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1 The *Schizosaccharomyces pombe* Map4 adhesin is a glycoprotein that can be extracted
2 from the cell wall with alkali but not with β-glucanases and requires the C-terminal
3 DIPSY domain for function.
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22
23 Abbreviations: aa, amino acid; ASL, proteins with alkali-sensitive linkages; GPI,
24 glycosylphosphatidyl inositol; PIR, protein with internal repeats; S/T, serine and
25 threonine.

Summary

In fungi, cell adhesion is required for flocculation, mating, and virulence, and it is mediated by covalently-bound cell wall proteins termed adhesins. Map4, an adhesin required for mating in *Schizosaccharomyces pombe*, is N-glycosylated and O-glycosylated, and is an endogenous substrate for the mannosyl transferase Oma4p. Map4 has a modular structure with an N-terminal signal peptide, an S/T-rich domain that includes 9 repeats of 36 amino acids (rich in serine and threonine residues, but lacking glutamines), and a C-terminal DIPSY domain with no GPI-anchor signal. Map4 can be extracted from cell walls with SDS/mercaptoethanol sample buffer or with mild alkali solutions. After extensive extraction with hot sample buffer, no more protein can be released by β-glucanases or alkali. Additionally, none of the cysteine residues of the protein is required for its retention at the cell wall. These results show that Map4 is not directly bound to β-glucans and point to the existence of alkali- and SDS/mercaptoethanol-sensitive linkages between cell wall proteins. The N-terminal S/T-rich regions are required for cell wall attachment, but the C-terminal DIPSY domain is required for agglutination and mating in liquid and solid media.

Introduction

In yeast and fungi, adhesion processes are mediated by cell wall proteins called adhesins, which include flocculins and mating agglutinins. The best-characterized adhesins belong to the genera *Saccharomyces* and *Candida* (see Lipke & Kurjan, 1992, Kapteyn *et al.*, 1999, Hoyer, 2001, Klis *et al.*, 2002, De Groot *et al.*, 2005, Klis *et al.*, 2006, Verstrepen & Klis, 2006, Dranginis *et al.*, 2007 for reviews). These proteins are covalently bound to the β-glucan in the cell wall and hence resist extensive washing with 2M NaCl and extraction with hot reducing agents. Cell wall proteins become attached to this structure in different ways. *S. cerevisiae* Aga1 and Sag1 mating adhesins and Flo flocculins, *C. albicans* Als, Hwp1 and Eap1 proteins, and *C. glabrata* Epa1 protein are glycosylphosphatidyl inositol (GPI)-bound cell wall proteins (Wojciechowicz *et al.*, 1993, Frieman *et al.*, 2002, Huang *et al.*, 2003, Li *et al.*, 2007 and references above). GPI-cell wall proteins are bound to β(1,6) glucan, which is in turn bound to the β(1,3)-glucan; accordingly, they can be released from the cell wall by digestion with either β(1,6) or β(1,3) glucanases. At least in the case of Aga1 the GPI anchor can be replaced by cysteine residues or transmembrane domains to mediate cell surface attachment (Huang *et al.*, 2003).

A second group of covalent cell wall proteins are the ASL (for Alkali-Sensitive Linkage) proteins. They can be extracted from the cell wall with mild alkali treatment (incubation in the presence of 30 mM NaOH at 4ºC; Mrsa *et al.*, 1997) after the cell wall has been boiled in the presence of reducing agents. Although the best-characterized ASL cell wall proteins are the *S. cerevisiae* PIR (Proteins with Internal Repeats) proteins (Mrsa *et al.*, 1997 and see also De Groot *et al.*, 2005, Klis *et al.*, 2006 for

reviews), other covalent alkali-extractable proteins have been found in *S. cerevisiae*, *C. albicans* and *S. pombe* (De Groot *et al.*, 2004, Yin *et al.*, 2005, De Groot *et al.*, 2007). Regarding the Ccw5/Pir4 PIR protein from *S. cerevisiae*, it has been shown that this protein is directly bound to the β(1,3)-glucan by a protein-carbohydrate linkage involving a glutamine residue present in the internal repeat of the protein (Ecker *et al.*, 2006). Since repeats bearing glutamines are present in all PIR proteins, it may be assumed that this new kind of linkage is universal for this family of cell wall proteins. Finally, Bad1/WI-1, an adhesin from the pathogenic fungus *Blastomyces dermatitidis*, is initially released to the medium but then re-associates with the cell wall through covalent and non-covalent bonds. Chitin seems to be the receptor required for the binding of the adhesin to the cell wall (Brandhorst & Klein, 2000).

All known adhesins show a signal peptide directing their secretion. The mature forms have a modular structure, with different domains serving different purposes (Hoyer, 2001, De Groot *et al.*, 2005, Verstrepen & Klis, 2006, Dranginis *et al.*, 2007, Linder & Gustafsson, 2007). Most adhesins have an N-terminal ligand-binding domain, which can adopt an immunoglobulin-fold configuration, as is the case of the *C. albicans* ALS family and the Sag1 agglutinin from *S. cerevisiae*. Others have lectin-like sugar-binding domains, as in the *S. cerevisiae* FLO and *C. glabrata* EPA proteins, or adopt other conformations, as occurs in the Flo11 protein from *S. cerevisiae* or the Eap1 and Hwp1 proteins from *C. albicans* (Lipke *et al.*, 1989, Wojciechowicz *et al.*, 1993, Chen *et al.*, 1995, Hoyer *et al.*, 1995, Cormack *et al.*, 1999, Hoyer, 2001, Dranginis *et al.*, 2007, Linder & Gustafsson, 2007). The central domains are typically rich in serine and threonine residues, and often consist of sub-domains showing varying numbers of tandem repeats and a stalk. In some cases it has been shown that this region is required

1 to expose the ligand-binding domain outside the cell wall, while in others it is required
2 to elicit strong binding to the ligand (Frieman *et al.*, 2002, Huang *et al.*, 2003, Rauceo *et*
3 *al.*, 2006, Dranginis *et al.*, 2007, Li & Palecek, 2008). The pre-GPI and GPI signal
4 sequences constitute the C-terminal domain, which is required for attachment to the cell
5 wall. In the case of PIR proteins, the modular structure differs from the one described
6 above. Thus, the signal peptide is followed by a pro-sequence, potentially cleavable by
7 Kex2, a number of internal repeats with several glutamine residues required for
8 attachment to the β(1, 3)-glucan (Ecker *et al.*, 2006), and a C-terminal domain with
9 several cysteine residues following a C-x(66)-C-x(16)-C-x(12)-C pattern (see De Groot
10 *et al.*, 2005).
11
12 Although the general rule seems to be the presence of an N-terminal ligand-binding
13 domain and a central or C-terminal cell wall attachment domain, a few exceptions have
14 been described. The *S. cerevisiae* a-mating agglutinin is composed of two different
15 proteins: Aga1 is an S/T (serine and threonine)-rich GPI protein with two internal
16 repeats and is the cell wall attachment subunit of the agglutinin (Roy *et al.*, 1991,
17 Cappellaro *et al.*, 1994, Huang *et al.*, 2003). Aga2 is the active subunit, which binds to
18 Aga1p through disulfide bridges and to the Sag1 α agglutinin through protein-protein
19 interactions (Cappellaro *et al.*, 1991, Cappellaro *et al.*, 1994, Shen *et al.*, 2001, Zhao *et*
20 *al.*, 2001). The Bad1 adhesin from *B. dermatitidis* displays a signal peptide, an N-
21 terminal domain consisting of several tandem repeats required for cell wall attachment
22 (Brandhorst & Klein, 2000), and a C-terminal epidermal growth factor (EGF)-like
23 domain required for yeast adherence to macrophages and for strong binding to the cell
24 wall (Brandhorst *et al.*, 2003).
25

The predicted protein sequence of the fission yeast Map4 adhesin contains a signal peptide, an S/T-rich N-terminal domain, a central region consisting of 9 tandem repeats (with 40% of serine and threonine residues and no glutamines) and a C-terminal DIPSY (Asp-Ile-Pro-Ser-Tyr) domain (Sharifmoghadam *et al.*, 2006). There is no GPI signal for cell surface anchorage. Recently, an *in silico* study has uncovered the existence of fourteen new potential adhesins in *S. pombe*. All of them share a general structure, with an N-terminal region, a central region consisting of several tandem repeats, and a C-terminal domain that can belong to one of three subgroups: the DIPSY domain, the Flo11 domain, and the GLEYA domain, which is related to the lectin-like ligand-binding domain present in other adhesins (Linder & Gustafsson, 2007). None of them have a GPI-anchor signal.

In this work, we further characterized the Map4 protein. We found that this adhesin is a heavily glycosylated protein that is the first-reported endogenous substrate for the mannosyl transferase *oma4*$^{+}$. Map4p is not bound to β-glucan, but it can be released from the cell wall by treatment with mild alkali as efficiently as by treatment with hot sample buffer. The S/T-rich regions, and not the cysteine residues present in the protein, are required for cell wall attachment. The internal repeats are not essential either for cell wall attachment or for function, although they are required for optimal agglutination. The C-terminal DIPSY domain is required for function. Elimination of the Asp-Ile-Pro-Ser-Tyr signature motif or of any of the four cysteines present in this region abrogates agglutination. Our results show that in fungi there are adhesion molecules bound to the cell wall through new kinds of linkages and that the computer-predicted DIPSY domain is indeed a functional adhesion domain.

1 **Results**

2 *Map4p is an N-glycosylated and O-glycosylated protein*

3 The expected size for the 8xA-GFP tagged Map4 protein is 140 kDa. However, when
4 we performed Western analysis of this protein, we reproducibly obtained a plethora of
5 bands ranging from ~40 kDa to more than 200 kDa. The pattern of bands in samples
6 obtained from the same strain varied slightly, depending on the culture and the gel
7 conditions. We wished to confirm that these bands were specific for Map4p and not a
8 non-specific reaction of the anti-GFP antibody. We also wanted to determine whether
9 the small-size bands were a consequence of protein degradation during the process of
10 extracting the cell wall samples. In order to do so, h^{90} *map4Δ* cells or h^{90} cells carrying a
11 GFP-fused or HA-tagged Map4p were induced to agglutinate in EMM-N (see
12 Experimental Procedures). In each case, the culture was split into two and centrifuged.
13 One set of cells were frozen in a dry ice-ethanol bath. The other set of cells were broken
14 and then centrifuged; the supernatants, corresponding to the cytosols plus membranes,
15 were frozen and the cell walls were washed as explained in experimental procedures
16 and frozen. Later, the total amount of protein was estimated and concentration-equalled
17 in all samples. Samples were boiled in SDS/mercaptoethanol buffer and centrifugued.
18 Finally, 50 μg of total protein from the supernatants from the selected samples (see
19 figure 1 A; whole cells -Cells- from the untagged strain and whole cells, cytosols plus
20 membranes -Cyt- and cell walls -CW- from the other strains) was loaded into a 4-20%
21 acrylamide gradient gel and decorated with anti-GFP or anti-HA antibodies.
22 Additionally, 50 μg of total protein from the cytosol samples was loaded into a gel and
23 decorated with a polyclonal antibody raised against the *Saccharomyces cerevisiae*
24 membrane ATPase Pma1p, which was used as a loading control. As shown in figure 1
25 A, no signal was detected in the lanes corresponding to the samples obtained from the

cells without a tagged Map4 protein. However, in the samples corresponding to whole cells, cytosols, or cell walls from cells carrying a GFP- or an HA-tagged Map4p, multiple bands were detected. The specific pattern of the bands varied depending on the tag and the nature of the sample. In any case, this result shows that all the bands observed with Western blot were Map4-specific. The low molecular weight bands were present in all the samples, suggesting that they were not a consequence of protein degradation during the process of obtaining the cell wall samples. Slow-mobility bands were observed in samples obtained from whole cells, cytosols and cell walls; however, the highest molecular-weight forms of the protein were more abundant in the cell wall than in the cytosolic samples, suggesting either that they are incorporated to this structure immediately after being produced or that they are more stable in the cell wall than in the cytoplasm.

In the Map4p sequence there are 12 potential N-glycosylation sites and multiple serine and threonine residues, which are susceptible to O-glycosylation. In order to understand the contribution of protein glycosylation to the posttranslational modification of Map4p, we used Western blotting to analyze the protein obtained from the control cells or from the O-mannosyl transferase mutants *oma1Δ* and *oma4Δ*, bearing the Map4-GFP protein, under conditions of agglutination. In the fission yeast, there is no *PMT3* homolog and the viability of the *oma2Δ* mutant is controversial (Tanaka *et al.*, 2005, Willer *et al.*, 2005). Interestingly, after 5 hours in EMM-N mating aggregates could be observed in the control culture but not in the *oma1Δ* or *oma4Δ* cultures. In both cases we compared the protein treated with endoglycosidase-H (EndoH +; figure 1B) and untreated protein (EndoH -). The amount of Map4 protein detected by Western blotting was lower in the *omaΔ* mutants than in the control strain so in figure 1 B the lanes corresponding to the

1 mutant strains were purposefully overexposed. When the extracts from the control strain
2 were treated with EndoH the mobility of some of the protein bands was reduced;
3 however, several bands of high molecular weight were still observed. Map4p obtained
4 from the *oma1Δ* mutant exhibited a smaller number of bands, most of them presenting a
5 high molecular weight. When this protein was treated with EndoH to eliminate N-
6 glycosylation, two prominent bands of about 200 and 100 kDa were observed. Finally,
7 the size of the protein bands obtained from the *oma4Δ* mutant was significantly smaller
8 than that of the protein bands obtained from the control strain, with a strong band of
9 about 160 kDa and some smaller bands. When this protein was treated with EndoH
10 most of the protein was detected as a band of about 100 kDa. In sum, Map4p is a
11 protein that is N-glycosylated and O-glycosylated, Oma4p being the mannosyl
12 transferase that contributes most significantly to its O-glycosylation.
13
14 The fact that in the absence of glycosylation Map4p showed a size smaller than that
15 expected indicated that it had undergone some additional posttranslational processing.
16 In the sequence of this adhesin there are two motifs for potential cleavage by Kex2-type
17 proteases: KK, at amino acid position 413, and KR, at amino acid position 1003 (see
18 asterisks in figure 5 A for their position in the protein). In order to determine whether
19 some of the bands detected by Western blot corresponded to cleavage of the protein at
20 those positions, we eliminated each of these sequences and both of them by site-directed
21 mutagenesis (producing $Map4_{kk}$, $Map4_{kr}$ and $Map4_{kkkr}$ proteins, respectively). All these
22 variants of the protein behaved like the wild-type protein both in the control strain (not
23 shown) and in the *oma4Δ* mutant (results not shown and figure 1 B), suggesting that the
24 protein is not processed by a Kex2-type protease.
25

1 *Map4 is bound to the cell wall through linkages sensitive to alkali*

2 Most adhesins described are attached to the β-glucan component of the cell wall by
3 covalent linkages. In order to understand how Map4 is linked to this structure, we
4 boiled cell wall samples from strain HVP1350, carrying the Map4 protein fused to the
5 GFP, with hot sample buffer containing SDS and reducing agents (see Experimental
6 procedures). Samples were centrifugued and an aliquot from the supernatant,
7 corresponding to the extracted material, was loaded into a 4-20% acrylamide gradient
8 gel (lanes "SB" in figures 2A and 2C). The cell walls extracted with sample buffer were
9 then treated with β(1,3)-glucanase, β(1,6)-glucanase, or with 40 mM NaOH (see
10 Experimental procedures). Then, the samples were centrifuged and the supernatants
11 were denatured by boiling for 5 minutes in sample buffer and loaded into the gel
12 (figures 2 A and 2 C). As a positive control for the activity of the glucanases, we treated
13 cell walls from a *S. cerevisiae* strain carrying an Aga1-HA protein in the same way
14 (figure 2 B). As expected, Aga1p was released from the extracted cell wall by β(1,3)
15 and by β(1,6) treatment. In contrast, hot reducing agents extracted Map4 very efficiently
16 from the cell wall, as shown in figures 2 A and 2 C, but neither further digestion with
17 glucanases nor incubation in the presence of mild alkali solutions released more Map4
18 protein from the sample buffer-extracted cell walls. These results rule out the possibility
19 that Map4p might be attached to the β-glucan and are in agreement with the absence of
20 a GPI anchor signal at the C-terminus of the protein and the absence of glutamine
21 residues in the internal repeats

22

23 The above results suggested that Map4p might be attached to structural cell wall
24 proteins through disulfide bridges. In order to identify the cysteine residue(s) required
25 for cell wall attachment, we used site-directed mutagenesis to eliminate each or several

1 of them (see figure 5 A for information about the relative position of the cysteine
2 residues in the protein, and Experimental procedures). Strains carrying *map4*$^{+}$ alleles
3 lacking single cysteines (Map4C77, Map4C124, Map4C950, Map4C954, Map4C1008,
4 or Map4C1009), cysteines located at the N-terminal ST-rich domain (Map4C77-124),
5 cysteines located at the C-terminal DIPSY domain (Map4C950-954-1008-1009), or the
6 6 cysteine residues of the protein (Map4C77-124-950-954-1008-1009) were induced to
7 agglutinate in EMM-N. Expression of the proteins was confirmed by observation of
8 green fluorescence under the microscope and by Western blot (results not shown and
9 figure 3). In order to determine wether these variants of the protein remained attached to
10 the cell wall or were released to the culture medium, cells were induced to agglutinate in
11 50 ml EMM-N for 5 hours (See Experimental procedures). After this time, the cultures
12 were collected by centrifugation. The culture media were concentrated to 200 μl using
13 Amicon Ultra-15 devices and were boiled in the presence of an additional 200 μl of 2X
14 Laemmli sample buffer to denature the proteins. The cells were broken and the cell
15 walls were washed as indicated in Experimental procedures and finally extracted in a
16 final volume of 400 μl with hot Laemmli buffer. In this way the culture medium and the
17 cell walls from each culture were in the same final volume. 50 μl from each sample
18 (medium -M- or cell walls -W-, see figure 3) was loaded into 4-20% gradient gels and
19 developed with anti-GFP antibody. As shown in figure 3, in the control strain the
20 amount of protein released to the medium was almost undetectable. Surprisingly, in
21 strains carrying Map4 variants lacking single cysteine residues (not shown), cysteine
22 residues at the N-terminal or C-terminal domains of the protein (C77-124 or C950-954-
23 1008-1009, respectively), or the six cysteine residues, the protein was still attached to
24 the cell wall (figure 3). These results demonstrated that Map4 is not attached to the cell
25 surface via disulfide bridges.

1

2 Map4 is a protein with a high proportion of serine and threonine residues (41% in the
3 complete mature protein). It also has some tyrosine, asparagine, and glutamine residues,
4 all of which are amino acids (aa) capable of forming hydrogen bonds. In all, 49% of
5 such protein residues are able to form hydrogen bonds, which can be cleaved in the
6 presence of ethanol (Mirsky & Pauling, 1936). Map4p also has some charged residues
7 (only 9% in the overall protein, but 23% in the C-terminal domain), which are
8 potentially involved in the formation of ionic bonds. This kind of linkage can be broken
9 by either acidic or basic treatments. Additionally, low-strength bonds can be cleaved by
10 high temperatures and by SDS. In order to understand the nature of Map4 protein
11 attachment to the cell wall, cells from strain HVP1350 were induced to agglutinate by
12 incubation in EMM-N. Cells were collected and cell wall samples were obtained,
13 washed as indicated in Experimental procedures, and treated in different ways. First, in
14 order to determine the effect of temperature on Map4p extraction, cell wall samples
15 were boiled with either SDS-mercaptoethanol sample buffer or water for 15 minutes. As
16 shown in figure 4 A, sample buffer extracted Map4p completely, but hot water was also
17 able to extract part of the protein, suggesting the participation of non-covalent bonds in
18 the anchorage of Map4p to the cell wall. Secondly, we observed that Map4p could be
19 partially extracted from the cell wall by either hot SDS or hot β-mercaptoethanol (figure
20 4 B). This result could be explained in two ways: i) both compounds are required to
21 break the linkages binding Map4p to the cell wall; ii) the linkages are broken by one of
22 the chemicals but part of the protein remains trapped in the cell wall matrix. Extraction
23 of other cell wall proteins by the other chemical would allow the release of these
24 molecules of the protein.

25

1 To analyze whether ethanol was able to release the protein, hence showing that the
2 protein was attached to the cell wall by hydrogen bonds, cell wall samples were
3 incubated in the presence of either water or 35% ethanol for 3 hours at 37ºC. We
4 observed that neither water nor 35% ethanol completely released the protein from the
5 cell wall, both being much less efficient than sample buffer in extracting Map4p (figures
6 4 A and 4 C). Higher concentrations of ethanol did not release more protein (not
7 shown). Finally, in order to investigate the role of ionic bonding in Map4p attachment
8 to the cell surface, we incubated cell wall samples in the presence of 150 mM NaOH or
9 150 mM HCl for 3 hours at 37ºC. As shown in figure 4 C, NaOH was able to
10 completely extract Map4p from the cell wall, but HCl was even less efficient than water
11 in releasing the protein. With a view to understanding how sensitive the linkage binding
12 Map4p to the cell wall was to alkali treatment, we incubated cell wall samples in the
13 presence of lower concentrations of NaOH. We observed that 50mM NaOH was able to
14 extract most of the protein from the cell wall when incubation was carried out at 37ºC
15 for 3 hours. Incubation of the cell wall samples in the presence of 25mM NaOH at 37ºC
16 for 3 hours extracted Map4p efficiently, but when the sample was boiled for 15 minutes
17 in the presence of 25 mM NaOH the protein was almost completely extracted from the
18 cell wall (figure 4 D), showing that the linkages that attach Map4p to the cell wall are
19 very sensitive to alkali.
20
21 Collectively, the results described above show that Map4p is attached to the cell wall
22 through linkages that can be broken by water at mild or high temperatures, but most
23 efficiently by NaOH or SDS-β-mercaptoethanol buffers.
24
25 *Serine and threonine-rich regions are required for Map4 cell wall attachment*

1 To further characterize the Map4 protein, we constructed a series of truncated proteins
2 to determine which regions of the molecule might be required for attachment to the cell
3 wall. The truncated proteins were as follows (see Experimental procedures and figure 5
4 A): Map4(ΔBam), in which only 4 of the internal repeats are present, so this region is
5 only 144 aa long. Map4(S/T), consisting of most of the N-terminal region (it lacks the
6 last 31 aa of this region). Map4(S/T+REP), which contains the N-terminal S/T-rich
7 domain, the internal repeats, and 19 N-terminal aa from the DIPSY domain, including
8 the cysteine residues at positions 950 and 954. Map4(REP), which includes 31 aa from
9 the S/T-rich regions, the internal repeats, and 19 aa from the DIPSY domain, including
10 the C950 and C954. Map4(REP+DIPSY), which contains 31 aa from the S/T-rich
11 regions, the internal repeats, and the complete DIPSY domain. Map4(DIPSY), which is
12 devoid of the first 19 aa of the C-terminal DIPSY domain domain so it has 133 out of
13 the 152 aa of this domain. Map4(Δrep), lacking the internal repeats and containing the
14 complete S/T-rich and DIPSY domains. Map4(Δrep+S/T), in which the 324 aa
15 corresponding to the 9 internal repeats have been removed and have been replaced by
16 202 aa from the S/T region.
17
18 Cultures from strains expressing Map4 or the different truncated proteins were
19 incubated in EMM-N to induce agglutination (see Experimental procedures). In order to
20 analyze the expression and the localization of the truncated proteins we observed the
21 cultures under the microscope. As shown in figure 5 B all of them, except for
22 Map4(DIPSY), localized to the cell surface. The small Map4(DIPSY) truncation was
23 retained at the endoplasmic reticulum. Additionally, we observed that all the truncated
24 proteins, except Map4(DIPSY), were polarized at the tips of the cells. This result shows
25 that the polarization of Map4p is not determined by any particular domain of the

protein. In order to estimate the abundance of the truncated proteins, except for Map4(DIPSY), in the cell walls, protein samples were obtained from cells under agglutination conditions. The amount of protein in the total cell extracts was concentration-equalled and aliquots from all samples were used to perform an anti-Pma1 Western blotting (used as a loading control, figure 5C) or to obtain cell wall samples that were boiled with sample buffer and used to perform Western blotting using monoclonal anti-GPF antibody (figure 5 C). We observed that all the proteins were expressed in the cell, although some of them gave a signal weaker than that of Map4p. This could be due to a lower expression/stability of the proteins -probably the case for Map4(S/T) and Map4(S/T+REP)- or to decreased attachment to the cell wall (Map4(REP), Map4(REP+DIPSY), Map4(ΔRep) and Map4(ΔRep+S/T), see below). We also observed that all truncated proteins exhibited a molecular weight higher than expected (see figures 5A, 5C and 5D), showing that glycosylation was present throughout the protein.

To assess cell wall retention of the truncated proteins, 50 ml cultures that had been induced to agglutinate for 5 hours in EMM-N were centrifuged; the supernatants were collected, concentrated to 200 μl, mixed with 200 μl of 2X Laemmli sample buffer and boiled. The cells were broken and cell wall samples were obtained as indicated in Experimental procedures and extracted in a final volume of 400 μl with sample buffer. 50 μl from each sample was loaded into 4-20% gradient gels. Thus, for each truncated protein, the amount of sample from the culture medium or from the cell wall came from the same volume of the original culture and could therefore be compared directly. As shown in figure 5 D, the Map4 protein was cell wall-attached in the control strain and in strains bearing the Map4(ΔBam), Map4(S/T), and Map4(S/T+REP) truncations. All

these proteins share the presence of the S/T-rich region. In the Map4(Δrep) and Map4(Δrep+S/T) truncations, lacking the internal repeats, a certain amount of protein was detected in the culture medium. Finally, most of the protein was detected in the culture media in the case of the strains carrying the Map4(REP) and Map4(REP+DIPSY) truncations, lacking the S/T-rich domain. These results show that the N-terminal S/T region is the part of Map4p that contributes most significantly to the attachment of the protein to the cell surface, and that the internal repeats also participate in this attachment.

The C-terminal DIPSY domain and cysteine residues are required for Map4 function

In order to gain information about the region(s) of Map4p required for the functionality of the protein, we determined the agglutination index and the mating efficiency (in liquid and solid media, respectively, see Experimental procedures) of strains carrying different mutated versions of the protein. We observed that no mating aggregates were formed in the cultures of cells carrying the Map4(S/T), Map4(S/T+REP), Map4(REP), Map4(REP+DIPSY) and Map4(DIPSY) truncated proteins. Additionally, as shown in figure 6 A, in these cultures the agglutination index (AI) was similar to that of the strain used as a negative control (see Experimental procedures for details). In the cultures from cells bearing the Map4(ΔBam), Map4(Δrep) and Map4(Δrep+S/T) truncations mating aggregates were detected. However, while in the cultures from the strain carrying the Map4(ΔBam) truncation the AI was fairly similar to that of the positive control strain (85% respect to 100%), in the case of the strains with the Map4(Δrep) and Map4(Δrep+S/T) versions of Map4 the aggregates were smaller and the AIs (65% and 60%, respectively) were significantly lower than in the control strain. To analyse

1 whether this partial function of the proteins lacking the internal repeats could be
2 improved with longer incubation times, the strains carrying the Map4(ΔBam),
3 Map4(Δrep) and Map4(Δrep+S/T) truncations were allowed to agglutinate for 8.5 hours
4 instead of 5 hours. Samples were taken along the experiments and the AI was compared
5 with that of the positive and negative controls and the strain carrying the Map4(REP)
6 truncation. As seen in the figure 6 B, the proteins lacking repeats were able to promote
7 agglutination. However, the extent of the agglutination was reproducibly lower than that
8 of the positive control, being higher in the case of the protein with more repeats.
9 Substitution of the internal repeats by a fragment of the S/T-rich unstructured region did
10 not result in a higher functionality of the protein. Even after incubation for 16 hours the
11 truncated proteins did not promote agglutination as efficiently as the Map4 protein (not
12 shown).
13
14 The above results showed that the proteins that were cell wall-attached but lacked the
15 DIPSY domain -Map4(S/T) and Map4(S/T+REP)- were not functional. This result
16 indicated that the C-terminal domain of Map4p was required for its agglutination
17 activity. In order to confirm this, we produced a full-length protein in which the
18 signature motif of the domain (the Asp-Ile-Pro-Ser-Tyr aa; see figure 5 A for
19 information about the position of these amino acids) was eliminated. This mutated
20 protein was termed Map4-dipsy. As shown in figure 6 C, this mutated protein was
21 unable to promote agglutination even though it was completely retained in the cell wall
22 (figure 6 D).
23
24 We also wished to investigate whether any of the six cysteine residues present in Map4
25 might play some role in functionality. To do so, we determined the AI in cultures from

1 cells carrying the Map4C77, Map4C124, Map4C950, Map4C954, Map4C1008,
2 Map4C1009, Map4C77-124, Map4C950-954-1008-1009 and Map4C77-124-950-954-
3 1008-1009 mutated proteins. As shown in figure 6 C, only the Map4C77 protein was
4 functional, showing that the cysteine at position 124 and the four cysteine residues in
5 the DIPSY domain were required for function. All these mutated versions of Map4p
6 were attached to the cell surface (figure 3 and results not shown).
7
8 We had previously found that cells lacking the *map4*$^{+}$ gene were defective in
9 agglutination in liquid medium and also in mating on plates (Sharifmoghadam *et al.*,
10 2006). In order to determine whether both functions depended on the same elements of
11 the protein or whether they were independent, we determined the mating efficiency of
12 cells carrying the different mutated Map4 proteins. As shown in figures 6 A and 6 C,
13 the ability of the different forms of Map4p to promote agglutination was fully correlated
14 with the ability of each protein to mediate zygote formation on plates. These results
15 therefore suggested that both functions depended on the same elements of the protein.
16
17 **Discussion**
18 In this work we have further characterized Map4 protein, an *h*$^{+}$-specific mating adhesin
19 from *S. pombe* (Mata & Bahler, 2006, Sharifmoghadam *et al.*, 2006). Treatment of cell
20 walls with Endoglycosidase H and expression of the protein in the *oma1Δ* and *oma4Δ*
21 mutants revealed that Map4p is N- and O-glycosylated. The level of Map4p detected by
22 Western blotting was lower in the mutants than in the control strain, in agreement with
23 the notion of O-mannosylation being required for the stability of some proteins (Weber
24 *et al.*, 2004). The results (figure 1 B) demonstrated that O-glycosylation contributes to
25 the high electrophoretic mobility of the Map4 protein. Protein modification was almost

1 completely lost when the protein produced in the *oma4Δ* mutant was treated with
2 EndoH. This result indicated that although Oma1p can participate in the process Oma4p
3 is the main O-mannosyl transferase for Map4p. It has been shown that the *S. cerevisiae*
4 Fus1 protein is not properly modified in the *ogm4Δ/oma4Δ* mutant (Tanaka *et al.*,
5 2005), but no endogenous substrate has been described for this mannosyl transferase. In
6 the budding yeast, the *PMT4* O-mannosyl transferase recognises S/T-rich membrane
7 proteins as a substrate (Hutzler *et al.*, 2007). The fission yeast Oma4p seems to have
8 different substrates since Map4p is an S/T-rich protein but is associated with the cell
9 wall. The Map4 protein extracted from the *omaΔ* mutants and treated with EndoH, had
10 a lower molecular weight than expected (figure 1 B). However, a specific processing by
11 Kex2-type proteases could be ruled out (results not shown and figure 1 B). In *S.*
12 *cerevisiae,* O-mannosylation controls N-glycosylation and also the proper processing of
13 some proteins (Ecker *et al.*, 2003, Lommel *et al.*, 2004). It is possible that some aberrant
14 processing of Map4p could occur in the *omaΔ* mutants. The low abundance of Map4p,
15 its altered O-glycosylation, its aberrant processing, or all these processes together,
16 probably contribute to the lack of agglutination in the *oma1Δ* and *oma4Δ* mutants
17 (Tanaka *et al.*, 2005 and our unpublished observations)
18
19 All fungal adhesins described to date are proteins covalently bound to the cell wall (see
20 Lipke & Kurjan, 1992, Mrsa *et al.*, 1997, Brandhorst & Klein, 2000, Hoyer, 2001, De
21 Groot *et al.*, 2004, De Groot *et al.*, 2005, Yin *et al.*, 2005, Ecker *et al.*, 2006, Klis *et al.*,
22 2006, De Groot *et al.*, 2007, Dranginis *et al.*, 2007). With the exception of the Bad1
23 adhesin from *B. dermatitidis*, which is bound to chitin (Brandhorst & Klein, 2000),
24 adhesins are linked to β-glucan. In a comprehensive analysis of the *S. pombe* cell wall
25 proteins it has been found that treatment with β(1,6)-glucanase releases very little

protein but β(1,3)-glucanase and mild alkali solutions are able to extract some proteins from the cell wall (De Groot *et al.*, 2007). Map4 is not released from the sample buffer-extracted cell wall by either β(1,6)-glucanase, β(1,3)-glucanase or mild alkali solutions. Since there is no chitin in the cell wall of *S. pombe* (Sietsma & Wessels, 1990, Arellano *et al.*, 2000), the most plausible explanation for these results is that Map4p would be linked to other cell wall proteins. We found that the protein could be completely released from the cell walls with hot SDS-mercaptoethanol buffer or with mild NaOH solutions and that the cysteine residues present in the protein are not required for cell wall attachment. With the data in our hands we cannot determine the nature of the linkages between the Map4 protein and the cell wall. However, our results did show that Map4p is attached to the cell wall in a different way to all previously described adhesins.

We also addressed the role of the different regions of Map4 in cell wall attachment and function. We found that the Map4 S/T-rich region is required for cell wall attachment and that the C-terminal DIPSY domain is the adhesin-binding domain. Additionally, elimination of each of the four cysteine residues from this domain abrogated its functionality, suggesting the participation of disulfide bridges in the binding between Map4 and Mam3, the h^--specific agglutinin (Mata & Bahler, 2006). This situation, in which the functional domain is located at the C-terminal end of the protein, is the reverse of that of most known adhesins (Lipke *et al.*, 1989, Wojciechowicz *et al.*, 1993, Chen *et al.*, 1995, de Nobel *et al.*, 1996, Shen *et al.*, 2001, Frieman *et al.*, 2002, Huang *et al.*, 2003, Rauceo *et al.*, 2006). An exception to this general characteristic of adhesins is the *B. dermatitidis* Bad1 adhesin (Brandhorst *et al.*, 2003).

Regarding the role of the internal repeats, we found that they contribute to cell wall attachment. In this respect, Map4 appears be similar to the *Saccharomyces* PIR proteins (Castillo *et al.*, 2003, Sumita *et al.*, 2005, Ecker *et al.*, 2006) and the *B. dermatitidis* Bad1 adhesin (Brandhorst *et al.*, 2003). However, we found that they also play a role in modulating activity. A role of the internal repeats in adhesion has been reported for *C. albicans* Als5 (Rauceo *et al.*, 2006). In the case of the *C. albicans* Eap1p, the tandem repeats mediate adhesion to polystyrene but they also seem to help to project the N-terminal binding domain to the extracellular environment (Li & Palecek, 2008). The repeats in the *C. glabrata* Epa1p and some sequences in the central region of the *S. cerevisiae* Aga1p are required for function because they provide the protein with the length required to expose the functional domain outside the cell wall (Frieman *et al.*, 2002, Huang *et al.*, 2003). We replaced the central region of the protein by part of the S/T-rich region. Although the Map4(Δrep+S/T) protein is longer than the Map4(ΔBam), it is less functional, its behaviour resembling that of the Map4(Δrep) protein. This result shows that the internal repeats do not act merely as spacers to expose the DIPSY domain to the surface.

A model with a hypothetical organization of Map4p in the cell wall is shown in figure 7. The unstructured S/T region would be included in the cell wall, bound to different proteins of the cell wall matrix. The cysteine at position 124 would be close to the internal side of the cell wall, where it could bind a protein to transmit a signal to the cell interior so that a more stable cell-cell contact, corresponding to the copulation phase of mating (Calleja *et al.*, 1977), can be established. The internal repeats would also be bound to the cell wall matrix but would be accessible for binding Mam3p. Finally, the DIPSY domain would be exposed at the cell surface.

A striking feature of the Map4 protein is the presence of two Arg-Gly-Asp (RGD) domains (see figure 5 A). RGD are integrin-ligating motifs present in human adhesion molecules such as fibrillin and fibronectin (Salsmann *et al.*, 2006). The presence of these domains in a yeast adhesin drew our attention, and we therefore eliminated each of these domains separately and both of them simultaneously. We found that neither of the domains had any obvious role in the anchoring or function of the protein (results not shown).

In sum, we have found that Map4 agglutinin has characteristics that differentiate it from the well-characterized adhesins. Overall, the Bad1 adhesin from the pathogenic fungus *Blastomyces dermatitidis* is the one most similar to Map4p. Both share the presence of the functional domain at the C-terminal end of the protein and are attached to the cell wall through some as yet uncharacterized mechanism (Brandhorst & Klein, 2000, Brandhorst *et al.*, 2003, Sharifmoghadam *et al.*, 2006, this study). A computer-assisted analysis of the *S. pombe* genome carried out by Linder and Gustafsson (Linder & Gustafsson, 2007) has predicted the existence of new families of adhesins in *S. pombe* and other Ascomyceta. Our results confirm that the computer-predicted DIPSY domain is indeed a functional domain, which validates Linder and Gustafson's analysis of the C-terminal ends of these proteins. Additionally, in a recent study it has been shown that closely related adhesins show different carbohydrate-binding specificities (Zupancic *et al.*, 2008). Therefore, the appealing possibility of finding new biological functions for computer-predicted adhesins remains open.

Experimental procedures

1 *Strains and growth conditions*

2 All strains used are derivatives of HVP1214 (h^{90} *map4::ura4*$^{+}$ *leu1-32 ura4D18 ade6*.
3 Sharifmoghadam *et al.*, 2006). This strain was transformed with the integrative vector
4 pJK148 to create strain HVP1461 (a negative control for the experiments), with the same
5 vector carrying the *map4*$^{+}$ gene (strain HVP1619, a positive control) or the HA- or GFP-
6 fused version of the gene (strains HVP1428 or HVP1350, respectively; Sharifmoghadam
7 *et al.*, 2006). The media used to grow the strains were YES (rich medium) and EMM
8 (Edinburgh minimal medium) with supplements and the strains were mated on plates of
9 EMM plus supplements (Moreno *et al.*, 1991). EMM-N was EMM without NH_4Cl. All
10 general techniques have already been described (Sambrook *et al.*, 1989, Moreno *et al.*,
11 1991; http://www.biotwiki.org/bin/view/Pombe/NurseLabManual).

12

13 *Mating analysis*

14 Mating efficiency was quantified as the number of zygotes plus asci with respect to the
15 total cell number (zygotes plus asci plus vegetative cells; Arellano *et al.*, 2000).
16 Agglutination tests were carried out as follows. $2x10^{8}$ cells, grown overnight in EMM
17 with supplements, were washed three times with water and inoculated in 10 ml of EMM-
18 N (minimal medium without nitrogen; Moreno *et al.*, 1991). Cultures were incubated at
19 25ºC with gentle shaking for 5 hours in 100-ml flasks. Then, the cultures were transferred
20 to 10 ml centrifuge tubes and were allowed to sediment on the bench for three minutes.
21 After this time, a sample was withdrawn from the central part of the tube, diluted and
22 used to estimate OD at 600 nm. All experiments were performed a minimum of three
23 times. The agglutination index (AI) was calculated as AI = $1/OD_{600}$. Unless stated, the
24 final values in the figures represent the percentage of each value with respect to the value
25 of the positive control, which was given a value of 100%.

1

2 *Molecular and genetic manipulations*

3 Plasmid KS+*map4*$^{+}$(*Apa*IATG *Not*ISTOP. Sharifmoghadam *et al.*, 2006) was used as a
4 template to perform site-directed mutagenesis by a previously described method (Kunkel
5 *et al.*, 1987). DNA sequencing was used to confirm the accuracy of the sequence in the
6 alleles constructed. Nucleotide sequences were analysed using the DNASTAR and
7 Clonemap programs. Then, the mutated DNA fragments were cloned in a modified
8 pJK148 vector (lacking the *Not*I restriction site) and digested with the *Nsi*I enzyme to
9 integrate them in the *map4*$^{+}$ promoter. In all cases, the 8xA-GFP green fluorescent
10 protein coding sequences (Sharifmoghadam *et al.*, 2006) were introduced before the stop
11 codon. Oligos C77(BglII), C124(Spe)F3, C950(Pst)WT, C954(PvuII), C1008(Pml) and
12 C1009(Cla) (see List of oligos in Supplemental material) were used to eliminate the
13 Map4 cysteine residues at amino acid positions 77, 124, 950, 954, 1008 and 1009,
14 respectively. Oligo C950Pst(954) was used to eliminate the cysteine at position 950 in a
15 mutant allele lacking the cysteine at position 954. Oligo C1008C1009(Hpa) was used to
16 eliminate cysteine residues at positions 1008 and 1009 simultaneously. Oligo21D-dipsy
17 was used to eliminate the Asp-Ile-Pro-Ser-Tyr signature aa in the DIPSY domain. Oligos
18 RGD1 and RGD2 were used to replace the RGD motif by ASI. Finally, oligos
19 21DKKnhe-f2 and 21DKRSpe-F were used to eliminate the KK and KR sequences,
20 potentially cleavable by Kex2-type proteases.

21

22 Different truncated proteins were created as follows. Map4(ΔBam) is a truncated version
23 of the protein lacking 5 of the internal repeats. It was created by digesting the
24 KS+*map4*$^{+}$(*Apa*IATG *Not*ISTOP) plasmid with *Bam*HI and religating. To create the
25 Map4(S/T) truncation, which includes from aa 1 to aa 586, a restriction site for the *Not*I

1 enzyme was created at position 1758 of the ORF on the KS+*map4*$^+$(*Apa*IATG
2 *Not*ISTOP) plasmid using oligo 21DNot3048. Then, the *Not*I-*Not*I DNA fragment was
3 eliminated by digestion with *Not*I and religation. The Map4(S/T+REP) truncation, which
4 includes aas 1 to 959, was produced by creating a *Not*I site at position 2877 using oligo
5 21DNot4167. Then, the *Not*I-*Not*I DNA fragment was eliminated. To create the
6 Map4(REP) truncation, which carries from aa 1 to aa 37 plus from aa 587 to aa 959, the
7 plasmid carrying the Map4(S/T+REP) truncation was used as a template to create
8 restriction sites for the enzymes *Apa*I and *Nde*I after nucleotide 1758 (oligo
9 21DApNd3048). Then, the *Apa*I-*Apa*I DNA fragment was eliminated. Finally, aa 1-37,
10 which include the signal peptide and had been PCR-amplified using oligos
11 21DApa(ATG) and 21DSigPep, were cloned as an *Apa*I-*Nde*I DNA fragment.
12 Map4(REP+DIPSY) is a truncation including aa 1 to aa 37 plus aa 587 to aa 1092. It was
13 constructed by creating restriction sites for the enzymes *Apa*I and *Nde*I after nucleotide
14 1758 (oligo 21DApNd3048) in the KS+*map4*$^+$(*Apa*IATG *Not*ISTOP) plasmid. Then, the
15 *Apa*I-*Apa*I DNA fragment was eliminated and the signal peptide was introduced as
16 described above. Map4(DIPSY), which includes aa 1 to aa 37 plus aa 960 to aa 1092 was
17 constructed by creating *Apa*I and *Nde*I sites after nucleotide 2877 (oligo 21DApNd4167)
18 in the KS+*map4*$^+$(*Apa*IATG *Not*ISTOP) plasmid. Then, the *Apa*I-*Apa*I DNA fragment
19 was eliminated and the signal peptide was introduced. Map4(Δrep), which includes aa 1
20 to 610 plus aa 945 to aa 1092, was created as follows. In order to eliminate only the
21 internal repeats, restriction sites for *Sph*I were created after nucleotides 1830 and 2835.
22 Then, the *Sph*I-*Sph*I DNA fragment was eliminated by digestion with *Sph*I and plasmid
23 religation. To create the Map4(Δrep+S/T) truncation, a DNA fragment corresponding to
24 nucleotides 1243 to 1849 from the ORF was PCR-amplified using oligos S/TSph-5' and
25 S/TSph-3', which introduced *Sph*I sites at both ends of the fragment. This fragment was

1 cloned at the *Sph*I site in the Map4(Δrep) truncation plasmid. Orientation of the insert
2 was assessed by restriction analysis and was confirmed by sequencing.
3
4 *Microscopy*
5 A Leica DM RXA microscope equipped with a Photometrics Sensys CCD camera using
6 the Qfish 2.3 program was used to perform the microscopy analyses.
7
8 *Protein analysis*
9 As a general procedure, cells from 50-ml cultures (about 10^9 cells) were collected by
10 centrifugation after 5 hours of incubation in EMM-N with gentle shaking in 500-ml
11 flasks. Cells were washed with Buffer B (50 mM Tris HCl, pH 7.5; 50 mM EDTA; 150
12 mM NaCl) supplemented with protease inhibitors (1 mM PMSF; 1 μg/ml Aprotinin,
13 Leupeptin and Pepstatin) and broken in the same buffer in a FastPrep (Savant). Total
14 protein was estimated using the *Biorad protein assay kit* (Bradford method). Cell extracts
15 were adjusted to the same protein concentration with Buffer B and centrifuged (5 minutes
16 at 3000 rpm) to separate cell walls from the rest of the cell extracts. Cell walls were
17 washed twice with 1 ml of 2M NaCl and 3 times with 1 ml of Buffer B. Then, they were
18 boiled in a final volume of 400 μl of Laemmli sample buffer (50 mM HCl-Tris, pH 6.8;
19 1% SDS; 143 mM β-mercaptoethanol; 10% glycerol). Depending on the purpose of the
20 experiment, the cell walls were treated in different ways. To determine protein extraction
21 by hot reducing agents, SDS, or both, cell wall samples were boiled for 10 minutes in the
22 presence of 143 mM β-mercaptoethanol in 50mM Tris pH 6.8, 1% SDS in 50 mM Tris
23 pH 6.8 or Laemmli sample buffer (containing both 143 mM β-mercaptoethanol and 1%
24 SDS in 50 mM Tris pH 6.8). Protein extraction with different concentrations of NaOH,
25 HCl, and ethanol, or with water, was performed by resuspending the washed cell walls in

200 μl of these solutions and incubating them for 3 hours at 37ºC with shaking. In all cases, after incubation in the corresponding compound the samples were centrifuged; the supernatants were transferred to clean tubes, brought up to a final volume of 400 μl with 2X sample buffer, and boiled. The pellets were washed three times with buffer B, brought up to a final volume of 400 μl in sample buffer, and boiled. 50 μl from each sample was loaded into 4-20% acrylamide gels.

In order to check whether Map4 was linked to the β-glucan, samples were extracted twice consecutively for 10 minutes with sample buffer, washed twice with Buffer A (50 mM Tris HCl, pH 7.5; 50 mM EDTA; protease inhibitors), and twice with the corresponding reaction buffer (see below). For β(1,3)-glucanase treatment, the cell wall samples were incubated with 100 units of Quantazyme (Q-Biogene) in the presence of 50 mM Tris HCl, pH 7.5; 40 mM β-mercaptoethanol and protease inhibitors for 16 hours at 37ºC with shaking. β(1,6)-glucanase digestion was performed using 10 μl of recombinant enzyme (Cabib & Duran, 2005) in the presence of 50 mM sodium acetate, pH 5.2, and protease inhibitors for 16 hours at 37ºC with shaking. In the case of the *S. cerevisiae* Aga1-HA protein, cells from strain YSE1092 were treated with α-factor as decribed (Huang *et al.*, 2003), and cell wall samples were obtained and treated as described above for Map4p. For NaOH extraction, the cell walls, extracted with sample buffer as described above, were washed twice with buffer A, resuspended in 40 mM NaOH, and incubated for 16 hours at 4ºC with shaking. In all cases, samples were centrifuged and the supernatant was boiled in sample buffer.

For endoglycosidase H (EndoH) treatment, cell walls were washed in Buffer A (see above), resuspended in 100 μl of denaturation buffer (0.2%SDS, 0.1M β-

1 Mercaptoethanol) and boiled for 10 minutes. Then, the samples were centrifuged and the
2 supernatants were divided into 2 sets of 50 μl. To one set (EndoH -) 10 μl of 10X EndoH
3 buffer (500 mM Sodium Acetate pH 5.2) and 40 μl of denaturing buffer were added. To
4 the other set (EndoH +) 10 μl of 10X EndoH buffer, 25 μl of denaturing buffer and 15 μl
5 endoglycosidase H (0.075 U endoglycosidase H from *Streptomyces plicatus*; Roche)
6 were added. All samples were incubated in a tube rotator for 36 hours at 37ºC. At the end
7 of the treatment they were boiled for 4 minutes.
8
9 When we wished to determine the attachment to the cell wall of some variants of Map4p,
10 cells carrying these variants fused to the GFP were induced to agglutinate as described
11 above. Cultures were centrifuged; the supernatants (corresponding to the culture media)
12 were concentrated to a volume of 200 μl using Amicon Ultra-15 (ultracel 10K,
13 Millipore); 200 μl of 2X Laemmli sample buffer was added, and the samples were boiled
14 for 4 minutes. The cells were broken and cell wall samples were obtained as described
15 above and boiled in a final volume of 400 μl in the presence of sample buffer. For each
16 mutated Map4 protein, 50 μl from the culture medium and 50 μl from the cell wall
17 sample were loaded in the gradient gel. Thus, the amount of protein loaded in the gel
18 corresponded to the culture medium or the cell walls from 6.25 ml of the culture, which
19 allowed us to determine how much of the synthesised protein was released to the medium
20 or remained attached to the cell wall.
21
22 The samples were loaded into 4-20% gradient gels (Biorad), transferred to Immobilon-P
23 membranes (Millipore) in Tris-Glycine buffer, and decorated with monoclonal anti-HA
24 (12C5A, Roche; 1:4000 dilution), anti-GFP (JL8, Nucliber; 1:500) or polyclonal anti-

1 Pma1 (1:5000) antibodies. Secondary antibodies (anti-mouse or anti-rabbit; 1:10000) and
2 ECL Advanced (Amersham) were used to develop the blots.
3

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12

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26

Figure legends

Figure 1- Map4p is an N- and O-glycosylated protein. (A) Western blot detection of Map4 protein. Strains HVP1461, HVP1350, or HVP1428 (*h^{90} map4::ura4^{+}* carrying an empty vector, a GFP-fused or an HA-tagged Map4 protein, respectively) were induced to agglutinate for 5 hours. 50 µg of protein from the whole-cell samples (Cells) from the three strains, or cytosolic (Cyt) and cell wall (CW) samples from strains HVP1350 and HVP1428 were detected by Western blotting using anti-GFP (left hand side of upper panel) or anti-HA (right hand side of upper panel). As a loading control, 50 µg of protein from the cytosol samples from the three strains were loaded into a gel and decorated with anti-Pma1p antibody (lower panel). (B) Map4p is N-glycosylated and O-mannosylated. The control strain (*h^{90} map4::ura4^{+}* carrying an integrative plasmid with the Map4-GFP fusion protein), the *oma1Δ* mutant carrying the Map4-GFP construct or the *oma4Δ* mutant carrying the Map4-GFP or the $Map4_{kkkr}$-GFP proteins were induced to agglutinate. Cell wall samples were treated (EndoH +) or not (EndoH -) with endoglycosidase H, as explained in Experimental procedures, denatured and run into a 4-20% polyacrylamide gradient gel. Anti-GFP was used to detect Map4-GFP. The lanes corresponding to the mutant strains were overexposed with respect to the lanes corresponding to the control strain (1 minute exposure for the control strain and 5 minutes exposure for the mutant strains). In all cases, samples were extracted and denatured in SDS/mercaptoethanol sample buffer.

Figure 2- Map4 protein cannot be released from extracted cell walls by either β-glucanases or by mild alkali treatment. (A) Cell walls from strain HVP1350 (carrying the Map4-GFP protein) were washed and boiled with hot SDS-Mercaptoethanol buffer.

1 The supernatant was loaded into the gel (lane SB). Pellets were extracted again in the
2 same way, washed, and incubated for 16 hours in the presence of β(1,3)-glucanase,
3 β(1,6)-glucanase (lanes +), or the corresponding buffer (lanes -). After incubation, the
4 samples were centrifuged and the supernatants were denatured and loaded into the gel.
5 (B) As a control for the glucanase treatments, *S. cerevisiae* cell walls were obtained
6 from strain YSE1092, carrying an Aga1-HA protein, and treated in the same way as
7 described above. (C) Cell wall samples were obtained as before. The samples loaded in
8 the gel correspond to the supernatants obtained after extraction with sample buffer (SB)
9 and subsequent incubation in the presence of water (-) or 40 mM NaOH (+). Proteins
10 were detected by Western blotting using monoclonal anti-GFP (A and C) or anti-HA
11 (B) antibodies.
12
13 Figure 3- Map4 mutant proteins lacking cysteine residues are cell wall-attached. Anti-
14 GFP Western blot analysis of the Map4 wild-type protein or proteins in which the
15 cysteine residues at the indicated positions had been eliminated. In all cases, the amount
16 of protein concentrated from the culture medium (M) or extracted from the cell walls
17 (W) loaded into the gels came from the same volume of the original culture (see
18 Experimental procedures) and can therefore be compared directly. Samples were
19 extracted and denatured in sample buffer. As a loading control, a sample of cell extracts
20 from each strain was loaded into a gel and decorated with anti-Pma1p antibody (lower
21 panel).
22
23 Figure 4. Extraction of Map4p from whole cell walls with different compounds. (A)
24 Cell walls were boiled in water or SDS-mercaptoethanol sample buffer (SB; see
25 Experimental procedures). (B) Cell walls were boiled in SDS-mercaptoethanol buffer,

1 SDS or β-mercaptoethanol. (C) Cell walls were incubated at 37ºC for 3 hours in the
2 presence of water, 35% ethanol (EtOH), 150 mM NaOH, or 150 mM HCl. (D) Cell
3 walls were incubated at 37ºC for 3 hours or at 100ºC for 15 minutes in the presence of
4 the indicated concentrations of NaOH. In all cases, the samples were centrifuged and
5 the supernatants were transferred to fresh tubes. Pellets were washed and then the
6 supernatants (SN) and pellets (P) were boiled in sample buffer, loaded into the gels, and
7 decorated with anti-GFP antibody.
8
9 Figure 5. Cell wall attachment of the different Map4 truncated proteins. (A) Diagram
10 representing the different domains in the Map4 wild-type protein and the indicated
11 truncations. The black-filled box, grey-filled box, striped box and hatched box represent
12 the signal peptide, the S/T-rich unstructured region, the internal repeats, and the DIPSY
13 domain, respectively. The open box represents the GFP preceded by the 8 alanine
14 residues. The circles mark the relative position of the cysteine residues; the white
15 triangle represents the DIPSY signature motif, the asterisks represent the sequences
16 potentially recognised by Kex2-type proteases, and the black triangles represent the
17 RGD motifs. The molecular weight (MW) of each mature protein, fused to the 8xA-
18 GFP, is given in kDa. (B) Micrographs of cells carrying the Map4 (1), Map4(ΔBam)
19 (2), Map4(S/T) (3), Map4(S/T+REP) (4), Map4(REP) (5), Map4(REP+DIPSY) (6),
20 Map4(DIPSY) (7), Map4(ΔRep) (8) or Map4(ΔRep+S/T) (9) proteins fused to the GFP,
21 under conditions of agglutination. (C) Upper panel: Anti-GFP Western blot analysis of
22 the indicated proteins fused to the GFP. Lower panel: anti-Pma1p Western blot analysis
23 of cell extracts from the same cells, used as a loading control. (D) Upper panel: Anti-
24 GFP Western blot. In all cases the amount of protein concentrated from the culture
25 medium (M) or extracted from the cell walls (W) that were loaded into the gels came

1 from the same volume of the original culture (see Experimental procedures) and can
2 therefore be compared directly. Lower panel: anti-Pma1 Western blot of cell extracts
3 from the same cultures, used as a loading control. In all cases, samples were extracted
4 and denatured in sample buffer.
5
6 Figure 6. Analysis of the elements of the Map4 protein required for function. (A)
7 Agglutination Index (AI) or Mating Efficiency (ME) in strains carrying a deletion in the
8 *map4*$^{+}$ gene and transformed with an empty integrative plasmid (*map4Δ*), an integrative
9 plasmid carrying the *map4*$^{+}$ gene (*map4*$^{+}$), or the indicated truncated alleles of the gene.
10 See Experimental procedures for details. Standard deviation is indicated for each value (
11 ┤). (B) Analysis of the agglutination process versus time in strains carrying the wild-
12 type *map4*$^{+}$ gene, the empty vector, or the indicated truncated alleles of *map4*$^{+}$. Open
13 symbols depict strains that did not agglutinate during the experiment, and closed
14 symbols indicate strains able to agglutinate. Arrows indicate the first time-point at
15 which aggregates could be observed in the cultures. Numbers represent the inverse of
16 the optical density (read at 600 nm) of a sample taken from the supernatant (see
17 Experimental procedures). A representative experiment is shown. The experiment was
18 performed three times and similar results were obtained. (C). Analysis of the
19 Agglutination Index (AI) or Mating Efficiency (ME) in strains carrying mutations
20 aimed at eliminating the DIPSY signature motif or the indicated cysteine residues.
21 Standard deviation is indicated for each value (┤). (D) Cell wall attachment of Map4 or
22 Map4-dipsy proteins. Upper panel: Anti-GFP Western blot. In both cases, the amount of
23 protein concentrated from the culture medium (M) or extracted from the cell walls (W)
24 that were loaded into the gels came from the same volume of the original culture (see
25 Experimental procedures) and can therefore be compared directly. Lower panel: anti-

Pma1 Western blot of cell extracts from the same cultures, used as a loading control. Samples were extracted and denatured in sample buffer.

Figure 7- Model of the possible distribution of Map4p in the cell wall. Filled line: S/T unstructured region; striped line: S/T-rich repeats; hatched line: DIPSY domain. The black circles indicate the position of the cysteine residues and the white triangle represents the DIPSY signature domain. The regions of the protein necessary for optimal cell wall (CW) attachment or adhesion are indicated.