Estrogen Effects on *Candida albicans:* A Potential Virulence-Regulating Mechanism

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Three *Candida albicans* strains were tested in the presence of 17-β-estradiol (10^{-6} *M* and 10^{-9} *M*) for increased growth and for enhanced survival during incubation at nonpermissive temperatures. All 3 test organisms showed increased growth in the presence of estradiol compared with estrogen-free controls. Likewise, all 3 strains, when treated with estradiol, survived incubation at 48°C better than did controls. Cytoplasmic extracts were probed with an anti-hsp90 antibody, and results suggested that intracellular hsp90 was up-regulated in the presence of 10^{-9} *M* 17-β-estradiol. The results were confirmed by reverse-transcriptase polymerase chain reaction with primers specific for *C. albicans* hsp90. A kinetic study revealed that peak *hsp90* expression occurred within 2 h of exposure to 17-β-estradiol. In addition, estrogen increased the amount of *cdr1* (*Candida* multidrug resistance) mRNA compared with cells not treated with estrogen. Coumarin and phenol also up-regulated *hsp90* and *cdr1* mRNAs, indicating that the estrogen-sensing and -response systems in *C. albicans* may lack specificity.

Microbial virulence is a regulated phenomenon that occurs among many bacterial species when the microorganism senses a change in the pH, temperature, or osmolarity of its environment [1, 2]. However, little information is available about virulence regulation in the most prevalent and clinically important fungal pathogen, *Candida albicans.* Several potential virulence factors have been identified in this organism [3–7], and it has been hypothesized that clinical infection involves the action of several of these factors operating in concert [8].

We had observed that 17-β-estradiol increased growth and toxin production [9] and increased germination [7] among clinical isolates of *C. albicans,* which led us to ask whether estrogen may serve as an environmental signal for yeast virulence induction. Because *Candida* may not have a true estrogen receptor or estrogen response elements homologous to those in the mammalian species [10], we sought alternative pathways by which estrogen could affect yeast cells. In a previous study [11], we had found that the pattern of cytoplasmic proteins seen on SDS-PAGE after brief estrogen treatment resembled that induced by heat stress. In the present article, we report the results of an investigation of the association between estrogen treatment and heat-stress protein (hsp).

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Materials and Methods

Yeast nitrogen broth base and BIGGY agar were purchased from Difco (Detroit), and all media were prepared according to manufacturers' directions. Yeast nitrogen broth was supplemented with 0.5% filter-sterilized glucose immediately before use.

We purchased a spin-column vacuum-extraction total RNA isolation kit and the Access reverse-transcriptase (RT) polymerase chain reaction (PCR) system kit from Promega (Madison, WI). All other reagents, except where otherwise indicated, were purchased from Sigma Chemical (St. Louis). 17-β-estradiol (1,3,5[10]-estratriene-3,17β-diol) was prepared as a stock solution of 10^{-3} *M* in methanol and stored at −20°C. Subsequent dilutions were prepared in yeast nitrogen broth, as needed.

C. albicans strains (GT 157 and JMMS 23) were obtained from patients undergoing gynecologic examination. These isolates were identified as *C. albicans* by observing microscopic morphology, germ-tube formation in human serum, and production of brown colonies on BIGGY agar. Isolates were maintained on Sabouraud dextrose agar (1% peptone, 2% glucose) slants at 4°C and were subcultured at 3-month intervals. Isolate ATCC 10231 was purchased from the American Type Culture Collection (Rockville, MD) and was handled in the same way as clinical isolates.

Induction of heat-stress response was evaluated, as reported elsewhere [11]. Cultures were prepared in yeast nitrogen broth and incubated overnight at 25°C in a shaking water bath at 110 oscillations per minute, which allowed organisms to reach logarithmic growth. At the time of the experiment, the starter culture was divided into aliquots for treatment with 17-β-estradiol (10^{-6} *M* or 10^{-9} *M*). Because the estradiol was prepared in methanol, methanol (1% final concentration) was added to controls to rule out effects due to the solvent. After overnight growth, control and estrogen-supplemented cultures were thermally challenged in a heat block set at 48°C. At intervals ≤60 min, duplicate 10-μL aliquots were allowed to dribble across the surface of a Sabouraud dextrose-agar plate.

Gene product	Sense primer	Anti-sense primer	Predicted product
CDR1	GAA GTG AGA GAA GCT CCA TC	TGA CAG AAT GCA CAA GAT CC	**562**
HSP	CGA TGA ATA TGC CAT GAC TC	TCC ATA GCA GAT TCT CCA GC	**605**
16S	ATG GCC GTT CTT AGT TGG TG	GCC AAG GCT TAT ACT CGC TG	**227**

Figure 1. Nucleotide sequences of primers

After all platings were completed, the plates were incubated until colonies were of countable size. The average colony count at each time point divided by the starting count (before the thermal challenge) was used to determine the percentage of survival of organisms under various growth conditions.

Cytoplasmic hsp90 was evaluated by a dot-blot procedure that used a commercial antibody (polyclonal rabbit anti–synthetic 12-mer [PEEVHHGEEEVECys], representing the N terminus of mouse hsp84) obtained from Neomarkers (Union City, CA). This antibody was reactive with the *Candida* antigen purified in our laboratory. To obtain cytoplasmic proteins from *Candida*, overnight cultures (50-mL volumes) of several *Candida* strains were prepared in yeast nitrogen broth and were in midlogarithmic phase at the time of the experiment. These cultures were grown at 25°C in a shaking water bath before the addition of 17-β-estradiol.

The experiment was begun by dividing the cultures into aliquots, adding estrogen or control solvent (methanol), and incubating at room temperature for 2 h. After the induction phase, the cells were centrifuged and resuspended in 100 μL of breaking buffer (2% Triton X-100, 1% SDS, 100 m*M* NaCl, 10 m*M* Tris [pH 8.0], and 1 m*M* EDTA) and 10 μL of Sigma protease-inhibitor cocktail. About 100 μL of acid-washed glass beads (425–600 μM; Sigma) was added to the tubes and mechanically disrupted at 3000 strokes per minute. After disruption, the tubes were centrifuged at 13,000 *g* at room temperature for 5 min, and the supernatant was collected. We used BCA protein reagent (Pierce, Rockford, IL) to determine protein content of the cytoplasmic extracts, and dilutions ($\leq$1 : 1000) of the cytoplasmic extract were applied to nitrocellulose membranes. The membranes were blocked for 1 h in 1% skim milk in TBS. Primary antibody was used at a 1 : 300 dilution in blocking buffer and reacted with the nitrocellulose-bound samples for 1 h at room temperature. Blots were washed 3 times with blocking buffer, reacted with secondary antibody (horseradish peroxi-

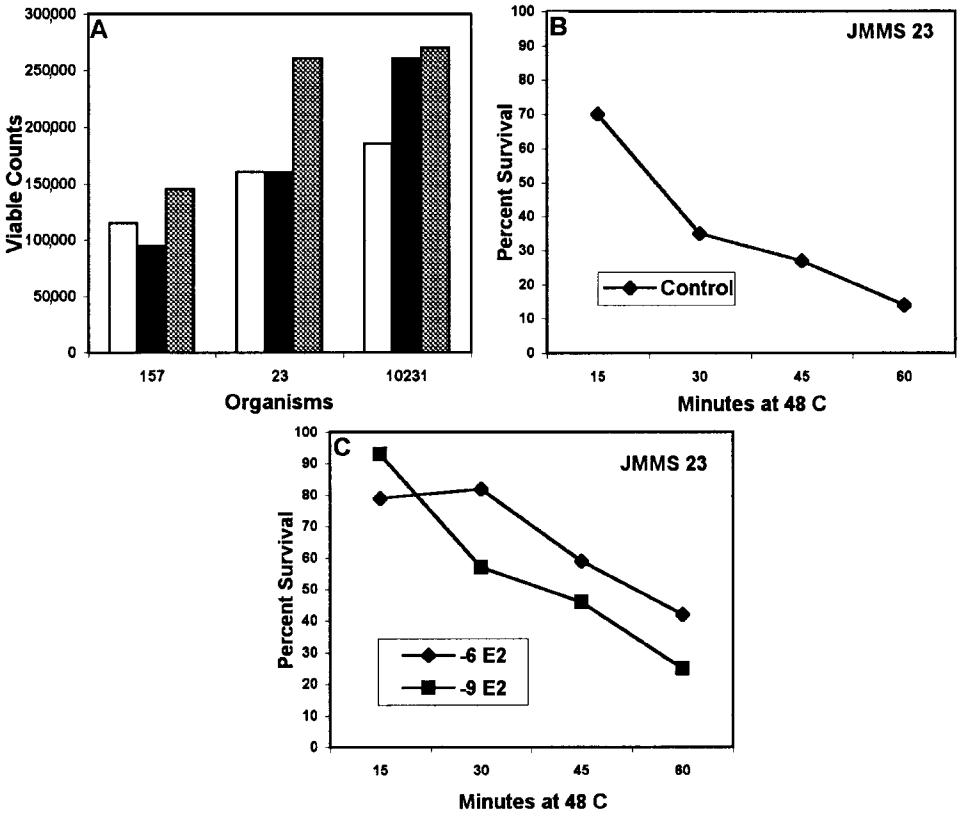

Figure 2. *A,* Effects of estradiol at micromolar *(solid bars)* and nanomolar *(shaded bars)* concentrations on *Candida* viable-colony counts 18 h after inoculation compared with cultures without added estradiol *(open bars)*. *B,* Survival of *C. albicans* (strain 23) during 60-min exposure at 48°C, as determined by viable-colony counts. *C,* Survival of strain JMMS 23 grown in the presence of micromolar (-6 E2) or nanomolar (-9 E2) estradiol. Estrogen-treated cells were less sensitive to the effects of elevated temperature than were control cells.

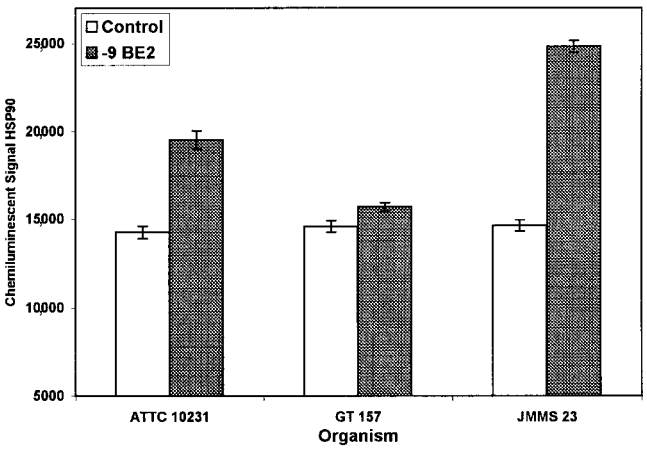

Figure 3. hsp90-reactive material of 3 *Candida albicans* strains cultured with 10^{-9} *M* 17-β-estradiol *(shaded bars)* or without estradiol *(open bars)*. Cytoplasmic extracts were reacted with anti-hsp90. Secondary antibody labeled with horseradish-peroxidase was detected by chemiluminescence. SDs were determined by use of imaging software (Multianalyst; BioRad, Hercules, CA).

dase–labeled polyclonal goat anti–rabbit IgG; Sigma) for 1 h, and washed 3 times in blocking buffer. The nitrocellulose-bound samples were flooded with ultrachemiluminescent detection reagent (Pierce) and were viewed in the dark mode in an imager (FluorS-Multi-Imager; BioRad, Hercules, CA). Chemiluminescent signal was measured by software (Multianalyst; BioRad). Final values for each dot were adjusted to normalize for differences in protein loading.

To obtain cells for total RNA preparations, starter cultures of *C. albicans* GT 157, JMMS 23, and ATCC 10231 were grown in yeast nitrogen broth for 17–20 h at 25°C with shaking, to reach midlogarithmic phase. Each starter culture was divided into 4 aliquots: 17-β-estradiol 10^{-6} *M* (final concentration) was added to 1 aliquot, 17-β-estradiol 10^{-9} *M* (final concentration) was added to the 2d aliquot, and the remaining 2 were treated with methanol (volume equal to that in 17-β-estradiol–treated cultures). After addition of 17-β-estradiol or methanol, tubes were incubated for 2 h at room temperature. During the final 20 min, 1 methanol-containing control was transferred to a 39°C water bath to induce a heat-shock response [11]. Cells were harvested by centrifugation at 4°C, and the pellets were collected for total RNA isolation.

Total RNA was isolated from *C. albicans* according to the manufacturer's protocol (SV total RNA isolation). Total RNA was measured by absorbance at 260 nm, and the RNA quality was established by visualization on agarose gel electrophoresis. We used the Access RT-PCR system (Promega) for RT-PCR products from total RNA, avian myeloblastosis virus RT for 1st-strand cDNA synthesis, and *Thermus flavus* thermostable DNA polymerase for 2d-strand cDNA synthesis and DNA amplification. Three pairs of primers (*Candida hsp90*, *Candida* 16S ribosome, and *Candida cdr1* [GenBank accession numbers: X81025, X53497, X77589, respectively]) were designed from the reported nucleotide sequence for RT-PCR products (cdr1, 562 bp; hsp90, 605 bp). These primers were prepared by the Marshall University DNA Core Facility (Huntington, WV) and are shown in figure 1. Initially, RT-PCR products were submitted for sequence analysis (Marshall University DNA Core Laboratory), which confirmed that products had the appropriate sequence. The 16S ribosome (227 bp) of *C. albicans* was used as an endogenous internal control.

RT-PCR reactions were carried out in total volumes of 50 μL and included 3 μg of total RNA template, 50 μM of primer pairs *(hsp90* or *cdr1),* and 3 μM of 16S ribosome primer pairs. Cycling profile was 48°C for 45 min for first-strand cDNA synthesis and 94°C for 2 min, followed by 40 cycles of 94°C for 30 s, 60°C for 1 min, and 68°C for 2 min for second-strand cDNA synthesis and PCR amplification, ending with a final extension of 7 min at 68°C. The size of the amplified cDNA fragments (including *cdr1, hsp90,* and 16S ribosome) was confirmed by electrophoresis in 1.5% agarose gel. Densitometric analysis of cDNA products was done by using Fluor-S Multi-Imager and Multianalyst software (both BioRad).

Results

To confirm that the *Candida* strains used in this study were responsive to 17-β-estradiol, we determined the growth and thermal-protective effect on strains JMMS 23, GT 157, and ATCC 10231. The growth of the 3 test organisms increased, as shown in figure 2*A*. For all 3 strains, the 10^{-9} *M* concentration of estradiol was most effective in increasing growth above control levels. We also showed that *C. albicans* JMMS 23 grown overnight in 10^{-9} *M* or 10^{-6} *M* 17-β-estradiol attained heat-stress protection, compared with controls (figure 2*C*). When viable-colony counts were made on yeast grown without estradiol, <15% survived for 60 min at 48°C. In contrast, survival

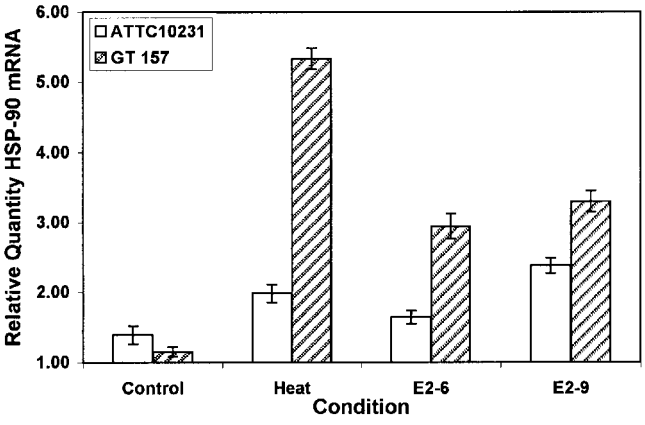

Figure 4. Induction of *hsp90* mRNA by heat and estrogen determined by reverse-transcriptase polymerase chain reaction (PCR) for 2 *Candida* strains. Total RNA preparations were made from yeast cells exposed to temperature up-shift (25°C–37°C) or 10^{-9} *M* 17-β-estradiol, and PCR products of *hsp90* mRNA and 16s mRNA were measured. Fluorescent intensity of *hsp90* bands was divided by fluorescent intensity of 16s RNA bands. SDs were determined by use of imaging software (Multianalyst; BioRad, Hercules, CA). E2-6, 17-β-estradiol (10^{-6} *M*); E2-9, 17-β-estradiol (10^{-9} *M*).

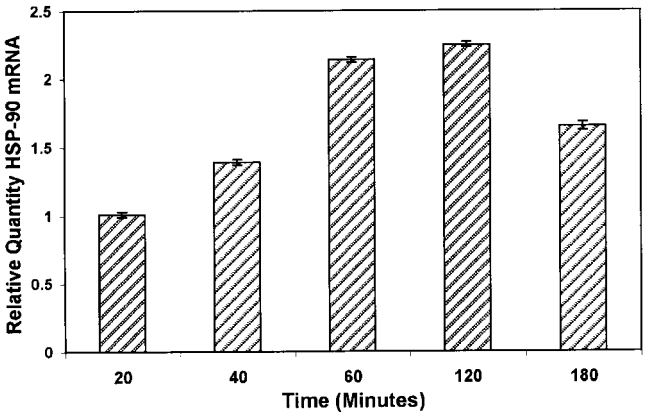

Figure 5. Relationship of time to *hsp90* expression after exposure to 10^{-9} *M* 17-β-estradiol. Highest induction was at 2 h in *Candida albicans* GT 157. Fluorescent intensity of *hsp90* bands was divided by fluorescent intensity of 16s RNA bands. SDs were determined by use of imaging software (Multianalyst; BioRad, Hercules, CA).

was >25% after 60 min at 48°C for cells grown in the presence of 10^{-9} *M* 17-β-estradiol and was >45% for those grown in 10^{-6} *M* 17-β-estradiol. The other yeast strains also showed heat-stress protection after growth in the presence of 17-β-estradiol.

To determine whether estrogen induced hsp90, we performed dot-blot experiments with the 3 *Candida* strains. Yeast cultures were grown overnight at 25°C in yeast nitrogen broth. The cells were incubated for 2 h with 10^{-6} *M* 17-β-estradiol, 10^{-9} *M* 17-β-estradiol, or methanol (1%) equal to the amount added with the estrogen solutions. Cytoplasmic extracts were diluted and applied to nitrocellulose and were probed with anti-hsp90. Figure 3 illustrates that 10^{-9} *M* 17-β-estradiol increased the amount of hsp90-reactive material in the cytoplasm of all 3 strains of yeast tested, although the increment with GT 157 was smaller than that for the other 2 organisms.

Because no anti-*Candida* hsp90 is commercially available, the dot-blot experiment relied on a commercial antibody raised against a synthetic peptide that was expected to be cross-reactive with the *Candida* protein. Our laboratory purified hsp90 from GT 157 and confirmed its identity through N-terminal sequencing (E.T.B. and B. L., unpublished data). We have determined that the commercial antibody used in our dot-blot experiment reacted with the pure *Candida* protein (data not shown).

Although our data appeared to support the concept that hsp90 is up-regulated by estrogen, a more specific method was needed to confirm that hsp90 expression is increased by exposure of *C. albicans* to estradiol. We used RT-PCR with primers for hsp90 and 16S rRNA (to normalize quantitation of hsp90 product) to amplify mRNAs from total RNA preparations of *C. albicans* (GT 157 and ATCC 10231). The relative abundance of hsp90 normalized for 16S rRNA product by the procedure described in Materials and Methods is shown in figure 4. Both heat (upshift from 25° to 37°C for 2 h) and exposure to 17-β-estradiol (10^{-9} *M* concentration) increased the expression of hsp90. A similar experiment was done by incubating aliquots of a starter culture of GT 157 with 17-β-estradiol for 20, 40, 60, 80, 120, and 180 min. Figure 5 shows that maximum expression of hsp90 mRNA occurred after 2 h of exposure to estrogen.

We next examined whether estradiol could up-regulate the mRNA for *cdr1,* an ATP-dependent pump that can serve as a multidrug-resistance factor [12]. Again, by using RT-PCR, we showed that *cdr1* mRNA was increased after a brief exposure to 10^{-9} *M* estradiol (figure 6). Of interest, for both *hsp90* and *cdr1,* 10^{-6} *M* estradiol was not stimulatory, an observation previously noted for such characteristics as stimulated germination [7].

Work reported by others [13] suggested that *ebp1,* which encodes the cytoplasmic protein that binds estradiol, may bind a variety of phenolic compounds. We investigated 2 compounds, phenol and coumarin, to determine whether they could induce increased levels of *cdr1* or *hsp90* mRNA. Figure 7 illustrates the result of this experiment and indicates that phenolic compounds can produce effects on *hsp90* and *cdr1* expression similar to those noted for estradiol. This study did not provide evidence to determine whether coumarin and phenol simply cause an effect similar to estrogen in these cells or actually bind to the same yeast cell–sensing molecule(s) that estrogen affects.

Discussion

Microbial virulence in opportunistic microorganisms is thought to involve many factors that are coordinately regulated. This kind of polygenic control is equally applicable to

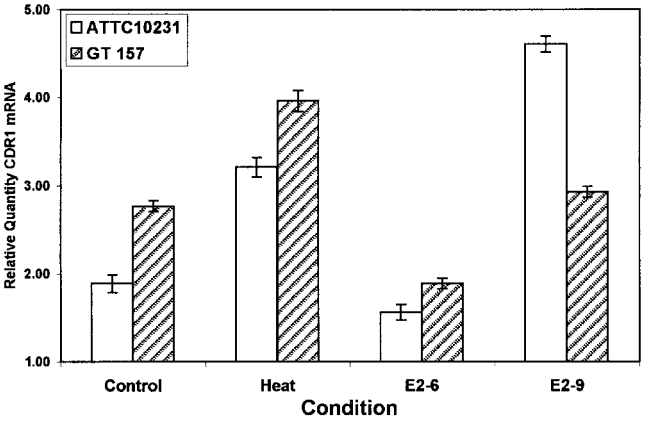

Figure 6. Effect of 10^{-9} *M* 17-β-estradiol on *cdr1* mRNA in 2 *Candida albicans* strains. Reverse-transcriptase polymerase chain reaction was used to measure *cdr1* mRNA, as was done for *hsp90* mRNA. Fluorescent intensity of *cdr1* bands was divided by fluorescent intensity of 16s RNA bands. SDs were determined by use of imaging software (Multianalyst; BioRad, Hercules, CA).

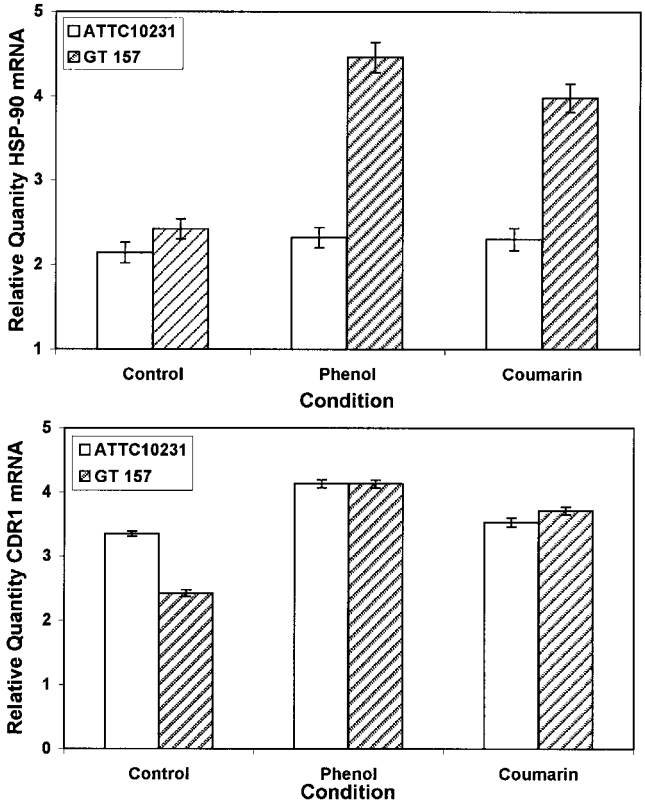

Figure 7. Nonestrogens induce *hsp90 (upper panel)* and *cdr1 (lower panel)*. Reverse-transcriptase polymerase chain reaction was used to show that exposure of 2 strains of yeast to phenol or coumarin produced responses similar to those elicited by estrogen. The fluorescent intensity of *hsp90* bands or *cdr1* bands was divided by fluorescent intensity of corresponding 16s RNA bands. SDs were determined by use of imaging software (Multianalyst; BioRad, Hercules, CA).

metabolic pathways and to pathways more directly involved with pathogenicity. Indeed, there is every reason to expect that a great deal of overlap between normal housekeeping functions and functions that promote infection should exist among microorganisms.

Although global regulation of virulence among fungal pathogens has not been investigated with the same completeness as among bacteria, complex regulatory pathways that involve known virulence factors, as well as apparently unrelated genes, should be anticipated. For example, Godon et al. [14] found that exposure of *Saccharomyces cerevisiae* to hydrogen peroxide resulted in the increased synthesis of 115 proteins and the simultaneous repression of 52 proteins. Many of the proteins were identified, and, although a minority were involved with protection against oxidative damage or were known stress-response proteins, others were involved with apparently unrelated pathways, such as carbohydrate metabolism. Of particular interest, among the many proteins listed as being induced by hydrogen peroxide was OYE3, an oxidoreductase that is apparently related to the estrogen-binding protein in *C. albicans* [15].

In view of the current findings, it is appropriate to ask whether estrogen could initiate some type of stress response in *Candida* that may in turn increase microbial virulence. Although changes in the fungus are consistent with increased virulence (e.g., hypha formation), they may simultaneously represent survival strategies. The discovery that phenolic compounds up-regulate *hsp90* and *cdr1* supports this concept, although it is not clear whether phenolic compounds bind to the same target as estrogen does or whether they trigger a similar response through an alternative pathway.

Interest in the relationship between stress response and virulence is based on several disparate findings. Matthews et al. [16] proposed that *hsp90*, or some fragment thereof, functioned as a virulence factor, because antibody to hsp90 was found in survivors of serious infection and was deemed to be protective. It has been impossible to produce an *hsp90* knock-out yeast strain, because such cells are nonviable [17]; therefore, evaluating the role of hsp90 as a virulence factor is challenging. However, overexpression of *hsp90* in *Saccharomyces* increased its virulence for mice [18]. Louvion et al. [19] showed that hsp90 plays an important role in the pheromone-signaling pathway in *S. cerevisiae* by interacting with Ste11, which is part of the yeast's MAP kinase pathway and is involved in steroid binding in the ergosterol pathway. Although *C. albicans* does not have a known pheromone-mediated mating pathway, recent reports indicate that at least some elements of a mating pathway are conserved in this asexual yeast [20].

It would be useful to understand whether hsp90 is directly related to virulence or whether it is merely a marker for other virulence mechanisms. Theoretically, elevated hsp90 could render microorganisms more resistant to host-defense properties, or it may have immunomodulating effects when released in the host. The study of virulence mechanisms of hsp90 may help to address virulence and virulence regulation in new ways.

The relationship of *hsp90* to the MAP kinase pathway provides a clue as to the mechanism whereby estradiol may activate gene function in *Candida*. Alternative signaling pathways for estrogen that involve MAP kinase have been identified in mammalian (breast cancer) cells and may act through calcium flux [21]. It is possible that estrogen also activates the MAP kinase pathway in *Candida*, particularly in view of the fact that this pathway plays an important role in morphogenic conversion from yeast to hyphal forms. Furthermore, our previous work [7] suggested that estrogen may promote yeast-to-hyphal transformation as well. We have yet to discover the key ligand that allows yeast to respond to estradiol, but the binding protein ebp1 is a reasonable candidate. The work by Buckman and Miller [13] demonstrated that binding is not limited to estradiol, and our finding that nonestrogen phenolic compounds stimulate *hsp90* and *cdr1* suggests that the estrogen-binding protein may be the initiation point for reactions that ultimately tran-

scriptionally activate *Candida* genes. The relatively long time needed to maximally induce hsp90 in *Candida* suggests that a number of other events may precede hsp90 production. In a plant model, *Arabidopsis,* the old yellow enzyme (homologous to the *Saccharomyces* estrogen-binding protein) has activity in an octadecanoate signaling pathway, which supports the possibility that ebp may function as an environmental sensor to regulate the MAP kinase pathway [22].

One of the practical questions elicited by our results is how estrogen influences the virulence of the organism. Clearly, if estrogen can promote hypha formation, it may, by such action, enhance the infectivity of the organism (although a role for other simultaneously produced virulence factors in clinical infections cannot be dismissed). The role of the hypha as a true virulence attribute has been debated, but recent studies show a lack of virulence for organisms unable to form hyphae [23] and provide important information on this issue. The induction of a "heat shock–like" reaction should enable the organism to survive some of the conditions that are encountered in the host, such as exposure to free radicals. We do not know all of the genes induced as a result of exposing *Candida* to estrogen, but it is likely that multiple virulence attributes are induced. Some experts dismiss the idea that housekeeping genes (e.g., *hsp90*) may have a role in virulence; however, the results of bacterial in vivo expression technology, which reveals genes or gene products only expressed in the host or host tissues [1], suggest that many genes with no reputation as virulence factors can contribute to in vivo survival, proliferation, and virulence.

The fact that *cdr1* appears to be up-regulated along with *hsp90* suggests that its potential role in clinical infection should be considered. Multidrug-resistance systems that are energy-dependent transporters are responsible for eliminating potentially damaging molecules from a wide variety of cells. Furthermore, *cdr1* has been associated with azole resistance in some *Candida* strains [24, 25] and, theoretically, if expressed at high levels, may contribute to the survival of the organism in the presence of antifungal drugs or other harmful substances. Future studies that more comprehensively examine gene regulation of *Candida* may reveal that it too involves housekeeping genes in the infectious process.

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