The cross-linking of polysaccharides to assemble new cell wall in fungi requires mechanisms by which a preexisting linkage is broken for each new one made, to allow for the absence of free energy sources outside the plasma membrane. Previous work showed that Crh1p and Crh2p, putative transglycosylases, are required for the linkage of chitin to β(1–3)glucose branches of β(1–6)glucan in the cell wall of budding yeast. To explore the linking reaction in vivo and in vitro, we used fluorescent sulfo-hodamine-linked laminari-oligosaccharides as artificial chitin acceptors. In vivo, fluorescence was detected in bud scars and at a lower level in the cell contour, both being dependent on the Crh proteins. The linking reaction was also shown in digitonin-permeabilized cells, with UDP-N-acetylglucosamine as the substrate for nascent chitin production. Both the nucleotide and the Crh proteins were required here. A gas1 mutant that overexpresses Crh1p showed very high fluorescence both in intact and permeabilized cells. In the latter, fluorescence was still incorporated in patches in the absence of UDP-GlcNAc. Isolated cell walls of this strain, when incubated with sulforhodamine-oligosaccharide, also showed Crhp-dependent fluorescence in patches, which were identified as bud scars. In all three systems, binding of the fluorescent material to chitin was verified by chitinase digestion. Moreover, the cell wall reaction was inhibited by chitooligosaccharides. These results demonstrate that the Crh proteins act by transferring chitin chains to β(1–6)glucan, with a newly observed high activity in the bud scar. The importance of transglycosylation for cell wall assembly is thus firmly established.

Fungal cells are endowed with a cell wall, external to the plasma membrane, which is essential for cell survival. The wall protects the cell against bursting caused by the internal turgor pressure and against mechanical injury. It also acts as a filter for large molecules that could injure the plasma membrane. Furthermore, the cell wall is the surface at which pathogenic fungi interact with the host, whether animal or plant, and against which host defenses are often mounted. Because the cell wall imparts shape to the fungal cell, we have used it for many years in Saccharomyces cerevisiae as a model for morphogenesis. On account of its essentiality and its composition, which includes substances not found in animal cells, the fungal cell wall is an obvious target for antifungal compounds. In fact, inhibitors of the echinocandin type against β(1–3)glucan synthase have recently entered clinical use.

The mechanical strength of the cell wall is explained by the linkage of its components to each other, which results in a tightly linked network. To build this structure, the cell needs to solve several problems as follows: how to synthesize the wall components, how to export them outside the plasma membrane, and how to assemble them in an orderly frame outside the cell.

In S. cerevisiae, the components of the cell wall are polysaccharides and mannoproteins. The latter are synthesized at the endoplasmic reticulum and the Golgi, followed by export in vesicles to the plasma membrane, to which they are tethered by glycosylphosphatidylinositol anchors. On the other hand, the polysaccharides, such as chitin (8), β(1–3)glucan (9), and probably β(1–6)glucan (10), are formed at the plasma membrane and extruded into the periplasmic space. Thus, no cross-linking of the components is possible intracellularly, because they do not become available to each other until they are outside the plasma membrane. Because in the cell wall the different components are covalently attached to each other, how are the cross-links made extracellularly? This poses a thermodynamic puzzle; inside the cell, ATP, directly or indirectly, provides the free energy for the formation of new chemical bonds. However, outside the plasma membrane there cannot be ATP or other small molecules, because they would be dissipated into the medium; so what is the source of the free energy? This problem is not limited to fungal cells. Bacterial and plant cells also have cell walls, and in many cases animal cells are endowed with an extracellular matrix, which may require some assembly.

Because small molecules are absent, the free energy for the formation of new bonds must come from the large components themselves, which can use up some of their preexisting linkages to make new ones or can enter into a reaction with some substance readily available outside the cell. Examples of both
**Crh1p and Crh2p as Transglycosylases**

Mechanisms have been described; thus, in bacteria, cross-linking of peptidoglycan chains occurs by transpeptidation, with the liberation of \( \delta \)-alanine (11). During formation of the fertilization envelope in sea urchin eggs, proteins are cross-linked peroxidatively by formation of intermolecular tyrosine bonds (12). About 20 years ago we proposed that linkages between different polysaccharides of the yeast cell wall might be generated by transglycosylation (13). A test for that hypothesis became possible only recently, when we developed a new procedure for the quantitative determination of some cross-links in the yeast cell wall (14). With that approach, it was found that the putative transglycosylases Crh1p and Crh2p are required for the formation of cross-links between chitin and \( \beta(1\rightarrow6) \)glucan but not between chitin and \( \beta(1\rightarrow3) \)glucan (15). In these cross-links, chitin becomes attached to \( \beta(1\rightarrow3) \)glucose branches of the \( \beta(1\rightarrow6) \)glucan (5) (Fig. 1A). Crh1p and Crh2p are members of a group of three highly homologous proteins (16) (Fig. 1B), the third being Crp1p, which appears to have a function in the formation of the spore wall (17). These proteins are endowed with motifs similar to the catalytic group of certain bacterial glucanases and plant transglycosidases (16). Introduction of a Crh1p mutated in that motif in a \( \textit{crr} \alpha \) \( \textit{crr} \beta \) strain failed to suppress the sensitivity of that strain to Congo Red (16). In addition to this presumptive catalytic site, both Crh2p and Crr1p contain a chitin-binding domain (CBM18), shown for Crh2p in Fig. 1C. Curiously, Crh1p is devoid of such a domain. It may have a previously unidentified chitin-binding site, or perhaps an appropriate binding site might be created by folding of the protein (19). All these data supported our hypothesis that the Crh proteins may link chitin and \( \beta(1\rightarrow6) \)glucan by transglycosylation, but it was desirable to buttress this proposition by better characterizing the activity of the Crh proteins and demonstrating it, if possible, in an \textit{in vitro} system. There is, however, a formidable obstacle to that endeavor, which is that chitin, as available to the researcher, is totally insoluble in water, because of hydrogen bonding between the sugar chains. Thus, it is unlikely that this material would serve for an \textit{in vitro} transglycosylation reaction. The situation may be quite different \textit{in vivo}, where individual chains emerge from the plasma membranes and may be captured by a transglycosylase positioned nearby, before they have an opportunity to interact with other chitin chains and form an insoluble fiber. To circumvent this problem, we used a stepwise approach, which started by offering artificial transglycosylation acceptors to growing cells and led to the use of permeabilized cells and finally of cell walls in an \textit{in vitro} system. Unexpectedly, in the course of these experiments we uncovered a very high activity of the system for chitin-\( \beta(1\rightarrow6) \)glucan linkage in bud scars.

**EXPERIMENTAL PROCEDURES**

**Strains and Growth Conditions**—The strains used are listed in Table 1. Cells were grown in minimal medium (2% glucose, 0.67% yeast nitrogen base) plus requirements, at 30 °C or 38 °C, as indicated in each case.

**Strain Construction**—To delete \textit{GAS1} in the LC355 background, yielding strain NBT001, the open reading frame was replaced by the \textit{HIS3} marker from plasmid pFA-HIS3MX6, with the long flanking homology PCR technique (22) and primers \texttt{5'}-CTGATAAAAACAAAAACAAACAAACAGCTAAAATCTCAACAATGCGTGACGAGTCGAC-3' and \texttt{5'}-GATACCATACCTCTGATGATTGATATGATGCAGAACATCGATATCCCGCAGCTCG-3'. Correct open reading frame replacement was verified by PCR, using the following primer pairs: \texttt{5'}-CTGACAAAAGAAGCTGCTCCTC-3'; \texttt{5'}-GCCAGATCCGAAGCTTGAAG-3' and \texttt{5'}-GCGAGCCTGCGTATGTCG-3'.
ride (AnaSpec, San Jose, CA, or Invitrogen) with a mixture of reductively aminated β(1–3)- or β(1–6)-linked glucose oligosaccharides or with individual reductively aminated oligosaccharides. The oligosaccharide mixtures, containing chains of 2–8 glucose in length, were obtained by partial hydrolysis, with 4 M trifluoroacetic acid at 100 °C, of laminarin (Sigma) for β(1–3)-linked oligosaccharides, or pustulan (EMD-Cabiochem) for β(1–6)-linked compounds, followed by fractionation on a Bio-Gel P-6 column. The single β(1–3)-linked oligosaccharides were obtained from Associates of Cape Cod, East Falmouth, MA. The concentration of SR-glucosidases was determined from the absorbance at 570 nm.

**Purification of Z-protease**—Four ml of a 10 mg/ml solution of zymolyase 100T (Associates of Cape Cod) in 1 M Tris, pH 7.5, was applied to a Sephacryl S-200 column (1.6 × 90 cm), previously equilibrated with the same Tris buffer. Elution was also carried out with 1 M Tris buffer with the help of a peristaltic pump. Fractions of 5 ml were collected. β(1–3)Glucanase in the fractions was determined with reduced laminarin as substrate, from the liberation of reducing power as described by Park and Johnson (24). Protease activity was measured with Azocoll (EMD-Cabiochem) as substrate. The reaction mixture contained, in a total volume of 300 μl, 6.25 mg of Azocoll and 50 μl of the column eluate. After 1 h of incubation at 37 °C, 0.35 ml of cold water was added, and tubes were centrifuged in the cold for 5 min at 18,500 × g. A 520, was measured in the supernatant. An A 520 > 1.0, as measured under those conditions, is taken as 1 unit of protease activity. β(1–3)Glucanase emerged from the column before protease, but the two activities partially overlapped. Fractions with protease activity but little glucanase contamination (in one batch, fractions 32–37) were pooled and concentrated to ~4 ml in an Amicon cell equipped with a YM-10 filter. The concentrate was applied to the same Sephacryl S-200 column, and the fractionation was repeated. The pooled fractions were concentrated to a final volume of 0.8 ml by ultrafiltration. The yield of protease activity varied between 20 and 40% in different batches. The purified protein still had some β(1–3)glucanase activity, but the ratio protease/glucanase increased between 60- and 120-fold with the purification.

**Uptake of Fluorescent Oligosaccharides by Living Yeast Cells**—To 0.5 ml of culture in a vial, 30 μl of either a 200 μM SR-oligosaccharide mixture or a 200 μM individual SR-oligosaccharide was added. Ampicillin was also added to the culture to a final concentration of 75 μg/ml. The cell concentration was such that the culture would be in early stationary phase after overnight incubation at 30 °C. Growth, from the A 660, was monitored in a parallel larger culture. After overnight growth, the cultures were transferred to Eppendorf tubes, which were centrifuged for 3 min at 6000 × g. Cells were washed three times with 1 ml of 20% ethanol and once with 0.5 ml of water, followed by suspension in 0.3–0.5 ml of water for fluorescence observation in the microscope.

For chitinase treatment, 150 μl of the gas1A (strain Y00897) fluorescent cells were incubated with 1.4 unit of Z-protease in Tris, pH 7.5, at a final 0.05 M concentration for 1 h at 37 °C. Tubes were centrifuged for 3 min at 16,000 × g. The cells were washed with 0.4 ml of 0.05 M MES, pH 6, and suspended in 0.2 ml of the same buffer. Purified Serratia marcescens chitinase (25) (20 milliunits) and 2 μl of 2% sodium azide were added, and the tubes were rotated overnight at 37 °C in the dark. Incubated cells were observed in the fluorescence microscope.

**Digitonin-permeabilized Cells**—Cells were permeabilized with digitonin as described previously (26). All cells were permeabilized, as verified by staining with methylene blue. The cells were suspended in twice as many milliliters of 25 mM MES, pH 6.3, as grams (wet weight) of cells used. A typical incubation mixture with SR-oligosaccharides contained 10 μl of cell suspension, 0.05 M MES, pH 6.3, 4 mM magnesium acetate, 2 mM UDP-GlcNAc, 32 mM N-acetylgalactosamine, 0.1% digitonin, and 20 μM SR-oligosaccharides, in a total volume of 50 μl. Incubation was on an Eppendorf shaker at 37 °C, usually for 1–3 h. If

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2 The abbreviations used are: SR, sulforhodamine; MES, 2-(N-morpholino)ethanesulfonate.
the incubation time was more than 1 h, a new addition of UDP-GlcNAc was made every hour. After incubation, 1 ml of cold 25 mM MES, pH 6.3, was added to each tube. Tubes were centrifuged 5 min in the cold at 18,500 × g. Cells were washed twice with 1 ml of 20% ethanol and once with 0.5 ml of 0.05 M Tris, pH 7.5 (centrifugations for 5 min at 15,000 × g). They were suspended in 0.5 ml of the Tris buffer, followed by incubation of 1.3 units of Z-protease, and incubated on a rotator at 37 °C for 1 h. After centrifugation for 5 min at 16,000 × g, the cells were washed once with 0.5 ml of 25 mM MES, pH 6.3, and suspended in 0.5 ml of 1% SDS. The tubes were rotated overnight in the dark at room temperature, followed by centrifugation as above. The cells were washed once with 0.5 ml of the MES buffer and suspended in 0.3 ml of the same buffer for observation in the fluorescence microscope.

For the time course experiments of Fig. 8, a 10-fold scaled-up mixture was used, with SR-hexasaccharide as substrate. After incubation at 37 °C with shaking for different times, 5 ml of cold 25 mM MES buffer, pH 6.3, were added and the tubes were centrifuged for 5 min at 15,000 × g in a swinging bucket rotor. Cells were washed twice with 5 ml of 20% ethanol and once with 2.5 ml of 50 mM Tris, pH 7.5, then suspended in 1 ml of the Tris buffer, and transferred to Eppendorf tubes. To each tube, 2.7 units of Z-protease were added, and tubes were rotated at 37 °C for 1 h, followed by centrifugation for 5 min at 16,000 × g. After two washings with 1 ml of 25 mM MES, pH 6.3, and suspension of the cells in 1 ml of 1% SDS, the tubes were incubated overnight in the dark on a rotator at room temperature. After incubation, tubes were centrifuged 5 min at 16,000 × g. Cells were washed with 1 ml of 0.05 M MES, pH 6, and suspended in 1 ml of the same buffer. A 10-μl sample was used for inspection and imaging in the fluorescence microscope. Of the suspension, 50 μl were set apart as controls, and to the remainder 50 μl (90 milliunits) of S. marcescens chitinase were added. Both the controls and the mixtures with chitinase were incubated overnight in the dark on a rotator at 30 °C. The tubes were centrifuged 5 min at 16,000 × g, and fluorescence was measured in the supernatants with a PTI Quantamaster spectrofluorimeter. The excitation wavelength was 566 nm, and the emission spectrum was acquired between 580 and 680 nm.

Cell Walls—For preparation of cell walls, 0.6 g (wet weight) of cells, or permeabilized cells from 0.6 g of intact cells, were suspended in 1.2 ml of 25 mM MES, pH 6.3. Of this suspension, 1.2 ml was transferred to a 15-ml Corex tube, followed by 4 g of glass beads (0.5 mm diameter). The tube was vortexed for 6 periods of 1 min, with 1 min cooling in ice in-between. The extract was withdrawn with a Pasteur pipette, and the beads were washed with six 0.5-ml portions of the MES buffer. The combined fractions were centrifuged 10 min at 3800 × g to sediment the cell walls, followed by one washing with 5 ml of the MES buffer and suspension in the same buffer to a final volume of 0.8 ml.

Incubation mixtures included 10 μl of cell wall suspension, 50 mM succinate, pH 5.5, and 20 μM SR-oligosaccharide, in a total volume of 50 μl. Incubation was on an Eppendorf shaker at 37 °C. After incubation, 1 ml of cold 25 mM MES, pH 6.3, was added, and the tubes were centrifuged 10 min in the cold at 18,500 × g. Cell walls were washed once with 1 ml of 1% SDS and once with 1 ml of the MES buffer, followed by suspension in 0.3 ml of the same buffer for observation in the fluorescence microscope.

For the time course experiments of Fig. 10, a 10-fold scaled-up mixture was used. The treatment was the same as for the time course of permeabilized cells, except that after the first incubation and centrifugation, the cell walls were washed twice with 5 ml of 50 mM Tris, pH 7.5, rather than with 20% ethanol followed by Tris. Also, in the Z-protease incubation, the amount of Z-protease added to each tube was 6.6 units.

**Fluorescence Observation and Imaging**—Samples were observed in the rhodamine channel of a Zeiss Axioskop microscope equipped for fluorescence. Images were captured with a Qimaging Retiga Exi SVGA digital camera and processed with iVision software (BioVision Technologies). Images to be compared were obtained with the same exposure and normalized with the same parameters.

For Calcofluor staining, cells were suspended in a 0.01% solution of Calcofluor White. After 5 min, they were observed in the UV channel (4′,6-diamidino-2-phenylindole channel) of the fluorescence microscope.
Wild type cells were grown in the presence of a mixture of fluorescent oligosaccharides, which would pinpoint where the reaction took place. Indeed, when the nascent chitin chains interact with the oligosaccharides through the agency of the transglycosylation system, we investigated whether sulforhodamine-labeled oligosaccharides would function as artificial substrates for the transglycosylases. Images to be compared were obtained with the same exposure and immersion. By comparing phase and fluorescence images, it was observed that the bright patches were close to the mother-daughter neck. Because in haploid cells each new budding occurs next to the previous one, bud scars remain close to the mother-daughter neck, which makes difficult to distinguish between the neck chitin ring and an adjacent bud scar. To clarify the precise location of the bright patches, we took advantage of the polar budding in diploids, which often results in bud scars at the opposite pole of the mother-daughter neck. A diploid (FY1679), isogenic with the haploid wild type previously used, was grown in the presence of fluorescent oligosaccharides. The cells were stained with Calcofluor White, which labels the chitin both in the neck ring and in bud scars, and were observed both in the UV (Calcofluor) and in the rhodamine channel (Fig. 2D). It could be seen that bud scars situated at the distal pole with respect to the bud showed both Calcofluor and rhodamine fluorescence (lower cell in Fig. 2D), whereas the mother-bud neck was only stained by Calcofluor (both cells in Fig. 2D). Statistical results confirmed the observations. Of 81 cells with bud scars but no bud, or with bud scars opposite to bud, 80 stained in both the Calcofluor and rhodamine channels and one in the Calcofluor channel only. Of 122 cells with bud, but no scar adjacent to the bud, in 115 the mother bud neck was visible in the Calcofluor channel only and in 7 it could be seen in both channels. Thus, the bright patches observed after growth in the presence of SR-labeled oligosaccharides are bud scars. This explains why the patches are not observed in all cells, because only about half of the cells in a culture have bud scars.

The synthesis of chitin-oligosaccharide linkages in bud scars was unexpected. The bud scar contains both the chitin ring made at the mother-bud neck during bud emergence and the chitinous primary septum formed at cytokinesis (31). In a previous study we found that most of the cross-linked chitin in the just synthesized chitin ring is bound to β(1–3)glucan, whereas about one-third of the chitin is free (14). In the primary septum,
made at cytokinesis, almost all of the chitin is free (14). We reexamined the distribution of chitin in bud scars in EM sections stained with wheat germ agglutinin attached to gold particles from a previous study (31) and found that, in addition to the two localizations already mentioned, often a chitin layer could be seen along the plasma membrane at the bud scar (Fig. 2E). Thus, there are three possible sources of chitin for cross-linking to \( \beta(1\rightarrow6) \)glucan during bud scar formation after cytokinesis as follows: the portion of the ring chitin that is still free, the chitin in the primary septum, and the newly formed chitin near the plasma membrane.

Whereas the requirement of the Crh proteins for the appearance of fluorescent patches was clear, the involvement of those proteins in the weaker fluorescence around the cell contour was more doubtful, because such fluorescence was also present to some extent in the chs3\( \Delta \) and crh1\( \Delta \) crh2\( \Delta \) mutants. We hypothesized that part of this fluorescence might be due to the Gas1 protein, which catalyzes transglycosylation between \( \beta(1\rightarrow3) \)glucan chains (32). Because \( \beta(1\rightarrow3) \)glucan is dispersed all over the surface of the cell, an exchange between that polysaccharide and the \( \beta(1\rightarrow3) \)-linked SR-oligosaccharides would result in widespread fluorescence over the cell wall. To test this supposition, we grew a gas1\( \Delta \) mutant (strain Y00897) in the presence of SR-oligosaccharides. Surprisingly, these cells became much brighter than the corresponding wild type, BY4741 (Fig. 3, A and B). In some cells, the fluorescence was still in patches, and in others it invaded most of the cell. Furthermore, the cell contour was also bright. However, when both CRH1 and CRH2 were deleted in the gas1\( \Delta \) mutant (strain GR011), all fluorescence, both in patches and in the cell cortex, disappeared (Fig. 3C). Expression of CRH2 in a high copy plasmid restored the fluorescence of the triple mutant (results not shown). This result shows that both the fluorescence in bud scars and the contour require the Crh proteins. The enhanced fluorescence of the gas1\( \Delta \) mutant may be accounted for by the overexpression of CRH1 (33) coupled to the high chitin content (34) in that mutant.

The high fluorescence of the gas1\( \Delta \) cells provided an opportunity to confirm that the oligosaccharides were bound to chitin. If this is the case, they should be solubilized when chitin is hydrolyzed by chitinase. Treatment of intact cells with chitinase led to only partial reduction of the fluorescence. In the assumption that the chitinase was not able to penetrate completely the cell wall, we treated the cells with a protease purified from zymolyase (Z-protease), which effectively removes the surface mannoproteins, thereby increasing the cell wall permeability (1). This treatment affected the contrast of the cells under phase but not their fluorescence (Fig. 3, D and E). However, the fluorescence was then readily removed by chitinase (Fig. 3F), as expected if the oligosaccharides were linked to chitin.

In all the experiments described so far, a mixture of SR-linked laminari-oligosaccharides of different lengths was used. To obtain some information about the specificity of the reaction, we synthesized individual oligosaccharides attached to sulforhodamine and tested them on wild type cells (supplemental Fig. S2). The intensity of the fluorescence clearly increased with the length of the oligosaccharide chain. Furthermore, the contribution of the cell cortex to the total fluorescence was higher in the longer oligosaccharides (supplemental Fig. S2). This effect may be partially due to a higher activity of Gas1p in catalyzing an exchange between the cell wall \( \beta(1\rightarrow3) \)-glucan and those oligosaccharides, because in the gas1\( \Delta \) mutant, although the general level of fluorescence was higher, there was little increase in the cell cortex with the hexa- and heptasaccharide (results not shown).
Permeabilized Cells as Containers for the Transglycosylase Reaction—The experiments with intact cells showed the feasibility of using SR-linked oligosaccharides as acceptors of chitin chains to mimic the in vivo transglycosylation. It also revealed the bud scars as sites of high transglycosylase activity. To move now toward an in vitro system, we made use of two of our previous observations. One is that when yeast cells are treated with digitonin and incubated briefly at 30 °C, the permeabilized (and inviable) cells acquire a very high chitin synthase activity (26), which can be measured by adding [14C]UDP-GlcNAc to the cells and determining incorporation of radioactivity into insoluble material. Thus, under these conditions, it appears that one or more of the usually zymogenic chitin synthases are activated.

The second observation was that nascent chitin is a much better substrate for chitinases than preformed chitin (35, 36). Our interpretation of this result was that the chitinase could act on nascent chitin before the chains could become cross-linked by hydrogen bonds and thus more impervious to enzymatic attack (35). On the basis of those two previous results, we decided to incubate digitonin-treated cells with both UDP-GlcNAc and SR-linked oligosaccharides. The idea was that the sugar nucleotide in the presence of the activated synthase would give rise to nascent chitin. Because the treated cells would be permeable to the oligosaccharides, they would be present at sites where the chitin was synthesized and, if the transglycosylases were also at the same location, a transfer reaction could occur. In other words, each permeabilized cell would act as an individual test tube for the transglycosylase reaction.

Initial results showed highly fluorescent cells, with little difference between experimental mixtures and controls. It was realized that the SR-linked oligosaccharides that permeated the cells could bind unspecifically to proteins and other substances and that it would be necessary to eliminate as much extraneous material as possible. Of the various procedures attempted to this end, the one that worked best was to first treat the cells with Z-protease (as for intact cells to be treated with chitinase, see above), followed by overnight extraction with 1% SDS. The partially emptied cells lost contrast under phase and showed a tendency to aggregate (Fig. 4A). However, cells incubated with UDP-GlcNAc and an oligosaccharide mixture still retained significant fluorescence after that treatment (Fig. 4A). When the nucleotide was omitted from the reaction mixture, most of the fluorescence disappeared, showing that chitin formation was necessary for oligosaccharide retention (Fig. 4B). Even dimmer were crh1Δ crh2Δ cells, although incubated with the complete system (Fig. 4C). Furthermore, overexpression of Crh2p in a wild type strain led to a clear increase in fluorescence (Fig. 4D). Again, most of the fluorescence disappeared when the nucleotide-sugar was omitted (Fig. 4E). As with intact cells, those with a gas1Δ deletion were very bright (Fig. 5C; compare with the corresponding wild type in Fig. 5, A and B). Here,
however, many bright patches persisted in the absence of UDP-GlcNAc (Fig. 5D), suggesting that at some locations (possibly bud scars) a limited reaction could occur with the utilization of preexisting chitin. On the other hand, deletion of CRH1 and CRH2 in a gas1Δ strain abolished the fluorescence almost completely (Fig. 5E and F).

For a better comparison among strains, it was desirable to obtain quantitative results for these experiments. To this end, cells from the reaction mixture were subjected to flow cytometry, and the percentage of cells that exceeded the fluorescence of a blank was measured (Figs. 4 and 5). Thus, the value for one of the wild types used, FY001, was about 37% (Fig. 4G), whereas in the corresponding control without UDP-GlcNAc, only 7% of the cells exceeded the blank limit (Fig. 4H). The double mutant crh1Δ crh2Δ behaved as the blank (Fig. 4I), whereas the SEC2R2 cells, overexpressing Crh2p, were substantially higher than wild type (Fig. 4J). Consistent with their bright fluorescence, almost all of the gas1Δ cells exceeded the blank range (Fig. 5I).

In addition, in this case two peaks were observed, suggesting two populations with different levels of fluorescence.

The controls, incubated without sugar nucleotide, gave much lower but still significant values, indicating that some reaction took place in the absence of chitin synthesis (Fig. 4H and K). The control value was much higher in the case of the gas1Δ control (Fig. 5J), in agreement with the presence of residual patches in the fluorescence picture (Fig. 5D).

The triple mutant gas1Δ crh1Δ crh2Δ graph shows some residual fluorescence, independent of the presence of nucleotide (Fig. 5, K and L). The source of this fluorescence is unknown.

When we found that digitonin-permeabilized cells showed high chitin synthase activity (26), it was not known that budding yeast contains three different chitin synthases (2). We used mutants in those chitin synthases, in addition to the corresponding wild type, to find out which enzyme(s) were responsible for the activity. Three of the four strain tested yielded high activity, whereas the chs1Δ mutant was totally inactive (Table 2). The conditions used, which were the same as for the incor-

| Strain          | Activity a |
|-----------------|------------|
| YPH499 (WT)     | 2132       |
| ECY46-1-8D (chs1Δ) | 30       |
| YMS11 (chs2Δ)   | 2311       |
| ECY46-4-1B (chs3Δ) | 4931      |
| Control (blank) | 38         |

a Counts/min incorporated in 30 min with [14C]UDP-GlcNAc as substrate.

**TABLE 2**

Chitin synthase activity in digitonin-permeabilized cells of different chs mutants

Activity was measured as described previously 23.
poration of SR-oligosaccharides, are optimal for Chs1p (37). When conditions for the measurement of Chs2p and Chs3p were tried, no activity was detected (results not shown). Accordingly, the chs1/H9004 mutant showed no incorporation of fluorescence (Fig. 6B). Surprisingly, the fluorescence of the chs2/H9004 strain and, even more, that of the chs3/H9004 strain, were much higher than that of wild type (Fig. 6, C–E). The high fluorescence of the chs3Δ mutant was totally eliminated by mutation of CRH1 and CRH2, as expected (Fig. 6, F–H). For the chs3Δ strain, the increased fluorescence may be due in part to a greater Chs1 activity (Table 2) and in part to enhancement of CRH1 expression (Fig. 6I). In the chs2 Δ strain there was no increase in chitin synthase activity, but again an augmented expression of CRH1 was detected. There was no increase in CRH2 expression in any of the mutants (results not shown). These results clearly show that Chs1p and not Chs3p, the physiologically active enzyme in vivo, is operating in permeabilized cells.

Enhanced Fluorescence of Glucanase and chs3 Mutants Allows Quantitation of the Transglycosylase Reaction—During this study, it was found, by the use of paper chromatography, that both intact and permeabilized cells degraded the SR-oligosaccharides (see supplemental Fig. S3 for an example in which the hexasaccharide was used as substrate during cell growth). The degradation occurred both in the wild type strains used and in the corresponding mutants. Because this degradation might affect the results by depleting substrate, we looked for strains with mutations in β(1–3)glucanase genes. Among different mutants, kindly provided by F. del Rey and C. Vázquez de Aldana, we chose strain LC355, which is defective in five glucanases (Exg1p, Exg2p, Eng1p, Eng2p, and Bgl2p). Both intact and permeabilized cells of LC355 did not degrade the oligosaccharides, even after prolonged incubation (supplemental Fig. S3 and results not shown). Cells of this strain grew well, although giving rise to small clumps, because in the absence of Eng1p the cells show a separation defect (38). Despite the lack of oligosaccharide degradation, intact or permeabilized cells of LC355 were not more fluorescent than those of FY001 after incubation with SR-oligosaccharides (see Fig. 7, top, for permeabilized cells). Based on our previous observation that the percentage of chitin bound to β(1–6)glucan increases at higher temperature, we tested different strains, including LC355, for fluorescence incorporation during growth at 38 °C rather than 30 °C, and we found only marginal increases (results not shown). However, when LC355 cells were grown at 38 °C, followed by permeabilization and incubation with SR-oligosaccharides, a dramatic increase in fluorescence was observed (Fig. 7). Note that omission of UDP-GlcNAc in the reaction mixture leads to almost total disappearance of the fluorescence (Fig. 7), in sharp contrast with the behavior of gas1Δ cells (Fig. 5D). Part of the increased fluorescence of the LC355 cells grown at 38 °C is because of the genetic background, because cells of the corresponding wild type (W303) also showed a similar, although less marked, effect (results not shown). Another wild type strain (FY001) only presented a small increase with growth temperature (results not shown).

The high fluorescence of the 38 °C-grown LC355 permeabilized cells suggested the possibility of measuring the extent of
the reaction as a function of time by fluorimetry. To this end, 10-fold scaled-up reaction mixtures were incubated for different times, followed by treatment with Z-protease and SDS, as outlined above. To enhance the fluorescence in this experiment, SR-hexasaccharide was used as substrate rather than an oligosaccharide mixture. The cells became brighter as the incubation time increased (Fig. 8, left). After chitinase treatment, almost all fluorescence disappeared, showing that in this case, as in intact cells, the oligosaccharides were bound to chitin (Fig. 8, upper right). The fluorescence of the chitinase digestion supernatants was measured in a spectrofluorimeter, yielding the curve of Fig. 8. It can be seen that the reaction proceeded linearly for the first 30 min and then became slower. However, the incorporation continued for at least 2 h.

Because of the high fluorescence of the chs3Δ mutant, the experiment was repeated with that strain. In this case, however, although the initial rate was similar to that of strain LC355, the time course of the incorporation flattened much earlier (results not shown). Part of this difference may be due to degradation of the substrate by the chitin synthase mutant (results not shown).

By the use of appropriate standards, it was possible to calculate the amount of SR-oligosaccharide incorporated. Surprisingly, this amounted to 19 pmol for the 2-h incubation mixture with LC355 cells, i.e. only 0.075% of the total amount of substrate added. The very small incorporation indicates that little substrate is required. This suggests that other factors, in addition to hydrolysis of the substrate, caused the flattening of the chs3Δ incorporation curve. In view of the small amount of substrate needed, it is understandable that in growth experiments the presence or absence of glucanases in the cells did not make much of a difference, because at the end of growth the ratio of cells to SR-oligosaccharides added initially was about 10 times lower than with permeabilized cells, and at early times much smaller.

Cell-free System: Incorporation of SR-oligosaccharide into Isolated Cell Walls—The results with the gas1Δ mutant permeabilized cells, where in the absence of UDP-GlcNAc fluorescent patches still arose (Fig. 5, C and D), suggested that in those cells two pathways for the formation of chitin-oligosaccharide complexes were functioning. One, giving rise mostly to peripheral fluorescence, utilized the nascent chitin formed during incubation. The other, possibly in bud scars, would take advantage of preformed chitin. We wondered whether this latter mechanism would still be functional in isolated cell walls. This was indeed the case. Incubation of cell walls, prepared either from intact or permeabilized cells of the gas1Δ strain, with SR-hexasaccharide but in absence of UDP-GlcNAc resulted in the appearance of bright patches on the cell walls (Fig. 9). In many cases, the patches could be identified as chains of bud scars (Fig. 9). In contrast, cell walls of the gas1Δ crh1Δ crh2Δ strain were uniformly dim (Fig. 9). On the other hand, incubation of cell wall supernatants, which included particulate material and soluble proteins, in the presence or absence of nucleotide-sugar, gave rise to little or no fluorescence, despite the presence of considerable chitin synthase activity in this fraction (results not shown).

To compare the cell wall results with those obtained with permeabilized cells, the time course of the reaction was determined in an experiment similar to that of Fig. 8. Here too, the fluorescence intensity of the patches increased with time and was totally abolished by chitinase treatment, again indicating that the oligosaccharide became linked to chitin (Fig. 10). The time course of incorporation, as determined by fluorimetry (Fig. 10A, solid circles), was not very different from that observed in permeabilized cells, except that it flattened out somewhat earlier.

Like intact and permeabilized cells, the gas1Δ cell walls degraded the oligosaccharide substrate (results not shown). To verify whether this degradation had an effect on the course of the reaction, we deleted GAS1 in the multiple glucanase mutant LC355 and repeated the experiments with cell walls of the resulting strain NBT001. Whereas the cell walls of LC355 showed only a few fluorescent spots, those of NBT001 acquired many bright patches (Fig. 9). Cell walls of either strain did not degrade the substrate (results not shown). However, the amount of fluorescent material incorporated and then released by chitinase for strain NBT001 was lower than in the case of strain Y00897 (Fig. 10A, open circles). This may have resulted from the different genetic background of LC355, which is the same as that of W303. Anyway, these data show that elimination of the glucanase activity does not result in a major change in the course of fluorescence incorporation.

An interesting corollary of the use of strain NBT001 came from the fact that its parent strain, W303, buds more or less randomly, because of a bud4 defect (39). Correspondingly, in contrast with strain LC355, an even distribution of fluorescent rings can be observed on the cell walls of NBT001, confirming nicely the nature of the patches as bud scars (Fig. 9). We also tested the effect of chitooligosaccharides on the cell wall reaction. Interestingly, these oligosaccharides were strong inhibitors of fluorescence incorporation, again implicating chitin as one of the substrates (Fig. 10B). We explored the pos-
sibility that the chitooligosaccharides might function as donors in this reaction, by subjecting reaction mixtures with the SR-heptasaccharide to thin layer chromatography and looking for fluorescent products of higher molecular weight. However, no such products were found (results not shown), suggesting that the oligosaccharides are too short to act as donors.

DISCUSSION

Faced with the impossibility of using chitin as a substrate, because of its insolubility, we initially left to the cell the task of providing the polysaccharide and supplied instead a possible artificial acceptor, in the form of SR-linked oligosaccharides. The fluorescence of these compounds was essential, not only to detect the products but also their localization. Thus, cells growing in the presence of the oligosaccharides accumulated fluorescence in the bud scars and, to a lesser extent, at the cell cortex (Figs. 2 and 3). The latter localization was expected (14), but the high fluorescence at the bud scars was a surprise. In a previous study of chitin cross-links at different locations in the cell, we found that chitin at the neck ring was preponderantly linked to β(1–3)glucan, whereas in lateral walls was mostly bound to β(1–6)glucan, and in the primary septum the chitin was free (14). The setup in those experiments did not include the bud scar, and we do not know of a way to find out what cross-links are made at the time of bud scar maturation. However, there is plenty of chitin in the bud scar, such as the chitin formerly at the neck ring, part of which was free at the time of ring formation, and the chitin we detected along the plasma membrane (Fig. 2E). Moreover, both Crh1p and Crh2p were previously detected at the bud scar, in addition to the cell cortex (15, 16). What the function could be of an enhanced cross-linking of chitin to β(1–6)glucan in the bud scar, is an open question. Because mannanproteins are attached to β(1–6)glucan (5), the chitin linkage may help to tether some protein to the bud scar.

We have previously shown that the deletion of CRH1 and CRH2 abolishes in vivo the binding of chitin to β(1–6)glucan, but it does not affect the chitin-β(1–3)glucan linkage. Thus, the Crh1p-Crh2p requirement for fluorescence incorporation in vivo and the solubilization of the fluorescent material by chitinase supported the view that the oligosaccharides were acting as β(1–6)glucan substitutes for a linkage to chitin and encouraged us to proceed to the next step. This was the use of digitonin-permeabilized cells as sources of chitin and enzymes, with again oligosaccharides as acceptors. Here, however, the system was much simplified, because the direct precursor of chitin, UDP-N-acetylglucosamine, was supplied externally and in its absence the extent of the reaction was dramatically decreased (Fig. 4, G, H, J, and K, and Fig. 5, G and H). It is interesting that the chitin synthase involved in the formation of polysaccharide here is Chs1p, not Chs3p, the physiological enzyme in vivo (Fig. 2B) (see also Ref. 14). This shows that we have set up here a truly artificial system. Because Chs1p is the enzyme accounting for most of the measurable chitin synthase activity (21), we believe that our old experiments on localization of the activity mainly dealt with this protein. We conclude that Chs1p is mostly attached to the plasma membrane, where it can catalyze a vectorial synthesis and extrusion of chitin through the membrane (8). Presumably, this is what occurs in permeabilized cells. To grab these nascent chitin chains and transfer them to β(1–6)glucan or oligosaccharides,
the Crh proteins must be placed nearby, either at the plasma membrane or at the cell wall. Although they have been detected only at the latter location (40), they must be attached to the plasma membrane through their glycosylphosphatidylinositol anchor at least transiently, before they are transferred to the wall. Thus, in permeabilized cells they may act at either location. All of this suggests that the local situation may be similar to that occurring in vivo, despite the use of a different chitin synthase. The free diffusion of the oligosaccharides, coupled to the even distribution of Chs1p on the membrane, explains the fairly uniform dispersion of fluorescence in the permeabilized cells. As in intact cells, here too the incorporation of fluorescence depends on the Crh proteins.

A very high incorporation of fluorescence was found in permeabilized cells of strain LC355, when they were previously grown at 38 °C. This high activity is probably because of several factors. One is that LC355 lacks several β(1–3)glucanases and does not degrade the oligosaccharides used as substrate. Second, at higher temperature the expression of Crh1p increases (15); the distribution of both Crh proteins becomes more diffuse (15); and in vivo, the proportion of chitin linked to β(1–6)glucan is higher (15). The migration of the Crh proteins from the bud scar to the cortex at 38 °C (15) may explain why little or no fluorescence was detected at the bud scar in the absence of UDP-GlcNAc (Fig. 7). Third, the effect of temperature seems to be higher in the genetic background of LC355, because the parent strain, W303, shows a similar, if somewhat less pronounced, behavior. The high fluorescence of the LC355 cells allowed us to follow quantitatively the course of the reaction, verifying at the same time that in this case too the fluorescent material was attached to chitin (Fig. 8). These results brought home the rather surprising result that less than 1% of the oligosaccharide was used in the reaction, showing that detection had been possible only thanks to the great sensitivity of the fluorescent label.

The permeabilized cells of a gas1Δ mutant differed from those of wild type strains in that even in the absence of UDP-GlcNAc fluorescent patches could be seen (Fig. 5, C, D, I, and J). Thus, it seemed that two processes were taking place here, one which used the nascent chitin formed inside the cell and another that was able to utilize preexisting chitin, possibly in bud scars. In the hope that this second reaction might still function in a cell-free system, we disrupted either the permeabilized cells or intact cells and isolated cell walls. In both cases, the cell walls, when incubated with an SR-oligosaccharide, acquired fluorescence in patches, which could be seen in many cases to correspond to bud scars (Fig. 9). That endogenous chitin is a participant in this reaction is borne out by the solubilization of the fluorescent material by chitinase as well as by the inhibition of fluorescence incorporation by chitooligosaccharides (Fig. 10). Finally, also in the cell wall system the Crh proteins were required (Fig. 9). It is not clear why in a preparation of cell walls from a gas1Δ mutant endogenous chitin of bud scars can be transferred to oligosaccharides, whereas with the corresponding wild type this reaction cannot be detected. Perhaps this result is related to the much higher chitin content of the mutant cell wall (34) and/or to the greater length of the chitin chains (15). Both reactions, the one occurring at bud

**FIGURE 9.** Fluorescence microscopy of cell wall preparations from different strains after 1 h of incubation at 37 °C with SR-hexasaccharide. LC355 is the strain with mutations in five β(1–3)glucanases; gas1Δ and gas1Δ crh1Δ crh2Δ are strains Y00897 and GR011, respectively, both in a BY4741 background, whereas NBT001 is similar to LC355 but with a gas1Δ deletion (see Table 1). Cell walls were treated for fluorescence microscopy as described under “Experimental Procedures.” The LC355 cells were grown at 38 °C, and those of the other strains were grown at 30 °C. The four small panels below the gas1Δ picture and below the NBT001 picture are magnified images of bright patches showing bud scar fluorescence. Scale bar in upper panel, 10 µm. Scale bar in small panel, 5 µm.
scars with endogenous chitin and that taking place at the cell cortex with nascent chitin, depend on CRH1 and CRH2, because their deletion abolished the fluorescence in both cases. Unless there are still fragments of membrane attached to the bud scar region of the cell wall, the results suggest that the transglycosylases acting here are attached to the cell wall, as previously reported (40). Therefore, we have here an in vitro system, consisting of components exterior to the plasma membrane, that can generate new chitin-glucan links. The inability of a cell wall-free fraction to generate fluorescence, despite the presence of chitin synthase activity, suggests that the structural organization of the system components must be maintained for the production of the peripheral fluorescence observed in intact permeabilized cells. If here too the Crh proteins are linked to the cell wall, a close juxtaposition of membrane and wall may be required.

All these results, together with those of a previous study (15), support the notion that the Crh proteins act as transglycosylases, transferring chitin chains to an acceptor, either oligosaccharides or, physiologically, β(1–6)glucan. Despite all the progress made, we were unable to achieve a simple system in which the reaction could be shown with a purified protein and appropriate substrates. In an attempt to attain that goal, we expressed Crh2p in Pichia pastoris and isolated the recombinant protein (results not shown). To obtain a water-soluble protein, the engineered Crh2p lacked the last 29 amino acids at the carboxyl terminus, where the glycosylphosphatidylinositol anchor is attached, whereas six histidine residues were attached to the same end to facilitate purification. Although the isolation of the protein was successful, we were unable to demonstrate any catalytic activity in many different systems. Either nascent chitin, synthesized in situ with a soluble chitin synthase preparation, or soluble derivatives of chitin, such as carboxymethyl-chitin or glycolchitin, were used as potential donors and oligosaccharides or β(1–6)glucans were the acceptors. Either radioactivity or fluorescence was employed to tag the substrates, but no reaction could be detected. Also, no chitinase activity of the recombinant protein was found, as measured by viscosimetry with carboxymethyl-chitin as substrate. The lack of activity of the recombinant Crh2p in these reconstructed systems might be explained by the need of some other proteins for the reaction to proceed; however, the recombinant Crh2p was also unable to rescue a cell wall preparation from the gas1Δ crh1Δ crh2Δ mutant, which should contain any such protein(s). Thus, it seems that either the missing amino acids were needed for activity or that the protein must be tethered to a solid support, as it is in vivo, to function. Still, we cannot exclude that some other protein is required, in addition to Crh1p and Crh2p, for the transglycosylation, although at least Crh2p seems to be equipped completely for the reaction, with both a chitin-binding domain and a catalytic site. Many of the events that occur in the periplasmic space remain unexplained, but it is now clear that transglycosylation is at least one of the mechanisms by which cell wall assembly takes place. Transglycosylation has been known for many years, but we believe this...
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is the first time that it has been shown to play an essential role in the linkage of two different polysaccharides to each other.

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