Specific Cell Wall Proteins Confer Resistance to Nisin upon Yeast Cells

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The cell wall of a yeast cell forms a barrier for various proteinaceous and nonproteinaceous molecules. Nisin, a small polypeptide and a well-known preservative active against gram-positive bacteria, was tested with wild-type Saccharomyces cerevisiae. This peptide had no effect on intact cells. However, removal of the cell wall facilitated access of nisin to the membrane and led to cell rupture. The roles of individual components of the cell wall in protection against nisin were studied by using synchronized cultures. Variation in nisin sensitivity was observed during the cell cycle. In the S phase, which is the phase in the cell cycle in which the permeability of the yeast wall to fluorescein isothiocyanate dextrans is highest, the cells were most sensitive to nisin. In contrast, the cells were most resistant to nisin after a peak in expression of the mRNA of cell wall protein 2 (Cwp2p), which coincided with the G2 phase of the cell cycle. A mutant lacking Cwp2p has been shown to be more sensitive to cell wall-interfering compounds and Zymolyase (J. M. Van der Vaart, L. H. Caro, J. W. Chapman, F. M. Klis, and C. T. Verrips, J. Bacteriol. 177:3104–3110, 1995). Here we show that of the single cell wall protein knockouts, a Cwp2p-deficient mutant is most sensitive to nisin. A mutant with a double knockout of Cwp1p and Cwp2p is hypersensitive to the peptide. Finally, in yeast mutants with impaired cell wall structure, expression of both CWP1 and CWP2 was modified. We concluded that Cwp2p plays a prominent role in protection of cells against antimicrobial peptides, such as nisin, and that Cwp1p and Cwp2p play a key role in the formation of a normal cell wall.

Nisin is an antimicrobial peptide produced by lactic acid bacteria and has been used in consumer products for many years (30). Although this lantibiotic is inhibitory to microorganisms, it is harmless to humans (15, 16). Nisin is the first antimicrobial peptide with “generally recognized as safe” status in the United States for use in processed cheese; in addition, its use in various food products is allowed in several countries (9). Nisin is also of interest to the pharmaceutical industry (10).

Nisin is known to inhibit the growth of a number of gram-positive bacteria and also the outgrowth of spores of bacilli and clostridia (15, 16). Furthermore, the gram-negative bacterium Escherichia coli becomes sensitive to nisin when its outer membrane is made permeable by osmotic shock (20). Inhibition of the growth of other gram-negative bacteria can be achieved by simultaneous treatment with nisin and an agent which modifies and chelates the outer membrane, such as EDTA (29). These findings are consistent with the notion that nisin acts on the cytoplasmic membrane. Indeed, the main antimicrobial activity of nisin seems to rely on the ability of the compound to form pores in the cytoplasmic membrane, which leads to a loss of small intracellular molecules and ions and a collapse of the proton motive force (1, 6, 14, 20, 24, 25, 34). To exert its antimicrobial activity, nisin does not seem to require a specific receptor but instead requires a sufficient trans-negative electrical membrane potential (24, 25). Driessen et al. concluded that nisin acts as an anion carrier in the absence of anionic phospholipids (12). It has been suggested that pore formation by nisin in vivo involves local perturbation of the bilayer structure and trans-membrane-potential-dependent reorientation from a surface-bound configuration to a membrane-inserted configuration. In this membrane-inserted form the hydrophilic side of nisin and the attached lipid head groups face the center of a water-filled pore. The hydrophobic surface of nisin and the fatty acid chains of the lipids point to the lipid bilayer (31).

Nisin has no antimicrobial effect on yeasts and filamentous fungi. These organisms each have a rigid cell wall, a complex structure consisting of glucan cross-linked with chitin and cell wall proteins (4, 18). The processing of mannoproteins is complex and has been partially characterized in yeasts (19, 21). A similar mechanism has been suggested for filamentous fungi (4). Because mannoproteins are generally considered one of the key wall components which determine cell wall porosity (8, 18), they may represent a major barrier preventing free permeation of nisin through the cell wall and thus access to the cytosolic membrane.

Initial experiments indicated that a yeast cell is prone to the antimicrobial activity of nisin in certain stages of the cell cycle, suggesting that cell cycle-regulated components of the cell envelope are involved. Recently, Caro et al. showed that specific cell wall mannoproteins are expressed in different stages of the cell cycle (7). In this study we assessed the importance of individual mannoproteins, β-1,3-glucan, and chitin in conferring resistance to nisin upon yeast cells. In addition, below we present data describing the role of cell wall protein 1 (Cwp1p) and Cwp2p in the structuring of a normal yeast wall.

MATERIALS AND METHODS

Strains, probes, and media. The E. coli strain used in this study was JM109 (endA1 recA1 gyrA96 thi-1 hsdR17 F’ [F traD36 proAB lacZ5M15] [35]).

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DNA probes were randomly labelled by using [32P]dCTP. All fragments were purified by QIAEX 150 gel extraction. The specific H2A BamHI, which resulted in probes FKS1 (2.1 kb), part of the SED1 gene (690 bp), or part of the TIP1 (455 bp) gene were isolated from an agarose gel. pHA2 was cut with SacI, and pCHS3 was cut with BamHI, which resulted in probes H2A (2.3 kb) and CHS3 (2.8 kb). The probe fragment of the FKS1 gene (2.1 kb) was isolated from pSB4 as a Xbal-XhoI fragment. All fragments were purified by QIAEX 150 gel extraction. The specific DNA probes were randomly labelled by using [32P]dCTP (Amersham) as a substrate (26).

Reagents. Nisin was obtained from Aplin & Barlet (Dorset, United Kingdom). Yeast nitrogen base, Bacto Peptone, Bacto Yeast Extract, and Bacto Agar were obtained from Difco Laboratories (Detroit, Mich.). DNA restriction enzymes were purchased from New England Biolabs Inc. (Beverly, Mass.) and Boehringer Mannheim Biochemicals (Mannheim, Germany). α-Factor was obtained from Bachem Feinchemikalien AG. 4,6-Diamidino-2-phenylindole (DAPI) was purchased from Sigma Chemical Co. (St. Louis, Mo.). Propidium iodide (PI), Calbiochem, and FUN1 were obtained from Molecular Probes Inc., European Bachem (Bachem Feinchemikalien AG. 4,6-Diamidino-2-phenylindole (DAPI) was purchased from Sigma Chemical Co. (St. Louis, Mo.). Propidium iodide (PI), Calbiochem, and FUN1 were obtained from Molecular Probes Inc., European Bachem (Bachem, Switzerland). 4,6-Diamidino-2-phenylindole (DAPI) was purchased from Sigma Chemical Co. (St. Louis, Mo.). Propidium iodide (PI), Calbiochem, and FUN1 were obtained from Molecular Probes Inc., European Bachem (Bachem, Switzerland).

Analytical procedures. The confocal scanning laser microscope (CSLM) used consisted of a Zeiss Axioplan inverted microscope, a Bio-Rad model MRC-1024 system, and Lasersharp software. The objective used was a 1.5× zoom objective (magnification, ×63) with an image width of 110 μm. The software used to determine the percentage of PI-positive cells was the Leica Q500 MC software, as adapted by Aat Don (Department of Analytical & Information Sciences, Unilever, Vlaardingen, The Netherlands).

Yeast spheroplast generation. Yeast cells were grown by centrifugation and washed twice in 10 mM Tris-HCl (pH 7.4) and once in spheroplasting buffer (50 mM Na2CO3, [pH 7.4], 1 M sorbitol). The washed cells were resuspended in 10 ml of spheroplasting buffer, and 20 μl of β-mercaptoethanol was added. After 10 min of incubation at room temperature, 200 μl of Zymolase 100T (5 mg/ml) was added, and the preparation was incubated at 37°C for an additional 40 min. The quality of spheroplast formation was assessed by diluting the preparation in 6.5 mM EDTA, 0.5% sodium dodecyl sulfate) and vigorously vortexed after incubation in water-bath incubated at 65°C for 1 h. The aqueous phase was extracted once with phenol and once with chloroform. The RNA was precipitated with ethanol.

For Northern blotting (7, 17), 10 μg of RNA was loaded onto a 1% RNA agarose gel containing formaldehyde and formamide. After blotting, the RNA was cross-linked to Hybond-N+ membranes by UV radiation. Northern hybridizations were performed in the presence of 50% formamide at 42°C by using α32P-labelled gene fragments. The blots were washed at 42°C with decreasing concentrations of SSC (1× SSC is 0.15 M NaCl plus 15 mM sodium citrate, pH 7.0) down to 0.5× SSC in the presence of 0.1% sodium dodecyl sulfate. Hybridization signals were quantified by scanning autoradiograms in the linear range of the films. Levels of expression were normalized to actin levels.

RESULTS

Yeast wall forms a barrier for small antifungal peptides. In order to determine whether the yeast cell wall forms a barrier to nisin, we first incubated cells with EDTA and dithiothreitol as described by de Nobelel et al. (11) in order to increase the wall permeability. We observed that treatment of log-phase yeast cells with EDTA and/or dithiothreitol made them significantly more sensitive (by a factor of 2 to 4) to treatment with small antimicrobial peptides, such as nisin, as measured by the increase in the percentage of PI-positive cells. To study the inferred barrier function of the yeast wall for nisin in more detail, we removed the cell wall by incubation with a wall-lytic enzyme preparation and incubated the spheroplasts with nisin. Spheroplasts did not stain with Calcofluor White but were stained when they were incubated with the viability dye FUN1. Cells with damaged membranes were stained red due to uptake of PI. Yeast spheroplasts rapidly lysed when they were incubated in the presence of nisin at concentrations which hardly affected intact cells (10 to 80 μg/ml). Apparently, the cell wall normally forms a barrier for nisin. As the composition of the cell wall varies during the cell cycle, we set out to analyze the nisin sensitivity of yeast cells during the cycle.

Nisin sensitivity during the yeast cell cycle. After incubation of a yeast culture with α-factor, the synchronous growth of this culture was checked (Fig. 1). Three synchronous consecutive cell cycles were observed. Confirmation of the cell cycle progression was obtained by measuring the fluctuation in H2A mRNA levels. The level of the mRNA of H2A, a prominent cell cycle marker gene which is actively transcribed in the S phase, peaked at 60, 150, and 240 min after the transfer to fresh medium without α-factor. This is consistent with the observation that cells had small buds at these time points.
The nisin sensitivity of the synchronous culture is shown in Fig. 2a. In the first cycle high percentages of PI-positive cells were recorded, and the culture was dominated by cells with small buds. In the second and third generations, however, cells with migrated nuclei seemed to be most sensitive to nisin. Perhaps the cells in the first cycle still suffered from direct effects of a-factor on the structure of their cell walls.

Figure 2b shows the transcription of CWP1 and CWP2 in relation to culture sensitivity to nisin. Maximum transcription of CWP1 was observed during the second and third genera-

**FIG. 2.** Correlation of cyclic nisin sensitivity with expression of genes coding for cell wall proteins and proteins involved in yeast cell wall biosynthesis. The levels of expression were determined relative to the level of expression of the ACT1 gene, which did not vary during the cell cycle. (a) Effect of nisin as assessed by determining the percentage of PI-positive cells. The different stages in the cell cycle are indicated at the bottom. (b) Expression of the mRNA of the CWP1 (●) and CWP2 (■) genes and nisin sensitivity (○) through three generations of synchronous growth. (c) Expression of the mRNA of the FKS1 (■) and CHS3 (●) genes and nisin sensitivity (○) through three generations of synchronous growth.

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CWP1 in a Cwp2p-deficient strain increased. This suggests that in the analysis of the antifungal effect of nisin on cwp1Δ described above, the effect of nisin on the mutant due to the absence of Cwp1p could be overestimated since the level of Cwp2p was also decreased. On the other hand, it was evident in the analogous analysis of cwp2Δ that the effect of nisin on this mutant could not be ascribed to a decrease in CWP1 expression.

Interestingly, in the Pmt1p-deficient strain expression of CWP2 was induced significantly, whereas in the Fks1p-deficient strain expression of both CWP1 and CWP2 was induced moderately. We found that in the fks1Δ mutant, in addition to both cell wall protein genes, chitin synthase expression was also induced (data not shown). In this mutant the cell wall proteins are anchored mainly in the cell wall to the extra chitin (17c). Neither SED1 nor TIP1 was induced in these strains. Together, the data strongly suggest that Cwp2p and Cwp1p have an important structural function in the yeast cell wall (17a).

**DISCUSSION**

Our studies show that specific glucanase-extractable cell wall proteins with no known physiological function are crucial in conferring resistance to the antimicrobial peptide nisin upon yeast cells. As the yeast cell wall mannoproteins are heavily glycosylated and therefore determining their specific levels im...
munologically is very difficult, we chose to measure the level of transcription of the genes involved in the expression of cell wall proteins rather than work with the proteins themselves. Although transcription levels do not necessarily correlate with the presence of the components in the cell wall, we know from preliminary studies performed with green fluorescent protein fusions that Cwp1p and Cwp2p appear in the cell wall at distinct points in the cell cycle, in agreement with Northern analysis data (17b). High levels of transcription of Cwp2p just before the stage in the cell cycle when the cells were very resistant to nisin suggested that this protein protects the cell from nisin and similar peptides. Upon depletion of both Cwp2p and Cwp1p, the cells were very sensitive to nisin, as demonstrated by the high percentage of PI-positive cells. The fact that expression of CWP1 was induced in a cwp2Δ strain whereas CWP2 expression was not induced in a cwp1Δ strain and the fact that cwp2Δ cells were slightly more sensitive to nisin than cwp1Δ cells support the conclusion that the Cwp2p protein seems to be more important than the Cwp1p in conferring nisin resistance upon yeast cells. van der Vaart et al. (32) showed that the exponentially growing cwp2 deletion mutant is more sensitive to Calcofluor White, Congo red, and Zymolyase than the cwp1, tip1, and srp1 deletion mutants. Furthermore, depletion of Cwp2p resulted in a thinner electron-dense layer around the glucan layer. The importance of Cwp2p for normal cellular physiology is also underlined by the observation that overexpression of this protein can partially compensate for the lack of sphingolipids. Sphingolipids are necessary for the growth of S. cerevisiae bypass mutants at low pH values (28). Survival of these mutants at low pH values is enhanced by overexpression of the Cwp2p protein.

In addition to nisin, there are similar small, membrane-perturbing peptides, such as histatins, cecropins, and magainins, as well as synthetically modelled peptides (2). We studied the antifungal activity of synthetically produced peptides during the yeast cell cycle with the approach described in this paper and found a pattern similar to that observed for nisin (11a). Yeast cell wall proteins are also involved in resistance to somewhat larger membrane-active plant antimicrobial proteins. Yun et al. (36) recently showed that Pr cell wall proteins are induced in yeast cells in response to a challenge with the plant antifungal PR-5 protein osmotin.

We are currently constructing fusion proteins which consist of α-galactosidase, a protease-processing site, and various Cwps. These constructs should allow us to purify Cwps with (parts of) their glycosyl phosphatidylinositol anchors for peptide binding studies. Furthermore, we plan to perform photo-labelling experiments with nisin (and other peptides) in incubations with yeast cells. Complexes formed upon cross-linking of the peptides to the yeast wall will be analyzed in order to characterize the in vivo binding of the peptides to wall components. These two approaches should allow us to distinguish between a direct protective effect of Cwps against peptides through specific binding and indirect protection through an influence on the structural organization of the wall.

Finally, how yeast cells respond to a constant challenge with an antimicrobial peptide is not yet clear. Another issue is whether the age of yeast cells influences their sensitivity to peptides; this could explain the apparent sensitivity of some wild-type cells to nisin, as indicated by the data in Fig. 3A. Current studies are aimed at answering these questions.

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