Inorganic Cations Mediate Plant PR5 Protein Antifungal Activity through Fungal *Mnn1*- and *Mnn4*-Regulated Cell Surface Glycans

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Antimicrobial activities of many defense proteins are profoundly altered by inorganic cations, thereby controlling disease pathologies in a number of mammalian systems, such as cystic fibrosis in humans. Protein-based active defense systems in plants also are influenced by cations; however, little is known of how these cation effects are mediated. Cytotoxicity of the pathogenesis-related protein osmotin against the model fungus *Saccharomyces cerevisiae* was progressively abolished by K⁺. By the use of *S. cerevisiae* mannosylation mutants, this effect was shown to require mannosephosphate residues in the cell wall. However, osmotin activity was not suppressed by even high concentrations of Ca²⁺. Rather, submillimolar levels of Ca²⁺ specifically facilitated osmotin's activity, as well as its binding to the cell surface. This effect also was dependent on mannosephosphate groups on the cell surface, and appeared to require negative charge on a portion of the osmotin protein. Results suggest that Ca²⁺ modulates osmotin action by facilitating its binding to the fungal cell surface, but that K⁺ blocks this interaction by competing for binding to mannosephosphate groups. Therefore, we have identified glycan interaction as a mechanism for antimicrobial protein activity modulation by cations, a pattern that may apply to diverse innate defense responses.

Additional keywords: antifungal, calcium, PR5.

Numerous plant defense proteins with antimicrobial activity have been described. However except for the presumed degradation of d-glucans and chitin (pathogenesis-related [PR] protein classes PR2 and PR3) and ribosome inactivating protein (RIP), little is known about the mode of action of these often very potent proteins. Some of these have been implicated to disrupt fungal plasma membrane function, possibly by forming pores. However, many show considerable specificity, having widely different activities against closely related fungi (Abad et al. 1996; Terras et al. 1992), sometimes attributable to only a few amino acid residues (DeSamblanx et al. 1997). These elements of specificity suggest the existence of functional targets on the pathogen cell surface that may interact

with the toxic proteins. Data consistent with the presence of a plasma membrane receptor exist for plant defensins (Thevisen et al. 1997). However, "receptors" for defense proteins are not necessarily proteinaceous. Many elements of host defense systems, such as mannose-binding proteins, selectins, and endocytotic receptors of hepatocytes and macrophages, recognize molecular patterns that are composed wholly or in part by carbohydrate moieties (Hoffmann et al. 1999; Weis et al. 1992). Even pathogen-encoded toxins such as Cholera toxin, Tetanus toxin, *Escherichia coli* enterotoxin, and Shiga toxin (MacKenzie et al. 1997; Ramegowda and Tesh 1996) recognize cell surface carbohydrates. Members of the plant PR protein osmotin (PR5) defense protein family recently have been shown to bind yeast phosphomannoproteins (Ibeas et al. 2000) and β -1,3 glucans (Osmond et al. 2001; Trudel et al. 1998), which are ubiquitous cell surface components in fungi, and these may serve as fungal pattern-recognition molecules for the proteins. Further, yeast cell surface components are known to act as initial recognition targets for the yeast killer toxins K1 (targeting β -1,6-D glucans) (Hutchins and Bussey 1983) and K28 (targeting mannoproteins) (Schmitt and Radler 1987). However, little information is available detailing how these toxin-target interactions occur, or how they may be affected by the environment or by other fungal or plant factors.

Many studies of antimicrobial protein action have been conducted in vitro under optimized conditions; however, solute and ion conditions within the infection court may substantially affect the activities of these proteins in situ (Terras et al. 1992). High levels of hexoses have been shown to materially increase the effectiveness of PR3 and PR5 proteins (Salzman et al. 1998), whereas inorganic cations dramatically suppress the effectiveness of many innate defense proteins from mammals (Lehrer et al. 1988) as well as plants (Abad et al. 1996; Broekaert et al. 1997). This cation suppression may be responsible for the relatively low level of protection seen in transgenic plants engineered to overexpress antifungal proteins (De Bolle et al. 1996).

In particular, Ca²⁺ concentrations as low as 1 mM dramatically reduce the antifungal activity of human defensins (Lehrer et al. 1988), hevein- or knottin-type peptides, and some plant defensins and lipid transfer proteins (LTPs), while other toxins of these classes are not significantly affected (Cammue et al. 1995). On the other hand, the pulmonary surfactant proteins

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SP-A and SP-D as well as the serum collectins mannose-binding protein and CL-43 bound in a Ca^{2+} -dependent manner to *Cryptococcus neoformans*, an opportunistic pathogen of the lung (Schelenz et al. 1995), and form part of the innate immunity system in animals (Hoffmann et al. 1999; Weis et al. 1992). Yeast killer toxin activity also was strongly stimulated by millimolar concentrations of Ca^{2+} (Kurzweilova and Sigler 1993), and Ca^{2+} dependence has been established for antifungal activity of the pradimicins and related compounds (Ueki et al. 1993).

The presence of monovalent cations such as K^+ or Na^+ almost universally reduces antimicrobial protein activity (Abad et al. 1996; Broekaert et al. 1997; Kurzweilova and Sigler 1993; Weis et al. 1992). However, monovalent cations generally are much less activity-suppressing on a molar basis than are divalent cations, and many proteins still retain some activity in the presence of 10 to 50 mM K^+ (Cammue et al. 1995; Terras et al. 1992) or 83 mM Na^+ levels commonly found in the plant apoplast (Grignon and Sentenac 1991) or in the extracellular fluids of mammalian tissues (Goldman et

Fig. 1. Extracellular Ca^{2+} mediates osmotin activity against *Saccharomyces cerevisiae* strain BWG, contingent on the presence of fungal phosphomannans. Yeasts of the indicated genotype were inoculated **A**, into synthetic medium with or without Ca^{2+} or **B–D**, into YPD (1% yeast extract, 2% Bacto peptone, and 2% glucose in distilled water) with or without 2 mM EGTA to chelate Ca^{2+} . Osmotin was added at stated concentrations. Yeast growth is shown as percentage of growth in respective medium without osmotin. Growth rate of BWG was not significantly affected by 2 mM EGTA, and growth rates of BWG and the mutant strains were equivalent in the absence of osmotin (data not shown). **A**, and **B**, BWG; **C**, *mnn4*; **D**, *Δmnn1*.

al. 1997), respectively. Although the biochemical basis of cation suppression has not been firmly established, an ionic interaction with either the antimicrobial proteins themselves or with their target molecules on the microbial cell surface often is presumed (Terras et al. 1992).

In recent studies of the plant antifungal protein osmotin (PR5), an initial target of osmotin's binding to yeast has been defined as the phosphate group contained in phosphomannoproteins of the yeast cell wall (Ibeas et al. 2000). We demonstrate here that Ca^{2+} apparently is instrumental in osmotin's binding to this target, resulting in enhanced antifungal activity. On the other hand, K^+ blocked osmotin activity strictly contingent on the presence of these mannosephosphate groups. We also show that osmotin is a Ca^{2+} -binding protein; a binding which is pH dependent and may depend on an acidic region of the toxin. Under physiological conditions, Ca^{2+} -mediated concentration of osmotin on the fungal cell surface likely is essential in allowing the protein an active role in plant defense, and Ca^{2+} may be essential to competitively titrate inhibiting monovalent cations.

RESULTS

Calcium facilitates osmotin antifungal activity and binding to the fungal cell surface.

We found that withholding Ca^{2+} from the incubation medium markedly reduced osmotin's action against *Saccharomyces cerevisiae* strain BWG (Fig. 1A). Extracellular chelation of Ca^{2+} with EGTA gave a similar effect (Fig. 1B). This suggested an extracellular role for Ca^{2+} in an interaction between the protein and a target on the fungal cell surface. To test the idea that Ca^{2+} facilitated osmotin's interaction with a cell wall recognition factor or factors, we used ^{35}S -labeled osmotin to monitor binding of the protein to the yeast cell surface. In vivo binding experiments indicated that removal of Ca^{2+} by EGTA reduced osmotin binding to the yeasts by approximately 40%

Fig. 2. Removal of Ca^{2+} reduces binding of osmotin to the yeast cell surface. Yeasts were precultured in YPD (1% yeast extract, 2% Bacto peptone, and 2% glucose in distilled water) with or without 2 mM EGTA, treated with ^{35}S -osmotin (10 $\mu\text{g}/\text{ml}$), and washed free of unbound protein after 60 min. Bound osmotin was determined by scintillation counting.

(Fig. 2), similar to the attenuation of osmotin-induced growth inhibition (Fig. 1B).

Calcium facilitation of osmotin activity is contingent on the presence of cell surface phosphomannans.

Several mannan-defective (*MNN*) genes have been shown to control glycosylation of mannoproteins in the *S. cerevisiae* cell wall (Ballou 1990). A knockout of the *MNN1* gene causes omission of terminal α 1-3-linked mannose residues in the outer chain portion of mannoprotein carbohydrate chains, leading to increased external exposure of mannosylphosphate groups, whereas knockout of *MNN4* causes reduction in the number of mannosylphosphate units. Growth inhibition experiments as described above performed on BWG mutated at *mnn4* showed that, contrary to the effect in BWG, the withholding of Ca^{2+} had no effect on osmotin's growth inhibition of the mutant (Fig. 1C). Further, the Ca^{2+} effect on osmotin's inhibition of BWG $\Delta mnn1$ mutant was even more pronounced than that on BWG (Fig. 1D). Calcium enhancement of osmotin action was found significantly greater against $\Delta mnn1$ than against BWG at dose levels of 5 and 10 $\mu\text{g}/\text{ml}$ by Student's *t* test ($P < 0.05$ at 5 $\mu\text{g}/\text{ml}$ and $P < 0.0001$ at 10 $\mu\text{g}/\text{ml}$).

Osmotin is a calcium-binding protein.

These data indicated that Ca^{2+} could be involved specifically in the binding of osmotin to the cell surface; therefore, we examined the possibility that Ca^{2+} could bind directly to osmotin, as is the case with certain mammalian antifungal proteins. Analysis of the osmotin sequence showed that the protein does not contain Ca^{2+} binding motifs of the EF-hand type or Ca^{2+} -dependent carbohydrate-recognition domains of the type characterized in C-type animal lectins (Drickamer 1993). However, osmotin was found to possess several regions with homology to epidermal growth factor (EGF) Ca^{2+} -binding domains (Selander-Sunnerhagen et al. 1992), as well as several type-III EGF signatures, and a sea urchin egg lectin (SUEL)-like domain (Fig. 3A). When resolved on sodium dodecyl sulfate polyacrylamide gel electrophoresis and electroblotted to nitrocellulose, osmotin appeared to exhibit no binding of ^{45}Ca (*data not shown*). Direct dot-blotting of the purified protein also gave no signal (Fig. 3B). However, after a demetalizing treatment, osmotin did indeed bind ^{45}Ca , indicating that it was necessary to first displace Ca^{2+} already bound to the protein, as has been shown for other proteins (Bouckaert et al. 1996; Dahlback et al. 1990). This binding occurred in the presence of 10,000-fold molar excesses of both Na^+ and K^+ , and a >1,000-fold molar excess of Mg^{2+} , and could be competed by unlabeled Ca^{2+} (Fig. 3B).

Enhancement of osmotin activity is Ca^{2+} and Mn^{2+} specific and is not revoked by high Ca^{2+} concentrations.

To determine whether the osmotin activity enhancement effect was specific to Ca^{2+} , we conducted growth-inhibition assays in the presence of other divalent cations. The Ca^{2+} -specific chelator EGTA was added to the assays specifically to prevent the already-demonstrated Ca^{2+} enhancement; then, individual divalent cations were tested for their ability to restore the osmotin activity lost due to the Ca^{2+} chelation. Mn^{2+} was able to restore osmotin action, but Mg^{2+} was not (Fig. 4A). Ba^{2+} and Sr^{2+} depressed growth of BWG generally, but did not alter osmotin sensitivity (*data not shown*). Growth of the yeast was not affected by Mn^{2+} or Mg^{2+} alone at the concentrations tested. The effect of elevated Ca^{2+} concentrations on osmotin activity also was tested. At 100 mM, Ca^{2+} showed essentially no osmotin activity-suppressing effect (Fig. 4B). This is a radical departure from the complete inhibition of activity by 0.5 to 5 mM Ca^{2+} seen with many other antifungal proteins.

Suppression of osmotin activity by monovalent cations is mediated by mannose phosphate targets on the yeast cell wall.

In contrast to Ca^{2+} , Na^+ (Abad et al. 1996) and K^+ (Fig. 4C) effectively blocked osmotin activity against susceptible fungi in a concentration-dependent manner. Interestingly, this blocking effect did not occur against *mn4*, indicating that K^+ could compete with osmotin for binding to yeast cell wall phosphate groups in the wild-type yeast (BWG). Accordingly, this blocking effect also occurred with Δste7 , a mutant in which increased resistance to osmotin is due to a mechanism unrelated to mannose phosphate deficiency (Yun et al. 1998). This further demonstrated that the K^+ blockage of osmotin activity operates specifically on the mannosephosphate residues of the cell wall.

Calcium enhancement of osmotin activity is pH dependent in BWG, but not in mannose phosphorylation mutant.

The great majority of osmotin's surface is positively charged, precluding Ca^{2+} binding. However osmotin contains one pronounced region of negative charge, an acidic cleft lined with Asp and Glu residues (Koiwa et al. 1999) at the boundary between a 10-stranded β -sheet region (domain I) and a set of

large, disulfide-rich loops (domain II), which could form a site for electrostatic Ca^{2+} binding. To test the possibility that Ca^{2+} bound to the acidic area of the protein surface could be contributing to the osmotin-enhancing effect, we conducted growth-inhibition assays over a pH range spanning the pK_a of Asp and Glu residues composing the cleft, such that crossing below the pK_a of acidic residues would render the cleft charge-neutral and remove the Ca^{2+} -enhancement effect. This was indeed the case. Ca^{2+} -enhancement of osmotin's action against BWG was progressively lost with declining pH (Fig. 4D), with the loss of effect centered near the pK_a of Asp and Glu (pH 3.9 and 4.2, respectively). However, no such response occurred when the assay was conducted on *mn4*, indicating that the Ca^{2+} -facilitated component of osmotin's action (i.e., cell wall binding) was not a factor in osmotin action against the mannosephosphate-deficient mutant.

DISCUSSION

For some time, Ca^{2+} and monovalent cations like K^+ have been implicated in modulation of antimicrobial protein activity. Investigation of the role of Ca^{2+} chiefly has been connected

A ATIEVRNNCPYTVWAASTPIGGGRRLDRGQTVWINAPRGTKMARVWGRNTNCFNA
 AGRGTCQGTGDCGGVLQCTGWGKPPNTLA**E**YALDQFSGLD**FWD**ISLVDGFNIPMTF
 APTNPSSGGKCHAIHCTANINGECPRELGVPPGCCNNPCTT**F**GGQ**Q**YCCT**Q**GPCG
 C-N---TFG---Y-C
 PTFFSKFFKQRCPDAYSYPQDDPTSTFTCPGGSTNYRVIFCPNGQAHNFPLEMP
 C-D SY D---TFTC
 GSDEVAK

B

Fig. 3. Osmotin is a Ca^{2+} -binding protein containing epidermal growth factor (EGF)-like calcium-binding domains. **A**, Sequence of osmotin was applied to a "Blocks" search for protein motifs (available through the Bioinformatics Center, Kyoto University) and also was compared to two consensus EGF calcium-binding regions derived from 22 EGF module-containing proteins (Selander-Sunnerhagen et al. 1992). XXX = mature osmotin protein sequence, **D/E** = osmotin acidic cleft residues, aligned stretches below osmotin sequence = EGF Ca^{2+} -binding consensus regions. XXX = Type III EGF signatures, XXX = sea urchin egg lectin domain. Dashes indicate residues differing from the EGF consensus. The boldface **D** in the consensus sequence contributes a side chain ligand for Ca^{2+} binding in factor X. **B**, Proteins (2 μg) were dot blotted onto nitrocellulose, incubated with ^{45}Ca and then washed. Calcineurin served as a positive control for Ca^{2+} binding. ^{45}Ca was competed off osmotin by including the concentrations of CaCl_2 shown in wash solutions.

Fig. 4. Osmotin activity modulation depends on cation identity, concentration, and pH. Effect of cations on osmotin action was measured in growth-inhibition dose-response assays. **A**, Control cultures contained YPD (1% yeast extract, 2% Bacto peptone, and 2% glucose in distilled water) with or without osmotin at 15 μg/ml, and with osmotin plus 2 mM EGTA (to block Ca²⁺ effect). Equivalent cultures including the stated concentrations of divalent cations were assayed for relative growth. Growth with added ions is expressed as a percentage of control growth. Data shown are the means of two replicates for each treatment. The entire assay was repeated twice, with similar results. Separate treatments established that Mn²⁺ and Mg²⁺ alone had no effect on growth rate (data not shown). **B**, Growth response of BWG to osmotin dose was measured in the presence or absence of 100 mM CaCl₂. **C**, Single doses of osmotin which gave approximately 70% inhibition of the stated strains (BWG at 15 μg/ml, *mnn4* at 40 μg/ml, and *Δste7* at 25 μg/ml) were added as indicated, plus increasing doses of KCl. Controls received only KCl. **D**, Yeasts were assayed for growth inhibition by single doses of osmotin as in C under varying pH conditions. Growth medium was YPD adjusted to stated pH with malic acid, with or without osmotin at concentrations stated. Data shown are the differences in growth: optical density at 600 nm (OD₆₀₀) without osmotin – OD₆₀₀ with osmotin. Osmotin activity was pH sensitive against BWG, but not against *mnn4*.

with Ca^{2+} influx into target fungi, which has been associated with growth cessation, altered fungal morphology, and other ion fluxes (Thevisen et al. 1996). We have demonstrated here that Ca^{2+} as well as K^+ have important extracellular roles in the interaction between osmotin and its fungal target.

Ca^{2+} exhibits contrasting effects on antimicrobial protein activity in different systems. Several proteins of the innate immune system in the lung and certain arthropod toxins show Ca^{2+} -enhanced activity. However Ca^{2+} enhancement of osmotin activity contrasts dramatically with the largely conserved pattern of Ca^{2+} -suppressed activity among mammalian defensin-type proteins (Lehrer et al. 1988) and the majority of plant antifungal proteins (Broekaert et al. 1997). Most of these lose activity completely when assayed at or below 1 mM Ca^{2+} , although *RsAFP1* and β -purothionin retained much of their activity at 1 mM Ca^{2+} (Terras et al. 1992), and activity of *AceAMP1*, a highly basic LTP from onion, was not significantly affected by up to 5 mM Ca^{2+} (Cammue et al. 1995). However, osmotin's activity against BWG was not significantly reduced by Ca^{2+} at concentrations at least up to 100 mM Ca^{2+} (Fig. 4B), and was enhanced by Ca^{2+} at levels (0.2 and 2.7 mM) similar to those occurring in the plant apoplast (0.02 to 1.3 mM) (Harker and Venis 1991), the site of interaction between osmotin and potentially invading fungi. The biological impact of a Ca^{2+} -facilitated binding of osmotin to the fungal cell surface may lie in an effect of concentrating osmotin in proximity to its primary site of action (the plasma membrane), to allow a localized buildup of osmotin to intoxicating levels. This conclusion is supported by the inverse relationship of osmotin dose to Ca^{2+} enhancement (Fig. 1A, B, and D). Higher levels of osmotin (e.g., 30 to 40 $\mu\text{g}/\text{ml}$) presumably do not require this cell wall targeting effect to reach inhibitory levels. These higher osmotin levels also are required to inhibit growth of yeast (*mn4*) defective in the cell wall mannosephosphate target of osmotin. Ca^{2+} binding by osmotin occurred even in the presence of physiological concentrations of K^+ and Mg^{2+} ; thus, Ca^{2+} also may be biologically important to maintain osmotin action in the presence of otherwise activity-suppressing ions in the plant.

The findings that Mn^{2+} (but not Mg^{2+}) enhances osmotin activity and, at higher doses, K^+ (but not Ca^{2+}) blocks osmotin activity, demonstrate that influences on osmotin action vary among specific cations. Similar activity-inhibiting effects specific to only certain divalent cation-protein combinations have been observed among mammalian defense proteins (Lehrer et al. 1988). Mn^{2+} is well known as an essential cofactor for glycan binding of concanavalin A, and Mn^{2+} dependence has been established for mannose-6-P receptor binding to phosphomannosyl residues in macrophages (Hoflack and Kornfeld 1985), suggesting a ligand-binding enhancement capacity similar to the Mn^{2+} effect on osmotin's mannosephosphate target binding.

The enhancement rather than suppression of osmotin activity by Ca^{2+} suggests that osmotin may utilize antifungal mechanisms unique from other proteins, such as hevein- or knottin-type peptides and most defensins and LTPs, which are suppressed by Ca^{2+} as well as K^+ (Broekaert et al. 1997). Osmotin action has been shown to involve a MAP kinase signal transduction pathway in yeast (Yun et al. 1998), consistent with the possibility that osmotin could interact with specific (unknown) signaling receptors. A positive influence of Ca^{2+} on such an interaction also is possible. For example, the spider venom α -latrotoxin is known to bind transmembrane proteins in a Ca^{2+} -dependent manner (Henkel and Sankaranarayanan 1999), leading to massive exocytosis similar to what has been observed in hyphal tips of fungi after PR5 treatments (Roberts and Selitrennikoff 1990; Vigers et al. 1991). Function of the transmembrane receptor Notch1 also is contingent on Ca^{2+} binding

(Oka et al. 1992). The conservation of cation modulation among many antimicrobial proteins probably is conditioned by a common mechanism of binding to carbohydrate recognition domains on molecules such as lipopolysaccharides, lipoteichoic acid, and mannans on the pathogen cell surface (Hoffmann et al. 1999), a mechanism which can be substantially influenced by cations.

By virtue of its apparent Ca^{2+} -enhanced interaction with fungal phosphomannans, osmotin appears to have lectin-analogous behavior (metal-mediated carbohydrate binding). Osmotin was found (Fig. 3) to contain a domain common to SUEL (Ozeki et al. 1991). The SUEL domain also is found in plant β -galactosidases (EC 3.2.1.23) and mammalian α -latrotoxin binding receptor (Krasnoperov et al. 1999). Further, several thaumatin-like proteins recently have been shown to bind fungal glucans (Osmond et al. 2001; Trudel et al. 1998). Specific structural determinants involved in Ca^{2+} binding thus far have not been demonstrated for PR5 proteins. The existence of EGF-like domains has not been widely reported among plant proteins; the only others to date are the thionins (Oka et al. 1992), which also have potent antifungal activity. Thionins are known to inhibit fungi by membrane permeabilization, a capacity shared by partially homologous mammalian complement and perforin proteins (Discipio et al. 1988; Shinkai et al. 1988), and which also has been proposed for osmotin (Abad et al. 1996), zeamatin (Roberts and Selitrennikoff 1990), and other PR5 proteins (Anzlovar et al. 1998). Calcium recently has been reported to complex with gamma-thionin SI α from *Sorghum bicolor* (deCastro et al. 2002), although no data regarding facilitation of the activity of plant antifungal proteins by Ca^{2+} have been reported. However, Ca^{2+} is known to promote the interaction of yeast cell wall mannans with lectin-like proteins, resulting in flocculation (Vinogradov et al. 1998). The subversion of this interaction by osmotin could appear to represent a novel strategy among plant defense proteins.

Fig. 5. Model of the molecular basis for Ca^{2+} and K^+ effects on activity of osmotin against yeast. Schematic view of the yeast cell surface shows the proposed behavior of osmotin in the presence or absence of available Ca^{2+} . Ca^{2+} is shown to tether osmotin to phosphomannans in the yeast cell surface, in proximity to the plasma membrane. This effect is blocked when Ca^{2+} is sequestered by EGTA, or when K^+ competes with osmotin for mannosephosphate binding sites on the yeast cell surface. It also is blocked in the *mn4* mutant, with absence of the terminal mannose phosphate residue. The precise mode of osmotin action on the plasma membrane is not completely understood at present.

Extracellular K^+ was shown here to inhibit osmotin action against BWG yeast in a dose-dependent manner, but this effect was completely blocked in the osmotin-resistant *mn4* mutant, indicating that K^+ competes with osmotin for cation-binding sites (specifically mannosephosphate residues) on the yeast cell wall. Further, K^+ also suppressed osmotin activity against other osmotin-resistant mutants, *Aste7* (Fig. 4C) and *Aste11* (Yun et al. 1998), confirming that the mannosephosphate residues (absent in the *mn4* mutant) are the target of the K^+ effect. The low-level residual activity of osmotin against *mn4* presumably is due to a cation-independent, membrane-acting component of osmotin action (Ibeas et al. 2000). Consistent with this idea, analysis of activity of a PR5 from flax against artificial liposomes showed that permeabilization of pure lipid bilayers was not affected materially by Na^+ (Anzlovar et al. 1998). Both of these findings suggest that the K^+ effects we have demonstrated on osmotin action against yeast cells are mediated through the cell wall.

K^+ efflux from fungi has been observed after treatment with a number of antifungal proteins (Broekaert et al. 1997). However, K^+ efflux from BWG was not observed after osmotin treatment, either by atomic absorption spectroscopy or by K^+ -selective electrode assays (*data not shown*). Taken together, these findings indicate that, at least in *Saccharomyces cerevisiae*, K^+ blocks activity of osmotin solely by a cell wall-mediated mechanism acting through phosphomannans, and not by, for example, balancing a K^+ efflux, such as that provoked by other antifungal proteins like defensins and thionins (Thevisen et al. 1996). Blockage of antifungal protein activity by K^+ has widespread implications, because all compartments in plant cells, as well as in the extracellular matrix, generally contain levels of K^+ that would attenuate activity of these proteins. Analogously, high levels of Na^+ in the extracellular space of the human lung and airway have been implicated to cause cystic fibrosis by blocking function of antifungal defensin proteins (Goldman et al. 1997).

As a basic protein, osmotin carries a mostly positive surface charge. Therefore, a direct interaction of the protein with Ca^{2+} at first seems unfavorable. However, an acidic cleft found in osmotin and other active PR5 proteins (Batalia et al. 1996; Koiwa et al. 1999; Osmond et al. 2001) could form a hollow for electrostatic binding of Ca^{2+} or other positively charged groups, as occurs in the S2 site of concanavalin A (Bouckaert et al. 1996), the EGF-like domain of factor X (Selander-Sunnerhagen et al. 1992) phospholipase A2 (Williams 1986), and a variety of other proteins. Osmotin's Ca^{2+} binding, and the finding that Ca^{2+} enhancement of osmotin activity is abolished when the net charge of acidic residues is removed (at approximately pH 4.0), support the possibility of an interaction of Ca^{2+} with this cleft. Further, the presence of the acidic cleft has been correlated with antifungal activity of PR5 homologs (Koiwa et al. 1999). Our data are consistent with Ca^{2+} ions acting as a direct ligand to both protein and target, "bridging" the negative charges of the acidic residues of osmotin and the phosphate moieties of yeast phosphomannans or other targets (Fig. 5), analogous to the Ca^{2+} crosslinking of antifungal mannose-binding proteins to their target mannans (Weis et al. 1992). K^+ presumably blocks this interaction by competing with Ca^{2+} for binding to mannose phosphate groups of the yeast cell wall, but probably not to the acidic residues of the protein, because ^{45}Ca binding to osmotin occurred in the presence of excess K^+ (Fig. 3B). The finding that low pH did not decrease PR5 membrane permeabilization in the absence of a cell wall (Anzlovar et al. 1998) also suggests that the pH effect on osmotin activity we observed is contingent on the cell wall. Osmotin has been shown to activate the STE MAP kinase cascade in yeast, resulting in changes to the cell wall

that further sensitize the cell to the protein (Yun et al. 1998). This process depends on presence of the cell wall, but is separate from the actual killing mechanisms of the protein. Therefore it is likely that tethering of osmotin to the cell wall by Ca^{2+} promotes signal flux through the STE pathway, in addition to facilitating its direct action on the plasma membrane or its stimulation of an independent fungal cell death pathway operating through cAMP and protein kinase A (Narasimhan et al. 2001). Alternatively to the Ca^{2+} bridging proposed above, binding of Ca^{2+} to the osmotin surface also could bring about a structural adjustment in the protein, increasing cell wall binding affinity (as Ca^{2+} and Mn^{2+} are known to act on concanavalin A) or could enhance stability of the protein to proteolytic degradation in the extracellular environment (Levine and Williams 1982; Weis et al. 1992). Beyond a direct interaction with the osmotin protein, the further possibility exists that the Ca^{2+} enhancement of osmotin activity may be brought about by an interaction of Ca^{2+} with elements of the fungal cell surface, leading to enhanced binding of osmotin; or to Ca^{2+} -dependent activation of cytoplasmic signal transduction by osmotin, as has been noted for α -latrotoxin (Henkel and Sankaranarayanan 1999) and Notch 1 (Aster et al. 1999).

MATERIALS AND METHODS

Media and culture.

SD medium was made according to Iida (Iida et al. 1990), including $CaCl_2$ to a final concentration of 2.7 mM. Ca^{2+} -free SD was the same except for omission of $CaCl_2$ and substitution of sodium pantothenate for the Ca^{2+} salt. YPD was composed of 1% yeast extract (Difco Laboratories), 2% Bacto peptone (Difco Laboratories, Detroit), and 2% glucose (Malincrodt AR, Phillipsburg, NJ, U.S.A.) in distilled water. The concentration of Ca^{2+} in YPD has been determined to be 0.22 mM (Prasad and Rosoff 1992).

Yeast strains and mutations.

Unless otherwise indicated, the background strain for all experiments was *S. cerevisiae* BWG7a. The *mn4* mutant was obtained after insertional mutagenesis using a *Tn3::lacZ::LEU2* transposon (Ibeas et al. 2000). Genomic DNA immediately adjacent to the *Tn3::lacZ::LEU2* insertion in the isolated mutant was cloned and analyzed as described (Burns et al. 1994). The *MNN1* and *STE7* loci were deleted by using the *kanMX* gene as described (Wach 1997). Precise gene disruption was confirmed by PCR.

Antifungal assays.

Growth inhibition assays were conducted by diluting yeast precultures from an optical density at 600 nm (OD_{600}) = 0.6 to 0.01 with the desired medium and additives. In all assays, growth controls lacking the factor being tested were included. Cultures then were incubated at 29°C with shaking until control growth reached OD_{600} = 0.6, and OD_{600} of treatments was recorded. Growth inhibition or enhancement due to the factor under test was expressed as percent of control growth.

Demetalization of osmotin, ^{45}Ca -autoradiography.

Osmotin purified according to Singh and associates (1987) was demetalized by a treatment combining low pH (Ono and Inoue 1988) and chelating resin (Bouckaert et al. 1996) as follows: 3 g of Chelex 100 beads (Biorad) were stripped with 1 M HCl and rinsed with 50 volumes of Ca^{2+} -free dH_2O (dH_2O previously run through a column of Chelex beads). The beads then were equilibrated to 10 mM malic acid and NaOH to pH 2.8, added to a 5-ml spin column, and centrifuged at $3,000 \times g$ for 1 min. A solution of 1 ml of osmotin (0.5 mg of protein)

adjusted to the same conditions as above was added, and the sealed column was placed on a rotator for 3 h. The column then was adjusted to pH 6.0 with NaOH and placed on the rotator for 30 min, after which the demetalized osmotin was eluted by centrifuging the column for 1 min at $3,000 \times g$. Ca^{2+} binding of osmotin was assayed after Maruyama and associates (1984). Briefly, purified proteins were dot blotted onto nitrocellulose membrane washed with incubation buffer (0.9 mM MgCl_2 , 4.8 mM KCl, 8.5 mM NaCl) for 10 min, followed by the addition of $^{45}\text{CaCl}_2$ (ICN, Costa Mesa, CA) at 1 $\mu\text{Ci}/\text{ml}$ (0.59 μM) to the solution. The blots were further incubated for 3 h, then rinsed with Ca^{2+} -free dH_2O four times for 5 min each, blotted dry, and autoradiographed.

Labeling of osmotin with ^{35}S and yeast-binding assay.

Osmotin was labeled with ^{35}S -methionine SLR general purpose labeling reagent (Amersham Pharmacia, Arlington Heights, IL) according to manufacturer's instructions. Briefly, reagent equivalent to 250 μCi of ^{35}S was dried of solvent with a stream of dry N_2 in a glass vial on ice. Next, 100 μl of osmotin in 0.1 M borate buffer, pH 8.5 (200 μg of protein total) was added and the vial was incubated for 30 min on ice. The reaction was stopped by adding 20 μl of 0.2 M glycine in 0.1 M borate buffer, pH 8.5, followed by a further 30 min of incubation on ice. A 1- μl aliquot was taken for scintillation counting, and unincorporated counts were removed from the remainder of the reaction by passing it through a column of Sephadex G25 (1 ml) equilibrated with 0.1 M borate and 0.02 M glycine, pH 8.5. Eluate (1 μl) was scintillation counted to determine percent incorporation of ^{35}S . For yeast binding assays, BWG cultures were grown in YPD or YPD at 2 mM EGTA to $\text{OD}_{600} = 0.6$, harvested by centrifugation, and resuspended to $\text{OD}_{600} = 6.0$ with the same fresh medium. For each time point to be measured, 300 μl of resuspension received 2 μl of ^{35}S -labeled osmotin or dH_2O and was divided into three 100- μl aliquots. These were incubated at 30°C without shaking. At each time point, aliquots were centrifuged for 2 min at $12,000 \times g$. Pellets were washed twice with 0.5 ml of YPD, resuspended in 100 μl of YPD, and counted with the scintillation counter.

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