

## Localization of Synthesis of $\beta$ 1,6-Glucan in *Saccharomyces cerevisiae*

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**$\beta$ 1,6-Glucan is a key component of the yeast cell wall, interconnecting cell wall proteins,  $\beta$ 1,3-glucan, and chitin. It has been postulated that the synthesis of  $\beta$ 1,6-glucan begins in the endoplasmic reticulum with the formation of protein-bound primer structures and that these primer structures are extended in the Golgi complex by two putative glucosyltransferases that are functionally redundant, Kre6 and Skn1. This is followed by maturation steps at the cell surface and by coupling to other cell wall macromolecules. We have reinvestigated the role of Kre6 and Skn1 in the biogenesis of  $\beta$ 1,6-glucan. Using hydrophobic cluster analysis, we found that Kre6 and Skn1 show significant similarities to family 16 glycoside hydrolases but not to nucleotide diphospho-sugar glycosyltransferases, indicating that they are glucosyl hydrolases or transglucosylases instead of genuine glucosyltransferases. Next, using immunogold labeling, we tried to visualize intracellular  $\beta$ 1,6-glucan in cryofixed *sec1-1* cells which had accumulated secretory vesicles at the restrictive temperature. No intracellular labeling was observed, but the cell surface was heavily labeled. Consistent with this, we could detect substantial amounts of  $\beta$ 1,6-glucan in isolated plasma membrane-derived microsomes but not in post-Golgi secretory vesicles. Taken together, our data indicate that the synthesis of  $\beta$ 1,6-glucan takes place largely at the cell surface. An alternative function for Kre6 and Skn1 is discussed.**

The cell wall of the yeast *Saccharomyces cerevisiae* consists of four classes of macromolecules organized in the form of supramolecular complexes (14, 22–24, 28, 29, 31). About 40 to 50% of the cell wall is accounted for by mannoproteins, and the other 50 to 60% is composed of  $\beta$ 1,3-glucan and  $\beta$ 1,6-glucan, with a small amount of chitin (12, 18). Chitin and  $\beta$ 1,3-glucan are synthesized by separate enzyme complexes located in the plasma membrane (7, 8, 41, 48), and cell wall proteins are processed and transported to the cell surface in a stepwise process by the secretory pathway (41). The biogenesis of  $\beta$ 1,6-glucan, which in its mature form consists of about 140 glucose residues (34), is less well known. By screening for increased resistance to K1 killer toxin, several genes (often designated *KRE* genes) that are required for normal cell wall levels of  $\beta$ 1,6-glucan have been identified (2, 3, 5, 6, 13, 21, 36, 42–44, 47, 50). The corresponding gene products operate in the endoplasmic reticulum (ER) (*CWH41*, *GLS2*, *KRE5*, and *CNE1*), in the Golgi apparatus (*KRE6* and probably *SKN1*), and at the cell surface (*KRE1*, *KRE9*, and *KNH1*). This largely genetic evidence has led to the proposal that the synthesis of  $\beta$ 1,6-glucan is a stepwise process that begins in the ER with the synthesis of protein-bound primer structures. These primer structures are believed to be extended by Golgi-located  $\beta$ 1,6-glucosyltransferases, encoded by *KRE6* and its functional counterpart *SKN1*, whereas remodeling and maturation of  $\beta$ 1,6-glucan are believed to take place at the cell surface (3, 26, 37, 41).

We have reinvestigated the role of Kre6 and Skn1 in the

biogenesis of  $\beta$ 1,6-glucan. Using hydrophobic cluster analysis (HCA), we found unexpectedly that Kre6 and Skn1 show significant similarities to family 16 glycoside hydrolases (11, 19). This called into doubt the putative functions of Kre6 and Skn1. Using advanced immunocytochemical techniques and affinity-purified antibodies raised against protein-bound  $\beta$ 1,6-glucan oligosaccharides, we could detect only  $\beta$ 1,6-glucan at the cell surface. In addition, post-Golgi secretory vesicles isolated by gel filtration did not contain detectable amounts of  $\beta$ 1,6-glucan, whereas plasma membrane-derived microsomes contained substantial amounts of  $\beta$ 1,6-glucan. Together, these findings seem to exclude Kre6 and Skn1 as genuine glucosyltransferases involved in the extension in the Golgi apparatus of  $\beta$ 1,6-glucan chains. The results also indicate that the majority of  $\beta$ 1,6-glucan is synthesized at the plasma membrane.

### MATERIALS AND METHODS

**Strains and media.** The strains used in this study were X2180A (*MATa*) and HMSF1 (*MATa sec1-1*). Cells were grown in YPD medium (1% [wt/vol] yeast extract, 1% [wt/vol] Bacto Peptone [Difco Laboratories, Detroit Mich.], 3% [wt/vol] glucose) at 28°C.

**HCA.** HCA (recently reviewed by Callebaut et al. [9]) uses a two-dimensional (2-D) plot in which the amino acid sequence of a protein is displayed as an unrolled and duplicated longitudinal cut of a cylinder, where the amino acids follow an  $\alpha$ -helical pattern. The duplication of the helical net allows the full sequence environment of each amino acid to be represented. On this representation, the clusters of contiguous hydrophobic residues (V, I, L, F, M, W, Y) correspond significantly to secondary structure elements in globular proteins. The segmentation of a protein into successive secondary structure elements becomes visible along the horizontal axis of the diagram, whereas the sequence itself can be read on an almost vertical axis. The analysis then involves the comparison of cluster shape (for instance, large horizontal clusters correspond predominantly to  $\alpha$  helices and short vertical ones correspond to  $\beta$  strands) and cluster distribution between several plots in order to find correspondences.

**Cryofixation, freeze-substitution, low-temperature embedding, and immunogold labeling of  $\beta$ 1,6-glucan.** Samples of *sec1-1* mutant cells were cryofixed in liquid propane by means of a Reichert-Jung KF80 apparatus and were freeze-substituted as described by Schwarz and Humbel (46) by placing the cryofixed samples in 0.3% uranyl acetate and 0.01% glutaraldehyde in methanol at –90°C

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for 2 days. The samples were subsequently warmed to  $-45^{\circ}\text{C}$  at a rate of  $5^{\circ}\text{C}/\text{h}$ , rinsed with methanol, and infiltrated with Lowicryl HM20. After 16 h, the specimens were transferred to an embedding mold filled with Lowicryl HM20 at  $-45^{\circ}\text{C}$ . Polymerization at  $-45^{\circ}\text{C}$  for 48 h was carried out in a CS auto apparatus using a UV light source attachment (360 nm); this was followed by a 2-day curing period using UV light at room temperature (51). Ultrathin HM20 sections of the yeast cells were mounted on nickel grids and incubated with affinity-purified anti- $\beta$ 1,6-glucan polyclonal antibodies (1:300) (27). The antigen-antibody complex was visualized with secondary goat anti-rabbit antibodies (1:20) conjugated with 10-nm gold particles (Aurion, Wageningen, The Netherlands) (39). The labeled ultrathin sections were viewed in a Philips EM420 electron microscope, and micrographs were taken at an acceleration voltage of 80 kV.

**Cross-linking and staining of carbohydrates.** Samples of *secl-1* mutant cells were fixed in 2% glutaraldehyde in 0.1 M phosphate-buffered saline (PBS; pH 7.4) for 16 h at  $4^{\circ}\text{C}$ . After being rinsed with PBS, the yeast cells were put for 1.5 h into a mixture of 0.1 M lysine-HCl, 0.05 M PBS, and 0.02 M sodium *m*-periodate to cross-link the carbohydrates (35). After a rinse with PBS, the yeast cells were postfixed with 1%  $\text{OsO}_4$  in 50 mM PBS for 2 h. After dehydration in graded acetone solutions, the yeast cells were embedded in Epon. Ultrathin Epon sections of the yeast cells were mounted on nickel grids, immunogold labeled as described above, and subsequently stained with alkaline bismuth for 30 min at  $37^{\circ}\text{C}$  as described by Shinji et al. (49). Sections were viewed in a Philips EM420 electron microscope, and micrographs were taken at an acceleration voltage of 80 kV.

**Fractionation and characterization of microsomes.** The protocols of Walworth and coworkers (55, 56) were used. *secl-1* mutant cells were grown at  $25^{\circ}\text{C}$  in rich medium containing 2% glucose and then transferred to a medium kept at the restrictive temperature ( $37^{\circ}\text{C}$ ) and containing only 0.2% glucose. This medium shift simultaneously imposes the secretory block, resulting in the accumulation of post-Golgi secretory vesicles within the cytosol, and derepresses the synthesis of invertase. The cells were converted to spheroplasts in 1.4 M sorbitol and lysed osmotically in 0.8 M sorbitol. The latter sorbitol concentration was maintained throughout the fractionation procedure to preserve the integrity of the organelles. The lysate was subjected to differential centrifugation to remove unlysed cells, cell wall debris, nuclei, and mitochondria, and the microsomal fraction was fractionated by passage through a Sephacryl S-1000 gel filtration column. Aliquots from each column fraction were assayed for protein content, plasma membrane ATPase activity, invertase activity, and  $\beta$ 1,6-glucan content. Protein was measured by bicinchoninic acid protein assay (Pierce, Rockford, Ill.) with bovine serum albumin (BSA) as a reference protein. Vanadate-sensitive plasma membrane ATPase activity was assayed as described elsewhere (4). Invertase activity was assayed with sucrose as substrate as described previously (17) except that the resulting reducing sugars were determined by the Nelson-Somogyi method (52). Distribution of  $\beta$ 1,6-glucan across the eluate was determined by dot blot analysis using affinity-purified  $\beta$ 1,6-glucan antibodies (27). An aliquot of 1  $\mu\text{l}$  from each column fraction was spotted on a polyvinylidene difluoride membrane and left for 30 min in a closed container. The membrane was incubated for 1 h with a blocking buffer containing 5% nonfat milk powder in PBS. For immunodetection, the membrane was treated as described below, and the staining intensities of the spots were measured by densitometric scanning.

**Conjugation of gentiobiose to BSA.** Gentiobiose was covalently linked to lysine residues of BSA by reductive amination (27, 45). Sodium dodecyl sulfate-polyacrylamide gel electrophoresis was performed on a linear 2.2-20% gradient (37). For Western analysis, proteins were electrophoretically transferred to polyvinylidene difluoride membranes overnight at 45 V. The membranes were washed three times with PBS and incubated for 1 h with a blocking buffer containing 5% nonfat milk powder in PBS. The membranes were washed three times with PBS and then incubated for 1 h with affinity-purified anti- $\beta$ 1,6-glucan antibodies (1:5,000) in 3% BSA in PBS. The membranes were washed five times with PBS and incubated with peroxidase-conjugated goat anti-rabbit antibodies (Bio-Rad Laboratories, Hercules, Calif.). The immunoblots were developed with ECL (enhanced chemiluminescence) Western blotting detection reagents (Amersham, Arlington Heights, Ill.).

## RESULTS

**Structural relationships of Kre6 and Skn1 with family 16 glycoside hydrolases and transglycosidases.** HCA is based on a 2-D helical representation of protein sequences (9, 15). It is a powerful tool for sequence comparison at low sequence identity levels and for the detection of secondary structure elements ( $\alpha$  helices,  $\beta$  strands, and loops). Using this method, we could not find sequence similarities with nucleotide diphospho-sugar glycosyltransferases (10) but instead detected significant sequence similarities with family 16 glycoside hydrolases and transglycosidases (11, 19). Figure 1 shows that throughout most of their luminal portions, Kre6 (and Skn1 [not shown]) display significant HCA similarities with well-characterized family 16 members. In particular, they share a conserved motif

with two glutamic acid residues separated by either three or four amino acids (20). These two residues are the catalytic amino acids in family 16 glycoside hydrolases. The 3-D structure of the  $\beta$ 1,3-1,4-glucanase of *Bacillus macerans* (Protein Data Base [PDB] entry 1BYH), which belongs to the same family, has been experimentally determined (25). An interesting feature of Kre6 (and Skn1 [not shown]) is the insertion of two segments (A and B in Fig. 2). The inserted elements are localized on two adjacent loops of the structure where they might form a protuberance in Kre6 without affecting the catalytic machinery. Pairwise BLAST analysis supported the results obtained by HCA analysis. Comparison of the full-length putative catalytic region of Kre6 (amino acids 321 to 660) with the clotting factor G alpha subunit resulted in the identification of two sequences with  $P = 8e-04$  (significant) for the first sequence (amino acids 573 to 660) and  $P = 0.94$  (not significant) for the other sequence (amino acids 325 to 433). When regions A and B (Fig. 1) were left out, a single sequence (amino acids 325 to 660) was identified with a probability of  $1e-12$  (significant). In other words, removal of regions A and B considerably improved the significance of the resemblance. Similarly, comparison of the full-length catalytic region of Kre6 with the full-length catalytic region of the  $\beta$ 1,3-glucanase II of *Oerskovia* again resulted in the identification of two sequences with probabilities of 0.43 for the first sequence and 0.63 for the second. Again, when regions A and B were left out of consideration, a single sequence (amino acids 329 to 659) was identified with a probability of  $2e-9$  (significant). Comparison of the full-length catalytic regions of Kre6 and  $\beta$ 1,3-1,4-glucanase from *B. macerans* resulted in a nonsignificant  $P$  value even when both regions A and B were left out. However, comparison between the full catalytic region of  $\beta$ 1,3-1,4-glucanase from *B. macerans* with the full catalytic region of  $\beta$ 1,3-glucanase II of *Oerskovia*, which as discussed above shows significant similarity with the Kre6 catalytic domain, resulted in a  $P$  value of  $8e-4$  (significant).

In summary, HCA and pairwise BLAST analyses point to a clear resemblance of Kre6 and Skn1 with glycoside hydrolases of family 16. This is difficult to reconcile with their postulated function as nucleotide sugar glucosyltransferases and suggests instead that Kre6 and Skn1 have either glycosidase or transglucosidase activity.

**Detection of  $\beta$ 1,6-glucan at the cell surface by immunogold labeling.** As the results of the HCA analysis seemed to be inconsistent with the proposed functions of Kre6 and Skn1 as nucleotide sugar glucosyltransferases responsible for elongating  $\beta$ 1,6-glucan chains in the Golgi apparatus, we decided to look for intracellular  $\beta$ 1,6-glucan immunocytochemically. The  $\beta$ 1,6-glucan antiserum that we used was raised against  $\beta$ 1,6-glucan oligosaccharides with an average chain length of 15 glucose residues coupled to BSA (37). The specificity of the  $\beta$ 1,6-glucan antiserum has been confirmed in various ways. Recognition of the epitope is competitively inhibited by pustulan ( $\beta$ 1,6-glucan) but not by laminarin ( $\beta$ 1,3-glucan) or mannan (37). In addition, periodate treatment of the  $\beta$ 1,6-glucan epitope completely abolishes the signal (37). The anti- $\beta$ 1,6-glucan antiserum has been further purified by affinity chromatography using  $\beta$ 1,6-glucan oligosaccharides immobilized on an epoxy-Sepharose column (27). This raises the question of whether the affinity-purified anti- $\beta$ 1,6-glucan antiserum also recognizes short  $\beta$ 1,6-glucan oligosaccharides. To answer this question, we analyzed the effectiveness of the antiserum toward protein-bound gentiobiose (Glc $\beta$ 1,6Glc). Figure 3 shows that the antiserum efficiently bound to gentiobiose coupled to BSA, whereas it had no activity toward BSA itself (Fig. 3). This shows that our affinity-purified antiserum is capable of recognizing short  $\beta$ 1,6-glucan oligosaccharides.

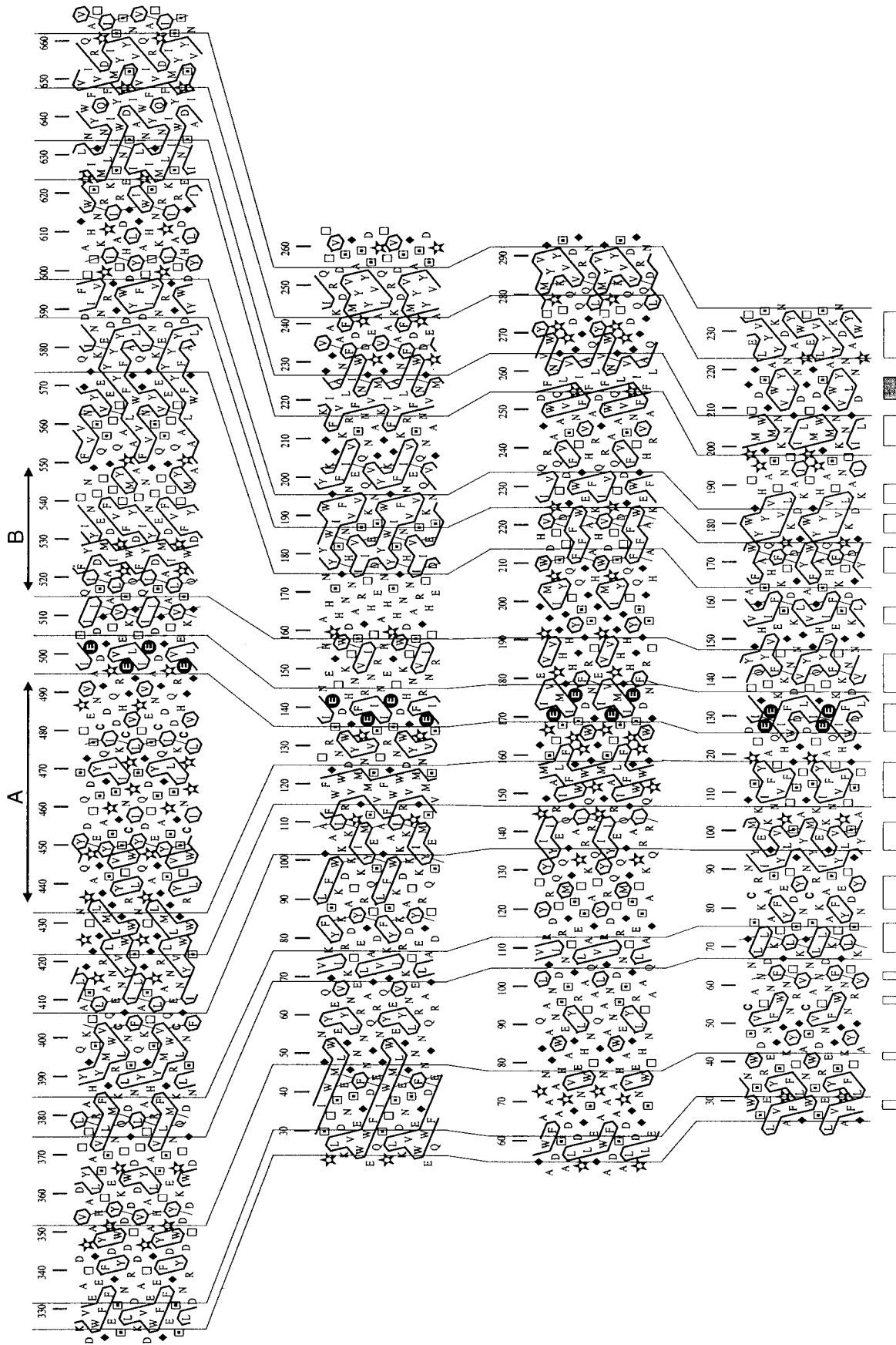


FIG. 1. HCA plots of selected members of family 16 glycoside hydrolases and transglycosidases (18, 20). From top to bottom: Kreb of *Saccharomyces cerevisiae* (SwissProt P32486), clotting factor G alpha subunit of *Tachypleus tridentatus* (GenBank D16622),  $\beta$ 1,3-glucanase II of *Oerskovia xanthicola* (GenBank AF052745), and  $\beta$ 1,3-1,4-glucanase of *Bacillus macerans* (SwissProt P23904; PDB 1BYH). The HCA plots were made, edited, and analyzed as described elsewhere (15, 19). To facilitate visual inspection of the plots, the symbols \*,  $\square$ , and  $\diamond$  are used for proline, glycine, serine, and threonine, respectively. Vertical lines show correspondences between proteins. The two catalytic glutamate residues are shown in white on black circles. The secondary structure elements of 1BYH are shown as open ( $\beta$  strand) and grey ( $\alpha$  helix) boxes under the corresponding plot. The two insertions found in Kreb6 are marked A and B.

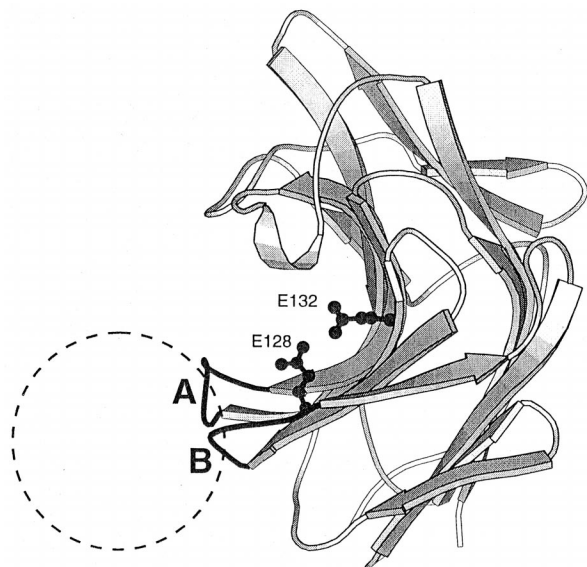


FIG. 2. Schematic 3-D structure of the  $\beta$ 1,3-1,4-glucanase of *B. macerans* (PDB 1BYH). The two glutamate residues that belong to the catalytic site are shown in ball-and-stick representation. The two loops which carry the insertions in Kre6 are labeled A and B. The location of the resulting putative protuberance in Kre6 is shown by dashed lines. The figure was prepared with the program MOLSCRIPT (30).

Yeast cells were first cryofixed and freeze-substituted. Ultrathin Lowicryl sections were immunogold labeled with affinity-purified  $\beta$ 1,6-glucan antibodies. In wild-type cells, the cell surface became clearly labeled, but no intracellular labeling was observed (data not shown). Since in wild-type cells only a few secretory vesicles are seen and intracellular  $\beta$ 1,6-glucan might for that reason have been overlooked, labeling experiments were also performed on *sec1-1* cells (40) kept at the restrictive temperature for 2 h. This is roughly equivalent to one generation time, implying that collectively the secretory vesicles are expected to contain sufficient cell wall precursor material to build an entire cell wall. In the section shown, about 360 gold particles are visible at the cell surface, whereas intracellular labeling is negligible (Fig. 4A). This is difficult to reconcile with the notion that the bulk of  $\beta$ 1,6-glucan synthesis is synthesized intracellularly. To exclude the possibility that  $\beta$ 1,6-glucan had leaked out of the secretory vesicles during the processing steps prior to electron microscopy, in the next experiment we introduced a cross-linking step, which results in the formation of aggregates of indiscriminately cross-linked carbohydrates and proteins (35). This was followed by specific staining of the carbohydrate cargo of the vesicles (49) (Fig. 4B). Although the vesicles were heavily stained under these conditions, indicating that possible losses of their contents were limited, still no immunogold labeling of the vesicles was observed. In summary, our data are consistent with the notion that the bulk of  $\beta$ 1,6-glucan synthesis takes place at the plasma membrane.

**$\beta$ 1,6-Glucan is absent from post-Golgi secretory vesicles.** Walworth and coworkers have developed an efficient and well-documented method to isolate intact post-Golgi secretory vesicles that are largely free from contaminating organelles including ER-, plasma membrane-, and vacuolar membrane-derived microsomes. An additional advantage of this method is that it results in a considerable purification of plasma membrane-derived microsomes which are relatively free from post-Golgi secretory vesicles and vacuolar membrane-derived microsomes (55, 56). Their method makes use of a late secretory

mutant which is allowed to accumulate secretory vesicles at the restrictive temperature. The microsomal fraction obtained by differential centrifugation of osmotically lysed spheroplasts is further fractionated by gel filtration in the presence of stabilizing concentrations of sorbitol, resulting in a clear separation of plasma membrane-derived microsomes and post-Golgi secretory vesicles. Using this approach, we analyzed the eluate for the presence of protein, invertase activity, plasma membrane ATPase activity, and  $\beta$ 1,6-glucan. As shown in Fig. 5A, protein eluted as three major peaks. The first protein peak coeluted with the first peak of the plasma membrane marker, vanadate-sensitive ATPase activity, and represented the plasma membrane-derived microsomes (Fig. 5C) (55, 56). The second protein peak cofractionated with the major peak of invertase activity, which marks post-Golgi secretory vesicles (Fig. 5B). The second ATPase peak, which coeluted with the major peak of invertase activity, probably represents ATPase transported by secretory vesicles (55, 56). The last protein peak cofractionated with an invertase peak. This probably represents material escaped from leaky secretory vesicles. Finally, the column fractions were analyzed by a dot blot assay using affinity-purified  $\beta$ 1,6-glucan antibodies (Fig. 5D). Although some signal (23%) was detected in the fractions corresponding to the post-Golgi secretory vesicles, most of the signal (77%) was found in the fractions containing plasma membrane-derived vesicles, suggesting the existence of a  $\beta$ 1,6-glucan-synthesizing protein (complex) associated with the plasma membrane. The presence of a weak positive signal in the fractions containing post-Golgi secretory vesicles might be due to contamination with plasma membrane-derived vesicles. However, we cannot exclude the possibility that secretory vesicles contain some  $\beta$ 1,6-glucan that cannot be detected by immunogold labeling.

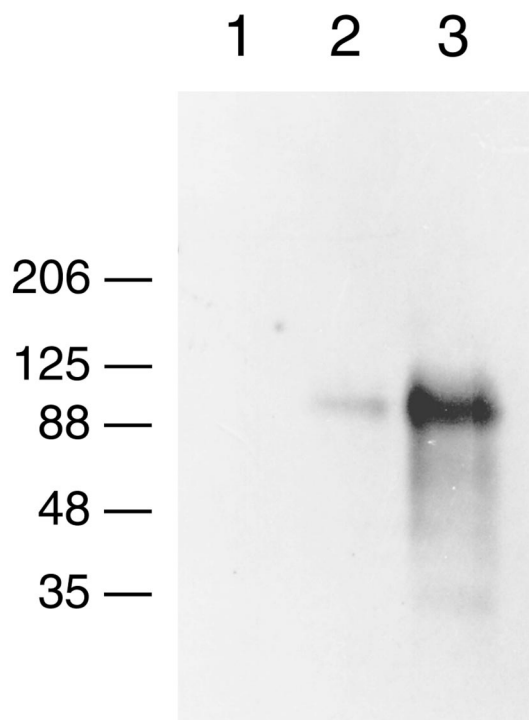


FIG. 3. Pustulan affinity-purified antibodies recognize protein-bound gentiobiose. Lane 1, 1  $\mu$ g of BSA; lane 2, 1  $\mu$ g of gentiobiose-BSA; lane 3, 5  $\mu$ g of gentiobiose-BSA. The blot was developed with ECL for 1 min. Sizes are indicated in kilodaltons.

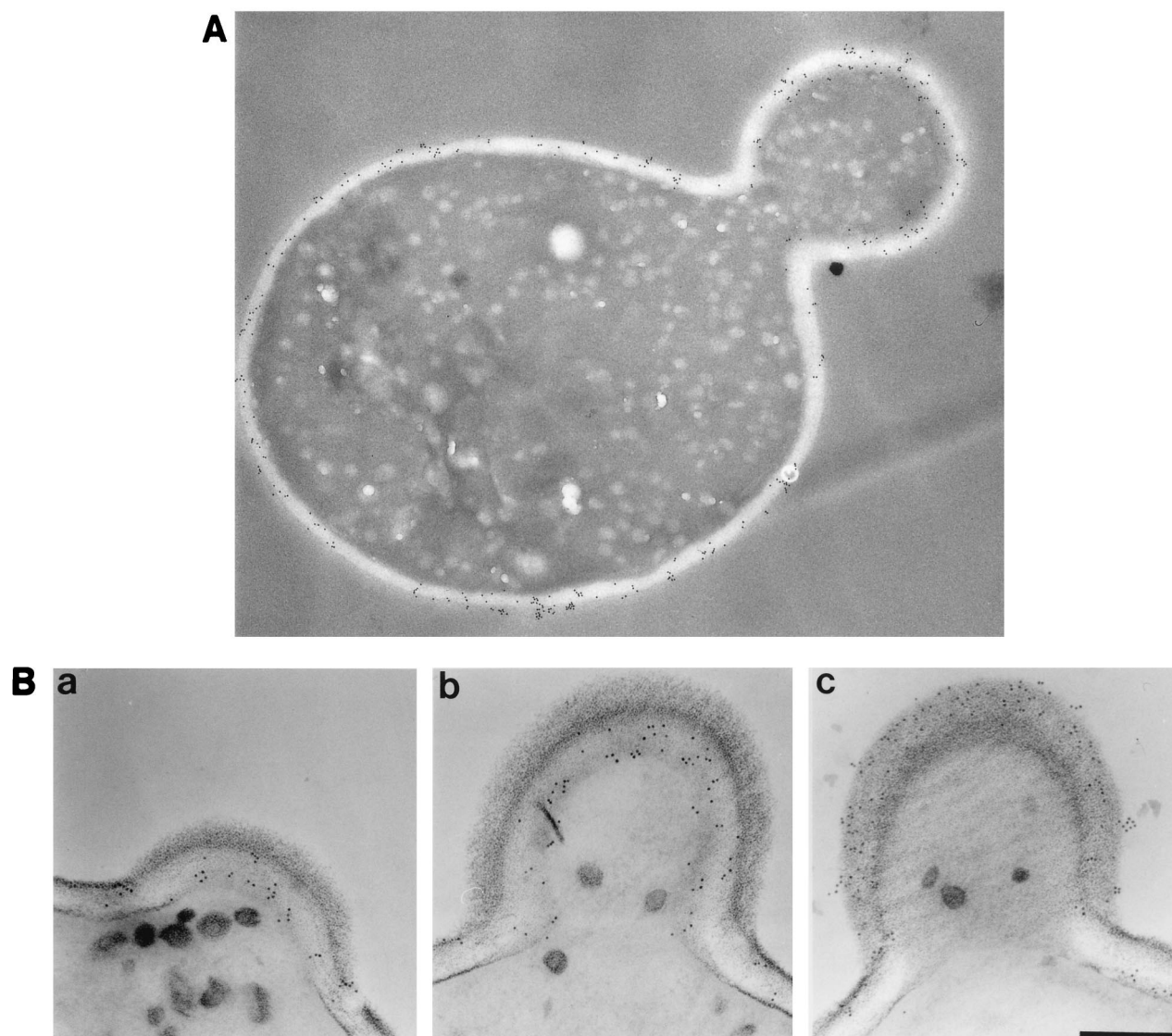


FIG. 4. Immunogold labeling of  $\beta$ 1,6-glucan in *sec1-1* cells. (A) To induce accumulation of post-Golgi secretory vesicles, *sec1-1* cells were kept at the restrictive temperature for 2 h. A representative cell is shown. About 360 gold particles are visible at the cell surface, whereas intracellular labeling is negligible. (B) The vesicles were visualized by cross-linking and staining of the carbohydrate cargo by the methods of McLean and Nakane (35) and Shinji et al. (49), respectively. Bar = 250 nm.

## DISCUSSION

*KRE* and *KRE*-related genes have been isolated as genes that are required for full levels of cell wall  $\beta$ 1,6-glucan and which, when nonfunctional, confer resistance to K1 killer toxin (reviewed in reference 41). As several of the corresponding proteins have been localized in the ER (Kre5 and Cwh41), the Golgi complex (Kre6 and possibly also its homolog Skn1), and at the cell surface (Kre1 and Kre9), it has been proposed that they might be involved in sequential steps of the biosynthesis of  $\beta$ 1,6-glucan (3, 26, 37, 41). Kre6 and Skn1 are predicted to have a single amino-terminal transmembrane domain and a long carboxy-terminal lumenal domain. It has been proposed that Kre6 and Skn1 are Golgi-located glucosyltransferases that elongate a protein-bound  $\beta$ 1,6-glucan primer structure formed in the ER (37, 41). Here we present evidence that Kre6 and Skn1 are not genuine glucosyltransferases and that the synthesis of  $\beta$ 1,6-glucan takes largely place at the plasma membrane. First, homology searches based on hydrophobic cluster analysis

show that Kre6 and Skn1 have the hallmarks of glycoside hydrolases or transglycosidases but not of nucleotide diphospho-sugar glycosyltransferases (19, 20; see also reference 1). Second, we were unable to detect any intracellular  $\beta$ 1,6-glucosylated proteins, either in wild-type cells or in *sec18*, *sec7*, and *sec1* cells kept at the restrictive temperature to accumulate ER, Golgi-like structures, and post-Golgi secretory vesicles, respectively (37a). As our antibodies efficiently recognize  $\beta$ 1,6-glucosylated cell wall proteins in yeast (37), intracellular  $\beta$ 1,6-glucan in the mycelial fungus *Trichosporum sporotrichoides* (38), and even protein-bound gentiobiose (this report), extensive  $\beta$ 1,6-glucosylation of intracellular proteins in yeast seems unlikely. Third, immunogold labeling of post-Golgi secretory vesicles in cryofixed cells gave negative results even after an additional cross-linking step to avoid potential losses of the vesicle contents during the processing steps prior to electron microscopy, whereas a strong signal was seen all over the cell surface, showing that our antiserum efficiently recognizes  $\beta$ 1,6-

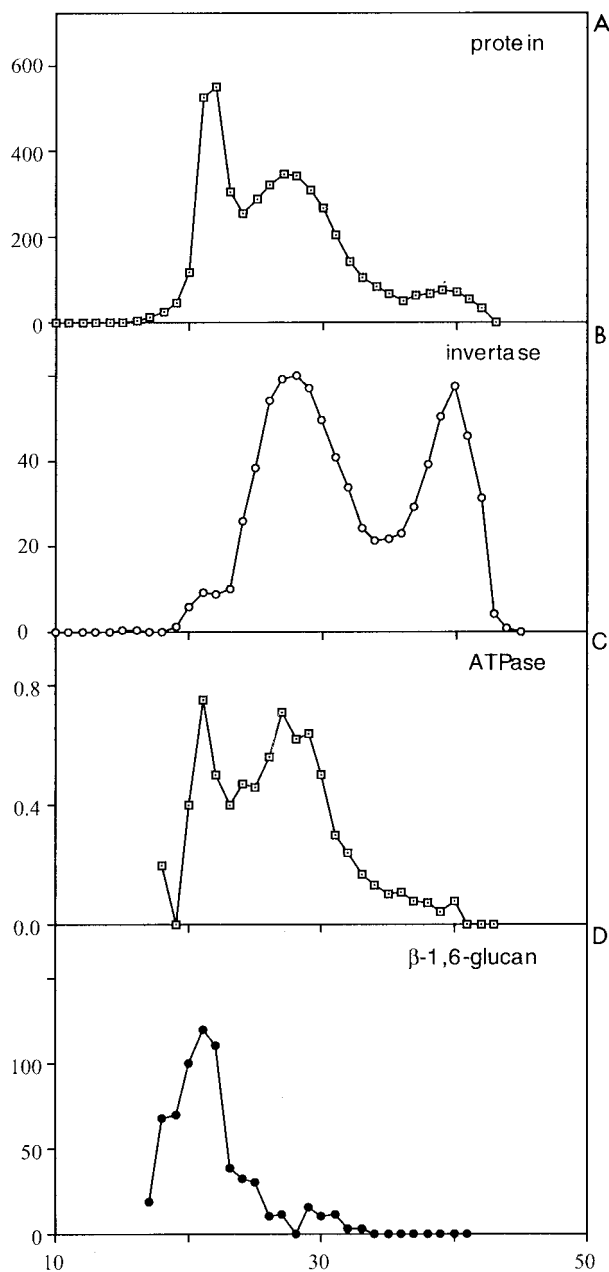


FIG. 5.  $\beta$ 1,6-Glucan colocalizes with plasma membrane-derived vesicles. To induce accumulation of post-Golgi secretory vesicles, *sec1-1* cells were kept at the restrictive temperature for 2 h. The cells were spheroplasted and gently lysed. The resulting homogenate was fractionated by differential centrifugation, and the microsomal fraction was separated by gel filtration on a Sephacryl S-1000 column (55, 56). Aliquots of each column fraction (x axis) were assayed for protein content, invertase, plasma membrane ATPase, and  $\beta$ 1,6-glucan content. y axes represent, from top to bottom, protein (micrograms), invertase (micromoles of Glc per minute), plasma membrane ATPase (micromoles of  $P_i$  per minute), and  $\beta$ 1,6-glucan (arbitrary densitometric units).

glucan. An alternative explanation of this result is that in contrast to wild-type cells, *sec1-1* cells immediately halt the production of  $\beta$ 1,6-glucan when transferred to 37°C. However, it is known that other cell surface components like plasma membrane ATPase, invertase, and acid phosphatase continue to be synthesized at this temperature (55, 56). Fourth, dot blot analysis of membrane vesicles fractionated by gel filtration revealed

only a small amount of  $\beta$ 1,6-glucan in the fractions containing post-Golgi secretory vesicles, possibly due to contamination with plasma membrane-derived vesicles. However, in the fractions that contained plasma membrane-derived vesicles, substantial amounts of  $\beta$ 1,6-glucan were present. As post-Golgi secretory vesicles are destined to become part of the plasma membrane, these data also suggest that the plasma membrane contains not only an activatable  $\beta$ 1,3-glucan synthase but also an activatable  $\beta$ 1,6-glucan synthase.

Still unanswered is the question of how the loss of function of Kre proteins in the secretory pathway, including Kre6 and Skn1, could lead to a reduction in cell wall  $\beta$ 1,6-glucan. One possibility is that the postulated plasma membrane-associated  $\beta$ 1,6-glucan synthase complex is for unknown reasons extremely sensitive to defects in N-glycosylation as observed in *mmm9 $\Delta$*  and *och1 $\Delta$*  cells do not result in decreased levels of  $\beta$ 1,6-glucan in the cell wall (47). Alternatively, Kre proteins in the secretory pathway may contribute to the construction of glucose-containing protein-bound carbohydrate structures, which may act as acceptor sites for the addition of  $\beta$ 1,6-glucan at the cell surface. For example, Kre6 and Skn1 could act as transglucosidases on a protein-bound glucan structure formed in the ER by Kre5. The nature of the postulated acceptor structures is unknown and could include modified glycosylphosphatidylinositol (GPI) anchors, N chains, and O chains, but not necessarily on the same protein. This is consistent with earlier observations by Van Rinsum and coworkers (54) (Fig. 4), who provided evidence for the presence of three different types of glucose-containing carbohydrate side chains in cell wall proteins, possibly corresponding with extended N chains, O chains, and GPI anchors. Indeed, chemical analysis has revealed a direct linkage between a processed GPI anchor and  $\beta$ 1,6-glucan (14, 29). Recently, a genetic analysis of ER-located Kre proteins has presented evidence that N chains may also be involved as alternative attachment sites for  $\beta$ 1,6-glucan (47). Finally, mutants defective in the first steps of O-glycosylation show partial resistance to K1 killer toxin (16, 33, 53), consistent with the notion that in some cases also O chains may function as attachment sites for  $\beta$ 1,6-glucan. In summary, we propose that the incorporation of  $\beta$ 1,6-glucan into the cell wall requires three critical steps: (i) the construction of glucose-containing protein-bound acceptor sites by Kre proteins in the early compartments of the secretory pathway for the later addition of  $\beta$ 1,6-glucan; (ii) the extension of these primer structures with  $\beta$ 1,6-glucan at the plasma membrane; (iii) the addition of  $\beta$ 1,6-glucan to cell wall proteins that have newly arrived at the cell surface, as has been described for  $\alpha$ -agglutinin and Tip1 (14, 32).

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