,2-linked M5. (iii) GPI proteins might get sorted according to their lipid domain, and diacylglycerol-based GPI proteins might have more access to the α1,3-mannosyltransferase of the early Golgi whereas ceramide-based ones have better access to the α1,2-mannosyltransferase of the late Golgi. (iv) Diacylglycerol-based GPI proteins might transit more slowly through the Golgi than ceramide-based ones so that the *cis*- or mid Golgi α1,3-mannosyltransferases have more time to attach a M5 and that the α1,2-mannosyltransferase of the late Golgi cannot find any suitable substrate any more. Enzyme specificities invoked in i and ii are rendered less likely by the high amounts of α1,3-linked M5 found on ceramide-based anchors of *mnn9*. This latter finding clearly shows that either α1,3-mannosyltransferase can act on ceramide-based anchors or that the lipid exchange enzyme can act on anchors with α1,3-linked M5. Sorting of GPI proteins according to their lipid moiety (possibilities iii and iv) would represent a novel mechanism. However, it has previously been demonstrated that the vesicular transport of yeast GPI proteins from ER to Golgi is dependent on ceramide biosynthesis whereas the vesicular flow of membrane proteins containing a hydrophobic, membrane spanning sequence is not dependent on ceramide biosynthesis (43).

The studies with mutants such as *mnn9, van1, anp1,* and *erd1* demonstrate that their deficiency only abolishes the capacity of *N*-glycan elongation but does not eliminate other glycosylation events of the Golgi such as addition of M5 to GPI proteins. Mannosylation of inositolphosphoceramides seems to be intact, since the pattern of inositolphosphoceramides and the mannosylated forms thereof are normal in all of these mutants (not shown). Further studies will be required to delineate the primary event leading to this specific deficiency in *N*-elongation.

Acknowledgments—We thank Clinton Ballou, Rowan Chapman, Michel Jaquet, Marc Lussier, Vivian MacKay, Sean Munro, Hugh Pelham, Phil Robbins, and Randy Schekman for yeast strains.

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