Characterization of the Wsc1 Protein, a Putative Receptor in the Stress Response of *Saccharomyces cerevisiae*

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ABSTRACT

Wsc1p, Wsc2p, Wsc3p, and Wsc4p, members of a novel protein family in the yeast *Saccharomyces cerevisiae*, are putative sensors or receptors in the stress response. Genetic characterization suggests that the *WSC* family are upstream regulators of the stress-activated PKC1-MAP kinase cascade and are required for the heat shock response and for maintenance of cell wall integrity. The Wsc proteins share sequence characteristics: at their N terminus they have a cysteine motif and a serine/threonine-rich domain predicted to be extracellular, a hydrophobic domain suggested to be transmembranous, and a variable, highly charged C terminus that may be involved in intracellular signaling. Although a role for the *WSC* genes in maintenance of cell wall integrity has been firmly established, little is known about the properties of the proteins. As reported here, to study its properties *in vivo*, we epitope tagged the Wsc1 protein. Wsc1p was found to fractionate with the membrane pellet after high-speed centrifugation. Extraction experiments show that Wsc1p is an integral membrane protein present in two forms: one solubilized by detergent, the other Triton X-100 insoluble. Our results also show that Wsc1p is glycosylated and phosphorylated. To characterize the contribution of different domains to the function of Wsc1p, we generated various deletion constructs. Analysis of the properties and function of the mutant proteins shows that the predicted extracellular serine/threonine-rich domain is required for Wsc1p functionality, as well as its glycosylation. A mutant Wsc1 protein lacking the putative transmembrane domain is not functional and partitions to the soluble fraction. Overexpression of full-length Wsc1p inhibits cell growth, with the N terminus alone being sufficient for this inhibition. This suggests that Wsc1p may function in a complex with at least one other protein important for normal cell growth.

IN the yeast *Saccharomyces cerevisiae*, progression from G_1 to the S phase of the cell cycle in vegetative growing cells is characterized by bud formation. Different sets of genes control site selection, emergence, and subsequent growth of the bud. These genes induce polarization of the actin cytoskeleton that results in recruitment of vesicles to the bud site for biosynthesis of the new cell wall and membrane (reviewed in Cid *et al.* 1995; Lew *et al.* 1997; Orlean 1997). Components of the PKC1-MPK1 pathway play a key regulatory role in maintenance of the cell wall integrity during budding (Cid *et al.* 1995) and during periods of environmental stress (Davenport *et al.* 1995; Kamada *et al.* 1995).

Protein kinase C (Pkc1p) controls the activity of a mitogen-activated protein kinase (MAPK) cascade. The MAPK cascade is composed of Bck1p/Slk1p (Costigan *et al.* 1992; Lee and Levin 1992), Mkk1p and Mkk2p (Irie *et al.* 1993), and Mpk1p/Slt2p (Lee *et al.* 1993; Mazzoni *et al.* 1993). Pkc1p is a target of the GTPase Rho1p (Nonaka *et al.* 1995; Kamada *et al.* 1996). Rho1p has two additional functions: it activates β-1,3 glucan synthase, which produces a major component of the cell wall (Drgonová *et al.* 1996; Qadota *et al.* 1996); and it regulates polarized growth by organizing the cytoskeleton at the bud site (Yamochi *et al.* 1994), possibly through its interaction with Bni1p (Kohno *et al.* 1996; Imamura *et al.* 1997). Rho1p function is controlled by a yeast phosphatidylinositol 3-kinase homolog, Tor2p (Schmidt *et al.* 1997). Rho1p activity is also regulated independently of *TOR2* in response to conditions that disturb the cell wall (Bickle *et al.* 1998).

We (Verna *et al.* 1997) and others (Gray *et al.* 1997) have identified the *WSC* family as putative upstream activators of the PKC1-MPK1 pathway. The Wsc proteins are encoded by at least three genes, *WSC1*/*HCS77*/*SLG1* (Sterky *et al.* 1996; Gray *et al.* 1997; Verna *et al.* 1997), *WSC2*, and *WSC3* (Verna *et al.* 1997), which have been shown to be required for maintenance of the cell wall integrity and for the heat shock response of *S. cerevisiae* (Gray *et al.* 1997; Verna *et al.* 1997). A fourth gene, *WSC4*, encoding a homolog of the Wsc proteins, has been identified but not characterized (Verna *et al.* 1997).

Deletion of *WSC1* leads to a cell lysis defect and to heat shock sensitivity. These phenotypes are suppressed

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by overexpression of *WSC2* or *WSC3*. Deletion of *WSC2* or *WSC3* exacerbates the lysis defect and the heat shock sensitivity of a *wsc1*Δ strain. The lysis defect of the *wsc*Δ mutants is rescued by adding osmotic stabilizers to the media and by overexpression of *PKC1* (Gray *et al.* 1997; Verna *et al.* 1997) or *RHO1* (Verna *et al.* 1997). It has been shown that the *WSC* genes are necessary for the activation of Mpk1p when cells are exposed to a mild heat shock treatment (Gray *et al.* 1997; Verna *et al.* 1997).

The Wsc family members have similar sequence characteristics. They have a predicted N-terminal signal peptide and are proposed to be type I membrane proteins. We have shown previously that Wsc1p localizes to the cell periphery (Verna *et al.* 1997). The putative extracellular domain of the Wsc proteins contain a cysteine motif, **C$_1$**-X-S-X$_{12\text{-}16}$-Φ-Q-S-X$_3$-**C$_2$**-X$_3$-**C$_3$**-X$_{5\text{-}8}$-A-L(I)-X$_{5\text{-}6}$-**C$_4$**-Φ-**C$_5$**-X$_{12\text{-}17}$-**C$_6$**-X$_3$-**C$_7$**-X-G-Φ-X$_4$-**C$_8$**-G-X$_{6(30)}$-VY, with the cysteine (C) residues numbered 1 to 8 and aromatic amino acids depicted by the symbol Φ (Verna *et al.* 1997). After this cysteine motif, there is a serine/threonine-rich region. This region is followed by a hydrophobic domain suggested to be transmembranous. The intracellular C-terminal domain of the proteins is highly charged and has the highest degree of divergence among the Wsc family members (Verna *et al.* 1997).

The function and cellular localization of Wsc1p suggest that the Wsc family of proteins may act as sensors or receptors that mediate intracellular responses to environmental stress in yeast. To further understand the function of the Wsc proteins, we epitope tagged Wsc1p and studied its properties *in vivo*. We also investigated the contribution of different domains to the function of Wsc1p by making deletion constructs coding for proteins that lack the various domains described above and by testing their ability to suppress the lysis defect of *wsc*Δ mutants. On the basis of our results we herein propose a model for the mode of action of Wsc1p.

MATERIALS AND METHODS

Media and strains: YPD, 1% yeast extract, 2% peptone, 2% dextrose; SC, synthetic complete media containing yeast nitrogen base at 0.67 g/liter, 2% dextrose, and amino acid supplements; and SC media lacking specific amino acids for selection (Rose *et al.* 1990). Yeast transformations were performed by the lithium acetate method, as described (Ito *et al.* 1983). The yeast strains used in this study are as follows: SP1, *MAT***a** *leu2 his3 ura3 trp1 ade8* (Toda *et al.* 1985); ALH7, *MAT***a** *leu2 his3 ura3 trp1 ade8 wsc1*Δ*::ADE8*; ALH718, *MAT***a** *leu2 his3 ura3 trp1 ade8 wsc1*Δ*::ADE8 wsc2*Δ*::URA3*; ALH758, *MAT***a** *leu2 his3 ura3 trp1 ade8 wsc1*Δ*::ADE8 wsc2*Δ*::URA3 wsc3*Δ*::TRP1* (Verna *et al.* 1997). Strains JVHw123Δ-1A *MAT***a** *leu2 his3 ura3 trp1 ade8 wsc1*Δ*::ADE8 wsc2*Δ*::HIS3 wsc3*Δ*::TRP1* and JVHwt *MAT***a** *leu2 his3 ura3 trp1 ade8* were generated by crossing strains isogenic to ALH758 and HRB718 (Verna *et al.* 1997). Diploid cells from these crosses were sporulated, and tetrads were dissected (Verna and Ballester 1999).

***WSC1* constructs:** To epitope tag the Wsc1 protein with the triple hemagglutinin (HA) epitope (Tyers *et al.* 1992) we amplified the open reading frame of *WSC1* by PCR using the clone pIRIS7 as a template (Verna *et al.* 1997). We used the primer 5′ WSC1 to introduce a *Hin*dIII site 5′ to the ATG, and the primer 3′ WSC1-HA to replace the endogenous stop codon with a *Not*I site, followed by a stop codon and, at the 3′ end, a *Sac*II site (see Table 1). The *Hin*dIII and *Sac*II sites were used to insert *WSC1* into pSKII, after which the HA epitope was introduced into the *Not*I site. The pSKII-WSC1-HA plasmid was used as a template for precise large deletions by the PCR-based overlap extension method (Senanayake and Brian 1995). The mutagenesis primers that we used are listed in Table 1; note that the T7 and the T3 universal primers were used as the flanking primers. Conditions for PCR reactions were as described for Pfu DNA polymerase (Stratagene, La Jolla, CA). Nonmutated WSC1-HA or the mutated PCR products were digested with *Hin*dIII and *Sac*II and introduced into the high-copy-number expression vector, pADNS (Colicelli *et al.* 1989), where expression is controlled by the alcohol dehydrogenase (*ADH*) promoter. All pADNS-WSC1-HA constructs were verified for the absence of unintended mutations by DNA sequencing using the Sequenase version 2.0 protocol from Amersham (Arlington Heights, IL). We used this vector to analyze expression, localization, and properties of Wsc1p and the mutant proteins.

TABLE 1

Primers used for the construction of WSC1-HA and the WSC1-deletion constructs

5′WSC1	ATCATCAAGCTTATGAGACCGAACAAAACAA
3′WSC1-HA	TATATACCGCGGTCAGCGGCCGCTATCAGCT TCGTCTGGATT
ΔCys-5′	TCGCAAGCGAACGCCGAAGATGCCTATTCT
ΔCys-3′	AGAATAGGCATCTTCGGCGTTCGCTTGCGA
ΔS/T-5′	GTGTACCAACTTGACGCTGGATCTGACTCT
ΔS/T-3′	AGAGTCAGATCCAGCGTCAAGTTGGTACAC
ΔNterm-5′	TCGCAAGCGAACGCCGCTGGATCTGACTCT
ΔNterm-3′	AGAGTCAGATCCAGCGGCGTTCGCTTGCGA
ΔTM-5′	AAGAAAGCCAATGTAAGACACATTAATATG
ΔTM-3′	CATATTAATGTGTCTTACATTGGCTTTCTT
ΔCterm-5′	GAACAAGACAGGATGAGCGGCCGCATCTTT
ΔCterm-3′	AAAGATGCGGCCGCTCATCCTGTCTTGTTC

To analyze the function of the epitope-tagged Wsc1p and the mutant proteins we subcloned the *Hin*dIII-*Sac*II fragments containing *WSC1* and the mutated genes from the pADNS plasmid into a low-copy-number vector containing the *WSC1* promoter and termination sequences. To generate this plasmid containing the *WSC1* promoter we amplified, by PCR using Pfu DNA polymerase, genomic DNA 5′ to the open reading frame of *WSC1*, from positions −633 to −1. We used a 5′ oligonucleotide (5′-ATATCTGCAGATGACACCAAGGA TACAA-3′), which introduces a *Pst*I site (underlined), and a 3′ oligonucleotide (5′-CCGGGGATCCTTCTAGAGTAAGCTT TATTTAAATAGAATTTTT-3′), which introduces, respectively, a *Hin*dIII and a *Bam*HI site (underlined). The PCR product was cloned into the *Pst*I-*Bam*HI sites of YCplac 22 (Gietz and Sugino 1998) lacking the *Hin*dIII site in the multicloning sequence. YCplac22 is a low-copy-number, *CEN* plasmid, with the *TRP1* gene as an auxotrophic marker. The resulting plasmid was named pCENWSC12. We next amplified genomic sequence 3′ to the open reading frame of *WSC1*, from positions +1 to +609 from the termination codon. We used the 5′ oligonucleotide (5′-ACTCTAGAGGATCCCCGGC CGCGGAGAAACCCAAAAAAAATT-3′), which introduces, respectively, a *Bam*HI and a *Sac*II site (underlined), and the

3′ oligonucleotide (5′-TATAGAATTCATACAGGAATCCGC AAA-3′), which introduces an *Eco*RI site. The PCR product was cloned into the *Bam*HI-*Eco*RI site of pCENWSC12 to generate pCEN1234. We next digested the resulting plasmid with *Hin*dIII and *Sac*II, and ligated it to the fragments containing the mutated genes isolated from pADNS.

Fractionation and membrane extraction assays: JVHw123Δ-1A (*wsc1*Δ *wsc2*Δ *wsc3*Δ) cells expressing the HA-tagged Wsc1 proteins were grown at 30° to midlog phase (OD_{600} of 0.8 to 1.2) in 5 ml of selective media, washed once in buffer A (20 mm Tris-HCl, pH 7.5, 200 mm NaCl, 5 mm $MgCl_2$) and resuspended in 400 μl of buffer A containing protease inhibitors (2 mm phenylmethylsulfonyl fluoride, 50 μg/ml aprotinin, 10 μg/ml leupeptin, 8 μg/ml pepstatin A). Cells were lysed with glass beads at 4° and then centrifuged at 250 *g* for 5 min to remove unlysed cells. The supernatant was removed and used for both fractionation and membrane extraction assays.

For fractionation assays, cell extracts were split into two equal aliquots. The first aliquot represents the total cell lysate, while the second aliquot was used to separate soluble and membrane fractions by ultracentrifugation at 100,000 × *g* for 1 hr at 4°. SDS-polyacrylamide gel electrophoresis (PAGE) sample buffer was added to equivalent amounts of protein and samples were boiled for 5 min at 95° before electrophoresis. Protein concentrations were determined using a bicinchoninic acid (BCA) protein assay reagent (Pierce, Rockford, IL). After electrophoresis, the proteins were electroblotted onto nitrocellulose membranes (Amersham), probed with anti-HA 12CA5 antibody at a dilution of 1:5000 (Field *et al.* 1989), and developed using sheep anti-mouse alkaline phosphatase-conjugated antibodies and an ECL chemiluminescence detection kit (Amersham).

For membrane extraction assays, the cell extract was divided into five fractions and treated as described by Ljungdhal *et al.* (1992) and Roemer *et al.* (1996). Four of the fractions were adjusted to a final concentration of either 0.1 m Na_2CO_3, pH 11.0, 1.0% Triton X-100, 1.6 m urea, or 0.6 m NaCl in a final volume of 200 μl. The fifth sample was diluted to 200 μl with buffer A plus protease inhibitors. The samples were then incubated on ice for 15 min and centrifuged at 100,000 *g* for 1 hr. The supernatant and pellet fractions were collected, separated by SDS-PAGE, and subjected to immunoblot analysis as described above.

For membrane extraction assays that used various reagents in combination, cell extracts were divided into four fractions and treated similarly as described above, but with some modifications. Three of the fractions were adjusted to a final concentration of either 0.5% Triton X-100, 0.5% Triton X-100 + 0.8 m urea or 0.5% Triton X-100 + 0.3 m NaCl. The fourth sample was diluted to 200 μl with buffer A. The samples were incubated on ice for 15 min and centrifuged at 100,000 *g* for 1 hr. The supernatant (S1) was removed and saved, while the pellet was resuspended in M buffer (20 mm HEPES, pH 7.4, 250 mm sucrose) with Triton X-100 alone or Triton X-100 in combination with urea or NaCl. The fraction was centrifuged again at 100,000 *g* for 1 hr and the supernatant (S2) was saved and the pellet collected. Equivalent amounts of each sample were resolved by SDS-PAGE and analyzed by immunoblotting as described above.

Preparation of cell extracts for localization, glycosylation and phosphorylation assays: ALH758 (*wsc1*Δ *wsc2*Δ *wsc3*Δ) cells expressing the HA-tagged Wsc1 proteins were grown at 30° to an OD_{600} of 0.7 in selective media containing sorbitol. Cells were harvested by centrifugation, washed with lysis buffer (50 mm Tris-HCl, pH 7.5, 150 mm NaCl, 0.2 mm sodium vanadate, 50 mm potassium fluoride, 30 mm sodium pyrophosphate, 2 mm phenylmethylsulfonyl fluoride, 50 μg/ml aprotinin, 10 μg/ml leupeptin, 8 μg/ml pepstatin A) and vortexed with glass beads. Cells were centrifuged at 100 × *g* in an Eppendorf centrifuge for 10 min and the supernatant was removed and centrifuged again at 16,000 × *g* for 30 min. The supernatant from this centrifugation was removed, and after a second centrifugation, used as the soluble fraction. The pellet was washed with lysis buffer, centrifuged again, and then resuspended in lysis buffer containing 1% NP-40. The samples were incubated on ice for 10 min followed by centrifugation at 16,000 × *g* in an Eppendorf centrifuge for 30 min. The supernatant from this centrifugation was used as the membrane fraction.

For the preparation of whole cell extracts, the cell pellet was washed once in buffer A (20 mm Tris-HCl, pH 7.5, 200 mm NaCl). The cell pellet was then resuspended in lysis buffer (as above) containing 1% Triton X-100, 0.1% SDS, and 1% sodium deoxycholate. The cells were then lysed by vortexing with glass beads. Samples were centrifuged for 10 min in an Eppendorf centrifuge at 16,000 × *g* for 10 sec and the supernatant was removed, transferred to a new tube, and centrifuged again for 30 min.

Glycosylation detection: For detection of glycosylation, 250 μg of soluble or membrane fractions (or 430 μg of the Wsc1p-ΔCterm extract) expressing the HA-tagged proteins were immunoprecipitated using standard procedures (Harlow and Lane 1988). Immunoprecipitates were resuspended in sample buffer and subjected to SDS-PAGE in a 11% gel and then transferred to nitrocellulose. The blot was first probed for glycoprotein using the ECL glycoprotein detection kit (Amersham). The membrane was then rinsed and the peroxidase inactivated as recommended by the manufacturer. The blot was reprobed with the anti-HA antibody as described above.

Calf intestinal phosphatase (CIP) assay: To treat cell extracts with CIP, we followed previously described procedures (Gross *et al.* 1992). A total of 5 μg of whole cell extracts were diluted 1:10 in phosphatase buffer [100 mm Tris-HCl, pH 9.6, 1% Triton X-100, 2 mm $MgCl_2$, 0.1 mm $ZnCl_2$, and protease inhibitors (as above) with or without phosphatase inhibitors (0.2 mm sodium vanadate, 50 mm potassium fluoride, 30 mm sodium pyrophosphate]. Five units of CIP (Bio-Rad, Hercules, CA) were added to each tube and incubated at 37° for 1 hr. Reaction mixtures were stopped by the addition of SDS-PAGE sample buffer and then boiled for 5 min before electrophoresis.

***In vivo* phosphorylation assay:** Cells expressing the Wsc1p-HA fusion protein were grown at 30° to midlog phase in low-phosphate selective media. Cells were harvested by centrifugation and then resuspended in selective media without phosphate (Kolodziej and Young 1991). [^{32}P]Orthophosphate was added to the culture to a final concentration of 500 μCi/ml and the cells were incubated at 28°. After 3 hr, the cultures were harvested and washed once with ice-cold stop buffer (0.9% NaCl, 1 mm sodium azide, 10 mm EDTA, 50 mm sodium fluoride) and then resuspended in cold lysis buffer. Whole cell extracts were prepared as described above. A total of 1.0×10^8 cycles per minute of trichloroacetic acid-insoluble radioactivity were used for immunoprecipitation with the anti-HA antibody (as above) in IP buffer (50 mm Tris-HCl, pH 7.5, 1% Triton X-100, 50 mm sodium fluoride, 5 mm sodium pyrophosphate, and 1 mm sodium vanadate). The protein was eluted from the immunoprecipitates, subjected to SDS-PAGE in a 8% gel, and followed by autoradiography.

RESULTS

***WSC1* deletion constructs:** To study the properties of Wsc1p in intact cells we first tagged the protein with

the HA epitope. We made a construct in which a triple-epitope tag-coding sequence was added in frame 3′ to the last codon of the open reading frame of *WSC1* (see materials and methods). The epitope was added at the C terminus because at the N terminus the Wsc1 protein has a putative signal peptide. Addition of the tag to the N terminus would result in its cleavage. A functional fusion protein was expected because a fusion between Wsc1p and the green fluorescent protein (GFP) at the C terminus is able to suppress the lysis defect of a *wsc1*Δ deletion mutant (Verna *et al.* 1997). The derived amino acid sequence of the epitope-tagged protein, Wsc1-HA, is shown in Figure 1. We expressed *WSC1-HA* under the control of the strong alcohol dehydrogenase (ADH) promoter in the multicopy 2μ vector, pADNS (Colicelli *et al.* 1989). We used this vector to attain high levels of protein expression, to study its localization and properties in intact cells, and to compare levels of expression between wild type and mutant proteins. The Wsc1p-HA fusion protein suppresses the lysis defect of a *wsc1*Δ *wsc2*Δ *wsc3*Δ mutant on YPD at 30° (data not shown).

To study the contribution of different domains of Wsc1p to its function, we made five deletion constructs (see materials and methods). A schematic representation of the full-length Wsc1p-HA and the mutant proteins are depicted in Figure 2. All the mutant proteins have the signal peptide at the N terminus (not depicted) and the HA epitope at the C terminus.

Signal Peptide

1 MRPNKTSLLL ALLSILSQAN AYEYVNCFSS LPSDFSKADS YNWQSSSHCN 50

Cysteine Motif

51 SECSAKGASY FALYNHSECY CGDTNPSGSE STSSSCNTYC FGYSSEMCGG 100

101 EDAYSVYQLD SDTNSNSISS SDSSTESTSA SSSTTSSTTS STTSTTSSTT 150

Serine and Threonine Rich

151 SSTTSSMASS STVQNSPEST QAAASISTSQ SSSTVTSESS LTSDTLATSS 200

Serine and Threonine Rich

201 TSSQSQDATS IIYSTTFHTE GGSTIFVTNT ITASAQNSGS ATGTAGSDST 250

Transmembrane

251 SGSKTHKKKA NVGAIVGGVV GGVVGAVAIA LCILLIVRHI NMKREQDRME 300

P P

301 KEYQEAIKPV EYPDKLYASS FSSNHGPSSG SFEEEHTKGQ TDINPFDDSR 350

P Triple HA epitope

351 RISNGTFING GPGGKNNVLT VVNPDEADSG RIFYPYDVPD YAGYPYDVPD 400

401 YAGSYPYDVP DYAAQCGR 414

Figure 1.—Sequence of the Wsc1-HA protein. The amino acid sequence of Wsc1p and its various domains are as in Verna *et al.* (1997). The general boundaries of the domains are delimited with the arrows at the end of the lines. The domains are as follows: a putative signal peptide; the cysteine motif; a serine/threonine-rich region; and a transmembrane domain. The triple hemagglutinin (HA) epitope is also shown. Indicated by underlining are the cysteine residues in the cysteine motif and in the transmembrane domain, three possible N-glycosylation sites (double lines at positions 4, 65, and 354), and an endocytosis signal in the C-terminal domain (double line at position 344). Three possible phosphorylation sites are depicted by the letter P enclosed in a circle. They are at positions 331, 349, and 353.

The Wsc family of proteins contains a cysteine motif at its N terminus, followed by a serine/threonine-rich region (Verna *et al.* 1997). These two domains are predicted to be extracellular. To establish the contribution of the extracellular domain to the function of Wsc1p, deletion constructs were made, coding for proteins lacking these domains individually or in combination. In Wsc1p (see Figure 1), the cysteine motif is composed of 81 amino acids from the first cysteine residue up to a pair of amino acids, VY, also conserved in all Wsc proteins. We generated a construct coding for a mutant protein lacking 79 amino acids in the cysteine motif (see Figure 2), WSC1-ΔCys. The serine/threonine-rich region (see Figure 1) in the Wsc1 protein is composed of about 143 amino acids. We generated a construct coding for a mutant protein, WSC1-ΔS/T, lacking 134 amino acids (see Figure 2) of this domain. We also generated a construct, WSC1-ΔNterm, coding for a protein lacking the extracellular domain (see Figure 2), but leaving behind 20 putative extracellular residues and the predicted transmembrane domain.

The Wsc proteins have a hydrophobic domain of about 26 amino acids, which is predicted to be transmembranous (Figure 1). To determine if this domain is necessary for the proper localization of the protein and for its function we made the construct WSC1-ΔTM (see Figure 2). The C terminus of the Wsc proteins is predicted to be intracellular. In Wsc1p, the smallest of the Wsc proteins, the C terminus is about 83 amino acids (Figure 1). In the WSC1-ΔCterm construct we deleted this sequence (see Figure 2) to determine if it is required for function.

We expressed these constructs in various mutant *wsc*Δ deletion strains and analyzed the properties of the mutant proteins by gel electrophoresis. We also tested for their ability to suppress the lysis defect of *wsc*Δ mutants.

Localization of the HA-tagged Wsc1p: To characterize the properties of Wsc1p-HA, we prepared total cell lysates or soluble and membrane fractions from the JVHw123Δ-1A strain transformed with the plasmid expressing WSC1-HA. Equivalent amounts of protein were analyzed by SDS-PAGE followed by immunoblotting with the monoclonal antibody (12CA5) against the HA epitope. As shown in Figure 3A, Wsc1-HA fractionates predominantly with the membrane fraction. This result is consistent with the proposal that Wsc1p is a membrane-bound protein with a stretch of 26 hydrophobic amino acid residues predicted to be transmembranous. Extracts from JVHw123Δ-1A transformed with the vector alone were used as negative controls and no signal was detected in any of the fractions tested (data not shown). To further demonstrate that this stretch of hydrophobic amino acid residues functions to localize Wsc1p to the membrane, we prepared cell extracts from the JVHw123Δ-1A strain transformed with the plasmid expressing Wsc1p-ΔTM. Total cell lysates and soluble and membrane fractions were prepared as described for Wsc1p-HA. The Wsc1p-ΔTM protein lacking these

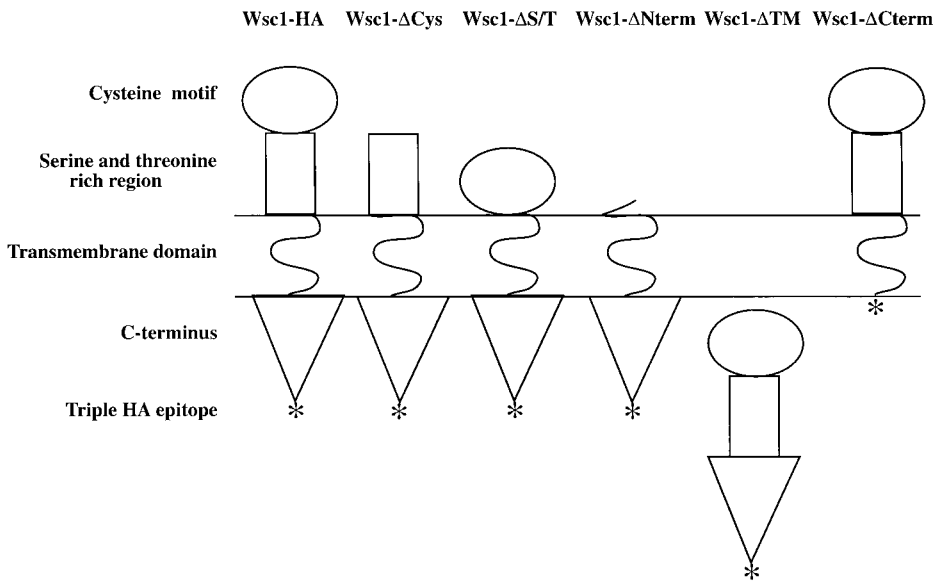

Figure 2.—Schematic representation of Wsc1p-HA and the mutant proteins. Wsc1p and the mutant proteins lacking various domains are shown schematically: a circle, the cysteine motif; a rectangle, the serine/threonine-rich region; a squiggle line, the transmembrane domain; an inverted triangle, the C-terminal domain; an asterisk, the triple HA epitope. The mutant proteins are depicted without the domain, which has been deleted in the constructs. Wsc1p-ΔCys lacks the cysteine motif from amino acids 22 to 100. Wsc1p-ΔS/T lacks the serine/threonine-rich region from amino acids 111 to 244. Wsc1p-ΔNterm lacks both domains from amino acids 22 to 244. The Wsc1p-ΔTM mutant lacks the transmembrane domain from amino acids 263 to 291. See materials and methods for description of the constructs.

hydrophobic amino acid residues localizes predominantly to the soluble fraction (Figure 3A). A small portion of Wsc1p-ΔTM is found to fractionate with the membrane fraction but this could be due to nonspecific interactions.

Wsc1p is tightly associated with the membrane: To characterize the association between Wsc1p and the membrane, total cell lysates prepared from JVHw123Δ-1A containing Wsc1p-HA were either mock treated (control) or incubated in 1% Triton X-100, 0.1 m Na_2CO_3, pH 11.0, 1.6 m urea, or 0.6 m NaCl. Lysates were separated by high-speed centrifugation at 100,000 *g* for 1 hr into supernatant and pellet fractions and then analyzed by immunoblotting. Wsc1p-HA is not solubilized by 0.1 m Na_2CO_3, pH 11.0, 1.6 m urea, or 0.6 m NaCl and is found to fractionate primarily with the membrane pellet after high-speed centrifugation (Figure 3B); thus Wsc1p-HA is not a peripherally associated membrane protein. Control mock-treated lysates also showed that Wsc1p-HA primarily fractionates with the membrane pellet. However, Wsc1p-HA is solubilized with 1% Triton X-100 and found in the supernatant fraction, indicating that Wsc1p is an integral membrane protein (Figure 3B).

Wsc1p-HA is solubilized by 1% Triton X-100; however, there still seems to be a significant amount of Wsc1p-HA in the pellet fraction. To address this, we prepared total cell lysates as described above and treated the cell extracts with either 0.5% Triton X-100 alone or 0.5% Triton X-100 in combination with 0.8 m urea or 0.3 m NaCl. Lysates were then centrifuged at high speed and separated into supernatant (S1) and pellet fractions as described above. In this experiment, the pellet was resuspended and treated a second time with Triton X-100 alone or Triton X-100 in combination with urea or NaCl to completely solubilize Wsc1p from the pellet. This resuspended pellet was centrifuged and separated again into supernatant (S2) and pellet fractions (P). Lysates that were mock treated show Wsc1p-HA to fractionate with the pellet (Figure 4A, Control). As we demonstrated above, a portion of Wsc1p-HA was solubilized with Triton X-100 (Figure 4A, Triton X-100-S1). We resuspended the pellet again in Triton X-100 and Wsc1p-HA was not solubilized upon this second treatment (Figure 4A, Triton X-100-S2) but maintained a tight association with the pellet (Figure 4A, Triton X-100-P). When lysates were treated with Triton X-100 and urea, Wsc1p-HA was completely solubilized and found in supernatant fractions (Figure 4B, Triton X-100 + Urea-S1 and -S2). Treatment with Triton X-100 and NaCl did not completely solubilize Wsc1p-HA, leaving a significant amount still associated with the pellet, similar to the Triton X-100 treatment alone (Figure 4B, Triton X-100 + NaCl). The same results were observed using higher concentrations of reagents, 1% Triton X-100 with or without 1.6 m urea or 0.6 m NaCl (data not shown). These results suggest that Wsc1p is an integral membrane protein and that a fraction of the protein in intact cells associates tightly with other cellular components.

Localization and apparent size of the HA-tagged Wsc1 and the mutant proteins: To characterize the properties of the different domains of Wsc1p we made cellular extracts from the ALH758 strain transformed with the plasmid expressing Wsc1p-HA or the Wsc1p mutants. We analyzed the membrane localization and the apparent size of the proteins by gel electrophoresis. Cellular extracts were prepared (as described in materials and methods) and equivalent amounts of soluble or membrane-extracted proteins were analyzed by SDS-PAGE followed by immunoblotting with the monoclonal antibody (12CA5) against the HA epitope. The soluble and membrane fractions were prepared at a centrifugation speed (16,000 × *g*) lower than the experiments in Figure 3. However, it has been shown that a significant propor-

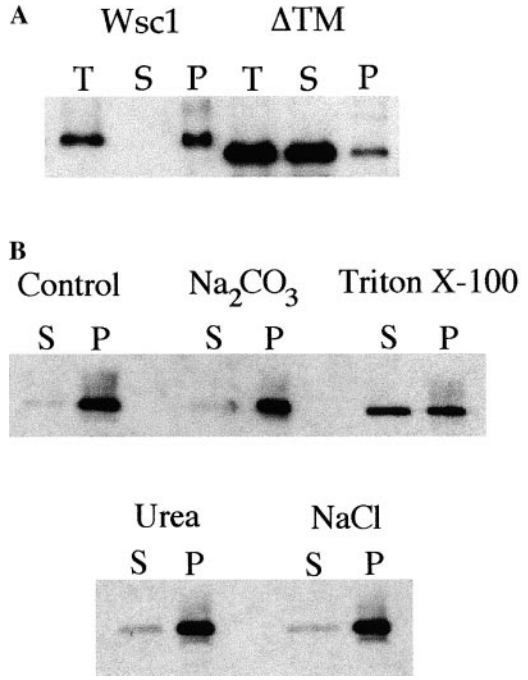

Figure 3.—Fractionation of Wsc1p to the membrane pellet and the mutant Wsc1p-ΔTM to the cytosol and extraction of Wsc1p from the membrane fraction. (A) Total cell lysates (T) and soluble (S) and membrane (P) fractions were prepared from yeast strains expressing Wsc1p-HA or Wsc1p-ΔTM as described in materials and methods. A total of 2.5 μg of protein for each sample was loaded, separated by SDS-PAGE, transferred to nitrocellulose, and subjected to immunoblot analysis using the anti-HA monoclonal antibody. (B) Total cell lysates from a yeast strain expressing Wsc1p-HA were separated into five aliquots. Aliquots were either mock treated (Control), or incubated in 0.1 m Na_2CO_3, 1% Triton X-100, 1.6 m urea, or 0.6 m NaCl. Lysates were centrifuged at 100,000 *g* for 1 hr and soluble (S) and pellet (P) fractions were collected as described in materials and methods. Wsc1p was detected by immunoblotting as in A.

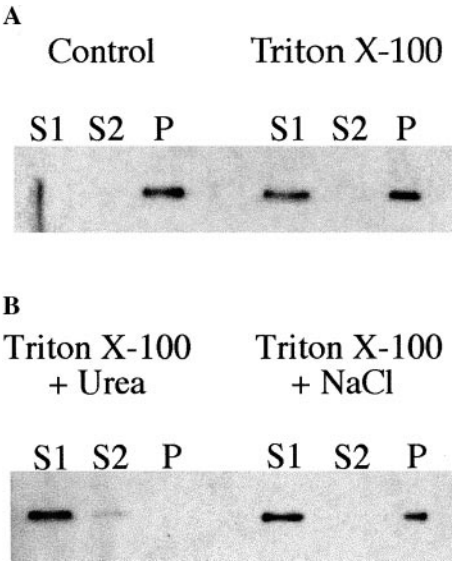

Figure 4.—Complete solubilization of Wsc1p with Triton X-100 and urea. Whole cell extracts were divided into four separate aliquots and either (A) mock treated (Control) or incubated in 0.5% Triton X-100, or (B) 0.5% Triton X-100 + 0.8 m urea or 0.5% Triton X-100 + 0.3 m NaCl. Treated extracts were centrifuged at 100,000 × *g* for 1 hr and supernatants (S1) were collected. The resulting pellets were resuspended again in the respective reagents and centrifuged for 1 hr at 100,000 × *g*. The supernatant (S2) was collected and the resulting pellet (P) was resuspended in M buffer (see materials and methods). Equal amounts of protein for each fraction (S1, S2, P) were separated by SDS-PAGE and subjected to immunoblot analysis as described in Figure 3.

tion of the plasma membrane pellets at speeds as low as 10,000 × *g* (Goud *et al.* 1988; Roemer *et al.* 1996). The results are shown in Figure 5, A and B. In the control lane, no signal is present in either the supernatant or pellet fraction. As before (Figure 3A), full-length Wsc1p-HA fractionates predominantly with the crude membrane fraction (Figure 5, compare A to B). The same fractionation pattern is seen for the mutant proteins, Wsc1p-ΔCys, Wsc1p-ΔS/T, Wsc1p-ΔNterm, and Wsc1p-ΔCterm. Consistent with the results shown in Figure 3A, deletion of the sequence encoding the putative transmembranous domain changes the solubility of Wsc1p (Figure 5, A and B). There is a low level of the protein in the pellet because, with a low speed centrifugation, some of the soluble proteins fractionate with the membrane fraction.

The apparent molecular weight of Wsc1p-HA is ~140 kD (Figure 5), which is three times higher than its predicted molecular weight of ~43 kD. This significant difference can be attributed to the serine and threonine-rich domain, which may contain sites for glycosylation (see below; Gray *et al.* 1997; Verna *et al.* 1997). It is also possible that these proteins are post-translationally modified by mechanisms other than glycosylation. Additionally, the C-terminal domain of the Wsc proteins is highly charged (Verna *et al.* 1997), which can also explain its aberrant mobility on SDS-PAGE. There are other low-molecular-weight bands detected by the antibody in the extracts from cells expressing Wsc1p-HA (Figure 5B). These bands are not present in extracts prepared from cells transformed with the empty vector (Figure 5B). They may be degradation products because the same bands are also seen in extracts prepared from cells expressing Wsc1p-ΔCys, Wsc1p-ΔS/T, and Wsc1p-ΔNterm, which are all predicted to have different molecular weights.

Deletion of the sequence coding for the cysteine motif in the Wsc1p-ΔCys mutant protein results in the appearance of multiple bands. There are two major and one minor bands of high molecular weight that migrate much higher than 34 kD, the predicted size of this mutant. This mutant protein may be more susceptible to degradation than the wild-type protein, or the various bands may be intermediate forms of post-translational modification. Proteins lacking the serine/threonine-rich domain (Wsc1p-ΔS/T) or the extracellular domain

(Wsc1p-ΔNterm) migrate closer to their predicted size of 30 or 21 kD, respectively. This suggests that the significant increase in the apparent molecular weight of the Wsc1 protein is contributed by the serine/threonine-rich domain. The two mutant proteins show a diffuse mobility and their sizes are still larger than predicted. In each case two major bands can be detected. The diffuse mobility may be a result of different degrees of phosphorylation (see below). As mentioned above, the C-terminal domain is highly charged (Verna *et al.* 1997) and this may explain the aberrant mobility. The mutant Wsc1p-ΔTM, lacking the transmembrane domain, and the Wsc1p-ΔCterm, lacking the C-terminal domain, also migrate with mobility higher than their predicted sizes of 41 and 33 kD, respectively.

Lower levels, but similar patterns, of protein expression are observed when the various constructs are under the control of the endogenous *WSC1* promoter in a *CEN* vector (data not shown).

Glycosylation state of Wsc1p and the mutant proteins: To establish if the Wsc1 protein is glycosylated *in vivo* and to compare the results with the mutant proteins we used the enhanced chemiluminescence (ECL) glycoprotein detection system from Amersham. With this glycoprotein detection system the carbohydrate portion of the protein is oxidized with sodium metaperiodate to form aldehydes that can react with hydrazides. Biotin-X-hydrazide is used to attach biotin to the oxidized carbohydrate. Biotin is then detected by streptavidin conjugated to horseradish peroxidase. Glycoprotein detection was performed for Wsc1p-HA and for the Wsc1p mutant proteins, using the same cell extracts as in Figure 5 (see materials and methods). Crude membrane extracts were used for all (Figure 5B) but Wsc1p-ΔTM, for which we used the soluble extract (Figure 5A). The HA-tagged proteins were immunoprecipitated, loaded onto a 13% gel, and transferred to a nitrocellulose membrane. The membrane was then used for detection of carbohydrates. The results are shown in Figure 6A. The same blot was then washed and probed with the anti-HA antibody as a control (see materials and methods) to determine the total amount of epitope-tagged protein present. The results are shown in Figure 6B.

The full-length Wsc1 protein is glycosylated as are the mutant proteins Wsc1p-ΔCys, Wsc1p-ΔTM, and Wsc1p-ΔCterm (Figure 6A). In Wsc1p-ΔCys the two major bands that are detected with the anti-HA antibody (Figure 5B) contain carbohydrates. No glycosylation is observed in Wsc1p-ΔS/T and Wsc1p-ΔNterm, which lack the serine/threonine-rich region (Figure 6A). When the immunoprecipitated protein is analyzed with the anti-HA antibody it can be seen that these two mutant proteins show a diffuse mobility with intensities similar to Wsc1p-ΔCterm and to the lower band of Wsc1p-ΔCys, which suggests that the lack of signal is not due to insufficient protein levels. These results show that the serine/threonine-rich domain is necessary for glycosylation of the Wsc1 protein and suggest that the increase in size is due, at least in part, to glycosylation.

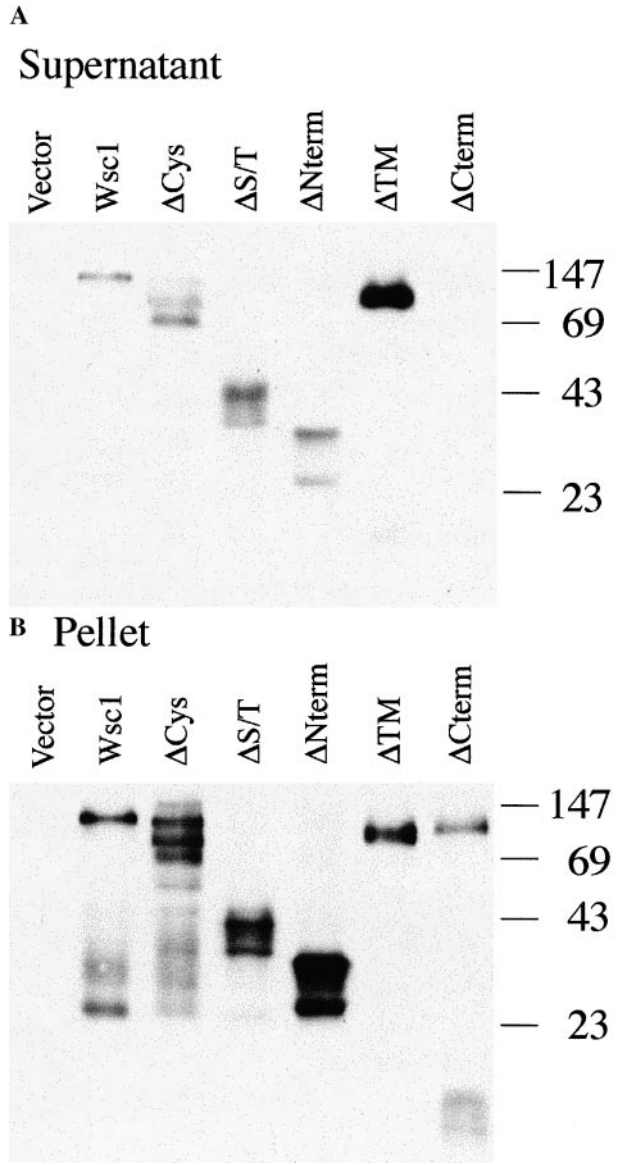

Figure 5.—Localization and apparent molecular weight of Wsc1p-HA and the mutant proteins. (A) Supernatant and (B) pellet fractions from ALH758 expressing full-length Wsc1p-HA or mutant proteins were prepared as described in materials and methods. A total of 10 μg of protein from each sample was loaded on a 13% SDS gel and transferred to nitrocellulose. The proteins were probed with the anti-HA antibody (12CA5). Lane 1: cell extract prepared from yeast cells transformed with the vector, pADNS.

Phosphorylation state of Wsc1p and the mutant proteins in intact cells: Noting that the Wsc1p-ΔS/T and Wsc1p-ΔNterm mutant proteins do not seem to be glycosylated and yet migrate on SDS-PAGE with a diffuse mobility, we tested if this may be because of phosphorylation. To characterize the phosphorylation state of Wsc1p *in vivo* and to compare it to the mutant proteins we prepared extracts from cells expressing the various

proteins and subjected them to treatment with CIP in the presence or absence of phosphatase inhibitors, as described in materials and methods. The results are shown in Figure 7A. Treatment of Wsc1p-HA with CIP in the absence of phosphatase inhibitor induces a small shift in the molecular weight of the protein (Figure 7A). Treatment of Wsc1p-ΔS/T (Figure 7A), Wsc1p-ΔNterm (Figure 7A), and Wsc1p-ΔCys (data not shown) with CIP induces a shift in the molecular weight, suggesting that these proteins are also phosphorylated in intact cells. The shift in molecular weight of Wsc1p-ΔS/T and Wsc1p-ΔNterm is significantly higher than that of Wsc1p-HA. This may be because of the lower molecular weight of these proteins, in which the difference in migration of the unphosphorylated and the phosphorylated forms is amplified. Treatment of Wsc1p-ΔTM (data not shown) and Wsc1p-ΔCterm (Figure 7A) does not seem to induce a change in their mobility. The shift in mobility after treatment with CIP suggests that the C-terminal domain of the Wsc1 protein is phosphorylated in intact cells.

To further demonstrate that Wsc1p is phosphorylated

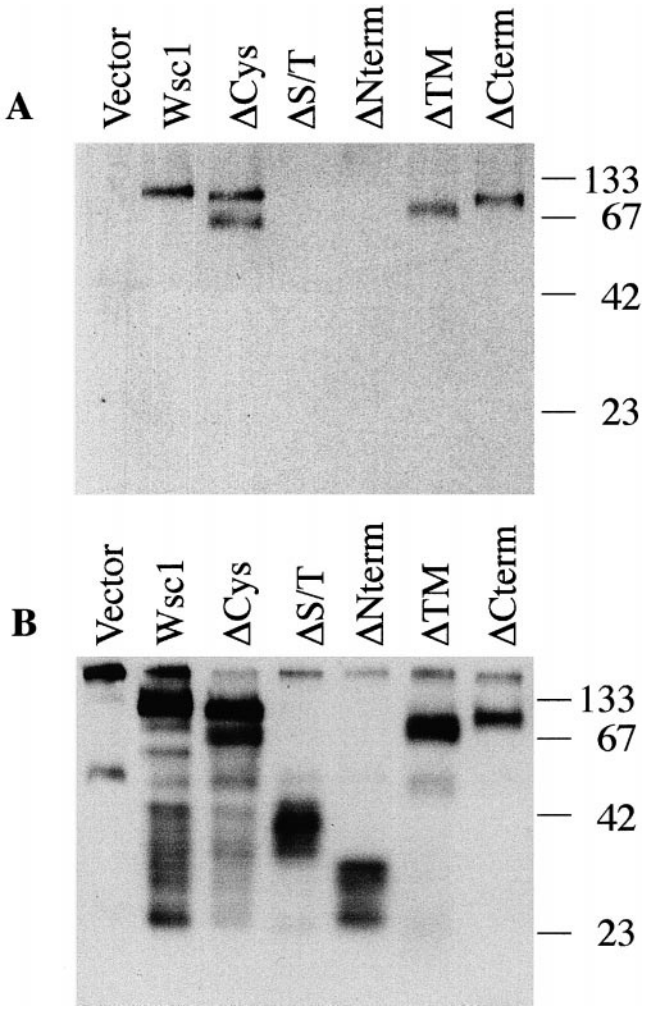

Figure 6.—Glycosylation of Wsc1p-HA and the mutant proteins. Wsc1p-HA and the mutant proteins were immunoprecipitated with the anti-HA antibody from cell extracts prepared as described in materials and methods, loaded on an 11% gel, and analyzed by immunoblotting. Lane 1 represents control yeast transformed with the vector, pADNS. (A) The blot was probed for the presence of glycoprotein. (B) The same blot was washed and reprobed with the anti-HA antibody as described in materials and methods.

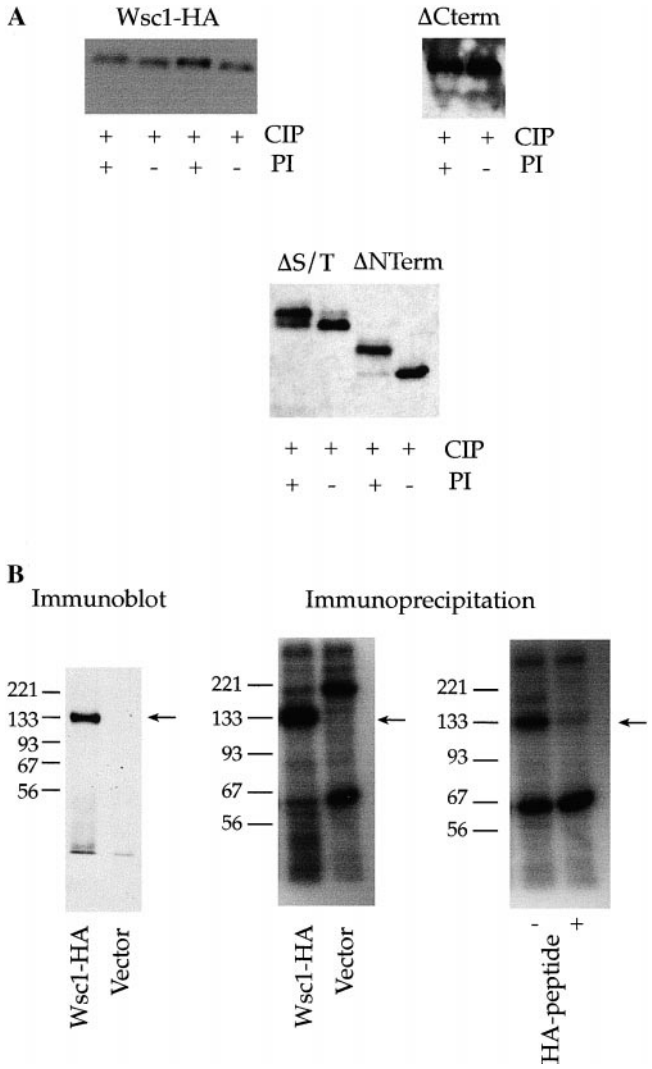

Figure 7.—Wsc1p-HA and the mutant proteins are phosphorylated *in vivo*. (A) Whole cell extracts from the *wsc*Δ strain (ALH758) expressing Wsc1p-HA and the Wsc1p-mutant proteins were prepared and subjected to treatment with calf intestinal phosphatase (CIP), with (+) or without (−) phosphatase inhibitors (PI) as described in materials and methods. Immunoblot analysis was performed with the anti-HA, 12CA5 antibody. (B) The *wsc*Δ strain expressing Wsc1p-HA or a control strain expressing only the vector was incubated with [^{32}P]orthophosphate and whole-cell extracts were prepared as described in materials and methods. The extracts were immunoprecipitated with anti-HA antibody. Extracts from cells expressing Wsc1p-HA were also incubated with anti-HA antibody in the absence (−) or presence (+) of 5 μg of the HA peptide. Samples were subjected to SDS-PAGE and the dried gel was subjected to autoradiography. Whole cell extracts were also prepared from the same strains without *in vivo* labeling and subjected to immunoblot analysis using the HA antibody.

in intact cells, we *in vivo* labeled the JVHw123Δ-1A strain expressing full-length Wsc1p-HA. We analyzed the phosphorylation state of Wsc1p-HA by determining the incorporation of ^{32}P by immunoprecipitation and autoradiography (as described in materials and methods). The results are shown in Figure 7B. The HA-directed antibody immunoprecipitates a radioactive protein in extracts from cells expressing Wsc1p-HA, but not from cells expressing the control vector. Immunoprecipitation of the labeled band is competed by addition of the HA epitope. The migration of the immunoprecipitated phosphoprotein on SDS-PAGE is very similar to the migration of the protein detected by immunoblotting using the 12CA5 antibody, suggesting that it may be Wsc1p-HA and not another protein that coimmunoprecipitates with Wsc1p-HA.

Suppression of the lysis defect of *wsc*Δ strains by expression of Wsc1p-HA and the mutant proteins: To determine the contribution of the different domains of Wsc1p to the function of the protein in maintenance of the cell wall integrity in yeast, we expressed the various deletion constructs in a *CEN* vector under the control of the *WSC1* promoter (see materials and methods) and tested for the ability to suppress the lysis defect of ALH7, a *wsc1*Δ deletion strain (Figure 8A). WSC1-HA is functional as a suppressor of the lysis defect of this strain when tested on YPD at 35° and 37° (Figure 8A). WSC1-HA suppression is similar to WSC1 without the epitope-tag. The *wsc1*Δ deletion mutant grows well when 1 m sorbitol is added to the media (Figure 8A, YPD 37° + 1 m sorbitol).

The cysteine motif is not essential for the ability of Wsc1p to suppress the lysis defect of the *wsc1*Δ strain. The WSC1-ΔCys construct suppresses the lysis defect of the *wsc1*Δ strain on YPD at 35°. However, removal of the cysteine motif has an effect on the protein's function because the WSC1-ΔCys construct does not suppress the lysis defect on YPD at 37°. The lack of suppression of the *wsc1*Δ strain at 37° is not the result of an increase in temperature because the WSC1-ΔCys construct is unable to suppress the lysis defect of a *wsc1*Δ *wsc2*Δ deletion strain on YPD at 35° (Figure 8B). This strain has a more severe lysis defect than the *wsc1*Δ strain (Verna *et al.* 1997). Lack of suppression is not a result of decreased expression because the Wsc1p-ΔCys protein levels are somewhat higher than those of full-length Wsc1p-HA, both in the multi-copy vector under the expression of the *ADH* promoter (Figure 5) and in the single-copy vector under the control of the *WSC1* promoter (data not shown). In multi-copy, the WSC1-ΔCys construct suppresses the lysis defect of *wsc*Δ mutants at all temperatures (data not shown).

The predicted extracellular domain is essential for the function of Wsc1p. Constructs lacking the serine/threonine-rich region (WSC1-ΔS/T) or the entire extracellular domain (WSC1-ΔNterm) are nonfunctional in suppression of the lysis defect (Figure 8A). Because the proteins are expressed highly (Figure 6) the lack of

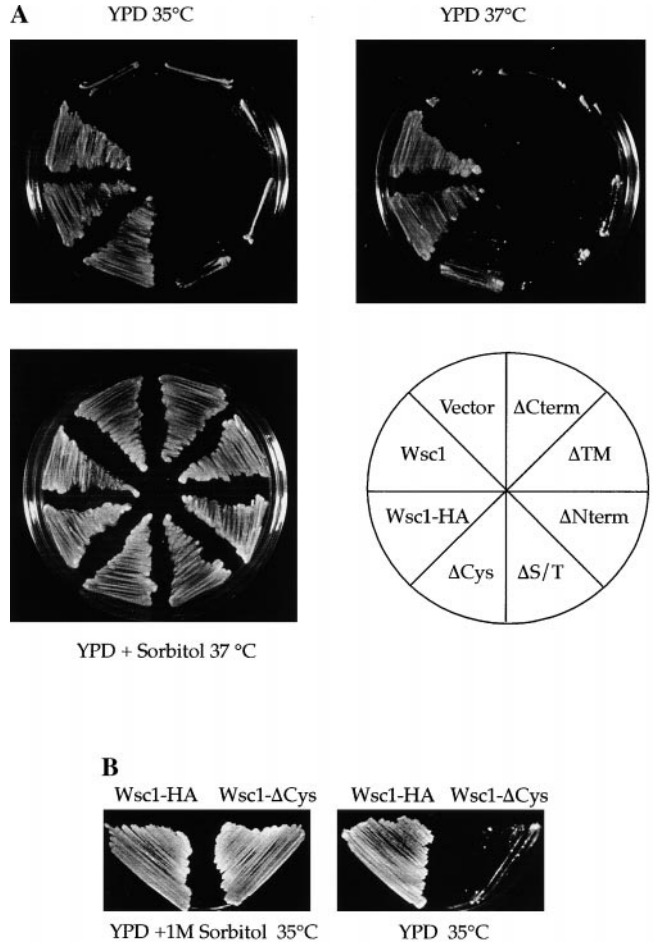

Figure 8.—Suppression of the lysis defect of *wsc*Δ strains by full-length Wsc1p-HA and the mutant proteins. The (A) *wsc1*Δ strain (ALH7) and (B) *wsc1*Δ *wsc2*Δ strain (ALH718) were transformed with the various plasmids and plated on selective media containing 1 m sorbitol. Transformants were streaked on YPD with or without 1 m sorbitol, grown at the indicated temperatures for 3 days, and photographed. The vector plasmid is pCEN1234.

suppression is not due to insufficient protein levels. The serine/threonine domain may be an important determinant for function in the extracellular domain, because lack of the cysteine motif does not entirely abolish function. The transmembrane domain is also essential for the function of Wsc1p-HA. The WSC1-ΔCterm construct that expresses a protein containing only the extracellular domain is not functional in suppression of the lysis defect of the *wsc*Δ mutant (Figure 8A). The same results are obtained when the mutant constructs are expressed in the multi-copy vector under the control of the *ADH* promoter (not shown).

Overexpression of *WSC1-HA* and *WSC1-ΔCterm* inhibit growth: During the course of our studies, we observed that, when cells are grown in liquid culture, overexpression of WSC1-HA under the strong *ADH* promoter in all *wsc*Δ strains tested causes a growth inhibition. To further characterize this observation we measured the rate of growth of wild-type cells expressing full-length

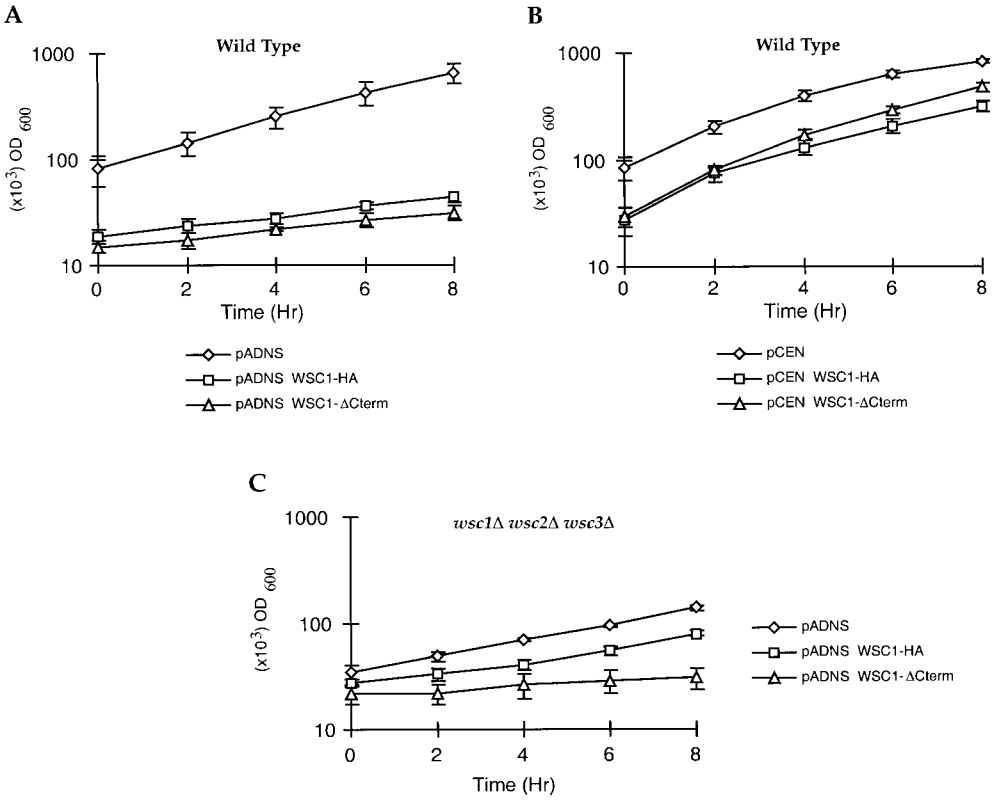

Figure 9.—Inhibition of the growth rate of wild-type and *wsc*Δ deletion strains by overexpression of Wsc1p-HA and Wsc1p-ΔCterm. Yeast cells were transformed with plasmids containing full-length Wsc1p-HA or Wsc1p-ΔCterm expressed under the control of the *ADH* promoter (pADNS) or the *WSC1* promoter (pCEN) and selected for growth on selective media. Cultures were grown to saturation in selective media, diluted, and then incubated at 30° overnight (~12 hr). After overnight incubation, the OD_{600} was determined at the indicated time points. The OD_{600} (OD_{600} = 1 represents 3×10^7 cells/ml) is plotted as a function of time. The mean from three independent experiments is shown. The error bars represent the standard error of the mean. (A and B) JVHwt (wild type). (C) JVHw-123Δ-1A (*wsc1*Δ *wsc2*Δ *wsc3*Δ).

Wsc1p-HA or the mutant proteins. Overexpression of WSC1-HA reduces the growth rate of the wild-type strain compared to the control vector (pADNS) (Figure 9A). Expression of WSC1-HA under the control of its own promoter and in a single-copy vector is slightly inhibitory to the growth rate of wild-type cells (Figure 9A), but to a lesser degree than expression of WSC1-HA under the control of the *ADH* promoter in a multi-copy vector. This would indicate that the effect of Wsc1p-HA is due to an increase in levels of expression. Significantly, overexpression of WSC1-ΔCterm is sufficient for the growth inhibition (Figure 9A). Similar to the expression of full-length WSC1-HA, expression of the WSC1-ΔCterm in a single-copy vector under the control of its own promoter reduces the growth inhibitory effect (Figure 9A).

When WSC1-HA or WSC1Δ-Cterm are overexpressed in a *wsc1*Δ *wsc2*Δ *wsc3*Δ strain, there is a reduction in the growth rate (Figure 9B). In this strain the effect of overexpression of WSC1-ΔCterm is more severe than that of full-length WSC1-HA, suggesting that deleting the C terminus of Wsc1p has produced a mutant protein with a negative effect on growth.

DISCUSSION

The results presented here show that Wsc1p is an integral membrane protein (Figures 3 and 4) found to be glycosylated (Figure 6) and phosphorylated (Figure 7) in intact cells and that a fraction of Wsc1p is Triton X-100 insoluble (Figure 4). Proteins that are not solubilized at 4° when treated with nonionic detergents are considered to be cytoskeletally associated (Tarone *et al.* 1984; Neame *et al.* 1995). It is possible that association of Wsc1p with the cytoskeleton prevents its complete solubilization. The results from this study, together with our previous localization of Wsc1p-GFP to the periphery of the cell (Verna *et al.* 1997), strongly suggest that Wsc1p is an integral membrane protein that localizes to the plasma membrane.

The transmembrane domain of Wsc1p may be required for other functions in addition to proper localization of the protein (Figure 3). In mammalian cells the transmembrane domain of the IL-6 cytokine receptor β-subunit gp130 has been shown to be important for signaling (Kim and Baumann 1997). Mutations in the transmembrane domain of the Neu/ErbB-2/HER-2 receptor (Bargmann *et al.* 1986) and other tyrosine kinase receptors (Ullrich and Schlessinger 1990) render the proteins constitutively active and oncogenic. The mammalian cell-surface receptor CD44, which is involved in the physiology of normal and tumor cells, has a cysteine residue in its transmembrane domain that is involved in dimerization (Liu and Sy 1997). In the protein ponticulin a cysteine in the β-barrel structure spanning the membrane is also involved in dimerization and is required for actin binding (Hitt *et al.* 1994). It is interesting to note that Wsc1p, but not the other Wsc proteins, has a cysteine residue in its transmembrane domain at position 282 (see Figure 1). It remains to be

determined whether this cysteine residue plays a role in the function of Wsc1p.

Glycosylation is believed to play a role in molecular recognition and has been shown to be important for cell adhesion and infection and antibody recognition in mammalian cells. Glycosylation is also important for maintaining proper folding and for the normal function of many proteins (Hounsell *et al.* 1996; O'Connor and Imperiali 1996; Gabius 1997; Rudd and Dwek 1997). The extracellular domain is essential for glycosylation of Wsc1p (Figure 6) with the serine and threonine residues as putative sites for *O*-linked glycosylation. In addition, there are three putative N-glycosylation sites in Wsc1p: one at the N terminus, 4-NKT, 5′ to the signal peptide; one in the cysteine motif, 65-NHS; and a third at the C terminus, 354-NGT (see Figure 1). Deletion of the sequences coding for the extracellular domain or the serine/threonine-rich region may abolish the function of Wsc1p (Figure 8) because it causes a misfolding of the protein, thereby impairing the ability of the C-terminal domain to signal. However, we find that both mutant proteins are phosphorylated in intact cells (Figure 7). This suggests that some of the normal folding of the protein is retained because they are recognized by the enzyme(s) responsible for the phosphorylation. The serine/threonine-rich domain of the Wsc1 protein may be required for the proper function of the protein because it mediates the interaction with other plasma membrane proteins, cell wall proteins, or signaling molecules. A possible interaction of Wsc1p with other cellular components is supported by our finding that overexpression of the extracellular domain alone inhibits growth of the wild type or mutant yeast strains (Figure 9).

Cysteine motifs are found in many classes of receptors or immunoglobulins where they function in oligomerization and ligand binding (Williams and Barclay 1988; Ullrich and Schlessinger 1990; Hynes 1992; Naor *et al.* 1997). The cysteine motif of Wsc1p may form a strongly structured domain that is placed at the end of a serine and threonine rod to sense signals and/or modulate the activity of the protein. Lack of this domain changes the ability to suppress the lysis defect of a *wsc1*Δ strain (Figure 8) and it results in an altered mobility on SDS-PAGE (Figures 5 and 6). This change in mobility may result from a change in the pattern of glycosylation or the pattern of phosphorylation.

The C-terminal domain of Wsc1p does not show any similarity to known kinases or phosphatases and it does not have any known motifs for interaction with other signaling molecules. This domain is the most divergent within the Wsc family, but in all the family members it is characterized by the presence of mostly negatively charged amino acid residues (Verna *et al.* 1997). The C-terminal domain is not functional in the lysis assay (Figure 8), but it is found to be phosphorylated (Figure 7). In the C terminus of Wsc1p there are several serine phosphorylation sites predicted by the Prosite database (Appel *et al.* 1994; see Figure 1): a casein-kinase-2 site (position 331, SFEE); a protein-kinase-C site (position 349, SRR); and a cAMP-dependent-protein-kinase site (position 350, RRIS). We have previously shown that there is a relation between the function of the *WSC* genes and the PKC1-regulated MPK1 cascade, as well as a relation between the function of *WSC* and the RAS-cAMP pathway in the stress response in yeast (Verna *et al.* 1997). The presence of putative cAMP-dependent protein kinase and protein-kinase-C phosphorylation sites suggests that there may be direct interaction between these proteins.

Interestingly, the intracellular domain of Wsc1p also shows an endocytosis signal motif NPFDD (at position 344). The NPFXD motif has been described as a new class of endocytosis signal in *S. cerevisiae* (Tan *et al.* 1996). This sequence is a clathrin-dependent endocytosis signal in the cytoplasmic domain of the a-pheromone receptor STE3, where it is important for recovery after mating. The a-pheromone receptor uses a different endocytosis signal (Hicke and Riezman 1996). It will be interesting to determine whether this sequence is important for the function of Wsc1p.

On the basis of the finding that overexpression of Wsc1p-HA induces an inhibition of growth (Figure 9), we propose that Wsc1p functions in a complex with at least one other protein. The putative protein, protein X, may be essential for growth. Overexpression of the full-length Wsc1 protein in a *wsc*Δ strain complements its lysis defect, but may also inhibit cell growth because it reduces the amount of available protein X in the cell. The interaction between Wsc1p and protein X is at least in part mediated by the N-terminal domain because its overexpression alone inhibits cell growth (Figure 9). Protein X is not Wsc4p, because the growth inhibition is also observed in a *wsc1*Δ *wsc2*Δ *wsc3*Δ *wsc4*Δ strain (data not shown).

The Wsc1 protein may function in yeast in a manner analogous to mammalian integrins. Integrins are type I plasma membrane proteins that interact with components of the extracellular matrix (ECM) and with the cytoskeleton. They function in cell growth, cell migration, differentiation, and programmed cell death (Hynes 1992; Howe *et al.* 1998). In coordination with the Ras and the Rho family of small GTPases and MAPK cascades, integrins function to regulate the organization of the actin cytoskeleton (Hynes 1992).

Given the conservation of the role of the Rho-like proteins and MAPK cascades in the regulation of the actin cytoskeleton and in the stress response (reviewed in Herskowitz 1995; and Lim *et al.* 1996), further studies of the function of Wsc will provide insights into the molecular basis of this fundamental process in yeast, as well as in all other organisms.

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