The CRH Family Coding for Cell Wall Glycosylphosphatidylinositol Proteins with a Predicted Transglycosidase Domain Affects Cell Wall Organization and Virulence of Candida albicans*

Giacomo Pardini1, Piet W. J. De Groot2, Alix T. Coste1, Mahir Karababa1, Frans M. Klis1, Chris G. de Koster1, and Dominique Sanglard2

From the 1Institute of Microbiology, University Hospital Lausanne, CH-1011 Lausanne, Switzerland and 2Swammerdam Institute for Life Sciences, University of Amsterdam, 1018 WV Amsterdam, Netherlands

In Candida albicans UTR2 (CSF4), CRH11, and CRH12 are members of a gene family (the CRH family) that encode glycosylphosphatidylinositol-dependent cell wall proteins with putative transglycosidase activity. Deletion of genes of this family resulted in additive sensitivity to compounds interfering with normal cell wall formation (Congo red, calcifluor white, SDS, and high Ca2+ concentrations), suggesting that these genes contribute to cell wall organization. A triple mutant lacking UTR2, CRH11, and CRH12 produced a defective cell wall, as inferred from increased sensitivity to cell wall-degrading enzymes, decreased ability of protoplasts to regenerate a new wall, constitutive activation of Mck1p, the mitogen-activated protein kinase of the cell wall integrity pathway, and an increased chitin content of the cell wall. Importantly, this was accompanied by a decrease in alkali-insoluble 1,3-β-glucan but not total glucan content, suggesting that formation of the linkage between 1,3-β-glucan and chitin might be affected. In support of this idea, localization of a Utr2p-GFP fusion protein largely coincided with areas of chitin incorporation in C. albicans. As UTR2 and CRH11 expression is regulated by calcineurin, a serine/threonine protein phosphatase involved in tolerance to antifungal drugs, cell wall morphogenesis, and virulence, this points to a possible relationship between calcineurin and the CRH family. Deletion of UTR2, CRH11, and CRH12 resulted in only a partial overlap with calcineurin-dependent phenotypes, suggesting that calcineurin has additional targets. Interestingly, cells deleted for UTR2, CRH11, and CRH12 were, like a calcineurin mutant, avirulent in a mouse model of systemic infection but retained the capacity to colonize target organs (kidneys) as the wild type. In conclusion, this work establishes the role of UTR2, CRH11, and CRH12 in cell wall organization and integrity.

1 This work was supported by a Howard Hughes Medical Institute grant (to D. S.) as an International Research Scholar and by a grant from the European Union (FUNGWALL) (to F. K.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

2 To whom correspondence should be addressed. Tel.: 41-21-3144083; Fax: 41-21-3144060; E-mail: Dominique.Sanglard@chuv.ch.

3 The abbreviations used are: GPI, glycosylphosphatidylinositol; MAP, mitogen-activated protein; CFW, calcifluor white; GFP, green fluorescent protein; ORF, open reading frame; CFU, colony-forming unit; PBS, phosphate-buffered saline; HPLC, high pressure liquid chromatography; CR, congo red; GH, glycoside hydrolase; CBM, carbohydrate-binding module.
Cell Wall Glycosidases of C. albicans

wall biogenesis. In Saccharomyces cerevisiae, the CRH family also consists of three GPI proteins, named CRH1, UTR2/CRH2, and CRR1, that are involved in cell wall construction (14). Deletion of CRH1 and CRH2 results in additive sensitivity to compounds (Congo red and calcofluor white) that interfere with cell wall assembly. Moreover, the double deletion mutant showed a 2-fold increase in the amount of alkali-soluble cell wall glucan in comparison to wild type, indicating that less glucan is bound to chitin (14). Studies with GFP fusion proteins indicated that Crh1p and Crh2p are located at the cell surface, particularly in chitin-rich areas, and that their temporal and spatial localization is specifically controlled during the cell cycle (14, 15). In addition, both proteins are shown to be covalently bound to the cell wall network (16). Recently, two Crh homologues of Yarrowia lipolytica were found to be important for cell wall construction and were shown to have endo-1,3-β-glucosidase activity in vitro (17).

Much less is known about the CRH family in C. albicans. In an earlier study, Utr2p/Csf4p was selected as a potential cell surface factor using a bio-informatics approach (18). Deletion of UTR2 in C. albicans resulted in altered colony morphology, attenuated cell adhesion, and reduced virulence. However, neither the role in cell wall assembly nor the function-ality of other members of the CRH gene family in C. albicans was investigated (18).

In this study, we have addressed the role of the putative transglycosidases encoded by UTR2, CRH11, and CRH12 in cell wall biolog and pathogenesis of C. albicans. First, we show that strains lacking UTR2, CRH11, and CRH12 exhibit phenotypes typical for cell wall defects. Second, alterations in cell wall polysaccharide composition in mutants lacking UTR2 suggest that Utr2p is involved in formation of the linkage between 1,3-polysaccharide composition in mutants lacking typical for cell wall defects. Second, alterations in cell wall strains lacking biology and pathogenesis of glycosidases encoded by UTR2, CRH11, and CRH12 were investigated (18).

EXPERIMENTAL PROCEDURES

Strains and Media—The C. albicans strains used in this study are listed in Table 1. C. albicans strains were grown either in complete medium YEPD containing 0.5% (w/v) yeast extract (Difco), 1% (w/v) bactopeptone (Difco), and 2% (w/v) glucose (Sigma) or in minimal medium YNB (yeast nitrogen base; Difco), 1% (w/v) bactopeptone (Difco), and 2% (w/v) glucose (Fluka). For growth on solid media, 2% (w/v) agar (Difco) was added to the media.

Escherichia coli DH5α was used as host for plasmid constructions and propagation (19). DH5α was grown in LB (Luria-Bertani broth; Difco) or LB plates, supplemented with ampicillin (0.1 mg/ml) when required.

Transformation Procedures—C. albicans strains were transformed by a one-step transformation method of yeast in stationary phase as reported previously (20).

Disruption of UTR2, CRH11, and CRH12 in C. albicans—Two different regions of UTR2 (orf19.1671 available on line) were amplified from C. albicans genomic DNA with the following pairs of primers (supplemental Table I): UTR2-1-XbaI and UTR2-2-XhoI and UTR2-1-XhoI and UTR2-2-XbaI/UTR2-2-XhoI in a PCR, and cloned into compatible restriction sites of pBluescriptKS+ to yield pGP01 and pGP03 (supplemental Table II), respectively. Deletions in the cloned UTR2 regions were created by PCR using outward directed primer pairs UTR2-1-PstI/UTR2-1-BglII and UTR2-2-PstI/UTR2-2-BglII (supplemental Table I) with pGP01 and pGP03 as templates. The products of these PCRs were digested with PstI and BglII and ligated to a PstI-BglII 3.7-kb fragment from pMB7 containing the hisG-URA3-lisG”Ura-blaster” cassette to yield pGP02 and pGP04 (supplemental Table II), respectively. The construction of two different UTR2 cassettes was aimed to enhance the recovery of targeted sequential genome deletions, because one cassette (pGP04) is internal to the first one (pGP02). Finally, disruption cassettes were linearized by digestion with Apal and SacI and used for sequential disruption of both UTR2 alleles with intermediate marker regeneration as described by Fonzi and Irwin (21).

The UTR2 revertant strain was obtained by reintroduction of UTR2 into the utr2Δ C. albicans mutant. A PCR was performed with the primers UTR2-5’-BamHI/UTR2-1-XhoI to yield a fragment containing the entire UTR2 ORF, including 3’- and 5’-flanking regions. The resulting fragment was cloned into pDS178 (22) to obtain pGP05. This plasmid was linearized with SalI, which cuts within LEU2 to favor integration at the LEU2 genomic locus, and was transformed into C. albicans. CRH11 (orf19.2706) and CRH12 (orf19.3966) disruption cassettes were obtained using the same strategy as outlined above. PCRs were performed with the pairs of primers listed in supplemental Table I. Plasmids used for CRH11 and CRH12 disruptions are listed in supplemental Table II.

Southern and Northern Blot Analysis—Genomic DNA was extracted from C. albicans and digested with appropriate restriction enzymes (supplemental Fig. 1). Northern blotting was performed as reported by Sanglard et al. (23). The TEF3 probe was used as internal standard and originated from a 0.7-kb EcoRI-PstI fragment from pDC1 described in Sanglard et al. (23).

Phenotypic Analyses—C. albicans strains were tested for their susceptibility to cell wall-perturbing agents and drugs were grown overnight in liquid YEPD medium. Cells were diluted to 1.5 × 10⁷ cells per ml with serial 10-fold dilutions. Of each dilution 5 μl was spotted onto YEPD plates or plates containing 100 μg/ml congo red (CR; Sigma), 40 μg/ml calcofluor white (CFW; Sigma), 0.06% SDS (Fluka), or 500 mM CaCl₂ (Fluka). Plates were incubated for 48 h at 34 °C.

Protoplast Formation, Cell Wall Regeneration, and Cell Wall Degradation Assays—Cells grown overnight in YEPD were washed twice in TE, pH 8.0. Aliquots containing 1.5 × 10⁷ cells were resuspended in PRO buffer (25 mM EDTA, 1 mM sorbitol, 20 mM Tris-HCl, pH 7.5) with 50 μg/ml 100T Zymolyase (Seikagaku, Tokyo, Japan) and 1% (v/v) β-mercaptoethanol (Sigma) and incubated at 37 °C until cell wall lysis occurred. Protoplast formation was checked by microscopic observation. To regenerate cell walls, protoplasts were suspended in a soft-top agar (YEPD broth containing 0.7% agar and 1.2% sorbitol, autoclaved and cooled to 45 °C). This suspension was poured evenly across the top of YEPD agar plates and allowed to solidify. To determine the number of intact cells present in the samples, the
same amount of protoplasts was plated onto YEPD agar without top agar. Plates were incubated at 37 °C for 48 h. Numbers of cells grown in YEPD with or without top agar ($n_{1}$ and $n_{2}$, respectively), and the starting inoculum ($n_{0}$) were determined as colony-forming units (CFU) by counting colonies of serial dilutions on YEPD agar. Cell wall regeneration was expressed as a percentage ($P_{cw}$) and determined by the following equation: 

$$P_{cw} = \frac{n_{1} - n_{2}}{n_{0}} \times 100\%,$$

Cell wall degradation was expressed as the percentage of intact cells after lysis caused by Zymolyase activity and was monitored by taking aliquots at 10-min intervals and measuring the optical density at 540 nm.

**Cell Wall Isolation and Analysis of Cell Wall Composition—*C. albicans* strains were grown overnight in liquid YPD at 30 °C to an $A_{600}$ of about 2. The detailed procedure for cell wall isolation is described by De Groot et al. (4). To determine alkali-resistant 1,6-β- and 1,3-β-glucans, cell walls (about 4 mg dry weight) were extracted by incubation (three times) in 1 ml of 3% (w/v) NaOH at 75 °C for 1 h (24). Extracted walls were washed three times with 1 ml of H$_2$O and freeze-dried. To release alkali-resistant 1,6-β-glucan, alkali-extracted walls were incubated with recombinant endo-1,6-β-glucanase (Prozyme, San Leandro, CA) as described (25). The supernatant after centrifugation represents the alkali-resistant 1,6-β-glucan fraction. The remaining cell wall pellet was washed two times with 1 ml of H$_2$O and freeze-dried. Alkali-resistant 1,3-β-glucan was solubilized by incubating with the recombinant endo-1,3-β-glucanase Quantzyme (Qbigenie Morgan Irvine, CA) as described (25). Supernatants containing either 1,6-β-glucan or 1,3-β-glucan were analyzed with the phenol-sulfuric acid assay using glucose as a reference (26).

For total cell wall glucan and mannann determination, cell wall carbohydrates were hydrolyzed to monomers using the sulfuric acid hydrolysis method (27). About 4 mg of freeze-dried walls were incubated in 100 μl of 72% (v/v) H$_2$SO$_4$ for 3 h at room temperature. The samples were then diluted with 575 μl of distilled H$_2$O to obtain a 2 μl H$_2$SO$_4$ solution and incubated for 4 h at 100 °C. Amounts of mannose and glucose in the samples were determined by HPLC (GE Healthcare) on a
REZEX organic acid analysis column (Phenomenex, Torrance, CA) at 40 °C with 7.2 mm H₂SO₄ as eluent, using an RI1530 refractive index detector (Jasco, Great Dunmow, UK). The chromatograms were analyzed using AZUR chromatography software and compared with chromatograms of known amounts of mannose, glucose, and glucosamine. Chitin was determined following the protocol described by Kapteyn et al. (28).

**Protein Structure Analysis**—Functional domains of Utr2p, Crh11p, and Crh12p, glycoside hydrolase (GH) domains and a carbohydrate-binding module (CBM), were identified using the SMART tool analysis software and the CAZy Carbohydrate-Active enzymes data base. Most likely, GPI addition of amino acid (ω)-sites (Ser⁴⁴⁰, Asn⁴³⁰, and Gly⁴⁷⁸) were predicted using big-PI Fungal Predictor software. The three-dimensional structure of the putative transglycosidase domain of Utr2p was obtained by homology modeling. The GH16 domain boundaries of Utr2p were predicted by alignment of multiprotein families using the Pfam protein families data base. The secondary structure of the Utr2p GH16 domain was predicted and aligned on template structures of a fold library using threading to identify a Protein Data Bank template for homology modeling. At the level of secondary structure, Utr2p favorably aligns with Bacillus licheniformis 1,3–1,4-β-D-glucan 4-glucanohydrolase (Protein Data Bank code 1GBG). This template also belongs to the CAZy GH16 family and cleaves 1,4-β-glycosidic bonds that are adjacent to 1,3-β-glycosidic bonds in mixed-linked glucans. Multiple sequence alignment with hierarchical clustering was used to align the Utr2p GH16 target and 1GBG template sequence for homology modeling. Optimization of this alignment was carried out manually.

**Detection of Mkc1p by Immunoblotting**—C. albicans cell extracts for immunoblotting were prepared from cells grown to mid-log phase as described by Navarro-Garcia et al. (29). Briefly, yeasts incubated for 2 h in the presence or absence of CR (20 μg/ml) were resuspended in lysis buffer and broken using glass beads in a Mini-Bead Beater-8 (Biospec Products, Bartlesville, OK) applying three 30-s rounds with intermediate ice cooling (1 min). Equal amount of proteins (150 μg), verified by the Bradford assay (30) and Ponceau S staining, were separated by SDS-PAGE and blotted onto a nitrocellulose membrane. Dually phosphorylated Mkc1p was detected using 1,000-fold diluted anti-phospho-p44/42 MAP kinase (Thr²⁰²/Tyr²⁰⁴) anti-serum (New England Biolabs, Ipswich, MA). Western blots were developed using an ECL kit following the manufacturer’s instructions (Amersham Biosciences).

**Construction of GFP Fusion Proteins**—To create a fusion protein of Utr2p with the GFP, a fragment of the LITR2 ORF (from +1 to +1263), lacking the ω-site and C-terminal GPI signal sequence, was amplified with the primer pair LITR2-5'—GFP-BamHI/GFP-3'—UTR2. GFP was amplified from yEGFP (31) with primers LITR2-5'—GFP and GFP-3'—Clal. These two fragments share a 30-bp overlap and were used as templates in a second PCR amplification step with external primers LITR2-5'—BamHI and GFP-Clal. The resulting LITR2—GFP fragment was cloned into pBluescriptSK⁺ digested with BamHI and Clal to yield pGP50. To obtain a functional Utr2p—GFP protein, a UTR2 fragment from +1263 to +2861 containing the ω-site and C-terminal sequence was amplified using primers UTR2-5’-Clal/UTR2-1-Xhol and cloned into a compatible site of pGP50 to yield pGP51.

The final UTR2—GFP fusion construct was amplified from pGP51 with the primer pair UTR2-5’—GFP-SalI/UTR2-3’—GFP-Nhel and cloned into pCMVACT-C-ZZ under the control of the ACT1 promoter to yield pGP52. This plasmid was digested with Stul and transformed into the utr2Δ strain (GPY04) to yield GPY101. The UTR2—GFP construct could yield a functional protein as it complemented the phenotypes (hypersusceptibility to cell wall damaging agents) of the utr2Δ strain (data not shown).

**Fluorescence Microscopy**—Fluorescence and contrast interference microscopy were performed using a Zeiss Axioskop microscope (Zeiss) equipped for epifluorescence microscopy with a 100-watt mercury high pressure bulb and Zeiss filter set 15. A Kappa DX30 digital camera with high resolution was used to record images, which were processed further using the computer program Adobe Photoshop 6.0. For chitin staining, cells were incubated for 5 min at room temperature in the presence of 0.5 μg/ml CFW, and cells were examined directly for fluorescence after washing off excess CFW.

**Mouse Infection Assays**—Animal experiments were performed as described previously (13). Briefly, C. albicans isolates grown overnight at 30 °C in YEPD were diluted to 10⁶ cells/ml into 50 ml of YEPD and incubated for another 24 h at 30 °C. Cells were washed twice with PBS, and cell concentrations were adjusted to 2 × 10⁶ CFU/ml in PBS. To confirm the accuracy of the infection dose, samples of inoculum suspensions were plated onto YEPD agar. Mice were infected intravenously with each strain at a dose of 5 × 10⁶ CFU in 250 μl of PBS via the lateral tail vein.

For reintroduction of UTR2, CRH11, and CRH12 into the utr2Δ/crh11Δ/crh12Δ mutant strain GPY103, pMK83 containing the CaSAT1 marker was used (32). UTR2, CRH11, and CRH12 were amplified by PCR using the primer pairs listed in supplemental Table I and cloned into compatible restriction sites of pMK83 to yield pGP55 (containing UTR2), pGP56 (containing CRH11), and pGP57 (containing CRH12). These plasmids were linearized by Hpal (pGP55 and pGP56) and MluI (pGP57) in order to facilitate integration into the UTR2, CRH11, and CRH12 genomic loci, respectively. Finally, Cip10 containing URA3 was linearized by digestion with Stul and introduced into different strain backgrounds as described by Brand et al. (33). Animal experiments were carried out at the University Hospital of Lausanne animal facility and were approved by the local ethical committee.

**RESULTS**

**UTR2, CRH11, and CRH12, a Family of GPI Proteins in C. albicans with a Predicted Transglycosidase Domain**—A previous study showed that Utr2p is a predicted GPI protein with high similarity to the GPI-modified cell wall protein Crh11p (18). Both proteins show significant similarity with the three S. cerevisiae CRH family proteins Crh1p (47% identity with Crh11p), Utr2p/Crh2p (42% identity with Utr2p), and Crr1p, which all contain a conserved transglycosidase domain (14). In the C. albicans genome, we also found a third putative member
of the family, called Crh12p, which has 33 and 21% identity to Crh11p and Utr2p from the same species, respectively, and is most closely related to Crh1p of S. cerevisiae (34% identity). Amino acid sequence analysis of these C. albicans proteins revealed common functional domains; all three proteins have a putative N-terminal signal peptide (amino acids 1–23 for Utr2p, 1–21 for Crh11p, and 1–18 for Crh12p), required for entry into the secretory pathway, a serine-/threonine-rich region, and a putative C-terminal signal sequence for GPI-anchor addition, which is characteristic for many glycoproteins that are linked to the cell wall (Fig. 1A). Utr2p, Crh11p, and Crh12p contain a putative transglycosidase domain with the active site motif DE(I/L)DXE (Fig. 1A). Transglycosidase domains are present in a widespread group of enzymes (34, 35). A classification system for Carbohydrate-Active EnZymes (CAZy) based on sequence similarity has led to the definition of 85 different families (36, 37). Utr2p, Crh11p, and Crh12p belong to glycoside hydrolase (GH) family 16, including the following enzymes with a number of known activities: lichenase, xylanoglucan xylanoglucosyltransferase, agarase; α-carrageenan, endo-1,3-β-glucanase, endo-1,3-1,4-β-glucanase, and endo-β-galactosidase. Homology modeling of the GH16 domain of Utr2p indicates that it has a β-jelly roll fold with the conserved DE(I/L)DXE motif situated on a curved β-strand. The two glutamic acid residues Glu168 and Glu172 are suitably disposed (7.0 Å from Cα to Cα) to have retaining glycosidase catalytic activity (Fig. 1B). Utr2p is distinct from Crh11p and Crh12p because it contains a putative chitin binding domain (CBM18) in its N-terminal segment (Fig. 1A). The existence of this particular domain suggests a specific role for this protein not shared by the two other members of the gene family. In conclusion, Utr2p, Crh11p, and Crh12p have typical features of GPI-anchored proteins, and because of their potential localization and proteins motifs, they are likely to be involved in the modification of cell wall sugar polymers.

Deletion of UTR2, CRH11, and CRH12—To explore the functions of UTR2, CRH11, and CRH12 and the relationship between these genes and calcineurin, utr2Δ (GPY03), crh11Δ (GPY80), and crh12Δ (GPY88) single mutant strains were analyzed for phenotypes (hypersusceptibility to cell wall-perturbing agents) exhibited by a cnaΔ mutant (DSY2091). To eliminate possible compensation phenomena from genes of this family, gene deletions of UTR2 CRH11 and CRH12 in all possible combinations were generated and analyzed as well. Southern blot analysis confirmed that the genes were deleted and that revertants had a correct genome insertion site (supplemental Fig. 1). As shown in Fig. 2, deletion of each of the three genes led to increased susceptibility to the cell wall-binding dye Congo red as compared with wild type. Of the three single mutants, growth of crh11Δ was most severely impaired by CR addition (Fig. 2, panel 1, rows 3, 5, and 7). Re-introduction of UTR2, CRH11, or CRH12 in single mutant strains restored growth in the presence of CR to the extent of the wild type (Fig. 2, panel 1, rows 4, 6, and 8), demonstrating that the absence of these genes is responsible for the observed phenotypes.

Mutant strains with two or three genes deleted displayed increased susceptibility to CR, CFW, calcium, and SDS (Fig. 2, rows 9–12) as compared with single gene deletants.
The role of the CRH family in cell wall integrity and cell wall regeneration

Moreover, \( \text{utr2}\Delta /\text{crh11}\Delta \) (GPY109), \( \text{utr2}\Delta /\text{crh12}\Delta \) (GPY23), and \( \text{crh11}\Delta /\text{crh12}\Delta \) (GPY100) mutant strains showed different phenotypes depending on the combination of deleted genes. For instance, the strains lacking \( \text{UTR2} \) in combination with another gene were almost not able to grow on YEPD plates containing calcium, whereas \( \text{crh11}\Delta /\text{crh12}\Delta \) grew as the wild type (Fig. 2, panel 4, rows 9–11). In accordance with these data, reintroduction of \( \text{UTR2} \) into a triple mutant lacking \( \text{UTR2}, \text{CRH11}, \text{and CRH12} \) restored growth to the extent of the wild type (data not shown). These data suggest that among the members of the CRH family, \( \text{UTR2} \) plays a major role in calcium-containing medium. On SDs-containing medium, growth of \( \text{utr2}\Delta /\text{crh11}\Delta \) was severely impaired, whereas \( \text{utr2}\Delta /\text{crh12}\Delta \) and \( \text{crh11}\Delta /\text{crh12}\Delta \) were only slightly more susceptible to SDS as compared with the wild type (Fig. 2, panel 3, rows 9–11). Finally, the \( \text{utr2}\Delta /\text{crh11}\Delta /\text{crh12}\Delta \) mutant strain GPY102 was not able to grow in the presence of any of the cell wall-perturbing agents (CR, CFW, SDS, and calcium) (Fig. 2, panels 1–4, row 12). In the presence of SDS or calcium, GPY102 displayed similar susceptibility profiles as observed in the \( \text{cna}\Delta \) mutant strain DSY2091 (Fig. 2, panels 3 and 4, rows 2 and 12). The additive effect of simultaneous deletion of \( \text{UTR2}, \text{CRH11}, \text{and CRH12} \) might reflect a common function for the three genes in the maintenance of cell wall structures. The severe phenotypes caused by various cell wall-perturbing agents led us to conclude that the CRH family plays a crucial role in cell wall biogenesis.

### Calcium- and Calcineurin-dependent Regulation of \( \text{UTR2}, \text{CRH11} \), and \( \text{CRH12} \)

As observed in Fig. 3A, deletion of \( \text{UTR2}, \text{CRH11}, \text{and CRH12} \) was first examined in single mutant backgrounds. As observed in Fig. 3A, deletion of \( \text{UTR2}, \text{CRH11}, \text{and CRH12} \) did not alter the expression of each other. However, expression of \( \text{UTR2} \) and \( \text{CRH11} \) was up-regulated in the presence of calcium, whereas \( \text{CRH12} \) expression was not detected by Northern blot analysis (Fig. 3A). The absence of \( \text{CRH12} \) expression under normal growth conditions and under calcium stress suggests that this gene may be of less importance to cell wall organization than \( \text{UTR2} \) and \( \text{CRH11} \). Further analyses were carried out to investigate if calcineurin was responsible for the increased expression of \( \text{UTR2} \) and \( \text{CRH11} \) in the presence of calcium. Consistent with published data (13), induction of \( \text{UTR2} \) by exposing strains to calcium was regulated by calcineurin, because no calcium-dependent up-regulation of \( \text{UTR2} \) was observed in the \( \text{cna}\Delta \) mutant (Fig. 3B). This was also the case for \( \text{CRH11} \), which further establishes a relationship between calcineurin and this family of cell wall proteins (Fig. 3B).

### Involvement of the CRH Gene Family in Cell Wall Assembly and Regeneration

To evaluate the importance of \( \text{Utr2p}, \text{Crh11p}, \text{and Crh12p} \) for cell wall assembly and integrity, cell wall degradation and regeneration assays were performed. Cell wall degradation was investigated by measuring cell lysis of \( \text{C. albicans} \) in the presence of Zymolyase, which is able to hydrolyze 1,3-\( \beta \)-glucan, the water-insoluble backbone of the cell wall. 1,3-\( \beta \)-Glucan chains are moderately branched polymers, with multiple nonreducing ends, which may function as acceptor sites for covalent addition of chitin and 1,6-\( \beta \)-glucans. Survival time courses after 5 h of incubation were measured for wild type, \( \text{utr2}\Delta, \text{crh11}\Delta, \) and \( \text{crh12}\Delta \) single mutants and the triple mutant \( \text{utr2}\Delta /\text{crh11}\Delta /\text{crh12}\Delta \). As summarized in Table 2, cell lysis by cell wall digestion with Zymolyase was significantly enhanced in all mutants compared with the wild type at all time points except at the last time point of 40 min after Zymolyase addition. Compared with single deletion mutants, the proportion of intact cells for the \( \text{utr2}\Delta /\text{crh11}\Delta /\text{crh12}\Delta \) mutant was decreased especially at the early cell wall digestion time point (10 min), thus providing further evidence for a role of \( \text{UTR2}, \text{CRH11}, \text{and CRH12} \) in cell wall integrity.

#### Table 2

| Strain          | Intact cells (% ± S.E.) at incubation time<sup>a</sup> | Cell wall regeneration (% ± S.E.)<sup>b</sup> |
|-----------------|---------------------------------------------------|---------------------------------------------|
|                 | 10 min | 20 min | 30 min | 40 min |                                             |
| CAF2-1          |        |        |        |        |                                              |
| GPY03 (\text{utr2}\Delta)<sup>c</sup>   | 66.6 ± 6.2<sup>2</sup>  | 35.3 ± 2.0<sup>2</sup>  | 24.2 ± 2.4<sup>2</sup>  | 15.7 ± 1.9<sup>2</sup>  | 80.2 ± 12.2<sup>2</sup> |
| GPY03 (\text{crh11}\Delta)<sup>c</sup> | 33.5 ± 3.0<sup>2</sup>  | 14.5 ± 6.0<sup>2</sup>  | 9.9 ± 2.3<sup>2</sup>  | 9.5 ± 3.0<sup>2</sup>  | 71.7 ± 7.6<sup>2</sup>  |
| GPY80 (\text{crh12}\Delta)<sup>c</sup> | 46.8 ± 5.1<sup>2</sup>  | 17.5 ± 2.1<sup>2</sup>  | 12.9 ± 2.6<sup>2</sup>  | 9.0 ± 1.4<sup>2</sup>  | 34.4 ± 6.2<sup>2</sup>  |
| GPY88 (\text{crh12}\Delta)<sup>c</sup> | 49.6 ± 4.2<sup>2</sup>  | 20.5 ± 0.7<sup>2</sup>  | 13.7 ± 0.6<sup>2</sup>  | 11.3 ± 2.9<sup>2</sup>  | 75.7 ± 23.7<sup>2</sup>  |
| GPY102 (\text{utr2}\Delta /\text{crh11}\Delta /\text{crh12}\Delta)<sup>c</sup> | 26.6 ± 1.8<sup>2</sup>  | 12.0 ± 2.3<sup>2</sup>  | 12.0 ± 2.3<sup>2</sup>  | 10.0 ± 1.4<sup>2</sup>  | 38.6 ± 8.8<sup>2</sup>  |

<sup>a</sup>Values are expressed as % of the initial \( A_{600}\) and are means (± S.E.) of three independent experiments.

<sup>b</sup>Regeneration of protoplasts was assayed after Zymolyase treatment for 1 h. See other details under “Experimental Procedures.” Values (%) are means (± S.E.) of three independent experiments.

<sup>c</sup>Revertant strains containing the wild-type gene (GPY07, GPY89, and GPY97) were not distinguishable from CAF2-1 in these assays.

<sup>d</sup>Values are statistically different \((p < 0.01)\) from those obtained with mutants GPY03, GPY80, GPY88, and GPY102.

<sup>e</sup>Revertant strains containing the wild-type gene (GPY07, GPY89, and GPY97) were not distinguishable from CAF2-1 in these assays.

<sup>f</sup>Values are statistically different \((p < 0.01)\) from those obtained with the wild-type CAF2-1, GPY03, and GPY88.
The role of Utr2p, Crh11p, and Crh12p in cell wall regeneration was addressed by incubation of protoplasts in YEPD containing 1.2 M sorbitol to allow cell wall regeneration. In this process, only Crh11p played an important role. Deletion of CRH11 resulted in a decreased ability to regenerate protoplasts as compared with the wild type (Table 2). Interestingly, this is consistent with recent transcript profiling data performed on C. albicans microarrays, which show a strong up-regulation of CRH11 but not of UTR2 or CRH12 in regenerating protoplasts (38). In the triple mutant utr2Δ/crh11Δ/crh12Δ, the ability to regenerate protoplasts was not decreased further in comparison to crh11Δ (Table 2). We conclude that CRH11 is important for cell wall regeneration in C. albicans, whereas UTR2 and CRH12 play only a modest or negligible role in this process.

**Cell Wall Alterations in Mutant Strains Lacking UTR2, CRH11, and CRH12**—Because the CRH family is putatively involved in cell wall construction, alterations in cell wall composition could be expected (14, 18). Possible differences in cell wall composition of the constructed mutants were examined by measuring the relative amounts of different cell wall components, in particular mannann, β-glucan, chitin, and cell wall proteins. Comparison of the three single gene deletants and the triple mutant utr2Δ/crh11Δ/crh12Δ with wild type indicated no statistically significant differences in cell wall protein and total glucan content (Fig. 4). However, the relative amount of mannann in the triple mutant utr2Δ/crh11Δ/crh12Δ was reduced (0.66 ± 0.02, p < 0.05), and this was accompanied by an increase in the amount of chitin (Fig. 4, 1.72 ± 0.1, p < 0.005). An increased cell wall chitin content is generally considered as a marker for increased activation of the Mck1 MAP kinase (or cell wall integrity) pathway in C. albicans in response to cell wall stress, confirming the importance of UTR2, CRH11, and CRH12 for the maintenance of cell wall integrity. Importantly, although no differences in the total cell wall glucan content were observed, separate analyses of 1,3-β-glucan and 1,6-β-glucan revealed a significant reduction in the amount of 1,3-β-glucan in the alkali-insoluble cell wall fraction of the utr2Δ (0.73 ± 0.05) and utr2Δ/crh11Δ/crh12Δ (0.7 ± 0.08) mutants (Fig. 4). This fraction was not significantly altered in crh11Δ (1.2 ± 0.07), crh12Δ (1.05 ± 0.1), and crh11Δ/crh12Δ (1.17 ± 0.15, not shown in Fig. 4). β-Glucan insolubility in alkali is because of its binding to chitin. Therefore, deviations from the normal concentration of alkali-insoluble 1,3-β-glucan may reflect either an altered proportion of glucan and chitin in the cell wall or an abnormal degree of cross-linking between these polymers. Because a reduction of alkali-resistant 1,3-β-glucan was measured in strains lacking UTR2, concomitant with increased chitin and normal glucan levels, this suggests that Utr2p could be involved in cross-linking of glucans and chitin.

Consistent with the unchanged cell wall protein content, a comparative inventory of covalently bound cell wall proteins in the utr2Δ/crh11Δ/crh12Δ mutant and various wild type strains revealed similar sets of proteins with only a few minor alterations (see supplemental Table III). As a confirmation of their gene deletions, Crh11p and Utr2p are absent in the triple mutant. In turn, the mutant contained two proteins, Sod5p and Pga3p1p, that are normally not found in mass spectra of wild type walls under these conditions. It seems likely that constitutive activation of cell wall integrity signaling pathway(s) (see below) has led to increased expression and incorporation of these two cell wall proteins. However, it should be noted that the mass spectrometric approach used here, despite being very sensitive and allowing some comparative analysis, does not provide solid quantitative data about individual protein concentrations, which requires a more extensive analysis.

**Deletion of UTR2, CRH11, and CRH12 Leads to Activation of the Cell Integrity Map Kinase Pathways**—The increased susceptibility of utr2Δ/crh11Δ/crh12Δ to cell wall-perturbing agents and the alterations in cell wall composition led us to investigate the activation of the cell integrity pathway in this mutant. Cell wall alterations sensed by the cell integrity pathway result in phosphorylation of the MAP kinase Mck1p through Pck1p activation (29, 39). Mck1p phosphorylation of different mutants was analyzed by immunodetection in cell extracts. Cells were incubated in the absence or presence of CR, whose cell wall-perturbing effect is known to induce Mck1p phosphorylation. As shown in Fig. 5A, simultaneous deletion of UTR2, CRH11, and CRH12 resulted in an increased phosphorylation of Mck1p as compared with the wild type, thus confirming that GPY102 has a weakened cell wall. Of the single gene deletants, only deletion of UTR2 led to Mck1p activation in the absence of CR. In crh11Δ and crh12Δ strains, Mck1p was activated only in CR-exposed conditions, like wild type (Fig.

**FIGURE 4.** Cell wall composition of C. albicans strains lacking UTR2, CRH11, and CRH12. Relative amount of protein, mannann, and chitin (left diagram), and total glucan- and alkali-resistant fractions of 1,3-β-glucan and 1,6-β-glucan (right diagram) of GPY03 (utr2Δ), GPY80 (crh11Δ), GPY88 (crh12Δ), and GPY102 (utr2Δ/crh11Δ/crh12Δ) in comparison to wild type strain CAF2-1. Amounts of cell wall components were determined as described under “Experimental Procedures.” Values obtained for CAF2-1, expressed as percentage of cell wall dry weight, were as follows: protein, 3.3 ± 0.3%; mannann, 23.1 ± 4.6%; chitin, 3.5 ± 0.3%; and total glucan, 74 ± 0.5% (this includes 26.2 ± 1.1% alkali-insoluble 1,3-β-glucan and 10.6 ± 0.8% alkali-insoluble 1,6-β-glucan). Each column represents the relative amount of a cell wall component in mutants strains in comparison to the wild type. Significant differences (t test) as compared with wild type are indicated by an asterisk next to the corresponding column.
5B). Consistent with these data, reintegation of a single UTR2 copy in the triple mutant utr2Δ/chr11Δ/chr12Δ restored the wild type phenotype, because Mkc1p activation was only observed in the presence of CR (Fig. 5C). Reintegration of CRH11 and CRH12 did not restore the wild type phenotype as Mkc1p activation also occurred in the absence of CR.

Using the anti-phospho-p44/42 MAP kinase (Thr202/Tyr204) antibody, an additional band corresponding to the phosphorylated form of the MAP kinase Cek1p, which is usually observed along with Mkc1p, was also detected (Fig. 5). Different studies have demonstrated that Cek1p and Mkc1p are co-activated along with Mkc1p, was also detected (Fig. 5). Different studies have demonstrated that Cek1p and Mkc1p are co-activated under the same experimental conditions, suggesting that Cek1p may act in an alternative cell integrity pathway (29, 40). In this context, cell wall defects may induce the activation of cell integrity pathways via Mkc1p and Cek1p. Taken together, we conclude that the CRH family, and UTR2 in particular, has a major impact on cell wall integrity MAP kinase signaling.

**Utr2p Localization during Yeast, Pseudohyphal and Hyphal Growth**—The above-mentioned results above suggest that Utr2p could be involved in creating the linkage between 1,3-β-glucan and chitin. Therefore, Utr2p is expected to be localized near regions where this assembly step is needed. To address Utr2p localization, a Utr2p-GFP fusion was constructed and expressed in GPY03 (utr2Δ). Utr2p-GFP and chitin localization were monitored during yeast, pseudohyphal and hyphal growth. As shown in Fig. 6 (A–C), Utr2p-GFP was clearly present at the cell surface under all tested growth conditions, but there were some differences in subcellular and temporal localization between the yeast, pseudohyphal and hyphal forms. In the yeast form, concomitant with bud emergence at early growth phase, Utr2p-GFP strongly accumulated around the presumptive bud site of pre-budding cells and at the new bud surface (Fig. 6A, arrows in 20- and 60-min incubation). As the bud grew, Utr2p-GFP appeared as a ring at the base of the bud neck (Fig. 6A, 120-min incubation). Observation of the same cells stained with CFW, which binds chitin polymers, indicated that Utr2p-GFP and chitin partially overlapped. Early in the cell cycle, chitin is located differently as CFW stained the neck.
structures rather than pre-budding sites and the new bud surface (Fig. 6A, arrows in 20- and 60-min incubation). Later, during bud growth, the staining of the chitin ring at the bud neck coincides with Utr2p-GFP localization (Fig. 6A, 120-min incubation). Concurrent with mother/daughter cell separation, GFP is still detected in the daughter cell but shortly thereafter decreased in intensity indicating breakdown or dilution of Utr2p in the cell wall (Fig. 6A, 180-min incubation).

A similar pattern of neck/ring localization was observed for cells grown in the pseudohyphal form (Fig. 6B). Pseudohyphae are described as chains of more or less elongated yeast cells joined end-to-end. Although the degree of elongation of pseudohyphal cells can vary considerably, they always display constrictions at their septa between individual cellular compartments (41, 42). During early pseudohyphal growth, Utr2p-GFP was detected in the area where new buds emerge (here weak CFW staining is observed) but also in the neck area between mature cells (Fig. 6B, arrows in 30-min incubation). However, during pseudohyphal elongation, GFP was detected essentially at the new ring area concomitant with prominent CFW staining (Fig. 6B, 60- and 90-min incubation). Finally, the pattern of Utr2p localization in the hyphal growth form differed from that observed in budding or pseudohyphal cells (Fig. 6C).

During hyphal elongation, Utr2p-GFP was detected at the tip of the germ tube (Fig. 6C, yellow arrowheads) as well as in a ring structure in the germ tube (arrows in 120-min incubation). The Utr2p-GFP in the ring co-localized with chitin (CFW) and represents the site of the septum in germ tubes. Chitin staining was less strong at the tip. Nevertheless, it is known that the incorporation of N-acetylglucosamine in the alkali-insoluble fraction of the cell wall, which is regarded as a measure for chitin synthesis, mainly takes place preferentially in the apical region (43). In conclusion, these results suggest that overall the pattern of Utr2p localization corresponds largely with the regions of chitin deposition. However, our data also indicate that Utr2p can adopt a transient localization that is dependent on the growth phase and that is not necessarily associated with chitin-rich regions of the cell wall. Utr2p localization was also analyzed during cell growth in the presence of FK506, a calcineurin inhibitor. Utr2p localization was not altered by calcineurin inactivation, suggesting that this protein was not able to control Utr2p localization during yeast, pseudohyphal and hyphal growth (data not shown).

Virulence of utr2Δ, crh11Δ, and crh12Δ Mutants in a Murine Model of Infection—Calcineurin is essential for virulence of C. albicans in the murine model of systemic infection (44–46). As UTR2 expression is strongly regulated by calcineurin and the cell wall is the first point of contact between fungus and host, the cell wall can be expected to play a crucial role in the infection process of C. albicans. To evaluate the possible implication of UTR2, CRH11, and CRH12 in virulence, we tested single deletion mutants, a utr2Δ/crh11Δ/crh12Δ triple mutant, as well as UTR2, CRH11, and CRH12 reintegrants in the background of the triple mutant. Wild type (MKY378) and avirulent calcineurin mutant (MKY379) strains were used as positive and negative controls in this assay (13). Ura– auxotrophy was complemented in these strains with the linearized URA3-containing Clp10 plasmid to favor integration into the genomic RPS10 locus. This procedure avoids positional effects of URA3 that can interfere with virulence as demonstrated by Brand et al. (33). As shown in Fig. 7A, all mice infected with wild type C. albicans died within 7 days of experimentation, whereas the mice infected with the calcineurin mutant survived during the entire experiment. In the group of mice infected with the utr2Δ/crh11Δ/crh12Δ mutant, no animal deaths were recorded during the entire surveillance period. In contrast, reintroduction of either UTR2, CRH11, or CRH12 led to increased virulence of C. albicans strains. However, only in the group of mice infected with the UTR2 re reintegrant, all mice died within 7 days, similar to wild type. Of the mice infected with the CRH11 and CRH12 reintegrants, 75 and 50%, respectively, of the mice were still viable after 30 days of post-infection. These data confirmed the role of the CRH family in C. albicans virulence and suggested that Utr2p is a major factor involved in the infection process. Consistent with these observations, single deletion mutants lacking UTR2, CRH11, and CRH12 showed different behavior in the murine model of infection (Fig. 7B). Mice infected with crh11Δ and crh12Δ died within 10 days post-infection, whereas 70% of the mice infected with utr2Δ survived during the entire surveillance period (Fig. 7B). This observation is in partial agreement with results obtained by Alberti-Segui et al. (18), who reported that 20% of mice infected with a utr2Δ mutant survived 10 days after infection. Several factors such as differences in strain background, URA3 positioning effect, and injected cell dose could explain this discrepancy. At day 3 post-infection, both kidneys and spleen from five animals per group were removed, and the tissue burdens of C. albicans were deter-
mined. Consistent with the proportion of mice that died, no significant differences were detected in the number of organisms recovered from kidneys and the spleens from animals infected with the wild type and with the UTR2 reintegrant (−4.5 log CFU g⁻¹ for kidney and 3 log CFU g⁻¹ for spleen). Surprisingly, tissues from kidneys and spleen of mice infected with the triple mutant contained similar CFU counts as compared with tissues from mice infected with the wild type (Table 3). Thus, although the triple mutant, like the calcineurin mutant, was not able to kill mice, it was still able to invade organs to a similar degree as wild type cells. The reasons for these differences were not analyzed in this study, but future work will be performed to elucidate this interesting behavior.

**DISCUSSION**

The Role of UTR2, CRH11, and CRH12 in Cell Wall Integrity and Synthesis—In this study we analyzed the possible role of the CRH gene family in cell wall organization. A previous study showed that UTR2 is a putative GPI protein with homology to CRH11 (18). In the C. albicans genome we found a third homologue, called CRH12. All three proteins have characteristics that are typical of GPI proteins. Furthermore, they all possess a putative catalytic domain shared by GH16 glycoside hydrolases (Fig. 1). In addition, Utr2p has a small putative carbohydrate-binding module belonging to CBM18, which in some cases has been shown to be involved in chitin binding (47).

Deletion of the three genes of this family resulted in an additive susceptibility to compounds interfering with the cell wall (Fig. 2), and mutant strains were also more susceptible to Zymolase, which is able to digest the 1,3-β-glucan network of the cell wall (Table 2). However, only deletion of CRH11 resulted in a decreased ability to regenerate protoplasts (Table 2), thus attributing a unique function to CRH11. Interestingly, CRH11 is up-regulated during cell wall regeneration in protoplasts (38), a feature also observed for its closest homologue CRH1 in S. cerevisiae (48). Previously, both proteins, as well as Crh2p of S. cerevisiae (4), have been shown to be covalently bound to the cell wall network in a GPI-dependent manner, and also Utr2p of C. albicans is covalently bound to the cell wall (supplemental Table III). This indicates the importance of these genes in cell wall construction. Our data therefore suggest that although Utr2p, Crh11p, and Crh12p share common functional domains, they might exert specific roles at different developmental stages of the cell wall. The participation of Utr2p, Crh11p, and Crh12p in cell wall biogenesis was investigated by more detailed cell wall analyses of mutant strains. Interestingly, 1,3-β-glucan levels detected in the alkali-resistant fraction of mutant strains lacking UTR2 were reduced as compared with the wild type. Glucan insolubility in alkali is because of its linkage to chitin. Because the total glucan and chitin levels in the utr2Δ mutant were not different from the wild type, a decrease of alkali-insoluble 1,3-β-glucan suggests a role for Utr2p in the cross-linking of 1,3-β-glucan and chitin. The triple deletion mutant utr2Δ/crh11Δ/crh12Δ, albeit in decreased amounts compared with wild type, still contains alkali-resistant 1,3-β-glucan. The applied conditions for alkali extractions may therefore have been insufficient to remove all glucan not bound to chitin. Alternatively, it cannot be excluded that additional enzymes are involved in the cross-linking of chitin to glucan. For instance, the (trans)glucosidase Bgl2p, belonging to glyco-side hydrolase family 17, is a homologue of cell wall proteins in both S. cerevisiae and C. albicans and was shown in a pulldown assay to bind to insoluble forms of chitin and glucan as well as to water-soluble glucan and chitosan (49).

We did not find a significant decrease in the amount of alkali-insoluble 1,6-β-glucan in mutants lacking UTR2. However, from these data we cannot conclude that Utr2p is less important for linking chitin and 1,6-β-glucan. In S. cerevisiae, mutants with a defective cell wall such as gas1Δ and fks1Δ showed constitutive activation of the cell integrity MAP kinase pathway, resulting in increased chitin levels and increased coupling of 1,6-β-glucosylated cell wall proteins to chitin (50). Therefore, a slight defect in the formation of the linkage between chitin and 1,6-β-glucan may be compensated by constitutive activation of Mck1p, the MAP kinase of the cell integrity signaling pathway, which has been observed in the utr2Δ mutants in our study.

Consistent with our hypothesis that the CRH family is important for connecting glucan and chitin, the fission yeast Schizosaccharomyces pombe lacks chitin in its wall (51, 52) and also lacks homologues of the CRH family. Moreover, two homologues of the CRH family in Y. lipolytica were shown to have specific endo-1,3-β-glucosidase activity in vitro (17). The putative catalytic glycoside hydrolase domain present in Utr2p (but also in Crh11p and Crh12p) is therefore likely to function in a trans-glycosidase reaction between polymers mainly present in the cell wall. The presence of a predicted chitin-binding module in Utr2p (but not in Crh11p and Crh12p; Fig. 1) further supports this hypothesis. The cell wall composition in the utr2Δ/crh11Δ/crh12Δ mutant also appeared to be altered in other aspects. Proteomic analyses were carried out in this mutant and revealed that Sod5p and Fga31p, which are normally not present in cell walls of wild type yeast cells growing under normal conditions (4, 53),

**TABLE 3**

Tissue burdens in spleen and kidneys of infected animals

| Strain          | Day 3 post-infection | Day 9 post-infection | Day 21 post-infection |
|-----------------|----------------------|----------------------|-----------------------|
|                 | Spleen Right kidney Left kidney | Spleen Right kidney Left kidney | Spleen Right kidney Left kidney |
| MKY378 (wild type) | 3.4 ± 0.3 4.5 ± 0.4 4.3 ± 1.1 | ND 5.6 ± 0.3 5.7 ± 0.1 | NA NA NA |
| MKY379 (cnaΔ)   | 3.1 ± 0.2 2.8 ± 0.2 2.7 ± 0.2 | ND ND ND | ND ND ND |
| GPY130 (utr2Δ/crh11Δ/crh12Δ) | 2.8 ± 0.3 4.4 ± 0.2 4.5 ± 0.2 | ND 5.9 ± 0.3 5.8 ± 0.2 | NA NA NA |
| GPY132 (utr2Δ/crh11Δ/crh12Δ/utr2Δ::UTR2) | 3.0 ± 0.5 4.0 ± 0.3 4.0 ± 0.4 | ND ND ND | ND ND ND |
were present in cell walls of the triple deletion mutant (see supplemental Table III). Furthermore, the chitin content in the cell wall was increased almost 2-fold as compared with wild type. Increased chitin levels are generally associated with cell wall stress; therefore, these data suggest an important role for Uttr2p in maintaining cell wall integrity. The role of the CRH family in cell wall integrity was confirmed by constitutive activation of Mkc1p in the triple deletion mutant. Mkc1p has been shown previously to be activated in different cell wall stress conditions to ensure cell wall integrity (29).

**Uttr2p and Chitin Accumulation in Septal Areas**—It would seem logical that Uttr2p localization is concentrated at sites where its activity is needed. Assuming a role in the linkage of 1,3-beta-glucan to chitin, Uttr2p would be expected to localize, at least temporarily, near regions where this assembly takes place. CFW reveals the distribution of cell wall chitin, which in yeast cells is mainly concentrated in the ring at the bud neck and at the subsequent bud scar. Using a GFP fusion construct, we found that Uttr2p also localized in these cell surface compartments with high CFW staining. However, Uttr2p was dissociated from CFW-rich staining regions essentially in small buds (Fig. 6, A and B). Uttr2p localization is also spatially controlled (from pre-budding area to bud surface and later to the ring of the bud neck) depending on cell cycle progression. Cell wall construction during the cell cycle is a dynamic process. Thus the temporarily and spatially controlled localization of Uttr2p seems to reflect the requirement of this protein at different cell wall compartments during growth. Co-localization with of Uttr2p with chitin-rich areas was observed also in pseudohyphae and hyphae (Fig. 6, B and C). On the basis of the localization of Uttr2p-GFP, it is possible that this protein could be involved in glucan-chitin assembly first at the budding site and later during formation of the septum between the mother and daughter cells. This hypothesis is consistent with localization of Uttr2p-GFP in pseudohyphae where Uttr2p-GFP is mainly localized to the septa between two cells (Fig. 6B). Finally, in hyphal cells Uttr2p-GFP also co-localized with chitin at ring structures in germ tubes (Fig. 6C). Taken together, these data strongly support the hypothesis that Uttr2p could be an important protein in the linkage of chitin to 1,3-beta-glucans.

Besides septal areas, Uttr2p-GFP is also persistently localized at the hyphal tip during hyphal formation and elongation (Fig. 6C). Importantly, it has been shown that N-acetylglucosamine, the precursor of chitin, is preferentially incorporated at the hyphal tip, suggesting that Uttr2p might be involved in interconnecting chitin and 1,3-beta-glucan at that location as well (43). This localization also coincides with actin, which is concentrated as patches at sites of polarized growth and at the position of developing septa (54, 55). In fact, in the yeast cell cycle, actin patches accumulate at the bud site although in hyphal formation similar actin patches were also seen at the apex of the germ tube (54, 55). The co-localization of actin patches and Uttr2p in hyphae suggests that Uttr2p is localized at polarized growth sites. This is consistent with the subcellular distribution of its closest homologue Crh2p/Uttr2p in *S. cerevisiae* (15), which is also reported to be localized at polarized growth sites.

**UTR2 and CRH11 Are Regulated by Calcineurin**—In this study we have investigated the possible involvement of the CRH family in cell wall construction and integrity. Phenotypic analyses of calcineurin mutants and previous studies on gene regulation point to a strong relationship between calcineurin and cell wall formation (44, 56). Uttr2p is a target of calcineurin when *C. albicans* is exposed to calcium. However, gene expression analysis undertaken here has shown that regulation of *UTR2*, *CRH11*, and *CRH12* is not identical. CRH12 expression was not detected, neither in wild type nor in the *cnaA* mutant under any of the tested conditions, indicating that this gene probably has a minor contribution in cell wall organization as compared with *UTR2* and *CRH11*. However, *CRH12* is important in the response of *C. albicans* to CR (Fig. 2), emphasizing that this gene is expressed under specific growth conditions. These questions need to be addressed in the future.

In contrast to *CRH12*, the transcript levels of *UTR2* and *CRH11* were regulated by calcineurin under calcium-exposed conditions (Fig. 3). Under these conditions, *UTR2* is one of the most strongly calcineurin-regulated genes (13). The relationship between calcineurin, *UTR2*, and *CRH11* was confirmed by analysis of a *utr2Δ/crh11Δ* double mutant, which like *cnaΔ* was not able to grow in the presence of CR, SDS, and calcium. Taken together, our data indicate that *CRH11* and in particular *UTR2* are strongly regulated by calcineurin. Nevertheless, it is known that the calcineurin pathway controls a large number of genes in *C. albicans* (13, 44, 56). Therefore, deletion of a single gene family coding for cell wall proteins cannot account for all calcineurin-documented phenotypes. Different phenotypes between the calcineurin mutant and a triple deletion mutant *utr2Δ/crh11Δ/crh12Δ* were indeed observed in a murine model of systemic infection. In this model, the triple deletion mutant was completely avirulent, like a calcineurin mutant, suggesting an active role of the *CRH* gene family in *C. albicans* virulence. However, in contrast to the calcineurin mutant, the *utr2Δ/crh11Δ/crh12Δ* mutant was still able to invade organs of the infected mice. These results suggest that the *utr2Δ/crh11Δ/crh12Δ* mutant had no defect in organ invasion but also indicated that host-pathogen interactions were altered. Interestingly, it has been shown that deletion of *CaOCH1*, a gene coding for an 1,6-alpha-mannosyltransferase involved in outer chain elongation of *N*-linked carbohydrate side chains of mannoproteins (40), resulted in a virulence phenotype similar to that observed for the *utr2Δ/crh11Δ/crh12Δ* mutant, which also has a decreased mannan level in its cell wall compared with wild type. To explain the behavior of the *och1Δ* mutant in the murine model of infection, it was proposed that alteration of cell wall surface epitopes derived from the lack of outer chain mannosylation can alter recognition of the fungus by cells of the immune system (40). A recent study confirmed that *N*-linked mannoproteins can be recognized by the mannan receptor of murine macrophages (57). Other cell wall constituents (*O*-mannans and *β*-glucans) are recognized by other macrophage receptors. Moreover, a separate study performed with *S. cerevisiae* mutants showed that altered cell wall composition can expose to the cell surface some cell wall constituents (*β*-glucans) that are otherwise embedded in cell wall structures.
Cell Wall Glycosidases of C. albicans

(58). Because β-glucans are potent stimulators of the immune system (57), their exposure to the cell surface and the resulting immune recognition may have a profound effect on the clearance of pathogens with altered cell wall structures. Deletion of UTR2, CRH11, and CRH12 may result in alterations of chitin or β-glucan chains that are present in the inner (rather than the outer) layer of cell wall. Such alterations may have subtle consequences in the presentation of cell wall structures to the host immune system, thus leading to the enhanced survival of both the host and the pathogen in the murine model of infection.

Even though this work establishes the CRH gene family as a calcineurin target, the deletion of UTR2, CRH11, CRH12 cannot explain all the phenotypes obtained in calcineurin mutant. It is known that calcineurin is able to regulate genes by activation of Crz1p and other proteins by dephosphorylation (13). Future work will be undertaken in our laboratory to investigate these additional calcineurin targets.

Acknowledgments—We thank Henk Dekker, Qing Yuan Yin, Martijn Bekker, and Johnny Hendriks (University of Amsterdam) for technical assistance with mass spectrometry, HPLC analysis, and homology modeling.

REFERENCES

1. Klis, F. M., Mol, P., Hellingwerf, K., and Brul, S. (2002) FEMS Microbiol. Rev. 26, 239–256
2. Sundstrom, P. (2002) Cell. Microbiol. 4, 461–469
3. Chauhan, N., Li, D., Singh, P., Calderone, R., and Kruppa, M. (2002) in Candida and Candidiasis (Calderone, R. A., ed.) pp. 159–175, American Society for Microbiology, Washington, D.C.
4. De Groot, P. W. J., De Boer, A. D., Cunningham, J., Dekker, H. L., De Jong, H., Lellingwerf, K. J., De Koster, C., and Klis, F. M. (2004) Eukaryot. Cell 3, 955–965
5. Lipke, P. N., and Ovalle, R. (1998) J. Bacteriol. 180, 3735–3740
6. Mouyna, I., Fontaine, T., Vai, M., Monod, M., Fonzi, W. A., Diaquin, M., Popolo, L., Hartland, R. P., and Latge, J. P. (2000) J. Biol. Chem. 275, 14882–14889
7. Martinez-Lopez, R., Monteloiva, L., Diez-Orejas, R., Nombela, C., and Gil, C. (2004) Microbiology 150, 3341–3354
8. Hoyer, L. L., Fundiga, R., Hecht, J. E., Kapteyn, J. C., Klis, F. M., and Arnold, J. (2001) Genetics 157, 1555–1567
9. Hoyer, L. L. (2001) Trends Microb. 9, 176–180
10. Hoyer, L. L., Payne, T. L., Bell, M., Myers, A. M., and Scherer, S. (1998) Curr. Genet. 33, 451–459
11. Sundstrom, P., Cutler, J. E., and Staab, J. F. (2002) Infect. Immun. 70, 3281–3283
12. De Groot, P. W. J., Hellingwerf, K. J., and Klis, F. M. (2003) Yeast 20, 781–796
13. Karabaca, M., Valentino, E., Pardini, G., Coste, A. T., Bille, J., and Sanglard, D. (2006) Mol. Microbiol. 9, 1429–1451
14. Rodríguez-Pena, J. M., Cid, V. J., Arroyo, J., and Nombela, C. (2000) Mol. Cell. Biol. 20, 3245–3255
15. Rodríguez-Pena, J. M., Rodriguez, C., Alvarez, A., Nombela, C., and Arroyo, J. (2002) J. Cell Sci. 115, 2549–2558
16. Yin, Q. Y., De Groot, P. W. J., Dekker, H. L., De Jong, L., Klis, F. M., and De Koster, C. G. (2005) J. Biol. Chem. 280, 20894–20901
17. Hwang, J. S., Seo, D. H., and Kim, J. Y. (2006) Yeast 23, 803–812
18. Alberti-Segui, C., Morales, A. J., Xing, H., Kessler, M. M., Willins, D. A., Weinstock, K. G., Gottrel, G., Fettler, K., and Rogers, B. (2004) Yeast 21, 285–302
19. Hanahan, D. (1985) in DNA Cloning A Practical Approach (Glover, D. M., ed) pp. 109–135, IRL Press at Oxford University Press, Oxford.
55. Hazan, I., and Liu, H. (2002) *Eukaryot. Cell* **1**, 856–864
56. Cruz, M. C., Goldstein, A. L., Blankenship, J. R., Del Poeta, M., Davis, D., Cardenas, M. E., Perfect, J. R., McCusker, J. H., and Heitman, J. (2002) *EMBO J.* **21**, 546–559
57. Netea, M. G., Gow, N. A., Munro, C. A., Bates, S., Collins, C., Ferwerda, G., Hobson, R. P., Bertram, G., Hughes, H. B., Jansen, T., Jacobs, L., Buurman, E. T., Gijzen, K., Williams, D. L., Torensma, R., Maccallum, D. M., Odds, F. C., Van der Meer, J. W., Brown, A. J., and Kullberg, B. J. (2006) *J. Clin. Investig.* **116**, 1642–1650
58. Wheeler, R. T., and Fink, G. R. (2006) *Plos Pathog.* **2**, e35
59. Navarro-Garcia, F., Alonso-Monge, R., Rico, H., Pla, J., Sentandreu, R., and Nombela, C. (1998) *Microbiology* **144**, 411–424