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Localization of an α 1,2 Galactosyltransferase Activity to the Golgi Apparatus of *Schizosaccharomyces pombe*

Thomas G. Chappell,*†‡ M.A. Nasser Hajibagheri,* Kathryn Ayscough,* Maria Pierce,* and Graham Warren*

*Imperial Cancer Research Fund, Lincoln's Inn Fields, London WC2 3PX, United Kingdom; and †Department of Cell Biology, Duke University Medical Center, Durham, North Carolina 27710

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We have cloned a gene encoding an α 1,2 galactosyltransferase activity from *Schizosaccharomyces pombe*. The open reading frame of the gene (*gma12* for **g**alacto**m**annan, **a**lpha **1,2**), combined with the previous protein purification (Chappell and Warren, 1989), predicts an O-linked glycoprotein with type II transmembrane topology. By homologous gene disruption, we have demonstrated that the *gma12* gene product (gma12p) is nonessential. The deletion strain (*gma12-D10::ura4*) has a significantly reduced level of galactosyltransferase activity relative to the parental strain, but both in situ lectin binding and in vitro biochemical assays demonstrate the presence of further galactosyltransferase activity in addition to gma12p. Although gma12p is not the only galactosyltransferase in *S. pombe*, it produces a unique carbohydrate structure on the surface of the yeast cells. We have generated a polyclonal antiserum against this carbohydrate epitope and shown that gma12p is capable of synthesizing the epitope both in vitro and in vivo. Electron microscopic localization of the *gma12*$^+$ specific epitope in *gma12*$^+$ cells revealed that gma12p synthesizes the carbohydrate structure in the Golgi apparatus, and subsequent intracellular transport distributes the epitope to later stages of the secretory pathway. The immunolocalization studies confirm the presence of one or more galactosyltransferase activities in the Golgi apparatus in fission yeast.

INTRODUCTION

All eukaryotic cells process glycoproteins and glycolipids by a series of sequential transferase reactions as molecules are transported through the secretory pathway. Our understanding of the secretory process has been strongly influenced by our ability to localize these transferase activities to specific subcellular compartments with biochemical, immunochemical, and histological techniques. In particular, these combined approaches have led to our current view of the Golgi apparatus as a series of distinct biochemical compartments through which secretory products pass in a vectorial fashion, finally to be sorted to their ultimate destinations from the trans-Golgi network.

‡ Corresponding author and present address: Medical Research Council, Laboratory for Molecular Cell Biology, University College London, London WC1E 6BT, United Kingdom.

Both Golgi morphology and interactions with cytosolic components necessary for transport vesicle formation and consumption have been highly conserved in evolution (for review, see Pryer *et al.*, 1992). We have been interested in the mechanisms by which the Golgi apparatus is able to retain diverse collections of glycosyltransferases, generating an organelle that is morphologically and functionally very similar from organism to organism, yet capable of producing highly divergent oligosaccharide structures. To this end, we have been studying the Golgi apparatus and oligosaccharide processing in the fission yeast, *Schizosaccharomyces pombe*.

It had previously been shown that N-linked oligosaccharides in *S. pombe* are processed in the secretory pathway by the addition of 50–100 sugar residues consisting of both mannose and galactose (Moreno *et al.*, 1985; Schweingruber *et al.*, 1986). Because >80% of the galactose is in terminal positions on galactomannan

(Bush *et al.*, 1974), our working hypothesis has been that a substantial mannose structure is built in early *S. pombe* Golgi cisternae and that a single galactosyltransferase activity, acting in late Golgi compartments, completes the oligosaccharide structure. Our previous work (Chappell and Warren, 1989) demonstrated the presence of a membrane localized galactosyltransferase activity in *S. pombe* with substrate specificity capable of generating the α-linked galactose residues found on the N-linked oligosaccharides of invertase and acid phosphatase. In this work we show that this galactosyltransferase activity is localized to the Golgi apparatus in fission yeast and that oligosaccharide processing in *S. pombe* is more complex than we had initially suspected, involving multiple galactosyltransferase activities along the secretory pathway.

MATERIAL AND METHODS

Media and Strain Construction

Yeast cultures were grown in rich growth (YEA) or *S. pombe* minimal (PMM) media as described by Moreno *et al.* (1991). Targeted gene disruption and plasmid transformations were performed using either lithium chloride (Moreno *et al.*, 1991) or lithium acetate (Okazaki *et al.*, 1990) protocols. Experiments were performed with TCP1002 (*h*$^-$, *leu1-32, ura4-D18, his3-237*), TCP3001 (*h*$^-$, *leu1-32, ura4-D18, his3-237, gma12-D10::ura4*), and TCP3002 (*h*$^-$, *leu1-32, ura4-D18, his3-237, gma12-D10::ura4*, pREP1::*gma12*).

Biochemical Assays

UDP-[^{3}H]Gal and GDP-[^{3}H]Man were purchased from New England Nuclear (Boston, MA). Galactosyltransferase assays were performed as previously described (Chappell and Warren, 1989). Mannosyltransferase assays were identical except for the substitution of GDP-Man for UDP-Gal. Triton X-114 (Sigma, St. Louis, MO) partitioning was performed as described by Pryde and Phillips (1986). Briefly, 10% (wt/vol) Triton X-114 was pre-equilibrated with water by mixing at 4°C, followed by an incubation at 37°C. The upper aqueous phase was removed and the cycle repeated several times. The equilibrated Triton X-114 was used to solublize *S. pombe* microsomes or dilute purified enzyme (Chappell and Warren, 1989) at 4°C with a final detergent concentration of 10% (wt/vol) in 100 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (HEPES) NaOH pH 7.0. The mixtures (1 ml) were phase partitioned by incubation at 37°C for 10 min followed by a brief centrifugation at 14 000 × *g*. The two phases were each adjusted to 1 ml by the addition of 100 mM HEPES NaOH pH 7.0 containing Triton X-100 to give a final concentration of 2% (wt/vol) Triton X-100. Purified protein was used to determine that the enzymatic activity was constant over a range of Triton X-114 concentrations from 0–10% in the presence of 2% Triton X-100.

Equilibrium density sedimentation of microsomes was performed in linear sucrose gradients. *S. pombe* microsomes were layered on top of 40–15% (wt/wt) sucrose gradients in 10 mM tris(hydroxymethyl)-aminomethane HCl (pH 7.0), 1 mM EDTA (TE) in SW 28.1 (Beckman, Fullerton, CA) ultracentrifuge tubes. Gradients were spun overnight (8–12 h) at 85 500 × *g* (r_{av}). Fractions were collected from the bottom of the gradients. The sucrose concentration in each fraction was measured by refractometry. Microsomes were reisolated from each fraction by adding an equal volume of TE to reduce the sucrose concentration, followed by centrifugation at 417 000 × *g* (r_{av}) in a TLA 100.3 (Beckman) rotor for 30 min.

Protein Microsequencing

Three preparations (~100 μg each) of α 1,2 galactosyltransferase from *S. pombe* were purified as previously described (Chappell and Warren, 1989). Protein sequences were obtained from the N-terminus of each batch of the purified protein using an Applied Biosystems (Foster City, CA) sequencer. Each sequencing run was typically 20–25 cycles. In addition, protein from one preparation was fragmented with cyanogen bromide (CNBr) and fractionated on a high-pressure liquid chromatography (HPLC) C-18 reverse phase column using an acetonitrile gradient. Protein sequence analysis was performed on three baseline resolved peaks from the CNBr fragmentation.

Genomic Library Screening and DNA Manipulations

Libraries of genomic *S. pombe* DNA in either lambda DASH (Stratagene, La Jolla, CA) (4 genome equivalents) or cosmid Lawrist 4 (Imperial Cancer Research Fund [ICRF] Reference Library System, Library #60) (40 genome equivalents) on Hybond N+ nylon filters (Amersham, Arlington Heights, IL) were used for all screenings. Oligonucleotides were synthesized on an Applied Biosystems DNA synthesizer. Oligonucleotide probes (20 pmol) were labeled with ^{32}P using T4 kinase (Boehringer Mannheim, Indianapolis, IN) and 50 μCi γ ^{32}P ATP (3000 Ci/mmol, Amersham). Filters were fixed with 0.4 M NaOH, and oligonucleotide hybridizations were performed in Church's buffer (Church and Gilbert, 1984) at 37°C. The filters were rinsed at 25°C in 6× SSC and washed (3 times for 5 min) in tetramethylammonium chloride buffer at 45°C. Screenings with DNA fragments were performed using a random oligonucleotide labeling kit (Boehringer Mannheim) to incorporate ^{32}P into the specific DNA fragment, followed by hybridization in Church's buffer at 65°C. Stringency washes in 2 to 0.2× SSC at 65°C were performed. Filters were exposed using Kodak XAR-5 film (Rochester, NY).

Positive lambda DASH phages were plaque purified by standard techniques. Positive cosmid colonies were obtained from the ICRF Genome Analysis Laboratory Reference Library #60 based on x,y filter coordinates. DNA was isolated from both phages and cosmids, analyzed by Southern blotting, and subcloned into pBluescript (Stratagene) using standard techniques. Sequencing was performed using either Sequenase or ΔTaq cycle sequencing protocols (United States Biochemical, Cleveland, OH) with universal forward and reverse primers and custom oligonucleotides.

The plasmid pREP1::*gma12* was constructed by polymerase chain reaction (PCR) mutagenesis of the *gma12* gene to introduce an *Nde* I site at the initial *Met* and an *Xba* I site directly after the termination codon of the gene. The internal *Nde* I site was removed by using parallel PCR reactions primed with oligonucleotides GT2 and GT4 and oligonucleotides GT3 and GT5 (Table 1). The products of these two reactions were isolated and mixed, and a further PCR reaction was carried out with GT2 and GT3 as primers. The sequence of the final 1134 basepairs (bp) *Nde* I/*Xba* I fragment was confirmed, and the DNA was cloned between the *Nde* I and *Xba* I sites in the polylinker region of pREP1 (Maundrell, 1990).

Antibody Production

Antibodies against the *gma12*$^+$ specific epitope were generated by immunization of rabbits with formaldehyde-fixed *gma12*$^+$ cells. Briefly, cells were grown in YEA and fixed by the addition of freshly prepared 30% formaldehyde to the media to give a final concentration of 3% fixative. After fixation, the cells were washed with water, and ~5 × 10^7 cells were resuspended in water and mixed with Fruend's complete adjuvant. The emulsion was injected subcutaneously. Boosts consisted of equivalent injections of cells in Freund's incomplete adjuvant. Bleeds were taken after several boosts. Pooled antiserum was adsorbed against formaldehyde-fixed *gma12-D10* cells until antibodies remaining in the serum no longer showed staining of the *gma*12-D10 cell surface. The antiserum was then affinity purified against fixed *gma12*$^+$ cells by binding and subsequent elution at pH 2.8 (Burke, *et al.*, 1982).

Fluorescence Staining

Fluorescence staining was performed on formaldehyde fixed cells washed with 100 mM piperazine-*N,N'*-bis(2-ethanesulfonic acid)

(PIPES) pH 6.9, 1 mM ethylene glycol-bis(β-aminoethyl ether)-N,N,N',N'-tetraacetic acid (EGTA), 1 mM $MgCl_2$ (PEM) and blocked with PEM + 2% fish skin gelatin (PEMF). For lectin staining, cells were incubated with fluorescein isothiocyanate (FITC)-conjugated *Bandeiraca simplicifolia* I B_4 isolectin (BSL I B_4) (Vector Labs, Burlingame, CA) at a concentration of 10 μg/ml in 100 mM HEPES pH 7.0, 1 mM $MgCl_2$, 100 μM $CaCl_2$, 100 μM $MnCl_2$. For immunofluorescence, cells were incubated with *gma12*$^+$ carbohydrate specific antibodies diluted 1:2 in PEMF at 25°C. The secondary antibody was FITC-conjugated goat anti-rabbit (Vector Labs) at a 1:100 dilution in PEMF. For both lectin and immunolabeling, cells were mounted in Citi-fluor antifade (Agar Aids, Stansted, UK) on coverslips coated with poly-L-lysine. Cells were photographed with T-Max 100 film (Kodak) using an 100× objective on a Zeiss Axioskop microscope (Thornwood, NY).

Electron Microscopy

Yeast cells were grown to midlog phase in YEA and then fixed with 1% monomeric glutaraldehyde in 0.1 M Sorensens phosphate buffer (pH 7.4) for 1 h at room temperature. After fixation the cells were treated with 0.5 M NH_4Cl in phosphate buffer for 4 h and washed in buffer overnight at 4°C. The cells were dehydrated in a series of graded methanols at progressively lower temperature and infiltrated with Lowicryl HM20 (Agar Aids) at −50°C. The resin was polymerized by UV light for 48 h at −50°C. Thin sections were mounted on carbon coated grids and were labeled as follows. After 5 min on a drop of phosphate-buffered saline (PBS) and 1 h preincubation on 5% normal goat serum plus 5% bovine serum albumin in PBS, the grids were transferred onto drops of antiserum (either *gma12* carbohydrate specific or fusion protein specific, 1:10 dilution in PBS) overnight at 4°C. The sections were then washed three times over a 15-min period with PBS and then followed by incubation with immunogold conjugates (goat anti-rabbit IgG, 10 nm gold, 1:50 dilution) for 2 h. After final rinsing in PBS (3 × 5 min) and distilled water (3 × 5 min), the sections were air-dried, contrasted with uranyl acetate and lead citrate, and examined with a Zeiss 10 C electron microscope. As negative controls, primary antibodies were omitted from the procedure to visualize any nonspecific binding of gold particles on cell organelles.

RESULTS

Cloning the Gene Encoding an α 1,2 Galactosyltransferase

Using material from multiple purifications of galactosyltransferase, an N-terminal sequence of MKEIIDQH was obtained. Repeated attempts to extend the sequence always resulted strong chromatographs for the first eight cycles followed by total loss of readable data on cycle 9. Occasional cycles between 9 and 20 showed weak chromatographic traces. Internal sequence information was also obtained from three HPLC-purified CNBr fragments of the protein. Codon degeneracy and sequencing gaps necessitated the use of the N-terminal sequence information for oligonucleotide probe design. We synthesized a degenerate 17mer antisense oligonucleotide corresponding to the six amino acid sequence MKEIDD. This oligonucleotide (GT1, Table 1) was used to screen an *S. pombe* genomic lambda DASH (Stratagene) library at low stringency. Two positive clones from this initial screening both contained the same partial open reading frame (ORF) with an internal MKEIIDQH sequence and two of the three CNBr fragment sequences. A 1-kilobase (kb) HindIII/*Bam*H I fragment of this ORF was used to screen an *S. pombe* genomic cosmid library at high stringency. Thirty-six positives were obtained from this second screening. Thirty-five of the 36 rescreened as positives with the GT1 oligonucleotide. A 2.8-kb *Cla* I/*Pst* I fragment was subcloned from one of the positive cosmids and used to sequence the entire ORF.

Table 1. Sequences of oligonucleotides used in this study

Name	Length	Sequence
GT1	17	TCIATIAT(TC)TC(TC)TTCAT
GT2	24	GATTATTTGCATATGCGGTTCGCT
GT3	24	CGTGACTGATCTAGACTAGGATGA
GT4	24	CATCAAAATCATAAGCGTTTATAG
GT5	24	CTATAAACGCTTATGATTTTGATG

In oligonucleotide GT1, deoxyinosine was introduced at positions 3 and 6 and 50/50 T/C degeneracy at positions 9 and 12.

Figure 1 shows the DNA and predicted amino acid sequence of *gma12* ORF. The partial protein sequences generated from the purified enzyme are indicated by double underlining. The ORF showed no significant homologies in the current DNA database on the National Institutes of Health (NIH) computer using a BLAST algorithm search (Altschul *et al.*, 1990). Using the BLAST algorithm for protein alignment (Gish *et al.*, 1993) resulted in high probability alignments of amino acids 43–95 with a group of proteins with high threonine content. An example of such a protein was human tracheal mucin (PIR #A37232). Dot plot alignments of gma12p with a three amino acid window did not generate any homologies to two known *S. cerevisiae* mannosyltransferases, och1p (Nakayama *et al.*, 1992) and mnt1p (Hausler and Robbins, 1992).

One very obvious observation that struck us about the ORF was the presence of 34 amino acids on the N-terminal side of what we thought would was initial *Met* of the protein. Figure 2 shows an hydropathy plot of the entire ORF, with the boundaries of the purified protein determined by microsequencing indicated. The 34 amino acid sequence at the N-terminus of the ORF absent from the purified protein contains the most hydrophobic region of the entire 375 amino acid ORF—a stretch of 25 uncharged amino acids. The two obvious possibilities for the loss of these 34 amino acids were either proteolytic cleavage during the protein purification procedure or cotranslational cleavage of an N-terminal signal sequence. Using the weight-matrix analysis method of von Heijne (1986) to predict signal sequence cleavage sites showed a very low probability of cleavage in the first 50 amino acids of the ORF. A related statistical analysis with MacPattern (Fuchs, 1991) predicted the hydrophobic region as a membrane-spanning domain.

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atg cgg ttc gct cct tat tta att agt gct gtt gta att aca acc ata atc ctg ggt gga gct tgg tgg act tca 75
 M  R  F  A  P  Y  L  I  S  A  V  V  I  T  T  I  I  L  G  G  A  W  W  T  S

gct atg gat acc aag ctt caa acg aag atg aag gag atc atc gac caa cac acg agt act tgg acc cca gtc gtt 150
 A  M  D  T  K  L  Q  T  K  M  K  E  I  I  D  Q  H  T  S  T  W  T  P  V  V

tcc tcc gta acc agt acc caa act gat act tta cgt gtt acc att agt gaa gtt gtt agt gtt act gcc act tta 225
 S  S  V  T  S  T  Q  T  D  T  L  R  V  T  I  S  E  V  V  S  V  T  A  T  L

acc gaa act ttc act gct act cca act gtc act tct gta gtt cat gct tta gct acc act gat cct cat ccg gat 300
 T  E  T  F  T  A  T  P  T  V  T  S  V  V  H  A  L  A  T  T  D  P  H  P  D

aac tct aaa atc gtc att ttg atg ggc tct aac ttt caa aat gat gct aac tct cct ctc cat cct ttc gct cag 375
 N  S  K  I  V  I  L  M  G  S  N  F  Q  N  D  A  N  S  P  L  H  P  F  A  Q

tcc att atc aaa aat cgt cgt gaa tat gct gaa cgc cat ggt tac aaa ttc gaa ttc tta gat gcc gat gcc tat 450
 S  I  I  K  N  R  R  E  Y  A  E  R  H  G  Y  K  F  E  F  L  D  A  D  A  Y

gct tct cgt gtt aca ggt cat tta atg ccc tgg gtt aag gtc cct atg ctt caa gat acg atg aag aaa tat cct 525
 A  S  R  V  T  G  H  L  M  P  W  V  K  V  P  M  L  Q  D  T  M  K  K  Y  P
                           X                                    ?  ?

gac gct gaa tgg att tgg tgg ctt gat cat gac gcg ttg gtc atg aac aaa gat ctt aac gtc gtc gac cat gtg 600
 D  A  E  W  I  W  W  L  D  H  D  A  L  V  M  N  K  D  L  N  V  V  D  H  V

ctt aag cac gac cga ttg aat acc att tta act agg gag gct gaa tat aag agt ggt gca ggt atc ccc gcc gat 675
 L  K  H  D  R  L  N  T  I  L  T  R  E  A  E  Y  K  S  G  A  G  I  P  A  D

gga ttt aga act cca aag gat cag gat gct aag gat gtt cat ttc atc atc tct caa gat ttt aat ggt att aac 750
 G  F  R  T  P  K  D  Q  D  A  K  D  V  H  F  I  I  S  Q  D  F  N  G  I  N

gct ggc agt ctt ttt att cgt aat agc gaa gtt ggc cgc tgg att gtt gat ttg tgg ttt gaa ccc tta tat ttg 825
 A  G  S  L  F  I  R  N  S  E  V  G  R  W  I  V  D  L  W  F  E  P  L  Y  L

gat cat att cag gga tat gct gag caa caa gcg ttt tct cac atg gta ttt tac cat ccc cag gtg tat aaa cat 900
 D  H  I  Q  G  Y  A  E  Q  Q  A  F  S  H  M  V  F  Y  H  P  Q  V  Y  K  H

gta ggt gtt gtc cca ctt aag gct ata aac gca tat gat ttt gat gat aat att tgg ggc tat gac gac ggg gac 975
 V  G  V  V  P  L  K  A  I  N  A  Y  D  F  D  D  N  I  W  G  Y  D  D  G  D

ttg tgt atc cat ttt gct gga tgt aac tat ttt aag aat tgt ccc gag aag ttc ctt aag tat gct caa att tta 1050
 L  C  I  H  F  A  G  C  N  Y  F  K  N  C  P  E  K  F  L  K  Y  A  Q  I  L

tct tct aaa caa gga tcc gat tgg atg tct gct caa gaa aag gat cat att caa aat ctt ttg aaa cca tca tcc 1125
 S  S  K  Q  G  S  D  W  M  S  A  Q  E  K  D  H  I  Q  N  L  L  K  P  S  S
                           ?                 ?
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Figure 1. DNA and translated protein sequence of the *gma12* ORF. The 1125-bp ORF encoded by the *gma12* gene contained all four of the sequences determined by microsequence analysis of the purified protein (indicated by double underlining). Residues marked with ? were unreadable on protein sequencing runs. The single mismatch, W_{161}, was read as an R on protein sequencing. The region indicated with the dashed underlining contains the majority of the serine and threonine residues in the protein and has a total amino acid composition that is 55% S/T/V. The longest stretch of uncharged amino acids (F_2 to M_{27}) is underlined. Proteolytic cleavage of gma12p occurred adjacent to K_{34} during protein purification, generating an unprotected N-terminus at M_{35}. The EMBL accession number is z30917.

To differentiate between signal sequence cleavage or proteolytic degradation experimentally, we used Triton X-114 partitioning to test the hydrophobicity of the galactosyltransferase activity before and after purification. Using microsomes isolated from *S. pombe*, we found that 60–75% of the galactosyltransferase activity partitioned into the Triton X-114 detergent phase. After purification, however, <10% of the activity partitioned into the hydrophobic phase. We concluded from these experiments that the hydrophobic nature of the galactosyltransferase activity is lost during the purification procedure, consistent with the loss of the 34 amino acid hydrophobic region of gma12p by proteolytic degradation. This hydrophobic nature of the activity in membrane extracts, in conjunction with the localization of this hydrophobicity to the 34 N-terminal amino acids, has lead us to conclude that the enzyme has a noncleaved signal sequence and thus has a type II membrane topology.

The molecular weight of the protein encoded by the ORF shown in Figure 1 is 42 600 Da. This prediction is significantly different from the 60 kDa molecular weight determined for the purified enzyme. Taking into account the proteolytic cleavage discussed above, there is a discrepancy of 21 kDa between the predicted and actual molecular weights. An mRNA splicing event that would add 21 kDa at the C-terminus of the ORF has been ruled out by two results. First, the protein microsequencing data extends to within two amino acids of the termination codon of the ORF (Figure 1), and there is no consensus 5′ splice site (GTANG; Russell, 1989) in the 10 nucleotides that separate our protein data and the termination codon of the ORF. Second, there is a second ORF in close proximity to the 3′ end of the *gma12* gene that encodes an *S. pombe* homologue of the *S. cerevisiae* Phe-tRNA synthetase α chain gene. The combination of these two results strongly argues that the extra 21 kDa is a consequence of protein modification.

We already knew from the protein purification studies that the galactosyltransferase is a glycoprotein. There are no N-X-S/T sequences within the ORF, ruling out the possibility of N-linked oligosaccharides. The distribution of serine and threonine residues within the ORF is strikingly asymmetrical. In the 53–amino acid stretch between T_{43} and T_{95} there are 25 Ser or Thr residues. This 15% of the ORF accounts for nearly 50% of the S/T amino acids. Our inability to microsequence past H_{42} can be explained by heavy O-linked glycosylation

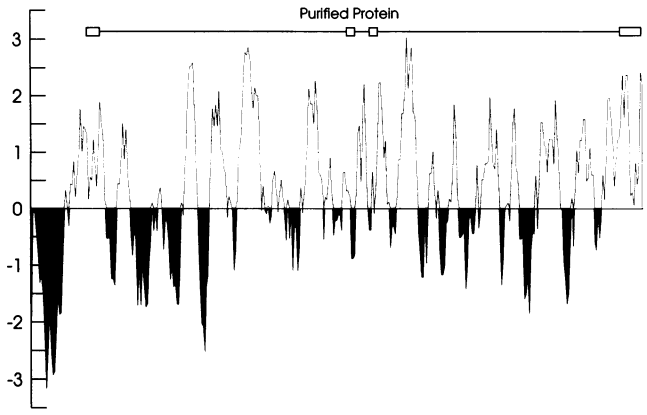

Figure 2. Hydropathy plot of gma12p. The 375 amino acid ORF of *gma12* from Figure 1 was plotted using standard Kyte-Doolittle hydropathy values (negative, hydrophobic; positive, hydrophilic) with running average window of seven amino acids. The most hydrophobic region of the protein corresponds to the uncharged amino acids underlined in Figure 1 (F_3 to M_{27}). The region spanned by the purified protein is indicated above the plot with sequenced regions of the protein marked by the four rectangles.

in this region of the protein. Using the ORF as a guide, we were able to find peaks on the sequencing chromatographs for W_{46}, P_{48}, V_{49}, and V_{50}. Gaps were present for T_{43}, S_{44}, T_{45}, T_{47}, and S_{51}. Given the overall sequence composition between T_{43} and T_{95} of 55% S/T/V, we suspect that O-linked glycosylation in this region of the protein accounts for much, if not all, of the posttranslational protein modification.

Disruption of the gma12 Gene in S. pombe

To demonstrate conclusively that the *gma12* gene encoded the galactosyltransferase activity in *S. pombe*, we deleted 90% of the ORF by homologous recombination using the linear *Cla* I/*Eco*RI DNA fragment diagrammed in Figure 3B. Our first expectation was that the enzymatic activity would be nonessential and that the deletion could be carried out in a haploid strain. This assumption proved to be correct. A second expectation was that disruption of the *gma12* gene would result in loss of galactosyltransferase activity. This assumption proved to be false. Each haploid transformant had significantly decreased levels of galactosyltransferase activity, but the specific activity (relative to both total protein and mannosyltransferase activity) was still ~15% of wild-type levels. One of the haploid transformants was selected for further characterization.

We used several criteria to demonstrate that we had in fact disrupted the *gma12* gene and deleted 90% of the ORF in the *gma12-D10::ura4* strain. High stringency Southern blotting with the 1-kb *Hin*dIII/*Bam*HI fragment deleted from the *gma12-D10* strain detected no homologous sequences in *gma12-D10* genomic DNA. Additionally, PCR reactions with oligonucleotides (GT2, GT3) (Table 1) situated just outside the deletion region resulted in single DNA fragments of the proper size in both *gma12*$^+$ (1.1 kb) and *gma12-D10* (1.9 kb) strains. Attempts to purify the residual galactosyltransferase activity from the *gma12-D10* strain using the protocol developed for the *gma12*$^+$ strain resulted in no detectable activity after the first anion-exchange chromatography step. By all three criteria, we concluded that by disrupting the *gma12* gene, we had, in parallel, removed the galactosyltransferase activity that we had previously identified. We had also, however, demonstrated the presence of further galactosyltransferase activities in *S. pombe*.

Demonstration of Two or More Galactosyltransferase Activities in Wild-Type S. pombe

Because our initial biochemical assays told us only that there was still galactosyltransferase activity present in the *gma12-D10* strain, we performed both lectin labeling and microsome fractionation experiments to address the questions of specific linkages and subcellular localization. Figure 4 shows that both wild-type and *gma12-D10* strains of *S. pombe* have binding sites for BSL I B_4 isolectin evenly distributed on their cell surfaces. Qualitatively, we do not see a difference in the level of staining between the two strains, indicating that there is still a significant amount of α-linked galactose on the cell surface in the absence of *gma12* gene expression. The presence of cell surface α-linked galactose indicates the

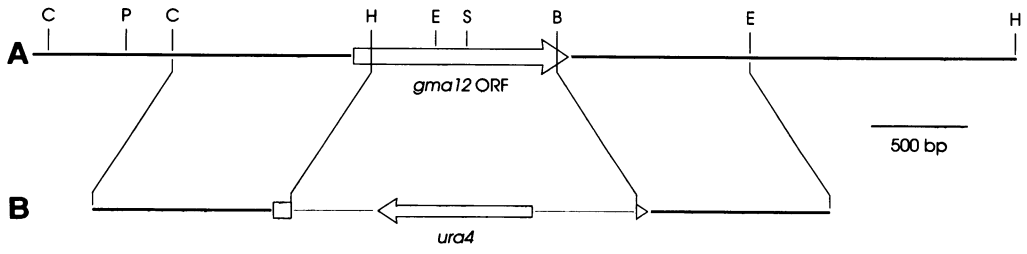

Figure 3. Restriction map of the *S. pombe* genomic region around *gma12* (A) and construct used for *gma12* gene disruption (B). DNA sequencing and restriction mapping of three cosmids spanning the *gma12* gene was used to generate a composite restriction map of the region of the *S. pombe* genome containing *gma12*. Abbreviations used: B, *Bam*HI; C, *Cla* I; E, *Eco*RI; H, *Hin*dIII; P, *Pst* I; S, *Sal* I. A 1.8-kb *Hin*dIII fragment containing the *S. pombe ura4* gene (Grimm *et al.*, 1988) was cloned between the *Hin*dIII and *Bam*HI sites of *gma12*. The 3.8-kb *Cla* I/*Eco*RI fragment shown in B was used in a one step gene disruption to remove 90% of the *gma12* ORF, as indicated.

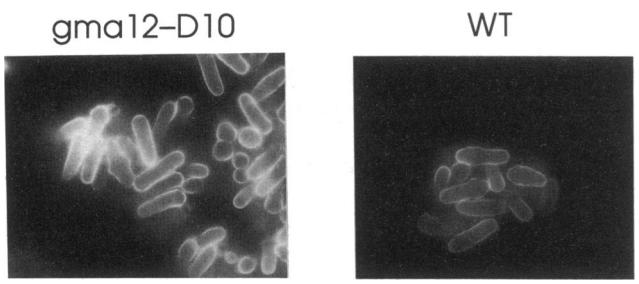

Figure 4. Cell surface staining of wild-type and *gma12-D10* strains of *S. pombe* with BSL I B_4 isolectin. Wild-type *S. pombe* cells (WT) and *gma12-D10* cells were grown to midlog phase in YEA at 32°C. After fixation with 3% formaldehyde, the cell surfaces were stained with FITC-conjugated BSL I B_4 isolectin, a lectin specific for terminal, α-linked galactose. Both strains showed extensive, uniform cell surface distribution of α-linked galactose.

presence of galactosyltransferase activity within the secretory pathway of the *gma12-D10* strain.

To quantitate the remaining galactosyltransferase activity and compare its intracellular localization with gma12p, microsomes from both wild-type and *gma12-D10* strains were fractionated on linear sucrose gradients. Figure 5 shows the activity profile of galactosyltransferase in each of the two strains. In wild-type cells, the peak of activity was present in fractions containing ~30% (wt/wt) sucrose. The activity profile of the deletion strain paralleled that of the wild-type strain across the gradient with a specific activity of ~15% that of wild-type in each fraction. The peak of activity in the deletion strain equilibrated a fraction higher in the gradient than the peak in the wild-type strain in the specific experiment shown. This result varied from experiment to experiment, probably in relation to the exact fractionation of each sucrose gradient. The residual activity in the *gma12-D10* strain is therefore present in membrane fractions of either similar or identical density to the fractions containing gma12p.

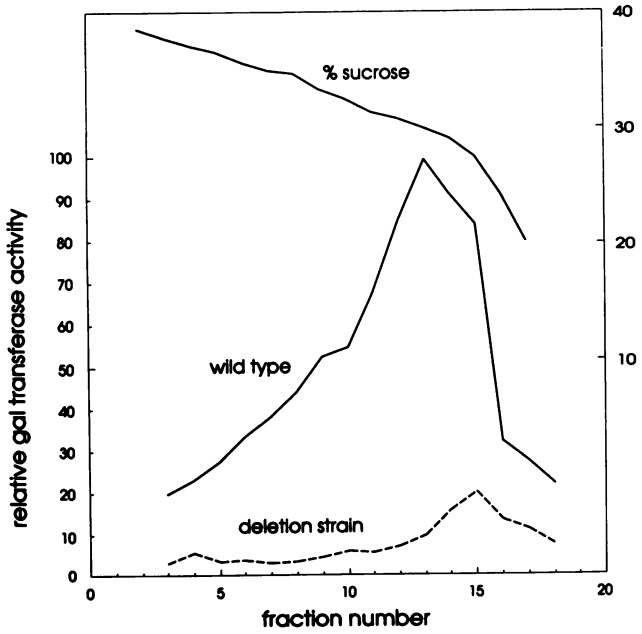

Figure 5. Equilibrium sedimentation fractionation of galactosyltransferase activity from wild-type and *gma12-D10* strains of *S. pombe*. Microsomes were prepared from wild-type and *gma12-D10* strains as previously described (Chappell and Warren, 1989). Samples containing 10 mg of total protein were loaded on top of 40–15% (wt/wt) sucrose gradients and centrifuged to equilibrium. Fractions were collected from the bottom of each gradient, and sucrose concentrations and galactosyltransferase and mannosyltransferase activities were quantitated. Both strains produced equivalent mannosyltransferase activity profiles.

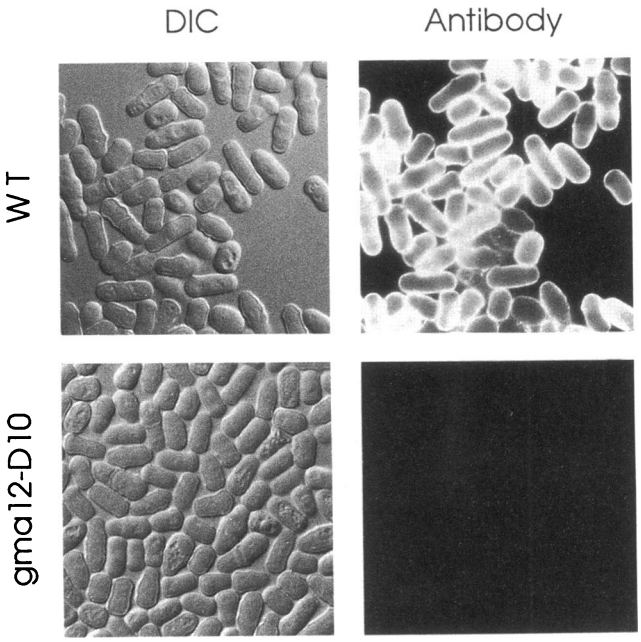

Figure 6. Immunofluorescence labeling of an antigenic epitope present on cells expressing gma12p. Wild-type *S. pombe* cells (WT) and *gma12-D10* cells were grown to late log phase in YEA at 32°C. After fixation with 3% formaldehyde, the cell surfaces were stained with the antiserum raised against wild-type cells and affinity depleted against *gma12-D10* cells. The portion of the antiserum that did not adsorb to *gma12-D10* cells contained antibodies that labeled wild-type cells but not *gma12-D10* cells.

The presence of two or more galactosyltransferase activities in wild-type *S. pombe* cells raised the direct question as to whether multiple activities were performing redundant roles in oligosaccharide processing. To answer this question, we raised a polyclonal antiserum to the cell surface of *gma12*$^+$ *S. pombe* cells. By removing all the antibodies from this antiserum that bound to the *gma12-D10* strain, we were able to generate a depleted antiserum containing antibodies that recognized epitopes unique to the wild-type strain. Figure 6 shows the cell surface staining of both *gma12*$^+$ and *gma12-D10* strains using this depleted antiserum in

conjunction with FITC-secondary antibodies. The wild-type cells can be seen to have an epitope uniformly distributed on the cell surface that is absent from the deletion strain. The staining pattern is identical to that of the wild-type cells labeled with BSL I B_4 isolectin (Figure 4), which shows the distribution of galactose molecules on the cell surface.

Demonstration that the Surface Epitope Is Synthesized by gma12p

We took two approaches to demonstrate that the surface epitope is generated by the enzymatic activity of gma12p. The first, an in vitro approach, is shown in Figure 7. An aliquot of *gma12-D10* cells were first fixed and washed. They were then incubated with purified galactosyltransferase in the presence of UDP-Gal and Mn^{2+}. The purified enzyme was capable of regenerating the epitope on the cell surface on the *gma12-D10* strain. The regeneration of the epitope was dependent on the presence of both UDP-Gal and Mn^{2+}, conditions that are required for enzymatic activity in vitro. These data demonstrate that it is the lack of gma12p, rather than the lack of an appropriate substrate, that results in the failure of the *gma12-D10* strain to synthesize the surface epitope.

Our second approach utilized the regulated *nmt1* promoter from *S. pombe*. The *gma12* ORF was cloned into an *nmt1* expression vector and transformed into *gma12-D10* cells under conditions where expression was repressed. After shifting to thiamine-free media, where expression of *gma12* was turned on, the cells were stained for the surface epitope. The result of this experiment is shown in Figure 8. Because growth in *S. pombe* occurs by cell cycle-dependent deposition of newly synthesized material at both ends of a septated cell (Miyata *et al.*, 1990), the surface staining parallels the expected elongation of the cell. Regulated expression of the *gma12* gene product results in regulated expression of the surface epitope.

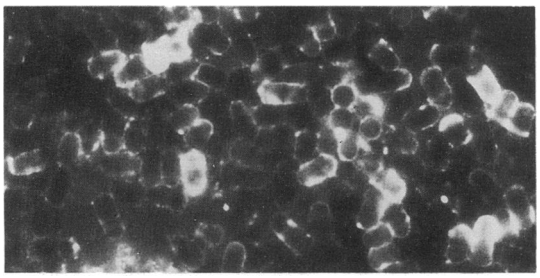

Figure 7. Regeneration of the surface antigenic epitope in vitro. Approximately 20 μg of purified galactosyltransferase (Chappell and Warren, 1989) was incubated for 2 h at 37°C with fixed *gma12-D10* cells in the presence of UDP-Gal (5 mM) and Mn^{2+} (1 mM). After extensive washing, the cells were stained with the *gma12*$^+$ specific antiserum (Figure 6). The enzymatic activity of the purified galactosyltransferase regenerated the gma12p specific cell surface epitope.

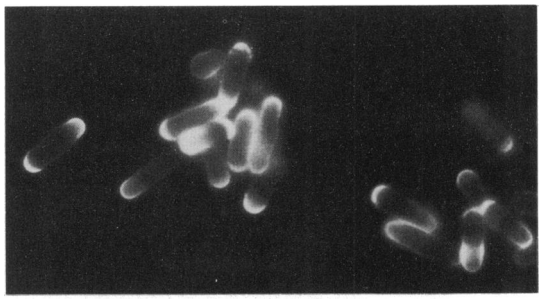

Figure 8. Regeneration of the surface antigenic epitope in vivo. A population of *gma12-D10* cells transformed with the pREP1::*gma12* plasmid was grown to midlog phase in PMM containing 1 mM thiamine. Under these conditions, expression of the *gma12* gene was repressed. The cells were washed twice with PMM and resuspending in PMM lacking thiamine to induce *gma12* expression. After four generations, the cells were fixed and stained according to the procedure used in Figure 6.

Together, Figures 7 and 8 demonstrate that it is the product of the gma12p enzymatic activity and not gma12p itself that is reacting with the antibodies in the depleted antiserum. Because the presence of the *gma12*$^+$ specific epitope on the cell surface could be the result of biosynthesis anywhere along the secretory pathway, we sought to determine precisely where along the pathway the epitope first appeared.

Immunolocalization of the Carbohydrate Epitope

To determine the intracellular distribution of the carbohydrate epitope, immunoelectron microscopy was performed. Cells were fixed in glutaraldehyde, embedded in Lowicryl, and sectioned. Sections were labeled with antibodies to the carbohydrate epitope followed by 10 nm immunoglod conjugates. As shown in Figure 9, A–D, most of the intracellular label was found over the Golgi stacks. All Golgi stacks in any given section were labeled, and the labeling was not restricted to any particular cisterna in the stack or part of a cisterna, though there was often a tendency for the dilated rims to be labeled more than the central parts. Labeling was specific for the carbohydrate epitope because the Golgi stacks in the *gma12-D10* strain were not labeled by the antibody (Figure 9, E–G).

There was no significant labeling of the endoplasmic reticulum (ER), neither the peripheral ER underlying the plasma membrane, nor the nuclear envelope (Figure 9A). This shows that the epitope is created in the Golgi stacks and not in an earlier compartment. Labeling was, as expected, found in compartments later in the secretory pathway, most notably the plasma membrane (Figure 9, A and C) and vacuoles.

DISCUSSION

A gene encoding an α 1,2 galactosyltransferase, *gma12*, has been cloned from *S. pombe*. The gene product is a

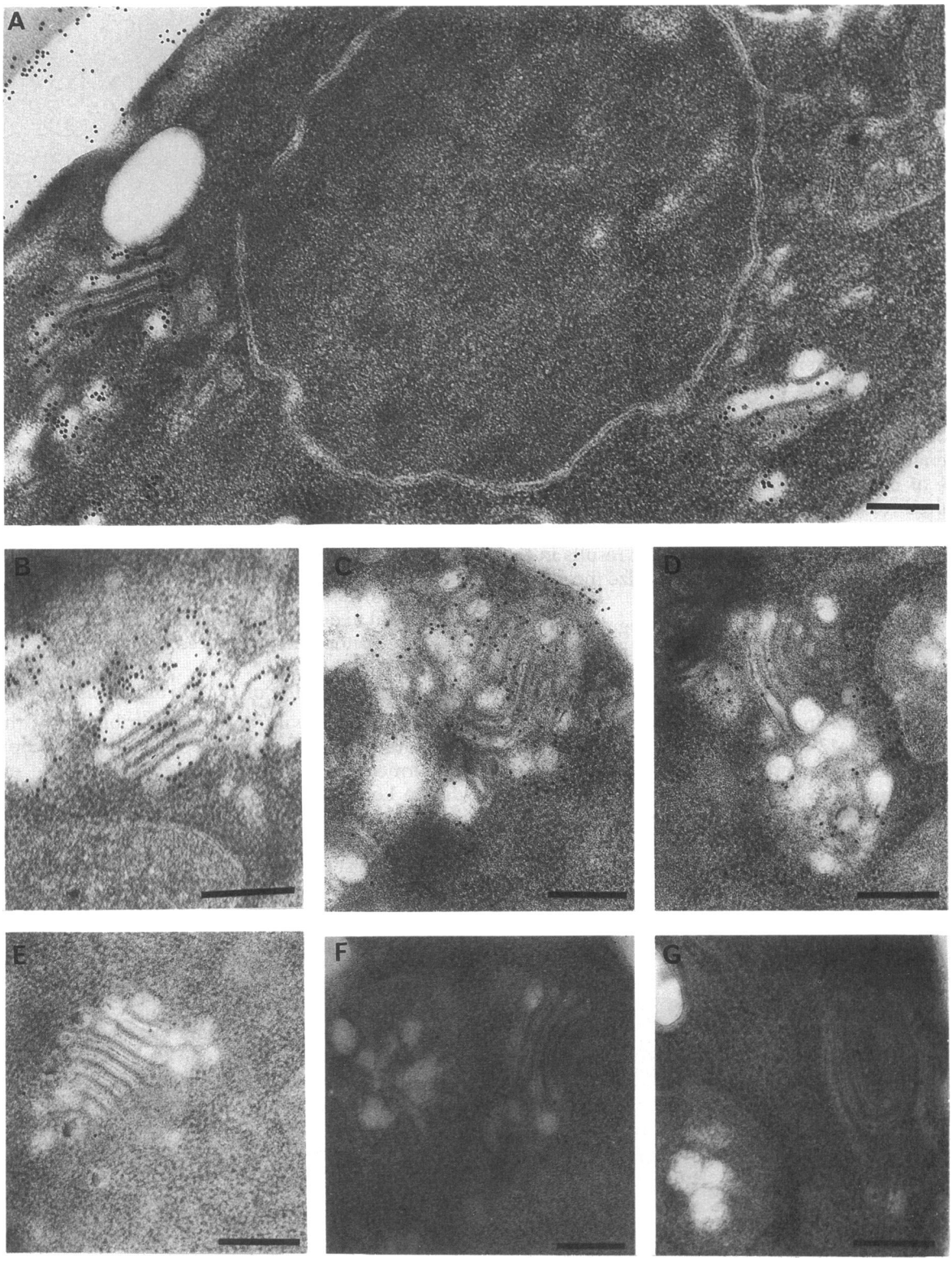

Figure 9. Immunolocalization of the *gma12* specific carbohydrate epitope. Electron micrographs of wild-type *S. pombe* cells (A–D) and *gma12-D10* cells (E–G), fixed with monomeric glutaraldehyde and embedded in HM20 Lowicryl resin. The ultrathin sections of these cells were incubated with the depleted rabbit polyclonal antiserum used to stain the cell surface in Figure 6, followed by immunogold conjugates (goat anti-rabbit IgG, 10 nm). Bars, A (0.25 μm); B (0.25 μm); C (0.25 μm); D (0.25 μm); E (0.25 μm); F (0.33 μm); and G (0.2 μm).

glycoprotein that contains a single membrane spanning domain near the N-terminus, producing a type II transmembrane topology—identical to that of mammalian glycosyltransferases (Paulson and Colley, 1989). The cytoplasmic domain appears to be the minimum necessary to generate and maintain a type II topology, consisting simply of the initial methionine and a single charged amino acid. The protein contains no consensus sites for N-linked oligosaccharide addition but does include a region with high serine/threonine content similar to the O-glycosylated domains of mammalian mucins (Carraway and Hull, 1991). We have therefore concluded that glycosylation of gma12p is exclusively O-linked. Because gma12p binds to both mannose- and galactose-specific lectins (Chappell and Warren, 1989), O-linked oligosaccharides in *S. pombe* must contain both types of sugar residues. This result suggests a more complex pattern of oligosaccharide processing in the Golgi apparatus of fission yeast than we had initially expected with terminal galactose added to both O- and N-linked oligosaccharides.

Deletion of the *gma12* gene by homologous recombination resulted in a significant decrease in galactosyltransferase activity. There was, however, still measurable intracellular enzymatic activity and cell surface-localized, galactose-specific lectin binding. Consequentially, oligosaccharide processing in *S. pombe* is the result of two or more galactosyltransferase activities located along the secretory pathway. The presence of multiple galactosyltransferases explains an observation we made early in our attempt to isolate the *gma12* gene; namely, our inability to generate mutant strains that lacked terminal galactose on the cell surface. By generating antibodies against a carbohydrate epitope produced by gma12p and demonstrating the absence of the epitope in a *gma12* deletion strain, we have concluded that these multiple galactosyltransferase activities are not providing overlapping activities in the fission yeast Golgi apparatus. One interpretation of these data would be the existence of distinct biochemical pathways for the maturation of N- and O-linked oligosaccharides, each involving galactose addition by functionally unique transferases.

Haploid cells lacking gma12p have normal growth rates and, as shown in Figure 9, contain Golgi structures that are morphologically indistinguishable from those in wild-type cells. The cells themselves appear to be slightly more rounded than wild-type, indicating a possible weakening of the cell wall. The contribution of gma12p-specific products to the overall cell wall composition is supported by both the uniform distribution of the gma12p-specific epitope on the cell surface and observations that spheroplasting enzymes affect *gma12-D10* strains differently than wild-type strains and that deletion strains show a higher degree of agglutination under a variety of growth conditions (Chappell, unpublished data).

The distribution of the gma12p-specific carbohydrate epitope throughout the Golgi apparatus raises several interesting questions. Methylation and hydrolysis of galactomannan from *Schizosaccharomyces* by Bush *et al.* (1974) led to the conclusion that *S. pombe* contained no nonreducing terminal mannose and only trace amounts of galactose in any position other than termini. This analysis requires the terminal position of every oligosaccharide branch to be occupied by a galactose residue. In conjunction with our in vitro regeneration of the gma12p-specific epitope at the cell surface, this suggests that terminal oligosaccharide structures are present throughout the Golgi apparatus.

If we impose a mammalian vectorial transport model on the Golgi stack of fission yeast—allowing a single visit to each cisterna during passage from the endoplasmic reticulum to the cell surface—gma12p would be required to act in a *cis* (early) compartment to guarantee distribution of the carbohydrate epitope throughout the stack. In this case, the galactosyltransferase would be finishing the biosynthesis of a portion of the oligosaccharides early in their transit through the Golgi apparatus. This model is most appealing if the action of gma12p is limited to the modification of O-linked oligosaccharides because these contain fewer hexose units than the extensive N-linked oligosaccharides and, more importantly, gma12p is itself modified by the addition of O-linked galactose. A *cis*-localized activity, devoted exclusively to N-linked modification, would lack O-linked galactose if the modification activities reside in distinct compartments within the Golgi apparatus.

If gma12p is responsible for O-linked oligosaccharide modification in the *cis* Golgi, the in vitro ability of gma12p to modify all substrates containing a terminal mannose residue (Chappell and Warren, 1989) would suggest that the majority of N-linked mannose addition occurs in later compartments. An initial α 1,6 mannan backbone could be synthesized before exposure to gma12p, but any branching from the backbone would be "capped" by the enzyme activity. Using the mammalian system as a model, we would postulate gma12p residing in a *cis* compartment with mannosyltransferase activity in the *medial* region followed by further N-linked specific galactosyltransferase activity in the *trans* compartment.

A more intriguing possibility is that the difference between mammalian and yeast oligosaccharide processing requires fundamentally different mechanisms of biosynthesis. In mammalian cells, final N-linked oligosaccharide structures are nonrepetitive—processing enzymes typically modify a specific structure only one to three times. In both *Saccharomyces cerevisiae* and *S. pombe*, however, N-linked oligosaccharide structures are polymeric and contain dozens of identical glycosidic bonds (Bush *et al.*, 1974; Ballou, 1982). One mechanism of creating such a polymer would be repetitive exposure

to a group of enzymes capable of synthesizing monomeric subunits. Such a model would allow for the distribution of the terminal gma12p-specific epitope throughout the Golgi apparatus even if the localization of the enzyme was restricted to specific compartments later in the Golgi.

Although our current study does not identify the precise localization of gma12p within the Golgi stack, the characterization and cloning of the first member of an emerging family of galactosyltransferases in *Schizosaccharomyces* provides additional tools to address questions of Golgi function in a genetically tractable organism. Specifically, the ability to tag cells containing active galactosyltransferase within Golgi compartments with antibodies provides an appealing screen for localization sequences. Elucidating the location and mechanism of retention of each of the transferases within the Golgi apparatus of *S. pombe* will help us to better understand both the route secretory proteins take through the organelle on the way to the cell surface and the function of the Golgi apparatus in general.

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