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review

Incorporation of Homologous and Heterologous Proteins in the *Saccharomyces cerevisiae* Cell Wall

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Summary

The yeast cell wall is a structure of pronounced complexity and principally consists of two structurally and functionally different moieties. The inner layer is predominantly built of glucan and it provides mechanical stability to the cell. The outer layer is composed of mannoproteins with their mannan chains shielding the cell against the surroundings. Mannoproteins play different roles including a number of enzymatic activities required for maintenance and remodelling of the wall, as well as proteins, whose role is interactions with the surrounding molecules or cells in the processes like mating or flocculation. The connection between the two layers is crucial for the cell wall architecture and physiology, and it is achieved by several types of linkages between glucan and protein parts of external glycoproteins. Some proteins are noncovalently adsorbed to β-1,3-glucan, some are covalently linked through the remnants of glycosylphosphatidylinositol anchors and β-1,6-glucan, some are attached covalently through alkali-sensitive ester linkages occurring between protein glutamines and glucan, and several proteins are attached also covalently but through a so far unexplained linkage.

Understanding of how yeast incorporates proteins in the cell wall can be used for biotechnological purposes to direct and immobilize heterologous proteins at the cell surface. In this way tedious chemical immobilization reactions which often result in a partial loss of biological activity or properties of the immobilized proteins can be by-passed and the yeast cell itself can both serve as an insoluble matrix and perform the immobilization of the protein of interest.

In this paper the present knowledge on the mechanisms for incorporation of both homologous and heterologous proteins in the model yeast *Saccharomyces cerevisiae* is reviewed with a survey of proteins so far found attached at the yeast cell surface.

Key words: yeast, cell wall, glucan, mannoproteins, protein incorporation

Introduction

The yeast cell wall is characterised by outstanding firmness and rigidity withstanding high osmotic pressures (*1*) but at the same time, it possesses flexibility needed for dynamic changes during cell growth, mating, sporulation, *etc.* (*2*). Such physiological requirements brought about evolution of a complex structure composed of β-glucans and mannoproteins with a small but essential amount of chitin. So far, more than 30 different mannoproteins have been identified in the wall (*3–5*) and about twice as many were postulated as a result of the *S. cerevisiae* genome screening (*6*). They can be divided into three groups according to the way they are linked to glucan. Some proteins are attached to the glucan network noncovalently (*3,4,7*) and can be released by hot SDS and β-mercaptoethanol. The second group comprises proteins glycosylphosphatidylinositol (GPI)

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linked to β-1,6-glucan/β-1,3-glucan moieties (*8–10*). They are usually extracted from the wall by either β-1,6- or by β-1,3-glucanases although restricted proteolysis has also been reported (*11*). Finally, a family of sequence-related proteins called Pir proteins (Proteins with internal repeats) are attached to β-1,3-glucan by a newly described type of alkali-labile ester linkage between the γ-carboxyl group of the specific glutamic acid residue within the protein repetitive sequence and the hydroxyl group of glucose (*12*). While the role of β-1,3-glucan and chitin is clearly shown to be mechanical stabilisation of the cell, the function of mannoproteins is to a large extent unclear. Several mannoproteins have been demonstrated to mediate cellular interactions during mating and flocculation, but most others can be depleted from the cell wall without any significant damage to the cell observed.

Yeast Cell Wall Architecture

Fungal cell walls have distinct layered structure demonstrated by both differential accessibility of different components to outer factors, and by electron microscopy. The inner layer of the cell wall is composed of β-1,3--glucan, a branched polymer with an average number of about 1500 glucoses. It forms elastic but strong helical structures capped by the addition of short chitin chains to the non-reducing ends of glucan (*2,13*). It is essential to the cell, thus any damage of this moiety leads to the loss of cell integrity. To some extent the damage can be repaired by the induction of the cell integrity pathway, a signal transduction chain involving MAP kinases, which begins with the mechanical sensory wall proteins Wsc1p and Mid1p and ends with an increased biosynthesis and deposition of wall chitin (*14*). If the damage of the inner layer of the wall is too extensive or if it occurs too fast, the cell will lose the capability to withstand turgor. The biosynthesis of carbohydrate components of the yeast cell wall has been extensively reviewed (*15*).

The outer layer of the yeast cell wall is composed of mannoproteins, most of which are both O- and N-glycosylated. Large N-linked glycosidic chains seem to be covering the cell, thus any molecule or cell approaching the yeast would first come in contact with mannan. A still puzzling fact about this structure is its pronounced complexity involving an unusually high number of different proteins required for its biosynthesis and a substantial amount of energy spent for this process.

Although structural homologies often characterise families of cell wall proteins, their variability still implicates many different functions, many of which are still unknown. Moreover, not only that there is a number of proteins building the mannoprotein wall layer, but there are at least three and most probably four different ways by which they are attached to β-1,3-glucan. Principally, some of the proteins are simply adsorbed to β-1,3-glucan chains, while the others are covalently linked, either through GPI anchors and β-1,6-glucan, or through an ester linkage between glutamate and glucose. Why there are more attachment mechanisms and how they are related to the function(s) of the proteins is still not explained mostly because of the lack of general understanding of the physiological roles of many of them.

Analysis of chemical properties of different layers showed that β-1,3-glucan was generally quite reactive at acidic pH and adsorbed *in vitro* many different proteins and also some polysaccharides including chitin and laminarin, forming predominantly hydrogen bonds (*16,17*). The latter interactions could have significance in the fortification of the fungal cell walls by glucan-glucan interactions. Mannan did not share the same chemical properties, indicating that the two sides of the wall have different chemical properties, the inner side attracting the proteins secreted from the cell and the outer side being chemically quite inert, except for ionic bond formed by the phosphate groups of phosphomannan, which are only partially protonated at acidic pH of the medium.

Noncovalent Attachment of Cell Wall Proteins

A number of yeast cell wall proteins were extracted from different wall preparations by SDS, mercaptoethanol, dithiothreitol, or a combination of these reagents, often at increased temperature (*3,4,7*). Therefore, these proteins seem to be noncovalently attached to glucans. It should be noted that although the extraction using reducing reagents implicates a linkage of proteins through disulphide bonds, the patterns of proteins obtained by dithiothreitol and SDS were very similar (*3*). In fact Aga2p, the active subunit of the a-agglutinin complex is the only protein reported in the *S. cerevisiae* cell wall that is attached to another wall constituent (Aga1p) by a disulphide bond (*3*). Thus the reason for extraction of other noncovalently bound proteins by thiol reagents must rely on some general changes in the wall structure, or extractive properties of the reagents, rather than on the direct reduction of the bonds. Structural analysis of extracted proteins revealed no similarities as potential signals defining their final location, which led to a generally accepted assumption that they were adsorbed to β-1,3-glucan by non-specific interactions. This was corroborated by a finding that β-1,3-glucan indeed had a strong hydrogen-bonding potential (*16*). Most of the noncovalently attached cell wall proteins have been found also in the culture medium, some even in a larger amount than in the cell wall, but the present knowledge on the physiological roles of these proteins does not allow a prediction if their activity is required in the cell wall or in the cell surroundings.

Although the physiological importance of most of the individual noncovalently attached wall proteins is not completely clear, it seems that most so far described proteins share significant primary structure homologies to glucan hydrolases or transglycosidases, which led to the assumption that they have roles in building, maintaining or remodelling of the wall glucan during growth, mating, sporulation and other cellular events involving changes in the cell wall. The first protein isolated from the cell wall whose primary structure has been elucidated was Bgl2p (*18*), a protein reported to possess either an endoglucanase (*19*), or a transglucosidase activity (*20*) in an *in vitro* assay, depending on the substrate concentration. Recently self-aggregation of Bgl2p in an amyloid fashion has been reported, but the physiological significance of this property is unclear (*21*). The protein shares significant sequence homology with three other nonco-

valently attached cell wall proteins, Scw4p, Scw10p and Scw11p (*4*), which are thus also postulated to be glucan--remodelling enzymes, but no enzymatic activities could have been demonstrated *in vitro*. Phenotypes of mutants lacking different combinations of these proteins suggest that some of them may have antagonistic activities leading to some sort of equilibrium state in the wall (*22*). This could explain the high rigidity and at the same time flexibility of the wall, which can be achieved by shifting the equilibrium of the remodelling activities but the exact physiological roles of these enzymes are still unknown. Besides, two other potential endoglucanases, Eng1p and Egt2p, were reported to be required for cell separation but only for the first protein its enzymatic activity could be verified *in vitro* (*23*). Apart from that, Scw3p/Sun4p protein was found in the SDS-soluble fraction of cell wall proteins (*3*) and it was found to be involved in septation (*24*) and other morphogenetic changes both in *S. cerevisiae* and in *Candida albicans* (*25*).

The second group of noncovalently attached proteins comprises two exoglucanases, Exg1p and Spr1p, which are functionally related to a third GPI-anchored exoglucanase, Exg2p (*26*). All three enzymes were reported to exhibit exoglucanase activity *in vitro* both with β-1,3- and with β-1,6-glucan, but Spr1p seemed to be sporulation specific and probably somehow involved in the spore wall formation (*27*).

Finally, the third glycosidase activity observed in the wall originates from two chitinases, Cts1p and Cts2p (*28*). Cts1p is primarily located at the daughter side of the bud neck and has a role in cell separation. Cts2p has sequence homology to chitinases but may be involved in sporulation since it shares most similarities with the *C. albicans* Cht4p and Cts2p from *Ashbya gossypii*, both sporulation specific chitinases (*29*).

An overview of noncovalently attached *S. cerevisiae* cell wall proteins is given in Table 1 (*3,4,8,9,12,23,24,26,28, 30–50*).

Table 1. *Saccharomyces cerevisiae* cell wall proteins

Protein	Mode of attachment	Biological activity	References
Bgl2p	noncovalent	endo- or *trans*-glucosidase	(*18–20*)
Scw4p	noncovalent/alkali sensitive	putative glucanase	(*4*)
Scw10p	noncovalent/alkali sensitive	putative glucanase	(*4*)
Scw11p	noncovalent	putative glucanase	(*4*)
Eng1p	noncovalent	endoglucanase/cell separation	(*23*)
Egt2p	noncovalent	endoglucanase/cell separation	(*23*)
Scw3p/Sun4p	noncovalent	septation	(*3,24*)
Spr1p	noncovalent	exoglucanase/sporulation	(*26*)
Exg1p	noncovalent	exoglucanase	(*26*)
Cts1p	noncovalent	chitinase/cell separation	(*28*)
Cts2p	noncovalent	chitinase/cell separation/sporulation	(*28*)
Gas1p-5p	GPI-anchored	glucanosyltransferases	(*30–34*)
Exg2p	GPI-anchored	exoglucanase	(*26*)
Crh1p	GPI-anchored	chitin-glucan cross-linking	(*35*)
Crh2p/Utr2p	GPI-anchored	chitin-glucan cross-linking	(*35*)
Crr1p	GPI-anchored	chitosan-glucan cross-linking/sporulation	(*36*)
Flo1p	GPI-anchored	flocculation	(*37,38*)
Aga1p	GPI-anchored	a-agglutinin subunit	(*39*)
Aga2p	S-S linked to Aga1p	a-agglutinin subunit	(*40*)
Sag1p	GPI-anchored	α-agglutinin	(*39*)
Cwp1p	GPI-anchored	unknown	(*8,41*)
Cwp2p	GPI-anchored	unknown	(*8,41*)
Dan1p-4p	GPI-anchored	anaerobiosis/sterol uptake	(*9,42,43*)
Tir1p-4p	GPI-anchored	anaerobiosis/Tir4p sterol uptake	(*9,42,43*)
Tip1p	GPI-anchored	anaerobiosis	(*9*)
Fit1p-3p	GPI-anchored	iron uptake	(*44*)
Sed1p	GPI-anchored	unknown/stress protection?	(*45*)
Spi1p	GPI-anchored	unknown/stress protection?	(*40*)
Ccw12p	GPI-anchored	unknown	(*46,47*)
Ccw14p	GPI-anchored	unknown	(*46*)
Yps1–3p, Yps6p, Yps7p	GPI-anchored	yapsins, proteolysis	(*48–50*)
Pir1p/Ccw6p	alkali-sensitive	unknown/wall integrity?	(*4*)
Pir2p/Ccw7p/Hsp150p	alkali-sensitive	unknown/wall integrity?	(*4*)
Pir3p/Ccw8p	alkali-sensitive	unknown/wall integrity?	(*4*)
Pir4p/Ccw5p	alkali-sensitive	unknown/wall integrity?	(*4,12*)

Attachment of Proteins *via* GPI Anchors

A treatment of cell walls from which noncovalently attached proteins have previously been depleted, with one of the commercial glucanase preparations like laminarinase or Quantazyme β-1,3-exoglucanase, liberates a set of proteins covalently linked to glucan (*4,8*). Most of them were found to be modified in the ER by the attachment of a glycosylphosphatidylinositol (GPI) anchor to their C-terminal amino acids (*10,41*). GPI anchors are assembled at the luminal side of the ER and transferred to proteins with a proper C-terminal amino acid sequence consisting of about 10–15 hydrophobic amino acids followed by about 10 amino acids of moderate polarity (*51–53*). The reaction consists of the proteolytic removal of the signal and the transamidation between the last amino acid remaining, a so called ω-amino acid, and the ethanolamine phosphate of the anchor. Usually the ω-amino acid is G, A, S, N or D and the position ω+2 is typically G, A or S. The membrane-anchored proteins migrate along the secretory pathway and are finally exposed at the outer side of the plasma membrane. The last step is then a postulated transglycosidation reaction by which the protein together with the remnant of the GPI anchor is transferred to β-1,6-glucan (*54,55*). The enzyme required for the last reaction, which presumably has to occur in the cell wall, has not yet been found. Besides, not all GPI-anchored proteins undergo this reaction but some of them, usually sharing a particular dibasic motif next to the ω-amino acid, remain anchored in the membrane (*56*). There are more than 50 genes in *S. cerevisiae* coding for proteins with putative GPI-anchoring signals (*6*). Only about half of them have actually been found in the cell wall or it has been reported that their fusion with heterologous proteins leads to the incorporation of the latter in the cell wall (*57*). This may either be a result of low quantities of some of them, or of different transcriptional regulation of their genes.

GPI-anchored cell wall proteins probably have a variety of functions, many of which are still unrevealed. It has been reported that some of them, like the five members of the GAS family, have glucan-remodelling activities (*30,31*). The proteins remain membrane bound, they are highly O- and N-glycosylated and have a C-terminal disulphide-stabilized domain important for the proper folding of the entire molecule (*32*). Mutation in *GAS1* results in abnormal β-1,3-glucan formation and secretion of a large amount of this polymer in the medium (*58*). *In vitro* experiments corroborated the enzymatic transglucosidase activity with the formation of a β-1,3--glucosidic bond between the reducing end of one glucan chain with the non-reducing end of another, suggesting that it has a role in β-1,3-glucan elongation (*33,34*). Other GAS proteins (Gas2p-Gas5p) share significant sequence homology with Gas1p but their exact physiological role is still under examination. Gas2p and Gas4p are sporulation specific and required for spore wall assembly and spore viability (*59*).

Other GPI-anchored wall proteins with enzymatic activities include an exoglucanase Exg2p and two proteins, Crh1p and Utr2p/Crh2p (*60,61*), which were found to have transglycosidase activities required for cross--linking of β-1,3-glucan with chitin (*35*). Both Crh1p and Crh2p are located at the cell cortex where they are also required for the cross-linking of Gas1p to the chitin ring (*62,63*). A homologue of these enzymes Crr1p was suggested to play a similar role in the formation of ascospore walls where it links β-1,3-glucan with chitosan (*36*).

Two sets of GPI-anchored proteins are required for cell-cell interactions under different conditions. The family of Flo proteins (Flo1p, Flo5p, Flo9p, Flo10p) or the so-called flocculins are lectin-like proteins required for flocculation (*37,38*), while the Aga1p/Aga2p complex and Sag1p are agglutinins, proteins which provide α- to a-cell adhesion during mating by direct protein-protein interaction (*39,40*).

Another set of wall proteins anchored through GPIs comprises members of the TIR family (*9*). These proteins were detected in the wall in different studies but they all have in common that they are differentially expressed under anaerobic conditions (*42*). Two members, Cwp1p and Cwp2p, are repressed, while Dan1p, Dan4p, Tir1p--Tir4p and Tip1p are induced. A mutant lacking Tir1p, Tir3p and Tir4p show growth defects under anaerobic growth conditions. In spite of the fact that Cwp1p and Cwp2p were the first GPI-anchored proteins described in yeast, little is known about the physiological functions of the members of *TIR* family although it has recently been observed that transcription and incorporation of Cwp1p, Cwp2p and Tip1p are cell cycle regulated (*64*). It has been suggested that Dan1p, as well as presumably Dan3p, Dan4p and Tir4p play a role in the intake of sterols under anaerobiosis by binding sterols from the surrounding medium in the wall (*43*). Similarly, three members of the *FIT* family, Fit1p-Fit3p are required for the iron uptake. It seems that these proteins bind iron from the medium, thus increasing the iron concentration close to the plasma membrane, facilitating the iron uptake by membrane transporters (*44*).

Two GPI-anchored proteins, Sed1p and Spi1p were induced under stress exerted by glucose limitation and their protective role during stress was proposed, but the exact functions of these proteins are not known (*45*). Besides, two other proteins, Ccw12p and Ccw14p, were purified from the cell wall preparations but their roles are still unknown although Ccw12p represents one of the most abundant GPI-anchored proteins and the *ccw12* mutant has an increased mortality in the stationary phase of growth (*46,47*).

In the late 90s a family of GPI-anchored aspartyl proteases called yapsins was found, corroborating much older observations that there was proteolysis occurring external to the cell membrane (*48,49*). Yapsins are anchored in the plasma membrane, they have substrate specificity closely related to the *trans*-Golgi Kex2p protease and play a role in maintaining the integrity of the cell wall. A mutant lacking all five members of the family undergoes lysis at 37 °C and has significantly decreased amount of β-1,3- and β-1,6-glucan although the primary synthesis of the two polymers is not affected (*50*). Thus the physiological substrate protein(s) for this class of proteases remains unknown.

A list of GPI-anchored proteins isolated from the *S. cerevisiae* cell walls is presented in Table 1.

Attachment of Alkali-Extractable Cell Wall Proteins

Extraction of biotin-labelled cell wall proteins with mild alkali resulted in four protein bands whose sequencing revealed four proteins encoded by the so-called *PIR* gene family (Proteins with Internal Repeats) (*4*). Pir1p/Ccw6p, Pir2p/Ccw7p/Hsp150p, Pir3p/Ccw8p and Pir4p/Ccw5p/Cis3p all share high sequence homology and consist of the signal sequence, part proteolytically removed by the Kex2p protease, a repetitive 12-amino-acid unit repeated between once (in Pir4p) and eleven times (Pir2p), and the C-terminal portion of the protein. All are highly O-glycosylated and all except Pir2p are also abundantly N-glycosylated in the C-terminal part. Deletion analysis of Pir4p revealed that the repeating unit is required for the attachment of the protein to glucan and mutagenesis of individual amino acids within the repeating motif SQIGDGQVQATS showed that the exchange of any of the three glutamines, or the aspartic acid resulted in the loss of the protein from the cell wall and its secretion into the medium (*12*). Furthermore, sequencing of the protein obtained by mild alkali treatment showed glutamic acid instead of the middle glutamine (Q_{74}) found in the *S. cerevisiae* genome version of *PIR4*. This led to a proposal of the putative reaction in which deamination of Q_{74} provided energy for the formation of the alkali-labile ester bond. Structural analysis of the linking region of Pir4p extracted from the cell wall by β-1,3-glucanase suggested that glutamine reacts directly with one of the hydroxyl groups of a glucose unit of β-1,3-glucan (*12*). The enzyme required for the formation of the ester bond is still unknown. Also, the role of Pir proteins is unclear although for some of them a protective role during osmotic stress or increased temperature was proposed and a mutant lacking all four *PIR* genes showed increased mortality both in the logarithmic and in the stationary phase of growth (*22*). Besides, the transcript levels of *PIR* genes are increased as a result of cell wall perturbations (*65*,*66*) indicating that these proteins might play a role in wall protection.

Alkali extracts of cell walls of the mutant lacking all four *PIR* proteins still contained a protein band of the size similar to Pir3p. Further elimination of two proteins previously described as noncovalently attached to glucan (Scw4p and Scw10p) resulted in the depletion of the remaining band showing that Scw4p and Scw10p were attached to the wall in two different ways. Proteins covalently attached are particularly interesting since they do not have the *PIR* repeating motif. Thus, there must be a fourth type of protein-glucan linkage by which Scw4p and Scw10p are attached.

Alkali extractable *S. cerevisiae* cell wall proteins are listed in Table 1.

Expression and Binding of Heterologous Proteins to the Yeast Cell Wall

In the past several years much effort has been devoted to the study of expression systems for the display of heterologous proteins on the surface of microorganisms, opening new perspectives in biotechnology. Heterologous proteins displayed at the host microbial surface can be used in a wide variety of applications such as production of pharmaceutical chemicals, detergent enzymes, food and feed enzymes, medical applications (diagnostic, immunoassays, development of vaccines, *etc.*) and many others. Recently a number of surface-engineered yeasts, displaying different heterologous proteins interesting for biotechnological or medical applications, have been constructed.

Conventional methods for covalent immobilization of proteins frequently result in changes of structure or characteristics of immobilized proteins due to often harsh treatment during the immobilization reaction. Immobilization of proteins through non-covalent interactions is less damaging but proteins immobilized in this way are more easily dissociated and subsequently lost during application. On the other hand, proteins immobilized at the microbial cell surface by regular cellular processes are bound covalently but are not exposed to potential damage by chemical treatment. Heterologous protein-encoding genes are generally fused with genes coding for host cell surface proteins or their fragments required for anchoring. A successful anchor protein should have an efficient signal sequence enabling transport of the fusion protein along the secretory pathway, and a strong anchoring structure to prevent detachment of the heterologous protein from the surface. Furthermore, it should be compatible with inserted or fused sequences to enable proper folding and stability of the heterologous protein. Depending on the characteristics of the protein to be immobilized, there are several possibilities for the construction. Fusion can be done either at the C-terminal or at the N-terminal end of the protein of interest, or the protein could be inserted within the 'carrier protein' sequence. The location of the insertion or fusion is important because it influences immobilization efficiency, stability, post-translational modifications of the protein and its specific activity.

The productivity of an expression system in the host cell could be limited in many ways. Potential bottlenecks are the gene copy number (*67*), the codon usage of the expressed gene (*68*), translocation determined by the secretion signal peptide (*69*), processing and folding in the ER and Golgi (*70*), secretion (*71*) and protein turnover by proteolysis (*72*).

Host microbial strains for surface display of heterologous proteins should be compatible with the displayed protein, have low activities of extracellular proteases and their cultivation should be easy and inexpensive. The most frequently used bacteria for this purpose are *Escherichia coli*, *Staphylococcus carnosus* and *Lactococcus lactis* (*73*). *Saccharomyces cerevisiae* has several advantages over bacteria as a potential host. It is a GRAS organism (generally recognized as safe) and can be used for food, biotechnological and pharmaceutical production. Yeast is a better host for expression of mammalian proteins than bacterial cells, since it possesses protein folding and secretory machinery similar to those of higher eukaryotic cells and enables proper post-translational modifications and folding of many mammalian proteins. Furthermore, secreted heterologous proteins are easily separated from other yeast proteins since it normally secretes few proteins and in low abundance. Moreover, it can be cultivated to a high cell density in cheap media. It is worth

noting that yeast genera other than *Saccharomyces*, like *Pichia* or *Kluyveromyces*, have also been extensively studied as potential hosts. Different applications of proteins displayed at the yeast cell surface are surveyed in the following paragraphs.

GPI-Anchored Heterologous Proteins in Yeast

Most heterologous proteins constructed for yeast surface display are GPI-anchored to the cell wall. Most frequently used GPI-anchored yeast protein for this purpose is α-agglutinin. It is transported to the outer side of the plasma membrane and then undergoes translocation to β-1,6-glucan typical for yeast GPI-anchored proteins (*10,74,75*). Thus, when a foreign protein is fused to the N-terminus of α-agglutinin, it will be anchored covalently at the cell surface. The protein can either be fused with the entire α-agglutinin, or with any of its parts providing it includes the GPI-anchoring signal. N-terminal fusions to α-agglutinin have widely been used to display various proteins used in biotechnology, medicine and for research purposes (Table 2, *76–102*).

Schreuder *et al.* (*76*) constructed a fusion protein consisting of the signal sequence of yeast invertase, guar α-galactosidase, and the C-terminal half of the α-agglutinin. The result demonstrated that the C-terminal half of the α-agglutinin contained the information needed to incorporate the protein into the cell wall.

In the conventional procedure of ethanol production the crude starchy material is heated at 140–180 °C prior to amylolysis. The energy consumed in this process is the cause of high costs. To save the heat energy, a non-cooking fermentation system using enzymes isolated from *Rhizopus oryzae* was developed (*103*). In the next step genetically engineered yeast producing secretory amylolytic enzymes was constructed. Finally, novel yeast strains were prepared that harboured the glucoamylase/α-agglutinin fusion gene on a multicopy plasmid and expressed the cell wall-associated glucoamylase (*77*). Engineered yeasts grew aerobically in 1 % soluble starch as sole carbon source to essentially the same level as yeast cultured in 1 % glucose. The optimal temperature for the anchored glucoamylase was somewhat lower than for the free enzyme, while no differences in thermal stability and pH optima were observed (*104*). Cells with glucoamylase/α-agglutinin fusion integrated into the chromosome exhibited glucoamylase activity at the cell surface as well, and their mitotic stability was much higher than that of the cells containing the plasmid (*104*). Sato *et al.* (*78*) showed that a long anchor enhances the activity of cell surface-displayed enzyme for polymer substrates by using a glucoamylase/Flo1p fusion.

Fermentation efficiency in direct fermentation of starchy materials with glucoamylase could be enhanced by the addition of α-amylase. Therefore, a yeast strain co-displaying glucoamylase and α-amylase at the cell surface was constructed in which the α-amylase/α-agglutinin fusion was integrated into the yeast chromosome as well as the glucoamylase/α-agglutinin fusion (*79*). The novel strain was able to grow faster in 1 % soluble starch as a sole carbon source, probably due to increased rate of glucose formation through the increased

Table 2. Heterologous proteins expressed and immobilized in the *S. cerevisiae* cell wall

Immobilized protein	Carrier	References
α-galactosidase (*Cyamopsis tetragonoloba*)	α-agglutinin	(*76*)
glucoamylase (*Rhizopus oryzae*)	α-agglutinin	(*77*)
glucoamylase (*Rhizopus oryzae*)	Flo1p	(*78*)
α-amylase (*Bacillus stearothermophilus*) glucoamylase (*Rhizopus oryzae*)	α-agglutinin	(*79*)
carboxymethylcellulase (*Aspergillus aculeatus)*	α-agglutinin	(*80*)
β-glucosidase (*Aspergillus aculeatus*) CM-cellulase (*Aspergillus aculeatus*)	α-agglutinin	(*81*)
β-glucosidase (*Aspergillus aculeatus*) endoglucanase II (*Trichoderma reesei*)	α-agglutinin	(*82*)
β-glucosidase (*Aspergillus aculeatus*)	α-agglutinin	(*83*)
glucoamylase (*Rhizopus oryzae*) CBD1 and CBD2 (*Trichoderma reesei*)	α-agglutinin	(*84*)
β-glucosidase (*Aspergillus acleatus*)	α-agglutinin	(*85*)
lipase (*Rhizopus oryzae*)	α-agglutinin	(*86*)
lipase (*Rhizopus oryzae*)	Flo1p	(*87*)
lipase B (*Candida antarctica*)	α-agglutinin	(*88*)
BFP	α-agglutinin	(*89,90*)
GFP (*Aequorea victoria*)	α-agglutinin	(*89,90*)
ZZ-domain of *Staphylococcus aureus*	α-agglutinin	(*91*)
ZZ-domain of *Staphylococcus aureus*	Flo1p	(*92*)
trans-sialidase (*Trypanosoma cruzi*)	Aga2p	(*93*)
CD47 (mammalian membrane protein)	Aga2p	(*94*)
polypeptide libraries	Aga2p	(*95*)
α-galactosidase (*Cyamopsis tetragonoloba*)	Flo1p Cwp1p Cwp2p Tip1p Sed1p Tir1p	(*96*)
GFP (*Aequorea victoria*)	Cwp1p Cwp2p	(*97*)
α-1,2-galactosyltransferase (*Schizosaccharomyces pombe*)	Pir1p	(*98*)
α-1,2-mannosyltransferase (*Saccharomyces cerevisiae*)	Pir1p	(*98*)
α-1,3-mannosyltransferase (*Saccharomyces cerevisiae*)	Pir2p	(*98*)
α-1,3-fucosyltransferase (human)	Pir1p Pir2p	(*99*)
xylanase A (*Bacillus* sp. BP-7)	Pir4p	(*100*)
α-2,3-sialyltransferase (human)	Pir2p	(*101*)
α-1,3-fucosyltransferase VII (human)	Pir2p	(*101*)
VP8* fragment of human rotavirus	Pir4p	(*102*)

production of molecules with non-reducing ends by α-amylase, which in turn served as a substrate for glucoamylase.

The most promising renewable carbon source that is available in large quantities is cellulose. Since *S. cerevisiae* is unable to utilize cellulosic material, yeast strains displaying cellulolytic enzymes at their surface were constructed. The enzymatic degradation of cellulose to glucose requires synergistic hydrolysis by different types of cellulolytic enzymes. CM-cellulase possesses endoglucosidase activity producing short-chain oligosaccharides that are converted to glucose by β-exoglucosidase. Murai *et al.* (*80*) constructed a yeast strain harbouring CM--cellulase/α-agglutinin on the cell surface. Subsequently, a yeast strain harbouring multi-copy plasmids for the co-expression of CM-cellulase/α-agglutinin and β-glucosidase/α-agglutinin fusion proteins in the cell wall was constructed (*81*) and it has been shown that this strain could grow in a medium containing cellobiose as a sole carbon source.

Similarly, a cellulose-degrading yeast strain for direct and efficient ethanol production from cellulosic materials was constructed by genetically co-displaying endoglucanase II (EGII)/α-agglutinin and β-glucosidase/α-agglutinin fusion proteins on the cell surface of *S. cerevisiae* (*82*). This strain could grow in media containing β-glucan as the sole carbon source and simultaneously produce ethanol.

Tokuhiro *et al.* (*83*) engineered a lactic acid-producing yeast to express β-glucosidase/α-agglutinin from genome-integrated plasmids for efficient fermentation of cellobiose to L-lactate.

A yeast strain that co-displayed *Rhizopus oryzae* glucoamylase and two kinds of *Trichoderma reesei* cellulose--binding domains (CBD1, CBD of cellobiohydrolase I (CBHI); and CBD2, CBD of cellobiohydrolase II (CBHII)) was constructed for enzymatic desizing of starched cotton cloth (*84*). By co-displaying glucoamylase and CBDs on the cell surface, the yeast cell acquired specific binding ability to cotton cloth that provided better activity than a strain displaying glucoamylase only.

The sulphuric acid hydrolysate of lignocellulosic biomass such as wood chips is an important material for fuel bioethanol production. Katahira *et al.* (*85*) constructed a recombinant yeast strain that could ferment xylose and cellooligosaccharides by integrating genes for the extracellular expressions of xylose reductase and xylitol dehydrogenase from *Pichia stipitis*, and xylulokinase from *Saccharomyces cerevisiae* and a gene for displaying β-glucosidase from *Aspergillus acleatus* on the cell surface.

The use of lipases in bioprocessing of lipids is energy saving since chemical reactions need high temperatures and pressures. Moreover, the resulting products require re-distillation and purification to remove impurities and degradation products. Lipases have high substrate and reaction specificity so that the separation of products is relatively easy. Lipases can be used in chemical and pharmaceutical industry, food industry, biodiesel production, wastewater treatment, production of laundry cleaning products and detergents, *etc*. At present, the production of lipases is usually achieved by secretion systems followed by enzyme purification. Therefore, cell surface display of immobilized lipases would lead to a reduction in the cost of enzyme preparation. Expression of lipase in *E. coli* resulted in enzymatically inactive and insoluble aggregates (*105*), but expression and secretion of active lipases were obtained in *S. cerevisiae* (*106*).

Washida *et al.* (*86*) have constructed a heterologous enzyme that contained the C-terminal half of α-agglutinin fused to *Rhizopus oryzae* lipase *via* a linker peptide. The transformant strain displayed lipase on the cell surface and could grow on triolein as the sole carbon source. Another construct showing high cell surface lipase activity was made by the fusion of Flo1p as a cell wall anchor and *Rhizopus oryzae* lipase (*87*). The lipase of *Rhizopus oryzae* exhibits relatively high activity among different available lipases and is suitable for human oral use so it could be suitable for food production as well.

Among different commercially interesting lipases, the lipase B from *Candida antarctica* (CALB) is one of the most famous and versatile ones. However, the high cost of the enzyme often becomes a problem in the industrial application. Tanino *et al.* (*88*) successfully constructed CALB-displaying yeast whole-cell biocatalysts using the Flo1p anchor system.

A green fluorescent protein (GFP) from the jellyfish *Aequorea victoria*, and GFP variant BFP (blue fluorescent protein) were used in the molecular design of a novel visible type of reporter to target the cell surface of *S. cerevisiae* (*89*). A combination of reporters and promoters to sense intra- and extracellular conditions is used for a construction of a novel cell-surface-engineered system for non-invasively identifying environmental changes (concentration of glucose, phosphate or ammonium ion) (*90*).

Nakamura *et al.* (*91*) and Shimojyo *et al.* (*92*) employed cell-surface engineering using the C-terminal half of α-agglutinin or Flo1p to construct a yeast strain displaying the ZZ domain derived from *Staphylococcus aureus*, which binds to the Fc part of immunoglobulin G (IgG) and has been used as an affinity tag to purify recombinant proteins and in immunoassays. *S. cerevisiae* cells displaying ZZ could therefore be widely used as immunoadsorbents in immunoassays and affinity purifications.

C-terminal fusions of heterologous proteins to a-agglutinin have also been performed although less frequently than N-terminal fusions to α-agglutinin (Table 2). a-Agglutinin is a complex of two subunits: Aga1p and Aga2p. Aga1p is anchored to the cell wall *via* GPI, while Aga2p is linked to the Aga1p subunit by two disulphide bonds (*39,40*).

The absence of sialylation on recombinant glycoproteins compromises their efficacy as therapeutic agents, resulting in rapid clearance from the human bloodstream. To circumvent this, C-terminal fusion of a *trans*-sialidase from *Trypanosoma cruzi* with Aga2p has been performed, and this yeast is used in the sialylation of synthetic oligosaccharides (*93*).

Besides, this approach has been used to display a ubiquitous mammalian membrane protein, CD47, which is implicated in cancer, immunocompatibility, and motility (*94*), and a polypeptide library on the yeast surface (*95*).

Other GPI-anchored yeast cell wall proteins such as Cwp1p, Cwp2p, Flo1p, Tip1p, Sed1p and Tir1p have been proven capable of displaying α-galactosidase (*96*), or GFP (*97*) on the yeast surface. Some of them, as Cwp2p and Sed1p, were shown to be better carriers than the commonly used α-agglutinin, giving six- to eightfold higher levels of displayed heterologous protein at cell surface (*96*).

It has also been shown that insertion of a 'spacer sequence' into the fusion significantly improves cell surface expression in *S. cerevisiae* (*107*).

Heterologous Proteins Immobilized on the Yeast Cell Surface by the Fusion with Pir-Proteins

Display systems with GPI anchors are usually not suitable for enzymes whose active sites are located near their C-termini. A possible approach for such enzymes is to use Pir-proteins as cell wall anchors (Table 2).

Moukadiri *et al.* (*108*) first reported that Pir4p can target recombinant proteins to the cell surface by constructing a fusion between Pir4p and part of protein A from *Staphylococcus aureus*. Abe *et al.* (*98*) fused three glycosyltransferases (α-1,2-galactosyltransferase, α-1,2-mannosyltransferase and α-1,3-mannosyltransferase) to the Pir1p or Pir2p respectively (Table 2). All three enzymes were successfully immobilized on the yeast cell surface and retained their activities. Furthermore, it has been shown that two fusion proteins, in this case two different mannosyltransferases, could be co-expressed and co-displayed in the cell wall. Whole yeast cells transformed in this way were successfully used as a biocatalyst for sequential synthesis of oligosaccharides identical to those synthesized *in vivo* (*98*). Deletion of endogenous *PIR* genes increased display efficiency of fusion proteins about threefold compared to wild type cells (*99*). Further increase in display efficiency is possible if two Pir proteins fused with the enzyme of interest are co-used and simultaneously expressed in the yeast cell (*99*).

Xylan is the second most abundant polymer of plant cell walls. The enzymes for depolymerization of xylan have potential biotechnological applications in food, feed and paper industry. Andrés *et al.* (*100*) used Pir4p for targeting of xylanase A from *Bacillus* sp. BP-7 to the cell wall or to the culture medium. Xylanase activity was successfully associated with the cell wall if constructs were made by insertion of a xylanase-encoding gene in the coding region of *PIR4* gene after the repetitive sequence or on the C-terminus. In contrast, xylanase was secreted to the medium if either the repetitive sequence or the C-terminal portion of Pir4p was missing.

Inflammation during organ rejection and other inflammation processes are mediated by leukocyte adhesion on endothelial cells through interaction of leukocytes with selectins, thus selectins can be used as targets for anti-inflammatory therapy. Binding of specific oligosaccharides (sLe^x) to selectins inhibits adhesion of leukocytes and decreases tissue damage. Pure glycosyltransferases isolated from human placenta and milk have been used previously in order to synthesize specific sLe^x oligosaccharides. Salo *et al.* (*101*) successfully fused two mammalian glycosyltransferases in the yeast cell wall for the synthesis of sLe^x.

Rotavirus infections result in high childhood mortality in developing countries and it is the most common cause of severe illness of infants worldwide (*109*). Some vaccines consisting of one or several attenuated rotavirus strains are in the process of development (*110*). An alternative approach for the development of a vaccine is the use of recombinant rotavirus antigens. For this purpose the antigenic VP8* fragment of the rotavirus spike protein was expressed as a fusion protein with Pir4p in yeast (*102*). Pir4p-VP8* fusion protein was successfully targeted to the cell wall or to the culture medium, depending on how the fusion had been performed. All mice immunized with heterologous Pir4-VP8* protein, purified from the medium, showed high titres of anti-VP8* antibodies. However, mice immunized with whole yeast cells showed no specific immune response.

Conclusion

In the past twenty years, researchers in a number of laboratories have contributed to the comprehension of the structure, biosynthesis and functions of the yeast outermost cellular compartment. After the first efforts to identify, clone and sequence cell wall proteins and elucidate polysaccharide structures participating in the formation of a rigid, yet flexible moiety, we are now starting to understand individual enzymatic activities involved in the synthesis and transformations in the wall enabling cellular processes like growth, budding, mating, sporulation, flocculation, *etc*. At the same time we are beginning to understand how processes involving cell wall remodelling are connected with the overall cell metabolism and to accumulate data on the signalling pathways by which a cell responds to changes in the surroundings reflecting on the cell wall structure and function. A large number of cell wall proteins still remains a puzzle and their biological roles still have to be explained. Moreover, the fact that different proteins are attached to polysaccharide moieties in different ways poses a question of the relation of the incorporation process and the roles of incorporated proteins. At the same time, an ever increasing number of papers clearly indicates how the knowledge gained on cell biology and biochemistry of the wall can successfully be employed for biotechnological purposes.

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