A family of covalently linked cell wall proteins of *Saccharomyces cerevisiae*, called Pir proteins, are characterized by up to 10 conserved repeating units. Ccw5/Pir4p contains only one complete repeating sequence and its deletion caused a release of the protein into the medium. The exchange of each of three glutamines (Gln\(^{69}\), Gln\(^{74}\), Gln\(^{170}\)) as well as one aspartic acid (Asp\(^{72}\)) within the repeating unit leads to a loss of the protein from the cell wall. Amino acid sequencing revealed that only Gln\(^{74}\) is modified. Release of the protein with mild alkali, changed Gln\(^{74}\) to glutamic acid, suggesting that Gln\(^{74}\) is involved in the linkage. Analysis by mass spectrometry showed that 5 hexoses are attached to Gln/Glu\(^{74}\). Sugar analysis revealed glucose as the only constituent. It is suggested that Pir proteins form novel, alkali labile ester linkages between the \(\gamma\)-carboxyl group of glutamic acids, arising from specific glutamines, with hydroxyl groups of glucans of \(\beta\)-1,3-glucan chains. This transglutaminase-type reaction could take place extracellularly and would energetically proceed on the account of amido group elimination.

Fungal cell walls are rigid albeit dynamic, complex structures, withstanding intracellular osmotic pressures of more than 10 bar (1). In *Saccharomyces cerevisiae* the cell wall amounts to about 20% of the total dry weight of the cell. Besides a small amount of chitin, it mainly consists of \(\beta\)-glucans and mannoproteins. The actual protein content comes to about 10% of the wall by weight (2). Almost all the proteins are highly O- and N-mannosylated (2–5). Cell wall studies in the past ten to fifteen years have focused on the various cell wall proteins, since the information concerning their function is expected to help understand the biogenesis as well as the dynamic rebuilding and dissolving of this highly intricate, extracellular organelle during budding and mating.

By now, more than 30 cell wall proteins have been identified (6–9). Some of them are solubilized from the cell wall by SDS under reducing conditions and are therefore called soluble cell wall proteins (10); however, most of them are covalently attached to the glucan layer and can be released from SDS-extracted cell walls by \(\beta\)-glucanases (11, 12). Two modes of covalent linkage can be distinguished. In the first case the cell wall protein is synthesized as GPI (glycosylphosphatidylinositol) modified intermediate. These are trans-mannosylated by hydrolyzing the oligomannosidic moiety of the GPI lipid anchor and by a subsequent transfer to \(\beta\)-1,6-glucan (12–16). This group of covalently attached cell wall proteins, called GPI-Cwps, are released from the wall both by \(\beta\)-1,6- and by \(\beta\)-1,3-glucanases, due to the attachment of GPI-Cwps to \(\beta\)-1,3-glucan via a short \(\beta\)-1,6-glucan bridge (4, 12). The second group of covalently linked cell wall proteins are the so-called Pir proteins, named originally according to three genes coding for putative proteins with internal repeats (Pir) (17) and subsequently identified as covalently linked cell wall proteins Ccw1, -2, -3, and -4p (8, 18, 19). For *S. cerevisiae* five PIR genes are known (Table I) coding for proteins with varying numbers of repetitive units (1 to 10). Pir proteins do not contain a C-terminal GPI-addition signal, they are all processed by Kex2 protease, and they are released from intact cells by very mild alkaline treatment (30 mM NaOH, 12 h, 4 °C) (8). Pir proteins are attached directly to \(\beta\)-1,3-glucan (20) and can therefore be released from *S. cerevisiae* by the corresponding \(\beta\)-1,3-glucanase, not however by \(\beta\)-1,6-glucanase (4).

The actual alkali labile linkage between the protein moiety and \(\beta\)-1,3-glucan is not known.

Pir proteins known to be involved in various biological phenomena are not essential for viability; a quadruple mutant, however, possesses a severe growth phenotype (21). Pir2/Ccw7/Hsp150p is strongly induced by heat stress and nitrogen starvation (22, 23). Pir proteins 1 through 3 have been shown to cause resistance to an antifungal protein of tobacco (18). Special functional importance of Pir proteins has also to be deduced from the fact that *PIR*1–3 are the most highly regulated genes in the cell cycle; the expression score of *PIR1* amounts to 16 with the activity peaking in M/G1, i.e. during cell separation (24).

In this report we characterize the linkage between Ccw5/Pir4p and \(\beta\)-1,3-glucan. Evidence will be presented that the amido group of a specific glutamine residue within the repetitive sequence is deamidated and a carboxyl ester with \(\beta\)-1,3-linked glucose is formed.

**EXPERIMENTAL PROCEDURES**

**Yeast Strains and Genetic Methods**—The following isogenic yeast strains were used: SEY6210 (MATa ura3–52 leu2–3,112 his3–Δ200 trp1–Δ901 lys2–801 suc2–Δ9), VMA5 (MATa ura3–52 leu2–3,112 his3–Δ200 trp1–Δ901 lys2–801 suc2–Δ9 ccw5Δ). Cells were grown at 30 °C in standard yeast media either YEPD (1% Bacto yeast extract, 2% Bacto peptone, 2% dextrose) or YNBD (0.67% yeast nitrogen base, 0.08% complete amino acid supplement mixture (from Bio 101, Inc.), 2% dextrose). Transformation into yeast and *Escherichia coli* was carried out using standard techniques.

**Plasmid Constructions**—Standard molecular biology techniques were used for all plasmid constructions. The correct sequence of PCR-amplified products was verified by DNA sequencing. The sequences of primers used in this study are available on request.

CCWS was amplified from yeast genomic DNA. The amplified gene was inserted into the Smal site of vector YEp351. To introduce a hemag-
Pir Proteins of Yeast Cell Walls

**FIGURE 1. Sequence of the Pir4/Ccw5/Cis3 protein.** The typical repetitive sequence of Pir proteins is boxed; a second pseudo-repetitive sequence present is shown within the dashed box. The arrow indicates the Kex2 cleavage site.

glutamin (HA) epitope at the C terminus of CCWS a NotI site was first created, and the fragment was inserted into the Sall restriction site at the very end of CCWS. Then, six HA epitopes were inserted into the NotI site in frame with CCWS and verified by Western blot analysis.

CCWS—plasmids containing N-terminal deletions, pCCWS-(Δ65–82)-HA, and pCCWS-(Δ83–95)-HA, respectively (see Fig. 2) were generated by two-step PCR technology.

Site-directed mutagenesis to exchange specific amino acids in pCCWS-HA was performed by the two-step PCR method to give pCCWS-(Q69A)-HA, pCCWS-(D72N)-HA, pCCWS-(Q74A)-HA, and pCCWS-(Q76A)-HA, respectively. The correct sequences have been confirmed by sequencing. Plasmid pCCWS-ZZ contains two C-terminal protein A epitopes in frame with CCWS inserted into the vector pRS425. In plasmid pCCWS-(S79M)-ZZ amino acid Ser^{19} has been replaced by Met as described above for the exchanges in the plasmid pCCWS/HA.

**Isolation and Purification of Pir4/Ccw5-ZZ—SEY6210, harboring pCCWS-ZZ, was grown in YNB without leucine to an A_{600} of 3. Cells (A_{600} 2000) were harvested and washed in 50 ml 0.1 M potassium phosphate buffer (pH 8), resuspended in 5 ml of ice-cold 0.1 M potassium phosphate buffer, containing the protease inhibitors N^{+}-p-tosyl-l-lysine chloromethyl ketone (0.1 mg/ml), l-tosylamido-2-phenylethyl chloromethyl ketone (0.05 mg/ml), benzamidine (1 mM), phenylmethanesulfonyl fluoride (1 mM), leupeptin, pepstatin, and antipain, each 1 μg/ml. The cells were homogenized twice for 25 s with intermittent cooling in a FastPrep Instrument (Qbiogene) in portions of 0.5 ml by glass beads. Cell walls were isolated by centrifugation at 3000 x g for 5 min. The cell wall pellets were washed twice with 20 ml of potassium phosphate buffer. Cell walls were boiled twice for 10 min at 100 °C in 20 ml of SDS Laemmli sample buffer, centrifuged at 3000 x g, washed with 20 ml of water and with 50 mM Tris-HCl (pH 7.5), and lyophilized. Extracted cell walls were resuspended in 2.5 ml of 50 mM Tris-HCl (pH 7.5), supplemented with protease inhibitors (see above), and incubated with 600 units of Quetzyme for 3 h at 37 °C. Then an additional 300 units were added and incubated for another 2 h. The homogenate was centrifuged at 21,000 x g for 20 min. The clear supernatant was removed and adjusted to 75 mM NaCl, supplemented with protease inhibitors (see above), and added to 0.5 ml of IgG-Sepharose 6 Fast Flow (Amersham Biosciences) prepared according to the manufacturer and equilibrated in TBST (0.5 M Tris-HCl (pH 7.4), containing 0.15 M NaCl and 0.05% Tween 20). The slurry was incubated by gentle rotation for 4 h at 4 °C and then loaded into a column and washed three times with 5 column volumes TBST and two times with 5 mM NH_{4}-acetate (pH 5). Ccw5p was eluted with 0.5 M NH_{4}-acetate (pH 3.4) and lyophilized from NH_{4}-acetate. For isolation of Ccw5p from the medium, this was lyophilized and extensively dialyzed against water, containing protease inhibitors, and subsequently subjected to IgG-Sepharose affinity chromatography, as described above.

**Analysis of Ccw5p-bound Carbohydrates—**Ccw5-ZZp isolated from cell wall and medium, respectively, and purified by affinity chromatography was treated overnight with 0.2 ml of 30 mM NaOH at 4 °C. The material was lyophilized, dissolved in water, centrifuged through a Millipore Ultraframe-MC cartridge, and separated by high performance anion exchange chromatography with amperometric detection (HPAEC-PAD) on a Dionex DX 500/ED40 system using a CarboPac PA1 column and a linear gradient from 16 mM NaOH to 500 mM sodium acetate in 150 mM NaOH within 45 min. For preparative purpose several runs were performed, and the respective fractions 1, 2, and 3 (see Fig. 8) were combined. The material was then desalted on Dowex 50W × 8 (H^{+}), lyophilized, and used for further analysis. As reference standards for β-1,3-linked gluco-oligosaccharides served laminariboise, triose, tetrose, and pentaose (Sigma) and for β-1,6-linked oligo-glucoses gentiobiose and pustulan (Calbiochem) partially hydrolyzed by 1 M trifluoroacetic acid for 1 h.

To determine the sugar composition of the factions 1, 2, and 3, glycans were hydrolyzed with 4 M trifluoroacetic acid and 4 N HCl, respectively, for 4 h at 100 °C, dried under nitrogen, dissolved in water, and analyzed by HPAEC on a CarboPac PA1-column with 16 mM NaOH as eluent.

**Isolation and Structural Analysis of CNBr Peptides—**CNBr digestion was performed overnight at 4 °C in 70% formic acid containing 5 mg CNBr/ml. The bulk of the solvent was evaporated under a stream of nitrogen, the oily residue was diluted with water and lyophilized. The peptides were separated by reverse phase HPLC using a Ydaci 214 RP18 column (250 x 2 mm) and identified by Edman degradation of collected peaks.

Sequencing was done using a Procise 492A sequencer (Applied Biosystems) with on-line detection of the PTH-amino acids according to the manufacturer’s instructions. Linear mode positive-ion MALDI spectra were recorded with a Ultraflex TOF/TOF (Bruker Daltonics) using 2,5-dihydroxybenzoic acid as a matrix.

**RESULTS**

**Identification of the Amino Acid of the Pir4/Ccw5 Protein That Is Linked to β-1,3-Glucan—**To study a possible role of the repetitive sequences of Pir proteins, we concentrated on the Pir4p/Ccw5p/Cis3p. This protein has only one repetitive sequence of the type present in all Pir proteins, whereas in a second, related sequence, next to the first one (Fig. 1), 5 of the 12 conserved amino acids are exchanged. As reported previously (25, 26), a deletion of the repetitive sequence (amino acids 65–82) resulted in the release of Pir4p to the medium. In Fig. 2 evidence is presented that HA-tagged Pir4 requires the conserved repetitive sequence, to get attached to the cell wall in a way withstanding extraction with hot SDS under reducing conditions. The following pseudo-repetitive sequence (amino acids 83–95) is neither sufficient nor necessary for covalent cell wall attachment. As seen in lane 2, the size of the Δ65–82 peptide is not reduced, which is due to the fact that the Kex2 cleavage site right next to the deletion is not used in this construct, as confirmed by N-terminal sequencing.

Pir4p is found to a considerable proportion also in the medium, which in part is due to the overexpression of the tagged protein. In addition, a certain amount of Pir4 remains non-covalently associated with the cell wall.
The typical repetitive sequence of Pir proteins is required for the covalent linkage between Pir4p and the cell wall. The various HA-tagged proteins constructed are shown (left side); only the portion C-terminal to the Kex2 cleavage site is shown. The black box indicates the canonical repetitive Pir sequence; the striped box represents the related Pir sequence. On the right side an immunoblot of mild alkali released material from SDS-treated cell walls and of material present in the growth medium after separation on SDS gels is shown (for details see "Experimental Procedures").

![Diagram of the typical repetitive sequence of Pir proteins](image1)

The typical repetitive sequence of Pir proteins is required for the covalent linkage between Pir4p and the cell wall. The various HA-tagged proteins constructed are shown (left side); only the portion C-terminal to the Kex2 cleavage site is shown. The black box indicates the canonical repetitive Pir sequence; the striped box represents the related Pir sequence. On the right side an immunoblot of mild alkali released material from SDS-treated cell walls and of material present in the growth medium after separation on SDS gels is shown (for details see "Experimental Procedures").

![Immunoblot showing Pir4p binding](image2)

The alkaline lability of the linkage of Pir proteins suggested that they may be attached to sugars via a hydroxy amino acid. However, when the serine and threonine residues in the repetitive sequence, as well as the neighboring ones (Fig. 3) were replaced by alanine, the attachment of Pir4p to the cell wall was not affected (data not shown). On the other hand, each individual exchange of the three glutamine residues within the repetitive sequence as well as aspartate 72 with alanine and asparagine, respectively, gave rise to a complete loss of cell wall-bound Pir4p (Fig. 3). The D65N exchange of an aspartate residue outside of the repetitive sequence had no effect. This result indicated that, although all 4 amino acids are necessary for the protein to get cell wall-bound, at most one could form the actual link.

Helpful information concerning the amino acid involved in the respective linkage was obtained by N-terminally sequencing Pir4p, which was released from cell walls by either β-1,3-glucanase or mild alkali treatment. As shown in Fig. 4, sequencing the protein released by glucanase, revealed that the expected PTH-amino acid for Gln in position 74 was missing, indicating the presence of a modified amino acid not detected by the standard analytical procedure. Surprisingly, however, in the protein released by alkali, there was a glutamic acid at position 74. This suggests that Gln74 is indeed the linking amino acid and that it is deamidated during the attachment to β-1,3-glucan. In accordance with this interpretation is the fact that Pir4p secreted to the medium does contain a glutamine at that position (Fig. 4).

A well known enzymatic reaction using protein-bound glutamine as substrate is the transglutaminase reaction (27). The responsible enzyme activates the ω-carboxamide group of the glutamine, which then reacts with primary amines, typically an ε-amino group of protein-bound lysines, thus cross-linking and polymerizing proteins in this way (28).

![Hypothetical reaction schemes](image3)

**FIGURE 3.** The typical repetitive sequence of Pir proteins is required for the covalent linkage between Pir4p and the cell wall. The various HA-tagged proteins constructed are shown (left side); only the portion C-terminal to the Kex2 cleavage site is shown. The black box indicates the canonical repetitive Pir sequence; the striped box represents the related Pir sequence. On the right side an immunoblot of mild alkali released material from SDS-treated cell walls and of material present in the growth medium after separation on SDS gels is shown (for details see "Experimental Procedures").

**FIGURE 4.** The N-terminal sequence of the Pir4 protein. Pir4/Ccw5 protein from wild-type cells was released from the cell wall by β-1,3-glucanase or by mild alkali. These proteins as well as the protein from the medium were purified (see "Experimental Procedures") and sequenced by Edman degradation.

**FIGURE 5.** Hypothetical reaction schemes. A, reaction scheme for a transglutaminase-type reaction attaching Pir4p to the cell wall. B, reaction scheme for an ester formation between a sugar hydroxyl and the ω-carboxyl of a glutamate arising from a glutamine by deamidation.
way of linking a protein-bound glutamine to β-1,3-glucan via the release of the amido group is suggested in Fig. 5B. The bond formed in this case would be an ester between the γ-carboxyl group set free from glutamine and an OH group of a glucosyl residue within the β-1,3-glucan chains. Energetically this bond would be formed on the account of the deamination and such an ester would definitely be alkali-labile.

Identification of the Oligosaccharide Bound to Gln/Glu⁷⁴ of the Pir⁴/Ccw⁵ Protein—To analyze the modification of the amino acid at position 74 of Pir⁴p, we used a C-terminal protein A-tagged variant, in which Ser⁷⁹ was replaced with a methionine residue by site directed mutagenesis (Fig. 6). This allowed the generation of peptide B with a C-terminal homoserine by treatment of the mutated Pir⁴ protein by cyanogen bromide (Fig. 6). Subsequent Edman degradation of the purified peptide and HPLC separation of the released PTH-amino acids gave no signals for Ser⁶⁸, Thr⁷⁸, and Gln/Glu⁷⁴ indicating that these amino acids are modified and escape detection under the separation conditions for standard amino acids. With our earlier finding that all hydroxyl amino acids of Pir⁴p are O-mannosylated (29), we can conclude that Ser⁶⁸ and Thr⁷⁸ are mannosylated, but the modification of Gln⁷⁴ remained obscure.

To further investigate the identity of this modification, an aliquot of peptide B was treated with mild alkali, resulting in formation of peptide A (Fig. 6). Residues Ser⁶⁸ and Thr⁷⁸ were still modified, but in position 74 a glutamic acid showed up, in agreement with the results obtained for the intact protein, as described above. Obviously, this treatment was

| Peptide (with E⁷⁴) | Theoretical average mass (M + H⁺) |
|--------------------|----------------------------------|
|                    | C-terminal homoserine | C-terminal homoserine lactone |
| unmodified         | 1532.7                | 1514.7                      |
| modified at S⁶⁸ and T⁷⁸ with 7 mannose residues | 2666.7                | 2648.7                      |
| additionally modified at E/Q⁷⁴ with 5 hexose residues | 3476.7                | 3458.7                      |

FIGURE 6. The two postulated variant peptides obtained after cyanogen bromide splitting of Pir⁴p followed by mild alkali treatment (A) or without treatment (B). X in the peptide sequences corresponds to hydroxy-amino acids modified with O-linked oligomannose residues; the bold X corresponds to the unknown modification of Gln/Glu⁷⁴; Hs means homoserine lactone.

FIGURE 7. Determination of the molecular mass of the moiety bound to Glu/Gln⁷⁴ of Pir⁴p. A, linear mode positive ion MALDI spectra of peptide B (not treated with alkali). The peak at m/z = 1749 was always present in our samples and seems to be nonspecific. B, assignment of mass spectrometry data to different modifications of Ser⁶⁸, Thr⁷⁸, and Glu/Gln⁷⁴.
sufficient to remove the modification of Gln\textsuperscript{74} but not sufficient to remove the mannose residues from Ser\textsuperscript{68} and Thr\textsuperscript{78}. Using mass spectrometry we obtained information about the size of the modifying groups. Linear mode positive ion MALDI mass spectrometry measurement of peptide B showed a prominent signal with a $m/z$ (mass/charge) value of 3458.8 and weaker one with $m/z = 2665.3$ (Fig. 7A), with a mass accuracy being better than 1 dalton. As outlined in Fig. 7B, these $m/z$ values correspond exactly to the molecular mass of singly charged, protonated peptides with homoserine lactone at the C-terminal end carrying 7 and 12 hexoses, respectively. In addition the signals at 2665.3 and 3458.8 show satellite peaks with masses higher by 18 mass units, which most probably correspond to the same molecules having a C-terminal homoserine instead of homoserine lactone, formed by opening of the lactone ring. After mild alkali treatment the peak at 3458.8 and its satellite completely disappeared and only the smaller mass remained (data not shown). Thus, together with the data obtained by Edman degradation, this is good evidence that Ser\textsuperscript{68} and Thr\textsuperscript{78} are substituted by a total of 7 mannose residues, and additional 5 hexoses are attached to the amino acid in position 74. Since the accuracy of the mass spectrometry analysis was better than 1 dalton, our data are consistent with attachment of the 5 hexoses to Glu and not Gln. Unfortunately, further structural analysis by MALDI-TOF/TOF was not possible, because due to decomposition during the time of flight the peptides could only be measured in linear mode and not in the reflector mode (data not shown).

To further analyze the oligosaccharide attached to Gln/Glu\textsuperscript{74}, we purified Pir\textsubscript{4}-ZZp after releasing it from the SDS-extracted cell walls by \textbeta\textsubscript{-1,3-glucanase}. As a control the protein was also purified from the medium. Both samples were treated with mild alkali, and the material released was separated by HPAEC. As can be seen from the elution profile shown in Fig. 8, the cell wall-bound material gave rise to three peaks, which were absent from protein purified from the medium. The three peaks coincided with laminaritriose, -tetraose, and -pentaose and clearly differed from the \textbeta\textsubscript{-1,6-oligomer standards (Fig. 8). When the three peaks were hydrolyzed, only glucose and a minute amount of mannose; however, no glucosamine has been detected; the latter should amount to at least 20% in the case of the pentasaccharide and to 33% in the case of the trisaccharide. It has to be concluded, therefore, that Pir\textsubscript{4}/Ccw\textsubscript{5p} is directly linked to \textbeta\textsubscript{-1,3-glucan. A laminaripentaose attached to the protein obviously cannot be further split by \textbeta\textsubscript{-1,3-glucanase. The treatment with 30 mM NaOH presumably caused some peeling off of glucoses, resulting in the smaller oligosaccharides. For mass spectrometry analyses the material was treated with 5% NH\textsubscript{4}OH, conditions that obviously did not cause pentasaccharide degradation.

**Lability of the Pir\textsubscript{4p}-Glucan Bond as Compared with a \textgamma-Isoglutamyl-Peptide Bond**—To release the covalently bound Pir\textsubscript{4}/Ccw\textsubscript{5p} protein from yeast cell walls, we normally used an overnight treatment with 30 mM NaOH at 4 °C. However, as shown in Fig. 9 the linkage is considerably more labile. Even at 10 mM NaOH more than half of the protein is released from the cell wall in 14 h at 4 °C, and at 30 mM NaOH 7 h are...
sufficient for complete release. A peptide linkage with the involvement of the γ-carboxyl group of glutamic acid, the type of linkage that would form in a classical transglutamination reaction, is known to be more easily hydrolyzed than a regular peptide linkage. To compare the lability of the Pir4-cell wall link with that of an isoglutamyl bond, we tested the alkali lability of isoglutaminyl-Ala-OH. At 30 mM NaOH even after 20 h of the Pir4-cell wall link with that of an isoglutamyl bond, we tested the easily hydrolyzed than a regular peptide linkage. To compare the lability with its reducing end may also be covalently bound to remained unknown. Furthermore, evidence was presented that chitin network, as for instance the peptidoglycan sacculus of bacteria. The This polymer does not, however, represent a covalently cross-linked formations have still to be uncovered.

**DISCUSSION**

The analysis reported herein shows that the Ccw5/Pir4 protein is attached to the cell wall via glutamine residue 74 within the repetitive sequence QIGDGQ/VQ. This motif is conserved in all Pir proteins. The data also demonstrate that the linkage at Gln74 is extremely alkali labile and that after the hydrolytic splitting, the glutamine has been transformed to a glutamic acid. This behavior points to the existence of a transglutaminase-type reaction. Since neither an amino sugar nor a primary amine containing molecule was detected as part of the β-1,3-glucan attached to the Pir4 protein, and since no potential gene encoding a transglutaminase can be identified in the S. cerevisiae genome, it is postulated that an ester linkage between the γ-carboxyl of a glutamate residue, arising from glutamine, and the sugar hydroxyl is formed. Such an ester would be expected to be extremely alkali labile.

Whereas the overall composition of S. cerevisiae cell wall is well known since quite some time, less is known about the problem how the various components are chemically linked and interconnected. Likewise, hardly any of the enzymes responsible for the corresponding reactions have been identified. About 50% of the chitin is β-1,4-linked to β-1,3-glucan chains, for which a periplasmic transglycosylation reaction has been postulated (30). An extensive molecular analysis of a yeast cell wall complex has been carried out in Cabib’s laboratory (13). From this and from previous work of the Klis’ laboratory and others (16), it was concluded that the GPI proteins are linked to β-1,6-glucan via one or several mannose residues of the GPI anchor; the linkage type remained unknown. Furthermore, evidence was presented that chitin with its reducing end may also be covalently bound to β-1,6-glucan. As a consequence cell wall proteins of the GPI type may be immobilized in the cell wall either due to their connection to β-1,3-glucan or to chitin and in either case a short β-1,6-glucan chain will constitute a bridging entity. The various transglycosidases required for these covalent bond formations have still to be uncovered.

The different linkages between cell wall components, described so far, give rise to a very complex and highly branched macromolecule. This polymer does not, however, represent a covalently cross-linked network, as for instance the peptidoglycan sacculus of bacteria. The bacterial covalently cross-linked macromolecule explains the resistance to high internal pressure. Truly cross-linked macromolecules could arise in the yeast cell wall, for example, if cell wall proteins formed intermolecular disulfide linkages. That these principle exist in fungal cell walls is well established, but there is no evidence for any extended protein network. The Pir proteins with up two 10 repetitive sequence domains (see Table 1) could be responsible for a different cross-linking principle. Since the repetitive unit forms a link to β-1,3-glucan as shown in the present paper, and since we can assume that this is the case for each repetitive unit, the Pir proteins could cross-link up to 10 β-1,3-glucan chains. The yeast strain deleted in four Pir genes does show a considerable increase in size as well as a changed morphology (21). They also show a pronounced susceptibility to cell wall synthesis inhibitors. On the other hand, assuming that Pir proteins play an important role in cell wall stability, their deletion would be expected to result in a lethal phenotype. According to the gene expression analysis (24), the synthesis of several Pir proteins increases massively during cytokinesis. This might indicate that the Pir proteins are especially required to stabilize both the newly synthesized walls separating mother and daughter cell; evidence that Pir1p and Pir2p are localized at the bud scars has indeed been presented (31).

A major open question is how the formation of the Pir-protein/β-1,3-glucan link is brought about. Which enzyme catalyzes the postulated peptide-ester transformation? From an energetic point of view, such an extra-cytoplasmic reaction would not create a problem, since the free energy of amide hydrolysis would be sufficient for the formation of the ester linkage. The reaction type being new does not allow, however, a guess concerning the potential gene and gene product involved. A daring speculation would be that the repetitive sequences of the Pir proteins themselves catalyze the formation of their attachment to the β-1,3-glucan. This certainly could explain the essential role of 2 glutamine residues and an aspartate one in the immediate neighborhood of the Gln74, to which the glucan gets attached. The possibility of such a mechanism is under investigation.

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