A Novel Endoplasmic Reticulum Membrane Protein Rcr1 Regulates Chitin Deposition in the Cell Wall of Saccharomyces cerevisiae*

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The fungal cell wall plays an important role in protecting the cell from various types of stress, including noxious chemicals and osmotic pressure. The cell wall of the budding yeast Saccharomyces cerevisiae is composed of β1,3- and β1,6-glucan, chitin, and mannoproteins (1). About half of the cell wall is made up of β1,3-glucan that has linkage with other polymers. β1,6-Glucan mainly links mannoproteins to β1,3-glucan. Chitin, a linear polymer of β1,4-linked N-acetylglucosamine, constitutes only 2–3% of the cell wall but has a vital role in S. cerevisiae (2). These components are under a dynamic and highly regulated control by stress or cell cycle and have a complementary role in which a decrease in one component is immediately compensated by an increase in others. In a defective mutant of fks1 that encodes a β1,3-glucan synthase catalytic subunit, the content of glucan greatly reduces, but the amount of chitin increases instead. Similar change in cell wall components was found in a gas1 mutant that releases soluble glucan in the medium and accumulates chitin and mannoproteins (3, 4).

Congo red binds to the cell wall and inhibits the growth of yeast. In a screening for multicopy suppressor genes of Congo red hypersensitivity of erd1Δ mutant, we found that a previously uncharacterized gene, YBR005w, makes most of the Saccharomyces cerevisiae strains resistant to Congo red. This gene was named RCR1 (resistance to Congo red 1). An rcr1Δ null mutant showed an increased sensitivity to Congo red. RCR1 encodes a novel ER membrane protein with a single transmembrane domain. Molecular dissection suggested that the transmembrane domain and a part of the C-terminal polypeptide are sufficient for the activity. We examined the effect of RCR1 in various null mutants of genes related to the cell wall. The resistance of mutants to Congo red correlates with a reduction of chitin content. Multicopy RCR1 caused a significant decrease in the chitin content while the amount of alkali-soluble glucan did not change. The binding of Calcofluor white to the cell wall significantly decreased in these cells. Our results show that RCR1 regulates the chitin deposition and add firm genetic and biochemical evidences that the primary target of Congo red is chitin in S. cerevisiae.

The mutant yeast cell that has an altered cell wall composition by the compensating system shows a different response to the external stress from the wild-type cell. A significant case is the sensitivity to K1 killer toxin or Calcofluor white. These compounds have a specific target in cell wall components and therefore have been used in the study of the cell wall. K1 killer toxin binds to its receptor, including β1,6-glucan, and forms fatal ion channels in the plasma membrane (5). A number of genes concerned in the synthesis of β1,6-glucan have been identified by studying the killer toxin-resistant (kre) mutants (6, 7). Calcofluor white preferentially binds to polysaccharides containing β1,4-linked d-glucopyranosyl units (8). In yeast, it binds to chitin and alters the assembly of its microfibrils (9). Therefore, the sensitivity against this compound closely relates to the chitin content. Mutants with increased chitin, by compensating for the defect of other components such as fks1 or gas1, show higher sensitivity to Calcofluor white (10, 11). On the other hand, reduction in chitin content makes cells more resistant to Calcofluor white. The mutants chs3, which encodes a major chitin synthase, and chs4-cha7, which help in the proper localization or activation of Chs3, show higher resistance to Calcofluor white (12–14). Similarly, overexpression of KNR4, which represses the chitin synthesis genes, makes the cell more resistant to Calcofluor white (15).

Although the stilbene-type dye Calcofluor white has been extensively used in many cell wall mutant studies, another cell wall-perturbing agent, benzidine-type dye Congo red, has not been widely used in identifying cell wall mutants. One of the reasons is that the effect of Congo red on the cell wall in S. cerevisiae is somehow ambiguous. Because Congo red stimulates chitin synthesis in S. cerevisiae like Calcofluor white (16), both compounds are thought to have similar effects on fungal cell wall. But it has also been known that Congo red binds to β1,3-glucan (17, 18) and interferes with the assembly of the β1,3-glucan filaments in S. cerevisiae (19). These effects would not be mutually exclusive, and the mechanism of growth inhibition by Congo red in S. cerevisiae remains somewhat unclear. Congo red as well as Calcofluor white binds to β1,4-linked d-glucopyranoside units, but the binding specificity to other polysaccharides, such as Curdlan, which mainly consists of β1,3-linked d-glucopyranoside units, was completely different from Calcofluor white (8). Therefore, the effect may be different among biological species. So far, there is little information on the relationship between the sensitivity to Congo red and the genes that are concerned in cell wall synthesis of S. cerevisiae. In this work, we report characterization of a previously uncharacterized gene RCR1 (resistance to Congo red 1) and firm evidence indicating that the primary target of Congo red in S. cerevisiae is chitin rather than glucan.
relA1) was used in plasmid propagation, and Escherichia coli was grown in an LB (1% Bacto-Tryptone, 0.5% Bacto yeast extract, 0.5% NaCl) medium with or without 100 μg/ml ampicillin. S. cerevisiae strains used in this study are listed in Table I. Yeast was grown in YPD medium (1% yeast extract (BD Biosciences), 2% peptone (BD Biosciences), 2% glucose) and SD medium (0.17% yeast nitrogen base without amino acids (BD Biosciences), 0.5% (NH₄)₂SO₄, 2% glucose, and appropriate supplements) (20). Solid media were made with 2% agar.  

Plasmid—Plasmids used in this study are listed in Table II. RCR1 (systematic name YBR005w) was amplified from genomic DNA by PCR using DNA polymerase Pyrobest (TaKaRa). A BamHI site was placed at 219 bp upstream from the initiating methionine codon of RCR1, and a XhoI site was placed at 633 bp downstream from the stop codon (sense primer, 5'-CTCCTGAGATCTATTGATCTCTGACGAGTA-3'; antisense primer, 5'-CGGGATCCCGCCTCCTCTCTCGAAGC-3'); an XhoI site was placed at 363 bp downstream from the stop codon (sense primer, 5'-CGGGATCCATGGTAGATGCTATCG-3'; antisense primer, 5'-CATCCTATTGATCTCTGAGGAGTA-3'); an XhoI site was placed at 363 bp downstream from the stop codon (sense primer, 5'-CCCTCTGGATCTATTGATCTCTGAGGAGTA-3'). The PCR product was digested with BamHI and XhoI and ligated into pRS426 (21) to obtain plasmid pK72. To obtain the N-terminal myc epitope-tagged Rcr1 in yeast, the RCR1 open reading frame was amplified by PCR from plasmid pK72, placing a BamHI site at the start codon and SacI site at 363 bp downstream from the stop codon (sense primer, 5'-CGGGATCCGACGGTTCATTCGAGGAGTA-3'; antisense primer, 5'-CGAGCTCATCCTATTGATCTCTGAGGAGTA-3'). The PCR product was digested with BamHI and SacI and ligated into pK8426 (21) to obtain plasmid pK972. To obtain a myc-coding sequence to generate pK948. The truncated Rcr1 constructs were made in the same way. The disruption plasmid pK106 (rrna1::kanMX4) was constructed by inserting the BamHI–PstI fragment obtained by PCR using the primers (sense primer, 5'-TGGCTAGCGAAGGGCATGGTAGATGCTATCG-3'; antisense primer, 5'-CGGGATCCGGTTCATTCGAGGAGTA-3') into the BamHI–PstI site of pRS426, and the remaining mixture was centrifuged at 100,000 g to remove unbroken cells. The plasmid was then digested with SacI and ligated into pK8426 (21) to construct plasmid pK972. The pK972 plasmids were transformed into BY4741. 

Protease Protection Assay—The KA31-1A spheroplasts harboring multicopy 6myc-RCR1 were prepared using lyticase in the presence of β-mercaptoethanol. Protease protection assays were performed as described previously (26, 27), with the use of protease K (Roche Applied Science). Spheroplasts (10 optical density unit equivalents) were incubated with SPP, including combinations of protease K and/or 1% Triton X-100, 0.1 M NaCl, 0.1 M Na₂CO₃ (pH 11), or 2 M urea. Then a portion of the mixture was taken as the total fraction (T), and the remaining mixture was mixed with 100,000 × g for 10 min and separated to the pellet (P) and the supernatant (S) fractions. Analyses of invertase, chitinase, and Gas1 protein were done as described in Hashimoto et al. (24) and Gentzsch and Tanner (25). 

Quantitative β1,3-Glucan Measurement—Amount of β1,3-glucan per cell was measured using aniline blue as described previously (29, 30) with some modification. In brief, cells were grown to an OD₆₀₀ of 0.5–0.8.
and 2.5 × 10^6 cells were harvested. The cells were washed twice with TE (10 mM Tris-Cl, 1 mM EDTA, pH 8.0) and resuspended in 250 μl of TE. To the cells 6 × NaOH was added to a final concentration of 1 N, incubated at 80 °C for 30 min followed by an addition of 1.05 ml of AB mix (0.03% aniline blue (Wako), 0.18 × HCl, and 0.49 N glyamine/NaOH, pH 9.5). The tube was vortexed briefly, then incubated at 50 °C for 30 min. Fluorescence of β-1,3-glucan was quantified using a spectrofluorometer (F-2500, Hitachi, Tokyo, Japan). The excitation wavelength was 400 nm, and the emission wavelength was 480 nm.

Measurement of Chitin Content—Total cellular chitin was measured as described by Bulawa et al. (31) and outlined by Ketela et al. (32) with some modification. In brief, washed cells (~65–100 mg wet cells) were resuspended in 1 ml of 6% KOH and incubated at 80 °C for 90 min. After cooling at room temperature, 100 μl of glacial acetic acid was added. Insoluble material was washed twice with water and resuspended in 450 μl of 50 mM sodium phosphate (pH 6.3) containing 0.2 unit of Serratia marcescens chitinase (Sigma) and incubated at 37 °C for 2 h. After centrifugation, 400 μl of supernatant was incubated with 0.25 mg of Helix pomatia β-glycosidase (Sigma) at 37 °C for 1 h, and then an aliquot of the mixture was assayed for N-acetylglucosamine content according to Reissig et al. (33).

Calcofluor White Staining—Calcofluor white staining was performed as described previously (34) with some modification. Briefly, log-phase cells grown at 30 °C were fixed with 3.7% paraformaldehyde and stained with 0.1 mg/ml Calcofluor white for 1 h. Cells were washed three times with phosphate-buffered saline and mounted on a slide in mounting medium (1 mg/ml p-phenylenediamine, phosphate-buffered saline, pH 9.0, 90% glycerol). Images were obtained using AX-80 microscope (Olympus).

RESULTS

Identification of RCR1/ YBR005w as a Multicopy Suppressor Gene of Congo Red Hypersensitivity of erd1Δ Mutant—ERD1 was previously found as a gene required for retention of the ER lumen proteins (35), and its mutants show temperature-sensitive growth in YPD medium, Genetin hypersensitivity,2 and glycosylation defect (36). We found that the erd1Δ mutant also shows hypersensitivity to Congo red and conducted a screening for multicopy suppressors that confer this to elucidate the function of the ERD1 protein.

S. cerevisiae YKI59 (erd1Δ) was transformed with a multicopy library YNL2 (URA3, 2μ). 82 out of 1,200,000 transformants formed colonies on SD medium containing 100 μg/ml Congo red. Seventy-six of them had ERD1. By sequencing and subcloning of the remaining six plasmids, we found that a previously uncharacterized open reading frame, YBR005w, was responsible for colony formation of YKI59 transformants on the SD plus Congo red medium (Fig. 1A). We named this gene RCR1 (resistance to Congo red 1). On the other hand, the other phenotype of erd1Δ, including the temperature sensitivity, glycosylation defect, and hypersensitivity to Genetin, did not recover in the RCR1 transformant. Fig. 1B shows that no difference was found in N-glycosylation of invertase, O-glycosylation of chitinase, and maturation of glycosylphosphatidylinositol-anchored Gas1 protein. Furthermore, the Congo red resistance was not restricted in the erd1Δ mutant, and introduction of the multicopy RCR1 made the wild-type and various mutant yeast strains more resistant to the growth inhibitory action of Congo red (Fig. 1C). Introduction of the multicopy RCR1 also made these strains more resistant to Calcofluor white (Fig. 1D).

Phenotypes of the rcr1Δ Null Mutant—Firstly, we examined the phenotype caused by an rcr1Δ null allele, because cells become significantly resistant to Congo red by the introduction of RCR1 in a CEN plasmid (data not shown). A heterologous diploid with RCR1/rcr1Δ was constructed from BY4743 and spores were dissected into tetrads. Sensitivity to SDS and growth at 37 °C were the same in the rcr1Δ and rcr1Δ haploid progenies. But the rcr1Δ progenies showed a higher sensitivity to 50 μg/ml Congo red or 40 μg/ml Calcofluor white in SD medium than the RCR1 progenies (Fig. 2A). The medium condition is important in determining the sensitivity, because all progenies grew similarly in YPD medium containing these compounds.

Characterization of RCR1—RCR1 encodes a polypeptide of 213 amino acids with a calculated mass of 23.9 kDa. Another open reading frame YDR003w encoding a polypeptide of 210 amino acids has a sequence identity of 46% with Rcr1. We named this gene RCR2 (Fig. 3A). However, the multicopy RCR2 did not have an activity to confer Congo red resistance in any strain tested so far. The rcr2Δ null mutant did not show increased sensitivity to Congo red or Calcofluor white, and the rcr1Δ rcr2Δ double disruptant showed similar sensitivity to these dyes as the rcr1Δ single null mutant (Fig. 2A). These null alleles had no effect on glycosylation of invertase (Fig. 2B) and maturation of Gas1 protein (Fig. 2C). Therefore, it is unlikely that RCR1 and RCR2 have an essential redundant role in S. cerevisiae.

The hydropathy plot suggests that Rcr1 and Rcr2 are integral membrane proteins with a single transmembrane domain (Fig. 3F), and the TMHMM program (available at www.cbs.dtu.dk/services/TMHMM/) predicts that amino acids 40–62 of Rcr1 form an α-helix that spans the membrane. It is noticeable that this transmembrane segment has an adjacent arginine-rich stretch (amino acids 64–71). The PEST find program (available at www.at.embnet.org/embnet/tools/bio/PESTfind/9) suggests that amino acids 99–114 is a region with a high PEST score (6.84). The PEST regions are thought to endow several proteins with sensitivity to ubiquitination or proteasomal digestion.

Localization of Rcr1 Protein—To make it clear that Rcr1 localizes in the membranes, we have first done a fractionation analysis. A 6myc epitope was added at the N terminus of Rcr1 for immunological detection, and the 6myc-Rcr1 protein was produced under the control of the SED5 promoter (pki94). By introduction of pki94, the yeast cells acquired a similar resistance to Congo red as the authentic RCR1 gene. The 6myc-Rcr1

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2 K. Imai, Y. Noda, H. Adachi, and K. Yoda, unpublished data.
Fig. 1. Effect of introduction of the multicopy RCR1 in the erd1Δ and other S. cerevisiae strains. A, the erd1Δ mutant having the multicopy ERD1 (mp34-8), RCR1 (pki72), or vector (pRS426) was streaked on SD agar medium containing 100 µg/ml Congo red and incubated at 30 °C for 3 days. B, invertase, chitinase, and Gas1 protein produced in the erd1Δ mutant having multicopy ERD1, RCR1, or vector were subjected to SDS-PAGE and detected by invertase activity staining, silver staining of chitinase, or Western blotting using anti-Gas1 antibody. Wild-type (BY4741) samples were used as the control. C, the wild-type (WT), pmt2Δ, rot2Δ, and swi6Δ strains having multicopy RCR1 or vector were grown as in A. D, the strains were grown as in C except the dye was 40 µg/ml Calcofluor white.

Fig. 2. Effect of rcr1Δ and rcr2Δ null mutations on the sensitivity to Congo red and Calcofluor white or glycosylation. A, the wild-type (RCR1 RCR2), rcr1Δ, rcr2Δ, or rcr1Δ rcr2Δ cells in a log-phase culture were collected and suspended at A600 nm of 1.0. The suspensions were 4-fold serially diluted, and 10-µl aliquots were spotted on SD agar plates with or without 50 µg/ml Congo red or 40 µg/ml Calcofluor white, and incubated at 30 °C for 2 days. B, invertase produced in these cells were examined by SDS-PAGE and activity staining. C, Gas1 protein produced in these cells was examined by SDS-PAGE and Western blotting using anti-Gas1 antibody. The erd1Δ cells were used as the control.
protein migrated in SDS-PAGE to the position corresponding to a protein of 54 kDa, although its calculated molecular mass is about 34 kDa (data not shown). The spheroplasts of wild-type yeast having pki94 were lysed, and the lysate was subjected to 100,000 \( g \) centrifugation. 6myc-Rcr1 was exclusively recovered in the pellet and not solubilized by treating with 0.1M Na2CO3 or 2 M urea (Fig. 4A), but it was solubilized by 1% Triton X-100. These results indicate that Rcr1 is a typical integral membrane protein.

Next, we examined the localization of Rcr1 by subcellular fractionation and immunofluorescent microscopy. A majority of 6myc-Rcr1 was recovered in P10 as well as the ER-marker protein Kar2, whereas a similar amount of the endosome-marker Pep12 or the Golgi-marker Gos1 was recovered equally in P10 and P100 (Fig. 4B). Immunofluorescent staining of myc epitope showed that 6myc-Rcr1 was present in a central ring and in peripheral ribbon-like structures beneath the cytoplasmic membrane. This immunological staining coincided well with that of the ER-marker Kar2 (Fig. 5).

**Topology of the Rcr1 Protein**—To make a topological issue clear, the membrane fraction was treated with proteinase K and protection of the N-terminal 6myc tag was examined by immunoblotting. In the absence of a detergent, a 32-kDa remnant of 6myc-Rcr1 was detected suggesting that the C-terminal cytosolic region was digested (Fig. 6). Kar2 protein in the lumen of the ER remained mostly intact. In the presence of a detergent, no myc signal was detected indicating the whole protein was digested and Kar2 became a smaller fragment that is intrinsically resistant to proteinase K digestion. When a tag was added to the C-terminal of Rcr1 to make Rcr1-6myc, no epitope signal was detected after proteinase digestion both in the presence and absence of detergent (data not shown). These results indicate that Rcr1 is a type I membrane protein with the N-terminal in the ER lumen and the C-terminal in the cytosol.

**Molecular Dissection of the Rcr1 Protein**—To get clues to reveal the mechanism that makes the cell more resistant to Congo red, we dissected the Rcr1 molecule and sought to find which region is responsible for the activity. The truncated polypeptides at the N or C termini were produced by a multicopy plasmid under the SED5 promoter in an rcr1/H9004 strain of KA31 genetic background (Fig. 7A). A similar amount of polypeptide was produced because a similar intensity of myc signal at the N-terminal was detected in each construct by immunoblotting (data not shown). We found that the N-terminal luminal region is dispensable for Congo red resistance, because Rcr1 (amino acids 40–213) is active (Fig. 7B). The
antibodies against endogenous Kar2 (A–C and D–F; multicycopy 6myc-Rcr1 or pRS426 (G–L vector) were double-labeled with a monoclonal anti-myc antibody (A, D, and G) and polyclonal antibodies against endogenous Kar2 (B, E, and H). C, F, and I show phase-contrast images.

The membrane fraction of cells having Rcr1 protein tagged with the 6myc epitope at the N-terminal was treated with proteinase K in the presence or absence of 1% Triton X-100. After precipitation with trichloroacetic acid, samples were analyzed by SDS-PAGE and Western blotting with anti-myc and anti-Kar2 antibodies. Positions of molecular mass markers are indicated at the left. Arrowheads indicate undigested proteins, and the asterisk indicates a major degradation product.

transmembrane domain is required for the activity, because Rcr1 (amino acids 63–213) and Rcr1 (amino acids 69–213) were inactive. A C-terminal region that has a low sequence similarity between Rcr1 and Rcr2 could be removed with a little decrease of activity and Rcr1 (amino acids 1–160) still had a significant activity. However, the cytosolic conserved region is essential, because Rcr1 (amino acids 1–86) and Rcr1 (amino acids 1–70) did not have an activity. A chimera protein consisting of amino acids 1–63 of Rcr1 and 64–210 of Rcr2 had activity (Fig. 7C), although Rcr2 itself did not confer Congo red resistance in the same expression construct. This suggested that the conserved region plays an important role for the activity of Rcr1. RCR2 may be a defective copy of a duplication of a common ancestor of the RCR1 gene.

Congo Red Sensitivity of the Cell Wall Mutants—Although it is well known that Congo red is a cell wall-disturbing agent, it is not firmly established which component of the cell wall is the most sensitive target in S. cerevisiae and which gene is responsible for it. For example, it was reported that disruption of RLM1 encoding a transcription factor (37) or LRG1 encoding a GTPase-activating protein of Rho-family GTPases (38) as well as overproduction of HOG1 (39) resulted in Congo red resistance, but there has been no explanation for the molecular mechanism. We therefore examined the effect of multicycopy RCR1 gene in various cell wall mutants in the S. cerevisiae disruptant collection. In the mutant of a gene that is necessary to make the cell resistant to Congo red, introduction of multicycopy RCR1 gene would have no significant response. In addition, multicycopy RCR1 would have induced some as yet unknown alteration in components of the wild-type cell wall and such alteration would not occur in non-responding mutants. By searching for “Calcofluor white” or “Congo red” in the keywords ofYPD data base, we randomly selected 200 genes to introduce multicycopy RCR1 gene in their null mutants and tested growth of the transformants on SD medium containing 100 μg/ml of Congo red.

As a result, these genes were classified into three groups (Figs. 1 and 8, and Table III); (i) the mutant became resistant to Congo red as the wild-type did, (ii) the mutant was hypersensitive to Congo red and multicycopy RCR1 did not make it grow on the test plate, and (iii) the mutant was intrinsically resistant to Congo red and no further effect of multicycopy RCR1 was found on the test plate. Most genes, which include PMT2 encoding glycosyltransferase, ROT2 encoding ER glycosidase II, and SWF6 encoding a transcription factor, belong to group I, although mutants of these genes show higher sensitivity to Congo red than the wild-type. The group II genes include those related to glucan synthesis (FKS1, ROM2, and KRE6), protein glycosylation (MNN9, MNN10, HOC1, and ANP1), and glycosylphosphatidylinositol-anchor protein (GASI), which are important for cell wall synthesis. Group III includes genes related to the activity of chitin synthase III (CHS3/CHS7).

Multicycopy RCR1 Affects Chitin Content in the Cell Wall—The above results suggest that a decrease in chitin correlates with Congo red resistance and RCR1 is likely to have an effect on the chitin content. As described above, N- and O-glycosylation and glycosylphosphatidylinositolanchoring were not affected (Figs. 1B, 2B, and 2C). Therefore, we analyzed the content of chitin and alkali-soluble glucan in the cell wall of wild-type, pmt2Δ and rot2Δ transformants with multicycopy RCR1 or vector plasmid. As shown in Table IV, the chitin content of the cells having multicycopy RCR1 was significantly lower than that of the control vector. In contrast, the content of alkali-soluble glucan did not change significantly. The amount of chitin in the pmt2Δ cells that had a little effect of RCR1 in Congo red resistance was intrinsically almost 3-fold of the wild-type. The fks1Δ cells in group II also had a significantly large amount of chitin in comparison with others. On the contrary, the amount of chitin in the chs7Δ cells in group III was only one-eighth of that of the wild-type cells. The colonies of wild-type and fks1Δ are red, but those of chs7Δ are white in Fig. 8. This shows that Congo red practically does not bind to cells lacking chitin made by chitin synthase III. These data clearly indicate a correspondence between the chitin content and Congo red resistance.

We microscopically examined cellular chitin by staining the cells with Calcofluor white. The fluorescent dye binds strongly to the bud necks and scars and less strongly but uniformly to the lateral cell wall as described in a number of previous reports (Fig. 9A). However, the cells with multicycopy RCR1 plasmid had a significantly different image. Fluorescence was totally reduced. The bud necks and scars still had bright fluorescence, but the lateral cell wall hardly had any detectable fluorescence in these Congo red-resistant cells (Fig. 9C).

Chitin Synthase III and Rcr1—It is likely that introduction of multicycopy RCR1 mainly affects the activity of chitin syn-
thase III. So, we examined intracellular localization of Chs3, Chs5, and Chs7 proteins by indirect immunofluorescence microscopy and subcellular fractionation. Chs3 and Chs5 proteins showed similar punctate immunofluorescent images in the presence and absence of multicopy *RCR1*. As shown in Fig. 10A, Chs3 and Chs7 were recovered equally in the P10 and P100 fractions similar to the Golgi/endosome-resident proteins. Chs5 was recovered in the P100 and S100. No difference was found in these distributions in the presence and absence of multicopy *RCR1*. Therefore, it is unlikely that overproduction of Rcr1 affects the localization of proteins, which affects chitin synthase III activity. Because Chs7 was reported to be an ER-resident protein (12), we examined if Rcr1 may interact with Chs7. We constructed a strain that produces both 6myc-Rcr1 and Chs7–3HA. 6myc-Rcr1 was collected from the detergent lysate, but Chs7–3HA was not detected in the immunoprecipitate (Fig. 10B). Therefore, it is unlikely that Chs7 and overproduced Rcr1 strongly interact in the ER membrane.

**FIG. 7.** Activity of the truncated or chimera Rcr1 proteins to increase resistance to Congo red. A, a schematic presentation of the truncated or chimera Rcr1 proteins and their activity to make *rcr1Δ* transformant Congo red resistance. The transmembrane domain, arginine-rich stretch, and PEST-like sequence are indicated. B, the *rcr1Δ* transformants were streaked on SD medium containing 100 μg/ml Congo red and incubated at 30 °C for 2 days. *RCR1* was the authentic *RCR1* gene, and all others were 6myc-tagged derivatives. C, the transformant having the Rcr1-Rcr2 chimera protein was tested as in B.

**FIG. 8.** Effect of multicopy *RCR1* in intrinsically resistant or sensitive mutants to Congo red. The wild-type (BY4741, WT), *fks1Δ*, and *chs7Δ* strains were transformed with pki72 (multicopy *RCR1*) or pRS426 (vector). Transformants were streaked on SD medium containing 100 μg/ml Congo red and incubated at 30 °C for 3 days.

**DISCUSSION**

During a multicopy suppressor screening for Congo red hypersensitivity of *erd1Δ* mutant, we found that a previously uncharacterized gene makes *S. cerevisiae* cells significantly resistant to this cell wall-perturbing agent. This gene, *RCR1* (resistance to Congo red 1), encodes a novel type I membrane protein of 32 kDa. A 6myc-tagged Rcr1 protein was active and exclusively localized in the ER membrane. The luminal N-terminal region was dispensable but the transmembrane domain and a part of the cytosolic region that is conserved in a homologous (46% identical) Rcr2 protein were essential to endow resistance to Congo red. The most C-terminal region that is not conserved in Rcr2 was also dispensable for Congo red resistance.

Our data indicate that the sensitivity to Congo red is closely related to the chitin content. In a number of mutants that were registered to have altered sensitivity to Calcofluor white, mu-
Table III

| Group | Gene | Vectora | RCR1a | Description of the gene product |
|-------|------|---------|--------|---------------------------------|
| I     | ERD1 | −       | ++++   | Protein required for luminal ER proteins |
|       | KNR4 | −       | ++++   | β1,3-Glucan synthesis protein |
|       | OST3 | −       | ++++   | Oligosaccharyltransferase subunit |
|       | PMT2 | −       | ++++   | Protein O-mannosyltransferase |
|       | ROT2 | −       | ++++   | Catalytic subunit of ER glucosidase II |
|       | SAC7 | −       | ++++   | GAP for Rho1 involved in β1,3-glucan synthesis |
|       | SW16 | −       | ++++   | Transcription factor involved in cell wall biosynthesis |
|       | CWH41| +       | ++++   | ER glycosidase I involved in β1,6-glucan assembly |
|       | ALG5 | ++      | ++++   | Doliol-P-glucose synthetase |
|       | ALG6 | ++      | ++++   | Glucosyltransferase |
|       | CHS1 | ++      | ++++   | Chitin synthase 1 |
|       | CHS4 | −       | ++++   | Chitin synthase 2 |
|       | CHS5 | ++      | ++++   | Chitin synthase 3 |
|       | CHS6 | ++      | ++++   | Protein involved in Chs3 activity |
|       | CHS7 | ++      | ++++   | Protein involved in the regulated transport of Chs3 from ER |

**a** Growth was examined on SD medium containing 100 μg/ml Congo red. ++++, colonies grew fully in 2 days; ++, colonies grew fully in 3 days; +++, colonies grew less than the wild-type; +, colonies grew as the wild-type; −, no growth.

Table IV

| Strain         | Plasmid | Chitin contentb (% | Alkali-soluble β1,3-glucanb |
|----------------|---------|-------------------|-----------------------------|
| Wild-type      | Vector  | 0.58 ± 0.05 (100) | 100                         |
| Wild-type      | RCR1    | 0.41 ± 0.03 (71)  | 106                         |
| pmt2Δ          | Vector  | 1.74 ± 0.30 (300)| 128                         |
|                | RCR1    | 1.25 ± 0.10 (216)| 116                         |
| rot2Δ          | Vector  | 0.93 ± 0.08 (160)| 130                         |
|                | RCR1    | 0.65 ± 0.08 (112)| 127                         |
| chs7Δ          | Vector  | 0.08 ± 0.01 (13) | ND                          |
| βks1Δ          | Vector  | 2.13 ± 0.19 (367)| 35                          |
| rom2Δ          | Vector  | ND                | 68                          |

**b** Values are represented in N-acetylgalactosamine content/wet weight of cells (μg/mg). All values are the mean of three independent determinations ± S.D.

**c** Level of alkali-soluble β1,3-glucan as a percentage of the level present in the wild type.

**d** ND, not determined.

**FIG. 9.** Fluorescent staining of chitin in the transformant cells by Calcofluor white. The wild-type (BY4741) cells having pRS426 (A and B; vector) or pki72 (C and D; multicopy RCR1) were fixed by formaldehyde and observed by fluorescence microscope after staining with Calcofluor white (A and C). Fluorescence images were obtained under a same capture condition. B and D show corresponding phase-contrast images.

Introduction of multicopy RCR1 made a number of cell wall mutants more resistant to Congo red, and the chitin content of all these cells decreased significantly while their alkali-soluble

The mutants having an increased amount of chitin by compensation mechanism for other defects in the cell wall (fks1Δ, gasΔ, kreΔ, rot2Δ, mnn9Δ, unp1Δ, among others) were significantly more sensitive to Congo red. In contrast to this, the mutants of the genes that are necessary for the activity of chitin synthase III, the major chitin-synthesizing enzyme (chs3Δ, chs4Δ, chs5Δ, chs6Δ and chs7Δ), were highly resistant to Congo red. These are consistent with the previous report that the growth inhibition of S. cerevisiae by Congo red was partially recovered by the addition of a competitive inhibitor of chitin synthase III, Nikkomycin Z (40).
Rcr1 Regulates Chitin Deposition

β1,3-glucan content did not change. The rcr1Δ null mutant that shows slightly increased sensitivity to Congo red had 115% of chitin compared with the wild-type in a different set of experiments as shown in Table IV. The effect of multicopy RCR1 was weak in the mutants with intrinsically higher chitin content such as pmt2Δ and was hardly recognized in mutants with a much higher chitin content such as fks1Δ (about 4 times that of the wild-type) (41). All these data suggest that chitin is the major target of Congo red in S. cerevisiae as generally recognized in other fungi. This is consistent with the fact that the cells with multicopy RCR1 were also more resistant to Calcofluor white.

It is well established that non-essential chitin synthase I engages in repair of the cell wall during the daughter cell separation (42). The chs1Δ mutant showed an increased resistance by multicopy RCR1 as the wild-type did (data not shown). Chitin synthase II engages in synthesis of chitin localized at the primary septum (43). The mutant is lethal or severely sick, and we could not examine the effect of RCR1. Chitin synthase III is the main enzyme that synthesizes 90% of chitin compared with the wild-type in a different set of experiments as shown in Table IV. The effect of multicopy RCR1 showed slightly increased sensitivity to Congo red had 115% of chitin deposition in a signal-dependent manner. Other genome-wide analysis, including yeast two-hybrids, coprecipitated protein analysis, and synthetic defective screening, have given us no information concerning RCR1 so far.

Another possible mechanism to reduce chitin synthesis at the lateral cell wall is to interfere with the supply or maturation of chitin synthase III that depends on vesicular transport. An ER membrane protein can engage in sorting the cargo into COPII vesicles, and so Rcr1 may function to retain an essential component for functional localization of chitin synthase III at the lateral cell wall. Therefore, we examined intracellular localization of the catalytic subunit Chs3 and a regulatory proteins Chs5 by immunofluorescent staining of their HA tag. These proteins were detected in a punctate pattern as reported previously, and we could not find a difference in the presence and absence of the multicopy RCR1. We also found no difference in the subcellular fractionation of Chs3, Chs5, and Chs7.

Our present results indicate that the synthesis of chitin is the primary target of Congo red, and this chitin deposition is regulated by an ER membrane protein Rcr1. The molecular mechanism of this regulation currently remains unclear. Existence of some novel mechanism by which the ER regulates a cell wall component deposition will attract interest in the basic research of the fungal cell wall biogenesis.

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Figure 10. Effect of overproduced Rcr1 on the proteins required for the chitin synthase III activity. A. Y1306 (Chs3–3HA), Y1304 (Chs5–3HA), and YK116–2 (Chs7–3HA), which produce tagged derivatives of proteins required for chitin synthase III, were transformed with pki72 (multicopy RCR1) or pRS426 (vector). Cell lysates were prepared and subjected to differential centrifugation, and the fractions were analyzed by SDS-PAGE and Western blotting using anti-HA monoclonal antibody. B. YK116–2 (Chs7–3HA) was transformed with pRS426 (vector) or pki94 (multicopy 6myc-RCR1), and the spheroplast lystate was solubilized in 1% Triton X-100 (S, start). After immunoprecipitation using anti-myc monoclonal antibody, the unbound (U) and bound (B) fractions were analyzed by SDS-PAGE and Western blotting to detect 6myc-Rcr1 and Chs7–3HA.

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On the other hand, the genome-wide expression analyses detected that the expression of RCR1 itself is regulated by several factors. First, mRNA of RCR1 increased more than 10-fold by the addition of calcium in CRZ1-dependent manner (46). Second, it also increased 2- to 3-fold by the treatment with Congo red or Zymolyase, which gives stress on the cell wall (47). These findings suggest that RCR1 functions to regulate chitin deposition in a signal-dependent manner. Other genome-wide analysis, including yeast two-hybrids, coprecipitated protein analysis, and synthetic defective screening, have given us no information concerning RCR1 so far.

Table IV. The effect of multicopy RCR1 on the proteins required for the chitin synthase III activity. A. Y1306 (Chs3–3HA), Y1304 (Chs5–3HA), and YK116–2 (Chs7–3HA), which produce tagged derivatives of proteins required for chitin synthase III, were transformed with pki72 (multicopy RCR1) or pRS426 (vector). Cell lysates were prepared and subjected to differential centrifugation, and the fractions were analyzed by SDS-PAGE and Western blotting using anti-HA monoclonal antibody. B. YK116–2 (Chs7–3HA) was transformed with pRS426 (vector) or pki94 (multicopy 6myc-RCR1), and the spheroplast lystate was solubilized in 1% Triton X-100 (S, start). After immunoprecipitation using anti-myc monoclonal antibody, the unbound (U) and bound (B) fractions were analyzed by SDS-PAGE and Western blotting to detect 6myc-Rcr1 and Chs7–3HA.
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