Kex1 Protease Is Involved in Yeast Cell Death Induced by Defective N-Glycosylation, Acetic Acid, and Chronological Aging*\(^{1,2}\)

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N-Glycosylation in the endoplasmic reticulum is an essential protein modification and highly conserved in evolution from yeast to humans. The key step of this pathway is the transfer of the lipid–linked core oligosaccharide to the nascent polypeptide chain, catalyzed by the oligosaccharyltransferase complex. Temperature-sensitive oligosaccharyltransferase mutants of Saccharomyces cerevisiae at the restrictive temperature, such as \(wbp1^{-1}\), as well as wild-type cells in the presence of the N-glycosylation inhibitor tunicamycin display typical apoptotic phenotypes like nuclear condensation, DNA fragmentation, phosphatidylserine translocation, caspase-like activity, and reactive oxygen species accumulation. Since deletion of the yeast metacaspase \(YCA1\) did not abrogate this death pathway, we postulated a different proteolytic process to be responsible. Here, we show that Kex1 protease is involved in the programmed cell death caused by defective N-glycosylation. Its disruption decreases caspase-like activity, production of reactive oxygen species, and fragmentation of mitochondria and, conversely, improves growth and survival of cells. Moreover, we demonstrate that Kex1 contributes also to the active cell death program induced by acetic acid stress or during chronological aging, suggesting that Kex1 plays a more general role in cellular suicide of yeast.

Apoptosis is a highly regulated suicide program crucial for the development and maintenance of higher eukaryotes, and its dysfunction is the cause of several diseases (1–7). For a long time, the opinion prevailed that apoptosis is a process characteristic only of multicellular organisms, but recent studies convincingly demonstrate that forms of programmed cell death with apoptotic-like phenotypes are also present in unicellular organisms such as bakers’ yeast (6, 8–13).

After the first discovery of apoptotic phenotypes in a \(cde48\) mutant (14), it was found that yeast undergoes apoptotic-like death after application of hydrogen peroxide (15), acetic acid (16, 17), salt or sugar stress (18–20), plant fungal peptides (21), exposure to killer toxins (22), valproic acid (23), or stress caused by defective N-glycosylation (24, 25).

Furthermore, orthologues of classical apoptotic regulators were identified and characterized in yeast, such as the HtrA2-like protein Nma111p (nuclear mediator of apoptosis) (26), apoptosis-inducing factor (Aif1p) (27), Ndi1p NADH dehydrogenase (yeast AMID) (28), mitochondrial fission factor Dnm1p (29), inhibitor of apoptosis protein (Bir1p) (30), Tat-D nuclease mediated in DNA fragmentation (31), Nuc1p endonuclease (32), and a caspase-like protein (Yca1/Mca1p) belonging to a new family of caspases, the so-called metacaspases (33).

In addition, physiological roles for yeast apoptosis are emerging during chronological aging, by providing a better regrowth of fitter, better-adapted cells (34) promoted through the release of nutrients (35). Similarly, regulated cell death has been shown to be essential during yeast development for the long term survival of the colony population in order to adapt to the environment, whereby ammonia acts as a signal for differentiation (36, 37).

We have recently shown that a defect in N-glycosylation exhibits typical apoptotic cellular phenotypes. N-Glycosylation is one of the most common types of eukaryotic protein modifications, and the pathway is highly conserved from yeast to humans (38). In humans, defects in N-glycosylation are the cause of congenital disorders of glycosylation, a new family of genetic diseases with a severe, multisystemic clinical picture (39). We demonstrated that yeast mutants with a defect in subunits of the oligosaccharyltransferase (OST),\(^{2}\) such as \(wbp1\) and \(ost2\), display morphological and biochemical features of apoptosis. OST2 from yeast is homologous to \(DADI\) (40), originally characterized in hamster cells as a “defender against apoptotic death” (41, 42). We observed nuclear condensation, DNA fragmentation, and externalization of phosphatidylserine. We also provided evidence for the production of reactive oxygen species (ROS) and a caspase-like activity, which could be diminished by heterologous expression of antiapoptotic human Bcl-2. Since deletion of the yeast metacaspase \(YCA1/MCA1\) did not seem to abrogate this activity, we postulated a different proteolytic

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\(^{2}\) The abbreviations used are: OST, oligosaccharyltransferase; AMC, 7-amino-4-methylcoumarin; DHR, dihydrorhodamine; DHE, dihydroethidium; D_2R, aspartate, rhodamine 110; FITC, fluorescein isothiocyanate; fnk, fluoromethylketone; GFP, green fluorescent protein; H_2DCFDA, 2′,7′ dichloro dihydrofluorofluorescein diacetate; PI, propidium iodide; ROS, reactive oxygen species; TM, tunicamycin; BisTris, 2-[bis(2-hydroxyethyl)amino]-2-(hydroxymethyl)propane-1,3-diol; mtGFP, mitochondrion-targeted green fluorescent protein construct.

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activity to be involved in cell death induced by a protein N-glycosylation defect (24).

Searching for a potential protease involved in this process, we came across Kex1. This protein has been characterized as a serine carboxypeptidase B-like protease, specific for basic amino acid residues, responsible for processing of prepro-α-factor (mating pheromone) as well as K1 and K2 killer toxin precursors (43, 44) while traversing the secretory pathway. Kex1 localizes to the Golgi apparatus and contains a membrane-spanning domain at the carboxyl-terminal side, whereas the large protease domain at the NH2 terminus is extended into the lumen of the secretory pathway (45).

In this study, we demonstrate that Kex1 protease is a new player in the yeast cell death cascade caused by defective N-glycosylation both in the glycosylation mutant wbp1-1 and in wild-type cells when exposed to the N-glycosylation inhibitor tunicamycin. Disruption of KEX1 diminishes caspase-like activity and ROS accumulation, retards the fragmentation of mitochondria, and leads to a better growth and survival of cells. Furthermore, we provide evidence that KEX1 is also involved in cell death provoked by acetic acid stress and during chronological aging.

**EXPERIMENTAL PROCEDURES**

**Yeast Strains, Media, and Genetic Methods**

The following strains were used: SS328 (MATα ade2-101 ura3-52 his3Δ200 lys2-801), SS328Δkex1 (MATα ade2-101 ura3-52 his3Δ200 lys2-801 Δkex1::kanMX), SS328Δyc1α (MATα ade2-101 ura3-52 his3Δ200 lys2-801 Δyc1α::kanMX), WCA (MATα his3-11,15 leu2-3,112 ura3), WCAΔpep4 (MATα his3-11,15 leu2-3,112 ura3 Δpep4::HIS3), WCAΔpep4Δkex1 (MATα his3-11,15 leu2-3,112 ura3 Δpep4::HIS3 Δkex1::kanMX), MA7-B (MATα ade2-101 ura3-52 his3Δ200 lys2-801 wpb1-1), MA7-BΔkex1 (MATα ade2-101 ura3-52 his3Δ200 lys2-801 wpb1-1 Δkex1::kanMX), MA7-BΔyc1α (MATα ade2-101 ura3-52 his3Δ200 lys2-801 wpb1-1 Δyc1α::kanMX), MA7-Δaif1 (MATα ade2-101 ura3-52 his3Δ200 lys2-801 wpb1-1 Δaif1::kanMX), MA7-Δaif1 Δkex1::kanMX, MA7-Δaif1 Δnma111 (MATα ade2-101 ura3-52 his3Δ200 lys2-801 wpb1-1 Δkex1::kanMX, MA7-Δnma111 (MATα ade2-101 ura3-52 his3Δ200 lys2-801 wpb1-1 Δnma111::kanMX). Strains were grown in YPD (1% yeast extract, 2% bacto-peptone, 2% glucose) or in selective media with 0.25 ml of YPD containing 0.2 mM D2R α-f antagonist for 200 min at 25 °C (16).

Induction of Programmed Cell Death by Temperature Shift and Treatment with Acetic Acid

For tagging genomic KEX1 with two ZZ-epitopes, a cassette was amplified by PCR with 5'-GGCTTCCGAAACACTGAA-TCGATGAATCTTTTGATGAGTGTGGCAAGCGCCGGTGC-3' and 5'-CCCAATATACATTGCAGCGCTGCGGACGCGCGGGTGC-3' and 5'-CCCTTTAAAGAATTTATCTTTTGATGAGTGTGGCAAGCGCCGGTGC-3' as primers and using plasmid pZZ-KAN as template. Transformants were selected on G418 sulfate medium, and the correct recombination at the chromosomal KEX1 locus was verified by PCR using the primers 5'-GTTAC-GCTGGCAATACATTACCC-3' and 5'-GAAATTTCTGGATTTTACGAAATATACATTGCAGCGCTGCGGACGCGCGGGTGC-3' and expression of the tagged protein by Western analysis. Mitochondria were visualized using a mitochondria-targeted green fluorescent protein in strains transformed with plasmid pVT100-mtGFP, as described previously (47).

Confluent Laser Microscopy and Flow Cytometry

For detection of caspase-like activity, 5×106 cells were harvested, washed in phosphate-buffered saline, and resuspended in 0.2 ml of staining solution containing 10 μM FITC-VAD-fmk in phosphate-buffered saline (CaspACE FITC-VAD-fmk in situ marker; Promega) or in 0.25 ml of YPD containing 0.2 mM D2R (25 mM stock solution in ethanol/dimethyl sulfoxide 2:1;
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Thermo Scientific). After incubation for 20 min at room temperature (for FITC-VAD-fmk) or 30 min at 30 °C (for D_{2}R) with low agitation in darkness, cells were centrifuged and washed twice with 1 ml of phosphate-buffered saline. Free intracellular radicals (ROS) were detected by dihydroethidium 123 (DHR123) and 2',7'-dichlorodihydro-fluoresceindiacetate (H_{2}DCFDA), which were added 1.5–2 h before harvesting the cells from a 2.5 mg ml^{-1} stock solution in ethanol to a final concentration of 5 μg ml^{-1}. Staining with dihydroethidium (DHE) was performed as described in Ref. 32. For staining with propidium iodide (PI), cells were treated as described for caspase staining. PI was added from a 50 μg ml^{-1} stock solution in water to a final concentration of 50 ng ml^{-1}.

For confocal laser microscopy, cells were concentrated by a short centrifugation step and immobilized by covering with 1% agarose. Analysis was performed using an LSM 510-META confocal laser-scanning microscope (Carl Zeiss, Jena, Germany).

For detection of FITC-VAD-fmk, D_{2}R, DHR123, H_{2}DCFDA, and GFP fluorescence signals, a 505–550-nm band pass emission filter was used with an excitation at 488 nm. To ascertain the presence of co-staining, probes were scanned sequentially.

Approximately 3 × 10^{8} cells were harvested at 4,000 g for 10 min at 4 °C, washed with 20 ml of 50 mM Tris-HCl (pH 7.5), and resuspended in buffer A containing 50 mM Tris-HCl (pH 7.5), 1 mM EDTA, 1 mM benzamidine, 1 mM MgCl_{2}, and 5% glycerol; per gram, wet weight, 1 ml of buffer was added. Cells were broken with glass beads using a Merckenschlager bead beater (Braun, Melsungen, Germany), and the cell lysate was filtered to remove beads. After centrifugation at 500 x g for 5 min at 4 °C, the supernatant was centrifuged at 48,000 x g for 30 min at 4 °C. The pellet was washed once and resuspended in buffer B containing 30 mM Tris-HCl (pH 7.5), 3 mM MgCl_{2}, 1 mM dithiothreitol, and 35% glycerol.

For flow cytometric analysis, cells were resuspended in 500 μl of phosphate-buffered saline, and 20,000 cells were probed. Analysis was achieved using the MoFlo (Cytomation) high speed sorter and Summit version 3.1 software. FITC-VAD-fmk, D_{2}R, DHR123, and H_{2}DCFDA fluorescence signals were determined using a 510–550-nm band pass filter; for PI and DHE, a 610–650-nm band pass filter was used (excitation 488 nm, argon laser).

Purification of KEX1-ZZ—For solubilization of KEX1-ZZ from the membrane fraction, 1.2 mg of protein in buffer B containing 0.5 M KCl and 1% octyl glucoside (added dropwise) were incubated in a final volume of 0.2 ml for 20 min on ice. The mixture was centrifuged at 150,000 x g for 40 min at 2 °C, and 0.1 ml of the supernatant was added to 50 μl of IgG-Sepharose equilibrated with 50 mM Tris-HCl (pH 7.5) and 150 mM NaCl. After incubation by rolling end over end for 30 min at 4 °C, the Sepharose beads were centrifuged at 300 x g for 3 min at 4 °C and washed three times with 25 mM Bis-Tris-HCl (pH 6.3).

Enzyme Tests—To determine Kex1-ΔZZ activity, 0.5 mM furylacryloyl-Ala-Arg-OH in methanol and 930 μl of 25 mM Bis-Tris-HCl (pH 6.3) were added to 50 μl of IgG-Sepharose beads to which Kex1-ΔZZ was adsorbed as prepared above and incubated for the times indicated. Beads were centrifuged at 13,000 x g for 15 s, and from the supernatant, the absorption at 340 nm was determined. To investigate a caspase-like activity, 40 μl Ac-VEID-AMC (2 μM stock solution in DMSO) was added to Kex1-ΔZZ-adsorbed IgG-Sepharose beads as described before. After incubation for 2 h by rolling end over end, emission of the supernatant was determined at a wavelength of 440 nm, applying an excitation of 370 nm. Measurements were accomplished in a Join Yvon-Spec Fluoromax-2 spectrofluorimeter with DataMax software.

In Vivo Labeling with [35S]Methionine/Cysteine

Yeast cells were grown in YPD to a cell density of 7 × 10^{6} ml^{-1} overnight at 25 °C and then shifted to 37 °C for the times indicated. Prior to labeling, cells were transferred for 30 min to YNB glucose medium, and subsequently cells were labeled for 30 min at 37 °C and further processed for isolation and PAGE analysis of carboxypeptidase CPY as described in Ref. 48.

RESULTS

Disruption of KEX1 Causes Better Growth and Survival of N-Glycosylation-defective Cells and Decreases Caspase-like Activity as Well as ROS Accumulation—Wbp1 is an essential subunit of the oligosaccharyltransferase complex, the key enzyme of protein N-glycosylation (49, 50). When the temperature-sensitive mutant wbp1-1 is shifted from the permissive temperature of 25 °C to a restrictive temperature, an underglycosylation of glycoproteins occurs, and cells start to die, thereby exhibiting typical markers of apoptosis (24). As shown in Fig. 1A, disruption of KEX1 restores the temperature growth defect of wbp1-1 at 31 °C. Similarly, a better survival of cells is observed. For this purpose, cells were shifted for different time periods to the restrictive temperature of 37 °C and subsequently separated with a cell sorter, and single cells were spotted on full medium plates. After 4 days at 25 °C, survival of cells was determined (Fig. 1B). The positive effect on survival by deletion of KEX1 can also be observed at restrictive temperatures lower than 37 °C (see supplemental Fig. 1). Deletion of KEX1 in wild-type cells has no effect on growth (Fig. 1A) or survival (data not shown), indicating that the N-glycosylation defect in wbp1 is the cause. A terminal, visible phenotype occurring in yeast programmed cell death is an alteration of the cell morphology, characterized by cell shrinkage. As shown in Fig. 1C, in wbp1-1Δkex1 cells lacking Kex1 protease, the

Test for Determination of Cell Survival

To determine the survival rate, cells were separated by the MoFlo cell sorter and spotted onto YPD plates for each measuring point. 250 single cells were plated, and the number of colonies was determined after 3–4 days of incubation at the indicated temperature.

Assay for KEX1 Activity

Membrane Preparation—Yeast cells were grown in minimal medium (0.67% yeast nitrogen base, 0.5% casamino acids, 20 mg ml^{-1} tryptophan, 20 mg ml^{-1} adenine, 30 mg ml^{-1} tyrosine, 2% glucose) overnight to a cell density of 2 × 10^{9} ml^{-1}. Approximately 3 × 10^{9} cells were harvested at 4,000 x g for 10 min at 4 °C, washed with 20 ml of 50 mM Tris-HCl (pH 7.5), and resuspended in buffer A containing 50 mM Tris-HCl (pH 7.5), 1 mM EDTA, 1 mM benzamidine, 1 mM MgCl_{2}, and 5% glycerol; per gram, wet weight, 1 ml of buffer was added. Cells were broken with glass beads using a Merckenschlager bead beater (Braun, Melsungen, Germany), and the cell lysate was filtered to remove beads. After centrifugation at 500 x g for 5 min at 4 °C, the supernatant was centrifuged at 48,000 x g for 30 min at 4 °C. The pellet was washed once and resuspended in buffer B containing 30 mM Tris-HCl (pH 7.5), 3 mM MgCl_{2}, 1 mM dithiothreitol, and 35% glycerol.
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FIGURE 1. Disruption of KEX1 suppresses temperature-sensitive growth (A), survival rate (B), and cell shrinkage (C) of wbp1-1 cells. A, in total, 3 μl of a serial 1:10 dilution of 10^6 cells ml^{-1} were spotted on YPD plates and incubated at 25 and 31 °C, respectively, for 4 days. B, wbp1-1, wbp1-1Δkex1, and wbp1-1Δyca1 strains were shifted for 2–12 h as indicated, to the restrictive temperature of 37 °C. Single cells were spotted with the MoFlo high speed cell sorter on YPD plates and incubated for 4 days at the permissive temperature of 25 °C. C, cell morphology was investigated after a t-shift of the cultures for 4 h to the nonpermissive temperature of 37 °C. Cells were analyzed by confocal microscopy. The arrows indicate examples of shrunken cells, the percentage of which in the population at 37 °C is indicated at the bottom. Bars, 10 μm. Data shown in B are the average of three independent experiments; the data depicted in A and C are representative of at least three independent experiments.

amount of shrunken cells was strongly reduced from 20 to 5%, in comparison with wbp1-1 cells upon a shift to 37 °C for 4 h.

We have recently demonstrated a caspase-like, proteolytic activity both by cell flow cytometry and cell-free extracts of N-glycosylation-stressed cells using typical metazoan caspase substrates (24). The enzyme activity was reduced in the presence of specific as well as broad spectrum pancaspase inhibitors, such as Z-VAD-fmk, or by the addition of the antioxidant N-acetyl-l-cysteine. So far the only caspase identified in yeast is Yca1p, belonging to the so-called metacaspase family (51), which was shown to have a central role in yeast apoptosis. On the other hand, there is increasing evidence for other apoptotic pathways that are, in contrast to those previously mentioned, caspase-independent, both in vertebrates and yeast (26, 27, 29, 34, 36, 52–54). Therefore, we asked whether Kex1 contributes to the proteolytic activity measured during cell death of wbp1-1 and how it compares with Yca1. As shown in Fig. 2A, deletion of Kex1 protease in wbp1-1 leads to a strong decrease of the amount of cells labeled with cell-permeable FITC-valyl-alanyl-aspartyl-(O-methyl)-fluoromethylketone (FITC-VAD-fmk), when analyzed by cell flow cytometry. Although 32 and 64%, respectively, of wbp1-1 cells showed positive staining after a temperature shift to 37 °C for 4 and 8 h, this amounted to only 10 and 25% in wbp1-1Δkex1 cells. In wild-type cells and wild-type Δkex1, no staining at 37 °C was detectable. The positive influence by KEX1 occurs already at lower restrictive temperatures than 37 °C (supplemental Fig. 1B). In contrast to KEX1, disruption of YCA1 in wbp1-1 had no effect on the caspase-like activity under the same conditions (Fig. 2A).

In cells cultured at the permissive temperature of 25 °C, hardly any activity could be measured. Similarly, disruption of YCA1 affects neither growth (Fig. 1A), survival (Fig. 1B), nor cell shrinkage (Fig. 1C). Co-staining with PI (Fig. 2B, upper lane) revealed that after 4 h at the restrictive temperature, already a significant portion of wbp1-1 cells (27%) stained for both FITC-VAD-fmk and PI, and only 5% remained positive exclusively for FITC-VAD-fmk, indicating that not all cells maintained plasma membrane integrity anymore. Since it has been reported that FITC-VAD-fmk may have limitations, because it may also stain nonspecifically dead cells (55, 56), we also tested the FITC chroomophore alone without the peptide moiety and observed that only cells positive for PI were stained with FITC (Fig. 2C). This indicates that at least in those 5% of cells that stain with FITC-VAD-fmk but exclude PI, staining is caspase-specific (Fig. 2, B and quantification column on the right). To verify this, we also used D₂R as an alternative substrate to FITC-VAD-fmk, which was reported to be a more specific caspase probe in yeast. Also with this compound, a similar amount of cells (i.e. 5%) was exclusively apoptotic, precluding PI (Fig. 2, D and E). We postulate that the fraction of cells that are positive for PI represents cells that became secondary necrotic or die by another mechanism. Since glycosylation in yeast is important for cell wall formation and cell integrity (57, 58), disturbance of this process, above all at higher temperature as applied in this experiment, may indeed contribute to the observed phenomenon (also see “Discussion”). Irrespective of the question of which kind of cell death program occurs caused by the glycosylation defect, we used for further experiments the FITC-VAD-fmk probe, in order to follow the overall progress in the death cascade.

One of the key mechanisms by which cells trigger programmed cell death is the production of ROS, which can be detected by incubation of yeast cells with DHR123 or DHE, which are oxidized by ROS to the fluorescent chromophore rhodamine 123 and ethidium, respectively (32, 59, 60). As shown by confocal microscopy (Fig. 3A) and quantification by flow cytometry, 33% of wbp1-1 and 37% of wbp1-1Δyca1 were ROS-positive after a 4-h shift to 37 °C when probed with DHR123 compared with 9% of wbp1-1Δkex1 cells, whereas control strains grown at 25 °C were unstained. A similar value was obtained with DHE (supplemental Fig. 2). No ROS was produced in wild-type or in wild-type Δkex1 at the elevated
temperature of 37 °C (Fig. 3B). Co-staining with PI revealed that about 6% of the ROS-producing cells were exclusively positive for DHR123 (Fig. 3C), in agreement with the results of Fig. 2B, demonstrating that only a fraction of cells is still in an apoptotic stage. Thus, DHR123 seems to also stain postapoptotic and necrotic cells, respectively. Since DHE does not allow costaining with PI because of a similar absorption/emission spectrum, this point could not be analyzed with this probe. As a third assay for the detection of ROS, H$_2$DCFDA was used. Also with this method, about 5% of cells displayed ROS after a 4-h shift to the restrictive temperature. Interestingly, and in contrast to DHR123, only viable cells (PI-negative cells) were stained with H$_2$DCFDA, indicating that this probe is more specific. The latter substrate needs, compared with DHR123, activation by intracellular esterases to be modified by ROS, which may be impaired in postapoptotic and necrotic cells, respectively. As shown in Fig. 3D, the addition of the antioxidant glutathione efficiently reduced the amount of ROS as well as caspase-like activity in $wbp1-1$ cells and also had a beneficial effect on the survival rate.

**Abnormal N-Glycosylation Induces Fragmentation of Mitochondria**—Fragmentation of the mitochondrial network is an early step in mammalian apoptosis, although its actual function remains unclear (61). It has also been reported that the mitochondrial fission machinery is involved in yeast programmed cell death (29). To address the role of mitochondria in the context of defective glycosylation, a mitochondrion-targeted green fluorescent protein construct (mtGFP) was expressed in $wbp1-1$ and $wbp1-1/H9004\text{Kex}1$ to visualize mitochondria by confocal microscopy. We observed that the normal tubular mitochondrial network became fragmented when $wbp1-1$ cells were shifted from 25 °C (Fig. 4A) to 37 °C for 4–8 h (Fig. 4, B–D). This process was abolished and retarded, respectively, in $wbp1-1/\Delta\text{Kex}1$ cells (Fig. 4, E–G), indicating that Kex1 is involved in the fragmentation process.

**Tunicamycin-induced Cell Death in Wild-type Yeast**—We have previously shown that the N-glycosylation inhibitor tunicamycin in combination with temperature stress provokes caspase-like activity also in wild-type cells, and thus this action...
FIGURE 3. Disruption of KEX1 leads to a decrease in ROS accumulation. A, for analysis of ROS-stained cells by confocal microscopy, cultures were shifted for 4 h to the restrictive temperature of 37 °C and incubated with DHR123; the percentage amount of ROS-positive cells was quantified by flow cytometry. B, ROS in wild type (SS328) and wild-type Δkex1 were analyzed after 8 h at 37 °C and analyzed by flow cytometry. C, ROS and membrane integrity was analyzed by co-staining of the cells with PI either with DHR123 or H2DCFDA. D, GSH was added to wbp1-1 cells at the time of shift to the restrictive temperature of 35 °C in the concentrations indicated, and its effect on ROS staining (DHR123), caspase-like activity (FITC-VAD-fmk), and cell survival was measured. The data shown are representative of at least three independent experiments. The white arrows in C indicate cells that exclude PI and are indicative of membrane integrity. Bars, 10 μm (A) and 5 μm (C). DIC, differential interference contrast.
is not only restricted to the glycosylation mutant (24). Tunamycin (TM) blocks the first step in the biosynthesis of the lipid-linked precursor and prevents N-glycosylation (62). To investigate this in more detail, we also analyzed the production of ROS, the survival rate of cells, and the influence of KEX1 disruption in dependence of TM. As depicted in Fig. 5A, wild-type cells in the presence of TM not only showed an increase of caspase-like activity (FITC-VAD-fmk) but also accumulated ROS (DHR123 staining). Disruption of KEX1, but not of YCA1 (data not shown), reduced caspase-like activity from 46 to 23% and ROS accumulation from 80 to 43%. Conversely, cell survival was improved from 60 to 80% in Δkex1 cells (Fig. 5D). We also observed that the antioxidant N-acetyl-L-cysteine diminished both caspase and ROS staining when added to the culture during treatment with TM (Fig. 5B). In Fig. 5C, it is shown that the TM-mediated caspase activity and ROS staining in wild-type cells was dependent on protein synthesis and required cellular metabolism, since in the presence of cycloheximide, their amount was reduced.

Cell Death of wbp1-1 Is Abrogated by Osmotic Stabilization Subject to the Growth Phase—As mentioned above, protein N-glycosylation is crucial for cell wall assembly and cell wall integrity, and defects can be suppressed by osmotic stabilizers (57, 58, 63). It has been shown for the temperature-sensitive ost2 mutant that 1 M sorbitol added to agar plates abolished the ts-phenotype of the mutant at 37 °C (25). A similar stabilizing effect is demonstrated in Fig. 6A for wbp1-1. Cells were cultivated in YPD medium at 37 °C with and without 1 M sorbitol for 26 and 50 h, respectively, and serial dilutions were spotted onto plates with and without 1 M sorbitol followed then by incubation at the permissive temperature of 25 °C for 4 days. Quite surprisingly, as depicted in Fig. 6B, the stabilizing effect of 1 M sorbitol was found to be dependent on the growth phase of the culture when shifted to the restrictive temperature of 37 °C. In cultures shifted at a very early growth phase (here $A_{600}$ of 0.15–0.18), sorbitol has no effect on caspase-like activity (FITC-VAD-fmk), ROS accumulation (DHR123), or PI staining. However, in cells from somewhat later stages, sorbitol compensates for the glycosylation defect and diminishes significantly the above mentioned, detrimental effects. This is in agreement with our earlier finding, where no influence of sorbitol on cell death of wbp1 was detected, because in these experiments cells of the early growth phase were used (24). Thus, sorbitol in late growth phase has a similar effect as deletion of KEX1. However, whether there is a direct link between both cannot be inferred.

KEX1 Does Not Affect N-Glycosylation—The primary defect of wbp1-1 is a reduced N-glycosylation of proteins (50). We asked whether deletion of KEX1 is able to suppress the glycosylation defect in wbp1-1 cells. We examined the glycosylation status of carboxypeptidase CPY in cells metabolically labeled with $[^{35}S]$methionine/cysteine. CPY is a well characterized model glycoprotein of yeast and contains four N-glycan chains. As shown in Fig. 7, compared with wild-type yeast, in wbp1-1 already at 25 °C a minor hypoglycosylation is observed that increases in a time-dependent manner upon shift of the cells to 37 °C. This is indicated by the appearance of bands with higher mobility lacking 1–4 N-linked chains. However, disruption of KEX1 did not abolish the underglycosylation defect. This means that KEX1 is not directly involved in stabilizing the mutant Wbp1 protein rather than having a function in the death cascade.

Expression of Kex1 Protease and Substrate Analysis—Because deletion of KEX1 caused an abrogation of cell death induced by the N-glycosylation defect, we asked whether, conversely, KEX1, when expressed from a plasmid in a wbp1-1Δkex1 strain, is able to suppress the Δkex1 deletion effect. Also, we wanted to know whether Kex1 is directly responsible for the caspase-like activity. We therefore introduced at the COOH terminus of KEX1 a protein A (ZZ) epitope, both for detection by Western blot analysis and purification, and cloned the construct into the constitutive multicopy expression vector pVT100. As shown in Fig. 8A, expression of KEX1 prevents growth of wbp1-1Δkex1 at 31 °C. We observed an even somewhat lower growth when compared with wbp1-1 cells transformed with only the vector (compare lanes 5 and 7), which may be due to the overexpression of Kex1, as demonstrated in the Western blot (Fig. 8B, lane 1), in which also an identical amount of protein extract was applied from cells expressing a chromosomally tagged version of KEX1-ZZ (lane 2). On the other hand, overexpression of KEX1 in wild-type yeast does not cause cell death per se (data not shown), indicating that Kex1 is probably a nonlimiting factor of the death pathway.

It was shown that Kex1 protease has a significant preference for COOH-terminal arginine and lysine residues (64). We used the synthetic substrate furylacryloyl-Ala-Arg and determined...
the enzyme activity in immunoprecipitates of Kex1 following product formation by the decrease of absorption at 340 nm (65). A 7-fold higher Kex1 activity was detected in cells overexpressing KEX1 (Fig. 8C, column 2) in contrast to cells containing only the chromosomal form of the protease (Fig. 8C, column 1). We also tested the ability of purified Kex1 to cleave Ac-VEID-AMC, which has recently been shown to be the best substrate in the fluorimetric caspase assay, using a cell-free extract from wbp1-1 cells as enzyme source (24). However, we found that Kex1 was not able to cleave Ac-VEID-AMC (Fig. 8C, columns 3 and 4).

Kex1 Protease Is Also Involved in Programmed Cell Death Induced by Acetic Acid Stress and Chronological Aging—Acetic acid stress has been shown to induce in yeast an active cell death program exhibiting typical apoptotic markers (16). More recently, it was reported that besides Yca1, also a noncaspase route is involved in this process (66). Hence, we asked whether Kex1 also plays a role in cell death challenged by acetic acid. For these experiments, cells were incubated for 200 min in rich medium adjusted to pH 3 containing acetic acid concentrations from 40 to 80 mM, and the production of ROS was determined (Fig. 9A). We also performed in a further experiment co-staining of...
DHR123 and PI (Fig. 9C). Only a small amount of cells positively stained with both dyes (about 10%) was measured, whereas more than 50% of the cells were exclusively positive for DHR123. Interestingly, we observed a strong increase of ROS accumulation (from 2 to 91%) and also of PI-stained cells (from 4 to 37%) when cells, treated with 40 mM acetic acid, were challenged with tunicamycin or acetic acid.

The involvement of apoptotic regulators recently identified in Saccharomyces cerevisiae (reviewed in Ref. 11), namely apoptosis-inducing factor Ain (27, 53) and nuclear mediator of apoptosis Nma111 (26, 30), was analyzed. We tested cell survival, production of ROS, and caspase-like activity in the wbp1-1 mutant. Neither disruption of Ain (Fig. 10A) nor of Nma111 (Fig. 10B) had an effect on the parameters measured, indicating that the respective proteins do not seem to have a significant role in the signaling and/or execution of the cell death process triggered by a defect in N-glycosylation.

**DISCUSSION**

Various findings in the past several years indicate that programmed cell death processes with apoptotic phenotypes are induced in yeast in response to deleterious environmental and also intracellular defects. Despite the fact that various key players of the mammalian apoptotic cascade have been identified, such as HtrA2/Omi (26), the apoptosis-inducing factor Ain (27), yeast homologues of the mitochondrial fission factors Fis1 and Dnm1 (29) or the yeast metacaspase Yca1/Mca1 (33), and others, the existence of distinct pathways similar to those in multicellular organisms is still a matter of discussion. Independent of this, yeast has become a suitable model to identify components of an ancestral apoptotic machinery, since, for example, heterologous expression of proapoptotic protein Bax resulted in cell death (67, 68), which could be prevented by co-expression of antiapoptotic Bcl-2 (68, 69). Similarly, mammalian protein kinase C isoforms modulated Bcl-xl phospho-

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PeP4 protease, which is the main player in the proteolytic activation of several vacuolar proteases. As shown in supplemental Fig. 3, compared with the KEX1 deletion, PeP4 did not affect production of ROS, survival, and FITC-VAD-fmk in cells challenged with tunicamycin or acetic acid.

**Cell Death Induced by Defective N-Glycosylation Does Not Depend on the Known Apoptotic Regulators Ain and Nma111**—In addition, the involvement of apoptotic regulators recently identified in Saccharomyces cerevisiae (reviewed in Ref. 11), namely apoptosis-inducing factor Ain (27, 53) and nuclear mediator of apoptosis Nma111 (26, 30), was analyzed. We tested cell survival, production of ROS, and caspase-like activity in the wbp1-1 mutant. Neither disruption of Ain (Fig. 10A) nor of Nma111 (Fig. 10B) had an effect on the parameters measured, indicating that the respective proteins do not seem to have a significant role in the signaling and/or execution of the cell death process triggered by a defect in N-glycosylation.

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**FIGURE 7.** Kex1 does not improve N-glycosylation. Cells were metabolically labeled with [35S]methionine/cysteine, and CPY was analyzed by immunoprecipitation followed by PAGE (8% gel) and autoradiography. P1 CPY is the endoplasmic reticulum glycosylated form, and P2 CPY is the Golgi glycosylated form, which is processed to the mature CPY. These intermediates are visible at the lower labeling temperature of 25 °C.

**FIGURE 8.** Overexpression and enzyme activity of Kex1. A, cultures were incubated in liquid minimal medium at 25 °C to a cell density of 2.5 × 10⁶ ml⁻¹. In total, 3 μl of a serial 1:10 dilution of 10⁴ cells ml⁻¹ were plated on YPD plates and incubated at 25 and 31 °C for 4 days. B, aliquots of microsomal fraction corresponding to 0.5 OD cells expressing plasmid-encoded KEX1-ZZ and genomic KEX1-ZZ, respectively, were analyzed by Western blotting. The arrow denotes mature Kex1-ZZ; the smaller bands are assumed to be proteolytic breakdown products. C, cleavage of furylacryloyl-Ala-Arg and Ac-VEID-AMC by immunoprecipitated Kex1 protease isolated from wbp1-1pVT100 (empty vector) and wbp1-1pVT100-KEX1-ZZ (vector overexpressing KEX1). Values shown are representative of two independent experiments.
rylation in acetic acid-induced apoptosis when expressed in yeast (70).

We have recently demonstrated that a defect in protein N-glycosylation induces a programmed cell death (24). The dying cells displayed an apoptotic phenotype characterized by several mammalian apoptotic hallmarks, such as phosphatidylserine exposure, condensation of chromatin, DNA fragmentation, and caspase-like activity in vivo and in vitro using known metazoan caspase substrates. However, disruption of YCA1, the single metacaspase in yeast known so far, revealed that this proteolytic activity is not involved in this death cascade. Yca1p has been implicated in an apoptotic-like cell death program triggered by exposure to several adverse environmental stimuli (20, 22, 23, 33, 34, 71–76). So far, there are no specific targets known for Yca1, and in general its role is still poorly understood. In addition, heterologous expression of human Bcl-2 in wbp1-1 prevents and retards, respectively, the cell death program. How Kex1 fulfills this role and integrates into the cell death process remains to be determined. One of the possibilities, that deletion of KEX1 exerts its function by directly improving the glycosylation efficiency of wbp1, could be ruled out (Fig. 6). Hence, this result rather supports the idea that it is involved in the death cascade. Since it has been shown that some proteases different from the caspase family are able to cleave caspase substrates and are prone to interact with small peptide caspase inhibitors (56, 79, 80), we reasoned that Kex1 could have a function additional to its hitherto known substrate specificity. However, Kex1 was not able to directly cleave the caspase substrates; thus, we postulate that it affects in an indirect manner downstream targets, which are constituents of the death pathway. Also, how Kex1 gets access to these processes has to be answered in the future. It is known that aberrant protein glycosylation induces the adaptive unfolded protein response and thus may ultimately lead to apoptotic cell death (81–85). It is conceivable that the endoplasmic

![FIGURE 9. Deletion of Kex1 protease rescues wild-type yeast from acetic acid-induced programmed cell death and improves cell survival in chronologically aged cells. A, ROS accumulation of wild-type and Δkex1 strains after treatment with 40–80 mM acetic acid for 200 min at 25 °C. Quantification of staining was performed by cell flow cytometry. B, survival of wild-type and Δkex1 yeast cells after incubation with 85 mM acetic acid for 200 min at 25 °C. Single cells were spotted with the MoFlo cell sorter directly on YPD plates and incubated for 3 days at 25 °C. C, co-staining of wild-type yeast for ROS accumulation (DHR123) and membrane integrity (PI) after stressing the cells with 80 mM acetic acid for 200 min at 25 °C. D, survival during chronological aging. Wild-type yeast and Δkex1 cells were cultured at 25 °C. Aliquots were taken at the indicated times, and single cells were spotted with the MoFlo cell sorter directly on YPD plates and incubated for 3 days at 25 °C. Data in A and D are the average of three independent experiments.](http://www.jbc.org/content/283/27/19160/F1)
reticulum/Golgi stress situation due to improper folding of proteins by aberrant glycosylation leads to changes in the membrane permeability of the secretory compartment, thus making Kex1 available to execute its function in the death cascade. Efforts to demonstrate a direct release of Kex1 from the membrane compartment to the cytosol in response to the glycosylation defect were negative.\textsuperscript{3} Another example of the Yca1p-independent pathway in yeast is the nuclear serine protease HtrA-like protein Nma111 (26). Serine proteases different from Htra2/Omi have also been found to act in mammalian cells during endoplasmic reticulum stress signaling both in caspase-dependent and caspase-independent pathways (86).

The involvement of Kex1 in programmed cell death cascades does not seem to be restricted only to a defect in N-glycosylation. Thus, also during chronological aging or in cell death induced by acetic acid, the deletion of \textit{KEX1} caused a better survival of cells and, in the latter case, also a reduced production of ROS. In both instances, the involvement of Yca1 was demonstrated, but in addition, a caspase-independent route was postulated (34, 66). Kex1 may be responsible for and contribute, respectively, to this pathway, albeit its specific function in the cascade remains to be determined. We considered whether chromatin condensation and any degree and combination of apoptotic features. In most cases, it is caspase-independent, although cases of caspases-8 and caspase-1 involvement were reported (88, 89). Understanding of the exact mechanism and mode of yeast cell death induced by reduced glycosylation is still in the early stages. Since it depends on protein synthesis (Fig. 5C) (24) and can be prevented not only by removal of the external stimulus, but also, for example, by heterologous expression of human Bcl2 or substances interfering with the production of ROS, it has to be considered as an active process. Furthermore, it is accompanied by the occurrence of typical morphological apoptotic markers, such as nuclear condensation, DNA fragmentation, and phosphatidylserine exposure (24) or cytoplasmic shrinkage (Fig. 1C). On the other hand, we found that plasma membrane integrity is lost readily, since only a fraction of cells excluded propidium iodide. It cannot be decided whether this latter fact reflects a postapoptotic secondary necrosis, because cell wall integrity and as consequence also membrane integrity is severely affected due to the glycosylation defect, or whether this cell death should be considered rather a form of necrosis-like programmed cell death. The yeast cell wall consists of about 50% glycoproteins and has to withstand intracellular osmotic pressure of more than 10 bars. Interestingly, we observed that osmotic stabilization of older cells aborted

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caspase-like activity and ROS production. Nevertheless, cell death in yeast, contrary to multicellular organisms, will always end with the lytic disintegration of the doomed cells.

The field of yeast apoptosis has grown rapidly, and many triggers of apoptosis have been described meanwhile. In the future, the identification of the specific targets and unraveling of the underlying mechanism of execution will be critical for understanding this complex suicide network.

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