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The fission yeast Map4 protein is a novel adhesin required for mating

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Abstract **Cell adhesion is required for many cellular processes. In fungi, cell–cell contact during mating, flocculation or virulence is mediated by adhesins, which typically are glycosyl phosphatidyl inositol (GPI)-modified cell wall glycoproteins. Proteins with internal repeats (PIR) are surface proteins involved in the response to stress. In *Schizosaccharomyces pombe* no adhesins or PIR proteins have been described. Here we study the *S. pombe* Map4p, which defines a new class of surface protein that is not GPI-modified and has a serine/threonine rich domain and internal repeats that differ from those present in PIR proteins. Map4p is a mating type-specific adhesin required for mating in h^+ cells and enhances cell adhesion when overexpressed.**

Keywords: Cell adhesion; Mating; Cell wall proteins; *Schizosaccharomyces pombe*

1. Introduction

Fungal adhesins, which include sexual agglutinins, virulence factors, and flocculins, are surface proteins that mediate cell–cell and cell–environment interactions. The best characterised adhesins belong to *Saccharomyces cerevisiae* or *Candida* [4,6,7,10]. They are relatively large proteins rich in serine and threonine residues; are extensively glycosylated; present some repeated domains, and are maintained on the cell surface through a plasma membrane- or a cell wall-linked glycosyl phosphatidyl inositol (GPI) anchor [10]. In *S. cerevisiae*, Aga1p-Aga2p and Agα1p agglutinins (a- and α-specific, respectively) are required for agglutination during mating [10]. Aga1p has two highly similar domains separated by a serine/threonine-rich heptapeptide repeat and Aga2p is a 69-amino acid polypeptide that binds to Aga1p through a pair of disulfide bridges [8]. Agα1p is a glycoprotein with a N-terminal domain that shows similarity to the immunoglobulin fold sequences found in some mammalian adhesion proteins [20]. Agglutinin-like and Epa proteins from *Candida albicans* and *C. glabrata* (coded by the *ALS* and *EPA* genes, respectively) are large proteins involved in cell adhesion to host surfaces [6,7]. Als proteins show a conserved N-terminal domain, a central region with variable numbers of tandemly repeated copies of a 36-amino-acid motif, and a serine/threonine rich C-terminal domain (Pfam database: http://www.sanger.ac.uk/Software/Pfam/, and [7]). Epa1p exhibits an N-terminal ligand-binding domain and a serine/threonine-rich region [6].

A less abundant and less characterised group of cell wall proteins are the protein with internal repeats (PIR) proteins, which are not GPI-modified. They present a signal peptide, a domain with tandem repeats of amino acids that include several glutamines, and a conserved carboxyl end that exhibits 4 cysteine residues following a pattern of c-x(66)-c-x(16)-c-x(12)-c [4]. In some cases, they have been shown to be processed at the amino terminal end by the Kex2 protease [3]. In *C. albicans* the putative PIR proteins do not have proper Kex2 sites [4].

In fission yeast only one GPI-anchored protein has been characterized biochemically [15] and no PIR proteins have been found. Therefore, the characterization of particular cell wall proteins from *Schizosaccharomyces pombe* is interesting in terms of analysis of the data annotated in the genome project, but also in terms of comparative studies with cell surface proteins from other organisms.

In *S. pombe*, the mechanisms leading to agglutination and cell fusion during mating are almost unknown. Yamamoto et al. proposed that the *map4*$^+$ gene coded for a putative P-specific agglutinin [21] but that gene was never characterised. A systematic analysis directed to the study of gene expression during sexual differentiation was carried out [11], and the genes were grouped according to their time of expression. The SPBC21D10.06c open reading frame (ORF) belongs to the group of genes involved in the first steps of mating. The corresponding protein was described as a surface glycoprotein. We cloned this gene in order to investigate whether it played a role in mating. During the development of this work we knew that the SPBC21D10.06c ORF corresponded to the *map4*$^+$ gene (http://www.sanger.ac.uk/Projects/S_pombe/). *map4*$^+$ codes for a PIR that lacks a potential GPI anchor. Map4p has unique structural properties that differentiate it from other cell wall proteins. Here we show that Map4p is an adhesin required for mating in h^+ cells, and that a high expression of *map4*$^+$ enhances cell–cell contact.

2. Materials and methods

2.1. Strains and growth conditions

The strains used were HVP281 (h^{90} *leu1-32 ura4D18 ade6*), HVP30 (h^- *leu1-32 ura4D18 ade6 his3D1*), HVP117 (h^+ *leu1-32 ura4D18 ade6 his3D1*) and HVP289 (h^- *cyr1::ura4*$^+$ *sxa2::ura4*$^+$ *leu1-32 ade6*). These strains were grown on YES medium or EMM with supplements

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Abbreviations: GFP, green fluorescent protein; ORF, open reading frame; PCR, polymerase chain reaction; WT, wild-type; GPI, glycosyl phosphatidyl inositol; PIR, protein with internal repeats

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and mated on SPA plates [14]. Quantitative mating experiments were carried out as described [1]. All general techniques have already been described [14,18].

2.2. Agglutination analysis

Agglutination in the mutant strains was estimated as follows. 10^8 cells from each parental strain, growing exponentially in EMM with supplements, were mixed, washed three times with water, and inoculated in 10 ml of EMM-N. EMM-N was EMM without ammonium chloride [14]. Cultures were incubated at 25 °C with gentle shaking overnight. At the end of this mating time, a 1 ml-sample from each cross was fixed with formaldehyde for 1 h at room temperature. After fixation, OD at 600 nm from upper part of the samples was estimated. All samples were studied in triplicate. The agglutination index (AI) was calculated as $AI = 1 - (OD_{600}^{\text{Problem crosses}}/1.1 \times OD_{600}^{\text{WT crosses}})$.

In order to analyse agglutination in cells carrying overexpression plasmids, h^+ cells growing exponentially in EMM with supplements and thiamine (15 μM) were washed extensively with water and inoculated in the same medium without thiamine. After 22 h of derepression, 5×10^7 cells were mixed with an equal number of *h⁻ cyr1::ura4⁺ sxa2::ura4⁺* cells that had been treated with P factor for 3 h according to [19]. Cells were incubated with shaking at 25 °C. Samples were collected at the desired time points and treated as above.

2.3. Molecular and genetic manipulations

A *Pml*I/*Sal*I DNA fragment containing the SPBC21D10.06c ORF together with upstream and downstream sequences was cloned from the SPBC21D10 cosmid into the *Sma*I/*Sal*I sites of a modified KS vector that lacked the *Not*I site (KS-Not). In parallel, the Gap Repair technique [16] was used to obtain the same DNA fragment from the genome. In order to express the *map4⁺* gene from the strong *nmt1⁺* promoter – repressible by thiamine [12]– an *Apa*I site was created before the ATG by site-directed mutagenesis using the 21DApaATG oligonucleotide (5′-cattaatattaatattaataattgggcccatgaattcatacgcaattttattg-3′). The ORF and 3′ non-coding region were cloned as an *Apa*I/*Nco*I fragment into the pJR-L1 plasmid [13]. To construct a Map4-GFP fusion protein, a *Not*I site was created before the stop codon by site-directed mutagenesis using the 21DNotSTOP oligonucleotide (5′-gttgttcttagagctttggaatatggcggccgctaggctttatttctactgaaaagttgcagg-3′). Then, a *Not*I/*Not*I DNA fragment, containing the green fluorescent protein (GFP) preceded by a hinge of 8 alanine residues, was cloned into the *Not*I site. This fusion protein was cloned into the integrative vector pJK148. The functionality of this protein was assessed by complementation of the mutant phenotype. A *21D::ura4⁺* deletion cassette was generated by creating a KS+*map4⁺* (ApaATG NotISTOP) plasmid lacking the *Apa*I site in the polylinker and replacing the ORF by the *ura4⁺* gene as an *Apa*I/*Not*I DNA fragment. Polymerase chain reaction (PCR), using different sets of oligonucleotides that were external and/or internal to the cassette, was used to assess the replacement of the SPBC21D10 ORF by *ura4⁺*. The Crn1-GFP protein was recovered by gap repair from the RP001 strain [17] and cloned into pJK148.

DNA sequencing was performed using the Universal (5′-gtaaaacgacggccagtgaat-3′), Reverse (5′-ggaaacagctatgaccatgattac-3′), BsawF (5′-ccggtagtagttcagctttatct-3′), MnlF (5′-gtgacggaaacgacgacttc-3′) and AluR (5′-gatgtacccactcctacagc-3′) oligonucleotides. Nucleotide sequences were analysed using DNASTAR and Clonemap programs.

2.4. Microscopy

A Leica DM RXA microscope equipped with a Photometrics Sensys CCD camera using the Qfish 2.3 program was used to perform microscopy analyses.

2.5. Protein analysis

Cells growing in EMM were washed with water and transferred to EMM-N and incubated for 5 h at 25 °C with gentle shaking. Cells were collected, washed with Buffer A (50 mM Tris–HCl, pH 7.5; 50 mM EDTA; 1 mM PMSF; 1 μg/ml Aprotinin, Leupeptin and Pepstatin) and broken in the same buffer in a FastPrep (Savant). Cell walls were recovered by centrifugation (5 min at 3000 rpm) and washed 6 times with buffer A. Then, they were boiled in the presence of sample buffer (50 mM Tris–HCl, pH 6.8; 1% SDS; 143 mM β-mercaptoethanol; 10% glycerol). The samples were loaded on 4–20% gradient gels, transferred to Immobilon-P membranes in Tris–glycine buffer and decorated with monoclonal anti-GFP (JL8, Nucliber; 1:1000) antibody. ECL Advanced (Amersham) was used to develop the blots.

3. Results

3.1. Characteristics of the Map4 protein

According to the *S. pombe* Genome Database (http://www.genedb.org/genedb/pombe/index.jsp), the SPBC21D10.06c ORF has 2847 nucleotides and is predicted to have two *Kpn*I and two *Bam*HI restriction sites. However, when we cloned this ORF and the non-coding regions from the cosmid we found that the size of some restriction fragments was larger than expected and that there were more sites for those enzymes. The same result was obtained from a genomic DNA fragment cloned by gap repair. We found that the disagreement between the sequence in the database and that of the cloned fragments was restricted to a DNA region flanked by sites for *Sac*I and *Eco*RV. We sequenced this region and found that it was 432 bp larger than expected and that it had four *Bam*HI and five *Kpn*I sites, which suggested the presence of internal repeats. To confirm that the sequence we got corresponded to the cloned DNA fragment, we PCR-amplified this part of the gene using the BsawF and AluR oligonucleotides (see Section 2). As shown in Fig. 1B, this fragment was larger than expected from the reported sequence (1.1 kb instead of 0.6 kb). The PCR product was digested using *Xho*I, *Bam*HI or *Kpn*I. The number and size of the bands resulting from the digestions corresponded to that predicted by the corrected sequence and showed that in the DNA fragment there were more than two *Kpn*I and more than two *Bam*HI sites (Fig. 1B).

The protein encoded by the SPBC21D10.06c ORF has 1092 amino acids and, according to computer-assisted predictions, shows the following features (Fig. 1A): a signal peptide with cleavage site between residues 23 and 24, a serine/threonine-rich region (amino acids 24 and 616), a region with nine repetitions of the 36-aminoacid sequence S(W/Y)VTET(V/T)TSGSV(G/E)FTTTI(A/T)TP(V/I)G(S/T)TAGTV(L/V)(V/I)D(V/I)PTP (residues 617–940), and a carboxy-terminal region (amino acids 941–1092) that includes four cysteine residues and a DIPSY domain, present in some cell wall proteins from *S. pombe* (according to Pfam). The protein has multiple potential sites for phosphorylation and O-glycosylation, and 12 potential sites for N-glycosylation. These features suggest that it is a cell surface glycoprotein. Except for the amino terminal end, the protein is hydrophilic.

3.2. map4⁺ is required for mating in h⁺ cells

In order to study whether the *map4⁺* gene is required for mating, we constructed a null mutant in a homothallic h^{90} strain, which produces h^+ and h^- cells that are able to mate with each other. We observed that after a 36-h incubation period at 28 °C the h^{90} control strain had mated and sporulated efficiently (Fig. 2). In contrast, in the mating mixtures from the h^{90} *map4::ura4⁺* mutant mating was significantly reduced. It is noteworthy that the few zygotes that we could observe had sporulated, showing that Map4p is not required for cell fusion. The most striking observation was that in the mutant strain there was an accumulation of shmoos with long tips (Figs. 2A and 3). Next, we wished to investigate whether dele-

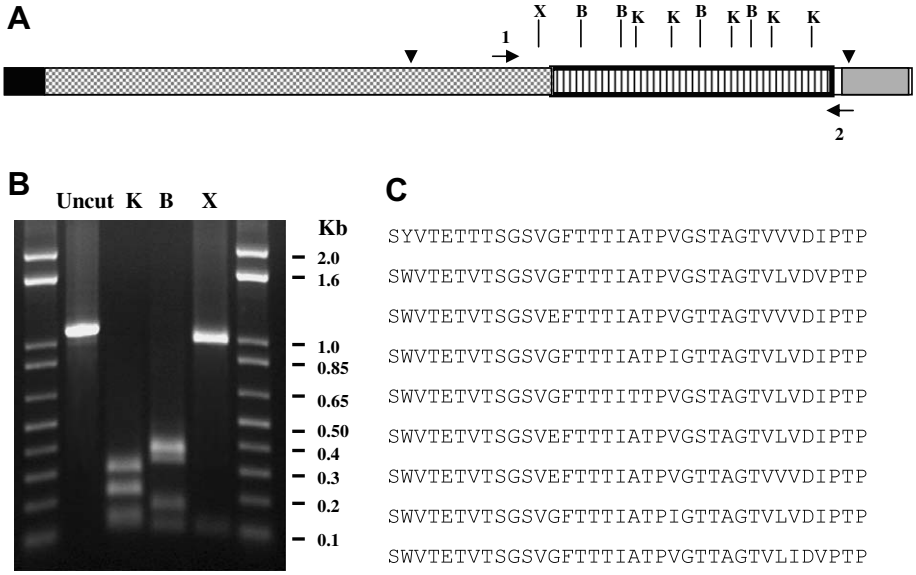

Fig. 1. Domain organization of Map4 protein. (A) Diagram of domain distribution in Map4p. The black box indicates the signal peptide, the hatched box indicates the serine/threonine-rich region, the stripped box corresponds to the internal repeats and the grey box indicates the DIPSY domain. Arrowheads indicate the position of the potential Kex2 sites. Arrows indicate the sites for hybridization of the BsawF (1) and AluR (2) oligonucleotides. X: *Xho*I, B: *Bam*HI, and K: *Kpn*I. (B) BsawF/AluR PCR product undigested or digested with the *Kpn*I, *Bam*HI or *Xho*I enzymes. (C) Amino acidic sequence of the internal repeats in Map4p.

tion of the *map4*$^+$ gene had the same effect in both mating types. Accordingly, we constructed h^+ and h^- deletants and performed quantitative mating assays. Mating efficiency – zygotes (white bars in Fig. 2B) with respect to total cell number – was similar for the wild-type (WT) $h^+ \times$ WT h^- and WT $h^+ \times$ *map4*Δh^- crosses, while for the *map4*$\Delta h^+ \times$ WT h^- and *map4*$\Delta h^+ \times$ *map4*Δh^- crosses it was reduced to 1.0% with respect to the WT $h^+ \times$ WT h^- cross. Additionally, shmoos (black bars in Fig. 2B) only accumulated in the crosses involving the *map4*Δh^+ strain. This result shows that Map4p is required for mating in h^+, but not in h^- cells.

3.3. Map4p is an adhesin

The facts that in the mating mixtures involving a *map4*Δh^+ parental mating was defective, but that a significant number of shmoos were observed, could be explained in different ways: (1) *map4*Δh^+ cells are not able to transmit the signal in response to pheromones and/or nitrogen starvation or to differentiate into shmoos, and (2) *map4*Δh^+ cells are defective in pheromone production. In the first case, the shmoos in the mating mixtures would correspond to the h^- parental strain, while in the later the shmoos would be mostly formed in the h^+ strain. In order to gain information about which the specific defect in the *map4*Δh^+ strain was, we introduced the actin-binding protein Crn1p, fused to GFP, into this mutant. In this way, the mutant cells could be tracked because of the fluorescence. When this strain was mated to a WT h^- strain, we observed a similar number of fluorescent and non-fluorescent shmoos (Fig. 3). This result shows that the *map4*Δh^+ cells are able to produce and respond to pheromone, and indicates that the *map4*Δh^+ shmoos are not able to mate because they do not establish cell–cell contact with the h^- shmoos.

To analyse cell adhesion, we performed mating assays in liquid medium (see Section 2). We observed that in the WT $h^+ \times$ WT h^- and WT $h^+ \times$ *map4*Δh^- crosses the cells were able to agglutinate, producing clumps of cells, while in the *map4*$\Delta h^+ \times$ WT h^- and *map4*$\Delta h^+ \times$ *map4*Δh^- crosses the cells did not form clumps (Fig. 4A). In order to quantify this defect, we estimated the agglutination index in the mating mixtures (see Section 2). After 20 h under mating conditions, the agglutination index was reduced to 30% in the crosses that involved the *map4*Δh^+ cells with respect to the WT $h^+ \times$ WT h^- (Fig. 4A). This result confirmed that *map4*Δ h^+ cells are defective in cell adhesion.

We then analysed whether a high expression of the *map4*$^+$ gene could increase cell adhesion. To do so, we expressed this gene, from its own promoter in a high-copy number plasmid (pAL+*map4*$^+$) or from the strong *nmt1*$^+$ promoter (pREP3X+*map4*$^+$), in h^+ cells. These cells were mated to h^- cells that had been previously treated with the pheromone *P* factor in order to stimulate production of their specific mating adhesin. We estimated agglutination after 3 h of mating and observed that at this time cell adhesion had increased twofold in cells carrying the pAL+*map4*$^+$ plasmid with respect to the control. In the cells expressing the gene from the *nmt1*$^+$ promoter the increase in agglutination was ninefold and large clumps of cells were observed in the culture (see the large precipitate in the corresponding tube in Fig. 4B). Finally, we performed time-course experiments to estimate the agglutination index over time in crosses involving h^+ cells that overexpressed the *map4*$^+$ gene. As shown in Fig. 4C, under these conditions cell adhesion was significant after only 15 min of incubation, while in the control cross agglutination was only observed after long mating times. These results show that Map4p promotes cell adhesion.

3.4. Map4p localizes at the cell wall of h^+ cells

A Map4-GFP protein was constructed to study Map4p localization. According to its ability to complement the mating defect of the *map4*Δh^+ mutant, this fusion protein was

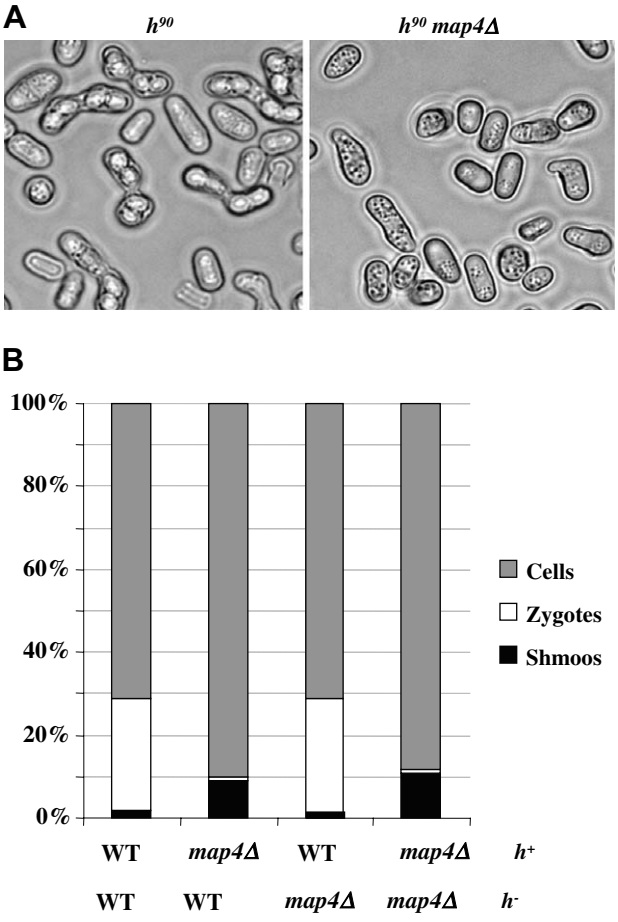

Fig. 2. *map4*$^+$ is required for mating. (A) Micrographs of h^{90} WT or *map4::ura4*$^+$ strains after 36 h under mating conditions. (B) Percentage of cells, zygotes and shmoos, with respect to the total cell number, in crosses involving the indicated h^+ and h^- strains. The results are the means of three independent experiments. A total of 1000 cells were counted in every case.

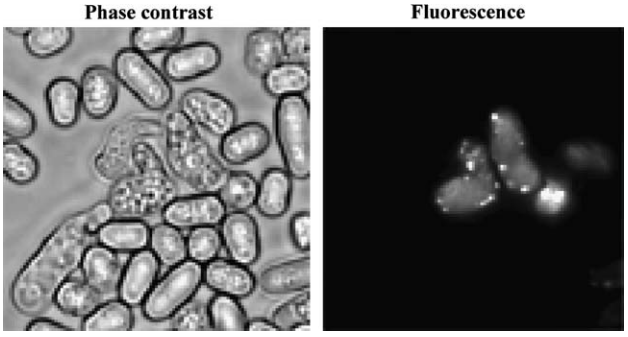

Fig. 3. *map4*Δ strains respond to pheromones. Phase contrast and fluorescence micrographs of cells from a cross between an h^+ *map4::ura4*$^+$, carrying the Crn1-GFP protein, and an h^-*map4::ura4*$^+$ strain.

functional. An h^{90} strain carrying Map4-GFP was induced to mate in liquid EMM-N. Observation under a fluorescence microscope allowed us to observe that the fluorescence was localized at the tip of the shmoos. When the mating partner got in touch the fluorescence concentrated at the projection of the shmoo in only one of the mating partners. Finally, when the cells fused the fluorescence could be observed at the conjugation bridge, but it still was asymmetrically distributed in the zygote (Fig. 5A). When the cell walls were isolated after cell breakage, Map4-GFP fluorescence could still be observed in the debris (Fig. 5B) even after incubation for 14 h at 25 °C in the presence of SDS and β-mercaptoethanol (not shown).

The pattern of fluorescence in the zygotes suggested that Map4p was only present in one of the mating partners. To gain information about this issue, we mated an h^+ *map4::ura4*$^+$ strain, carrying the integrated pJK148+map4$^+$-GFP plasmid (which confers leucine prototrophy), with an h^- *map4::ura4*$^+$ strain. We selected h^+ and h^- leu$^+$ ura$^+$ clones from the offspring. Map4-GFP was observed in the mating mixtures involving h^+ *map4::ura4*$^+$ *leu1:map4*$^+$::*GFP* cells but not in those involving h^- *map4::ura4*$^+$ *leu1:map4*$^+$::*GFP* (not shown). This result strongly suggested that Map4p is only produced in h^+ cells. This result was confirmed by Western blot. As shown in Fig. 5C, two protein bands were detected in the lanes corresponding to the purified cell walls from mating mixtures involving h^{90} or h^+ cells which carry the Map4-GFP fusion protein, but not in those involving h^- cells with Map4-GFP or untagged cells. The detected bands were larger than the expected size (147 kDa), indicating that Map4p is a modified protein.

4. Discussion

Cell surface glycoproteins are involved in cell–cell adhesion in many cellular processes. The fission yeast *map4*$^+$ is a sexually induced gene that is dispensable for sexual differentiation but is required for mating in h^+ cells. Additionally, overexpression of this gene increases cell agglutination in h^+ (Fig. 4) but not in h^- (not shown) cells. Fluorescence microscopy and Western blot analyses confirmed that this protein is only present in the cell wall of h^+ cells. Map4-GFP can be observed at the tip of the shmoos and in the conjugation bridge of the zygotes. However, the fact that cells bearing the pREP3X+*map4*$^+$ plasmid were able to form clumps of cells in a nitrogen-rich medium (a condition that does not induce the mating differentiation program) shows that polarization of the protein to the tip of the shmoo is not required for cell–cell contact. Probably, polarization guarantees a better mating efficiency.

Map4p is a protein with internal repeats that exhibits a domain distribution that differs from that of budding yeasts GPI or PIR proteins. It has a signal peptide followed by a long serine/threonine-rich region (which does not show internal repeats, according to Dotlet: http://www.isrec.isb-sib.ch/java/dotlet/Dotlet.html) and a domain with nine repeats of an amino acidic sequence that lacks glutamine residues (an amino acid present in the internal repeats of the *S. cerevisiae* PIR proteins that is required for linkage to β-1,3-glucan [5]). The carboxyl end exhibits a DIPSY domain and has four cysteine residues with a c-x(3)c-x(53)-c-c distribution, which differs substantially from that of PIR proteins [4]. In Map4p, the potential sites for digestion by Kex2 protease are not located at the amino terminal end of the protein, but at positions 414 (between the serine/threonine-rich domain and the internal repeats) and 1004 (88 residues before the last aminoacid). The DIPSY domain has only been found in four predicted *S. pombe* cell wall glycoproteins, but nothing is known about its function. While two of

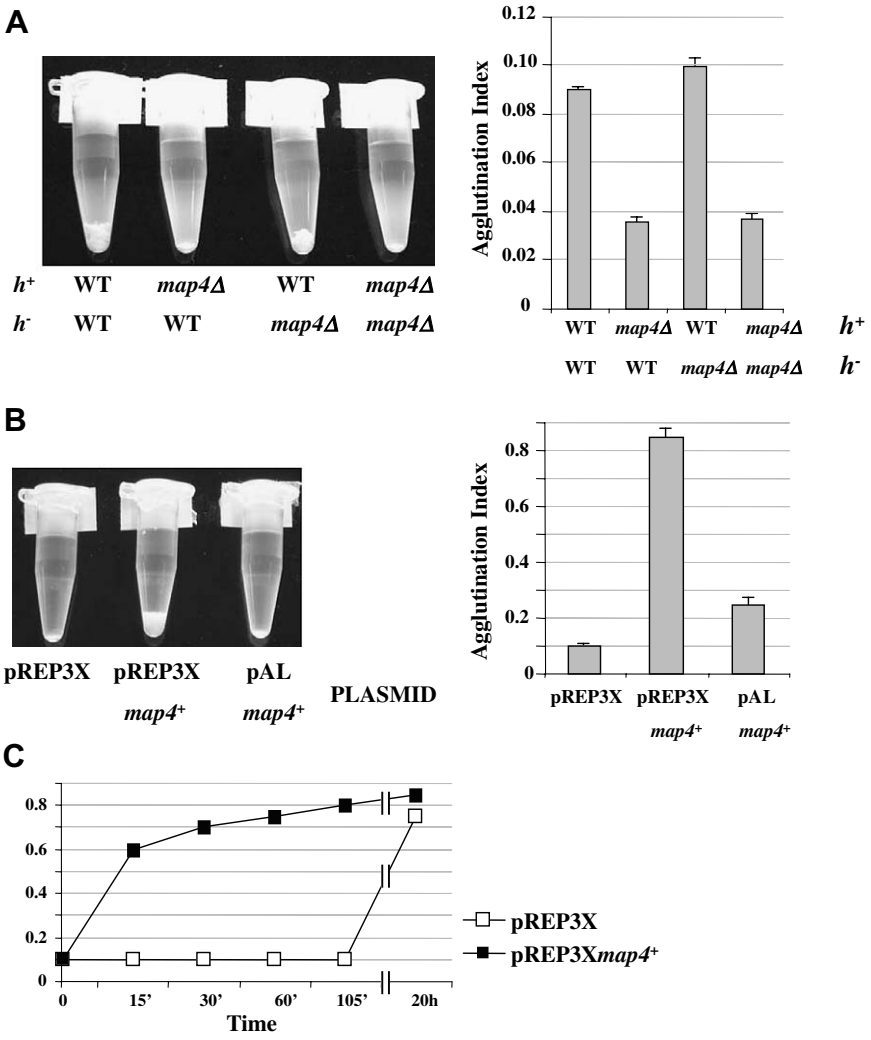

Fig. 4. Map4p is an adhesin. (A) Photograph (left panel) and quantification (right panel) of agglutination in crosses between the indicated h^+ and h^- strains. (B) Photograph and quantification of agglutination in crosses between h^+ strains, carrying the indicated plasmids, and an h^- strain treated with pheromone. In both cases, the agglutination index is $1 - (OD_{600}^{\text{Problem crosses}}/1.1 \times OD_{600}^{\text{WT crosses}})$. (C) A time-course analysis of agglutination in crosses between h^+ strains, carrying the indicated plasmids, and an h^- strain treated with pheromone. The time points correspond to the time from the moment when the h^+ and h^- strains were mixed.

them are small proteins (SPBC359.04c, SPAC186.01), the other two (*map4*$^+$ and SPBC947.04, upregulated by osmotic stress) are much larger. Notably, the latter protein carries 17 repeats of 35 amino acids, which are matching to the P-agglutinin repeats at 19 positions (53%). The short proteins each carry a single unit (33 or 35 amino acids), matching the Map4p repeats at 44% or 47%, respectively. Furthermore, the repeat unit sequence of the P-agglutinin is able to retrieve numerous other predicted cell wall-associated glycoproteins, when analysed by BLAST at the Sanger Center web site. The closest paralog match in the *S. pombe* genome is P11E10.02c (coding for the M-specific agglutinin), which carries 10–11 repeats of almost perfect match to the P-agglutinin, but no DIPSY domain is contained in this protein. Repeats of lesser similarity are present in various other predicted glycoproteins. In all these cases, internal similarity among the repeats of the same protein is much higher than between different proteins, indicating that the generation of repeats proceeded independently in different precursor genes, which initially were without repeated arrays.

Since Map4p is required for cell–cell contact it is expected to be exposed to the cell surface. It is possible that the long serine/threonine-rich domain, with multiple potential O-glycosylation sites, giving a "stiff rod" structure to the proteins [9], could be located at the internal side of the cell wall and helps the repeats and/or the DIPSY domain to be exposed to the cell surface, where the protein would interact with the cell wall from h^- cells. It is also possible that the cysteines in the DIPSY domain might strengthen the cell wall association by the formation of disulfide bridges. This raises the possibility that both the serine/threonine-rich domain and the cysteine residues participate in anchoring the terminal domains inside the wall, so that only the inner part of Map4p, including the repeat array, is sticking out as a fold-back loop.

The presence and distribution of domains make Map4p a novel surface protein, which suggests that new kinds of adhesion proteins might be involved in differentiation processes in other organisms. Because of the presence of internal repeats and its function in cell adhesion, *S. pombe* Map4p might be

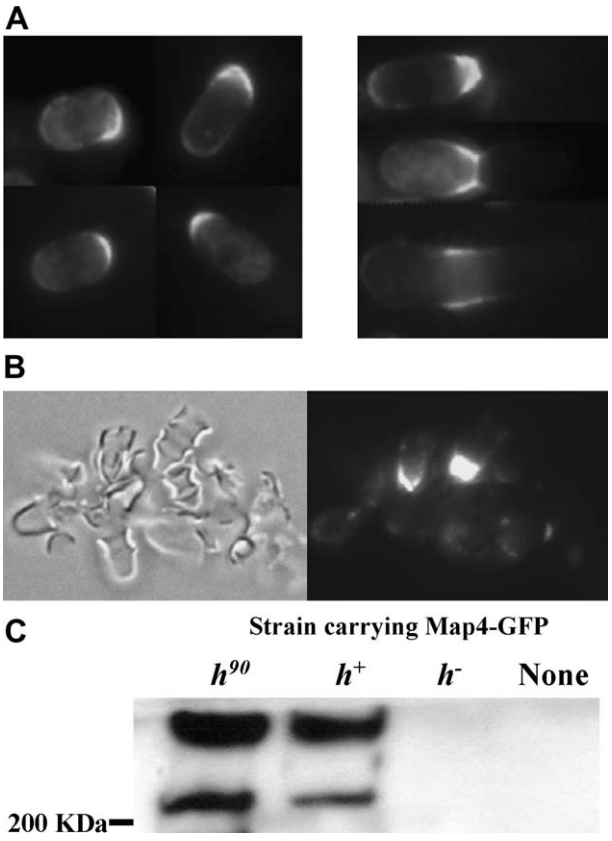

Fig. 5. Map4-GFP protein localizes at the cell wall of h^+ cells. (A) Fluorescence micrographs of shmoos and zygotes. (B) Bright field (left panel) and fluorescence (right panel) micrographs of purified cell walls from the h^{90}*map4::ura4*$^+$ strain carrying the Map4-GFP fusion protein. (C) h^+ or h^- cells carrying the Map4-GFP fusion protein and untagged cells were mated to untagged cells from the opposite mating type. The cell walls were purified, treated as indicated in Section 2 and analysed by Western blot analysis. A h^{90} strain carrying Map4-GFP was included as a control.

considered to be functionally related to human extracellular glycoproteins, such as fibronectin and neural cell adhesion molecules (NCAM), which participate in cell adhesion processes and have several tandems of amino acidic modules [2]. A detailed structure–function analysis of Map4p could provide useful information about the function of these proteins.

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References

[1] Arellano, M., Cartagena-Lirola, H., Nasser Hajibagheri, M.A., Durán, A. and Valdivieso, M.H. (2000) Proper ascospore maturation requires the chs1+ chitin synthase gene in *Schizosaccharomyces pombe*. Mol. Microbiol. 35, 79–89.
[2] Baldwin, T.J., Fazeli, M.S., Doherty, P. and Walsh, F.S. (1996) Elucidation of the molecular actions of NCAM and structurally related cell adhesion molecules. J. Cell. Biochem. 61, 502–513.
[3] Castillo, L., Martinez, A.I., Garcera, A., Elorza, M.V., Valentin, E. and Sentandreu, R. (2003) Functional analysis of the cysteine residues and the repetitive sequence of *Saccharomyces cerevisiae* Pir4/Cis3: the repetitive sequence is needed for binding to the cell wall beta-1,3-glucan. Yeast 20, 973–983.
[4] De Groot, P.W., Ram, A.F. and Klis, F.M. (2005) Features and functions of covalently linked proteins in fungal cell walls. Fungal Genet. Biol. 42, 657–675.
[5] Ecker, M., Deutzmann, R., Lehle, L., Mrsa, V. and Tanner, W. (2006) Pir proteins of *Saccharomyces cerevisiae* are attached to beta-1,3-glucan by a new protein-carbohydrate linkage. J. Biol. Chem. 281, 11523–11529.
[6] Frieman, M.B., McCaffery, J.M. and Cormack, B.P. (2002) Modular domain structure in the *Candida glabrata* adhesin Epa1p, a beta1,6 glucan-cross-linked cell wall protein. Mol. Microbiol. 46, 479–492.
[7] Hoyer, L.L. (2001) The ALS gene family of *Candida albicans*. Trends Microbiol. 9, 176–180.
[8] Huang, G., Zhang, M. and Erdman, S.E. (2003) Posttranslational modifications required for cell surface localization and function of the fungal adhesin Aga1p. Eukaryot Cell 2, 1099–1114.
[9] Jentoft, N. (1990) Why are proteins O-glycosylated? Trends Biochem. Sci. 15, 291–294.
[10] Lipke, P.N. and Kurjan, J. (1992) Sexual agglutination in budding yeasts: structure, function, and regulation of adhesion glycoproteins. Microbiol. Rev. 56, 180–194.
[11] Mata, J., Lyne, R., Burns, G. and Bähler, J. (2002) The transcriptional program of meiosis and sporulation in fission yeast. Nat. Genet. 32, 143–147.
[12] Maundrell, K. (1993) Thiamine-repressible expression vectors pREP and pRIP for fission yeast. Gene 123, 127–130.
[13] Moreno, M.B., Duran, A. and Ribas, J.C. (2000) A family of multifunctional thiamine-repressible expression vectors for fission yeast. Yeast 16, 861–872.
[14] Moreno, S., Klar, A. and Nurse, P. (1991) Molecular genetic analysis of fission yeast *Schizosaccharomyces pombe*. Methods Enzymol. 194, 795–823.
[15] Morita, T., Tanaka, N., Hosomi, A., Giga-Hama, Y. and Takegawa, K. (2006) An alpha-amylase homologue, aah3, encodes a GPI-anchored membrane protein required for cell wall integrity and morphogenesis in *Schizosaccharomyces pombe*. Biosci. Biotechnol. Biochem.
[16] Orr-Weaver, T.L., Szostak, J.W. and Rothstein, R.J. (1983) Genetic applications of yeast transformation with linear and gapped plasmids. Methods Enzymol. 101, 228–245.
[17] Pelham Jr., R.J. and Chang, F. (2001) Role of actin polymerization and actin cables in actin-patch movement in *Schizosaccharomyces pombe*. Nat. Cell Biol. 3, 235–244.
[18] Sambrook, J., Fritsch, E.F. and Manniatis, T. (1989) Molecular Cloning: A Laboratory Manual, Cold Spring Harbor laboratory press, Cold Spring Harbor, New York.
[19] Stern, B. and Nurse, P. (1997) Fission yeast pheromone blocks S-phase by inhibiting the G1 cyclinB p34^{cdc2} kinase. EMBO J. 16, 534.
[20] Wojciechowicz, D., Lu, C.F., Kurjan, J. and Lipke, P.N. (1993) Cell surface anchorage and ligand-binding domains of the *Saccharomyces cerevisiae* cell adhesion protein alpha-agglutinin, a member of the immunoglobulin superfamily. Mol. Cell. Biol. 13, 2554–2563.
[21] Yamamoto, M., Imai, Y. and Watanabe, Y. (1997) in: The Molecular and Cellular Biology of the Yeast Saccharomyces (Pringle, J.R., Broach, J.R. and Jones, E.W., Eds.), Cell Cycle and Cell Biology, Vol. 3, pp. 1037–1106, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York.