The Presence of an R467K Amino Acid Substitution and Loss of Allelic Variation Correlate with an Azole-Resistant Lanosterol 14α Demethylase in Candida albicans

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Azole resistance in the pathogenic yeast Candida albicans is an emerging problem in the human immunodeficiency virus (HIV)-infected population. The target enzyme of the azole drugs is lanosterol 14α demethylase (Erg16p), a cytochrome P-450 enzyme in the biosynthetic pathway of ergosterol. Biochemical analysis demonstrates that Erg16p became less susceptible to fluconazole in isolate 13 in a series of isolates from an HIV-infected patient. PCR–single-strand conformation polymorphism (PCR-SSCP) analysis was used to scan for genomic alterations of ERG16 in the isolates that would cause this change in the enzyme in isolate 13. Alterations near the 3′ end of the gene that were identified by PCR-SSCP were confirmed by DNA sequencing. A single amino acid substitution (R467K) that occurred in isolate 13 was identified in both alleles of ERG16. Allelic differences within the ERG16 gene, in the ERG16 promoter, and in the downstream THR1 gene were eliminated in isolate 13. The loss of allelic variation in this region of the genome is most likely the result of mitotic recombination or gene conversion. The R467K mutation and loss of allelic variation that occur in isolate 13 are likely responsible for the azole-resistant enzyme activity seen in this and subsequent isolates. The description of R467K represents the first point mutation to be identified within ERG16 of a clinical isolate of C. albicans that alters the fluconazole sensitivity of the enzyme.

Oral candidiasis is one of the earliest and most frequent opportunistic infections associated with immune system failure in human immunodeficiency virus (HIV)-infected individuals (8). Candidiasis is usually treated effectively with oral fluconazole or other azoles (28). Recently, the efficacy of these drugs has been compromised by the emergence of azole-resistant strains of Candida albicans and intrinsically resistant species such as Candida glabrata and Candida krusei (25, 29). One study estimated that >33% of isolates from patients with AIDS have MICs of fluconazole greater than 12.5 μg/ml, while standard susceptible strains have MICs of less than 4 μg/ml (18).

As the frequency of resistance has increased in the HIV-infected population, the mechanisms that result in azole resistance have become a focus of attention. Biochemical analysis of azole-resistant strains has documented changes in the target enzyme of the azoles, lanosterol 14α demethylase (Erg16p), and other enzymes in the ergosterol biosynthetic pathway (9). The azoles bind to the active site of Erg16p, a cytochrome P-450 enzyme in the biosynthetic pathway of ergosterol (9). The target enzyme of a drug is frequently altered as resistance develops in many biological systems (4). The purpose of the present study was to investigate the Erg16p enzyme in this series of 17 fluconazole-resistant C. albicans isolates by both biochemical and molecular techniques. Enzymatic assays were used to determine if the Erg16p enzyme activity is altered in this series. PCR–single-strand conformation polymorphism (PCR-SSCP) and DNA sequencing are used to recognize DNA sequences that have been mutated in the series. By using the 17 isolates, the timing of each genetic alteration within the resistance series was determined.

MATERIALS AND METHODS

Strains and growth of cultures. The C. albicans samples used in this study and the growth and storage of these cultures have been described previously (36, 38). As previously described, isolate 10 was not available for analysis.

Biochemical analysis. Inhibition of Erg16p enzymatic activity by fluconazole was evaluated as described previously (3, 20). Cell extracts were prepared as described previously (20). Briefly, cells were grown to mid- to late log phase in YEPD broth (36). Cultures were chilled on ice for 30 min, pelleted by centrif-
Ugation, and washed in phosphate buffer (0.1 M sodium phosphate, pH 7.5). Cells were resuspended in a small volume of phosphate buffer in a 50-mL conical centrifuge tube. Glass beads (0.5-mm diameter) were added to create a slurry, which was vortexed at high speed five times for 1 min each. Between each vortexing, the tube was cooled on ice for 1 min. Glass beads were removed by centrifuging the slurry through a disposable filter column (Fisher Scientific, Pittsburgh, Pa.) into a 15-mL conical centrifuge tube. The cell extracts were centrifuged for 10 min at 2,500 g and for 10 min at 10,000 g to remove whole cells and cellular debris. The resulting extracts were frozen at −20°C until use. Protein concentrations were determined by a Bradford assay (Bio-Rad [Her-2 cells and cellular debris. The resulting extracts were frozen at

mM MgCl₂, 0.25 mM dithiothreitol, 50 μM each deoxynucleotide (dATP, dGTP, dTTP), and 5 μCi of [α-32P]dCTP (10 mCi/mM), and 10 U of Taq polymerase. The PCR was performed under the following conditions: 15 cycles of 94°C for 1 min, 55°C for 1 min, and 72°C for 2 min.

A

FIG. 1. (A) Genomic region surrounding the ERG16 gene. The ERG16 coding region (16) and the partial THR1 coding region are diagrammed as shaded boxes. DNA fragments surrounding the ERG16 gene (including regions of the THR1 gene) that were used as probes and in PCR-SSCP analysis are shown above the line (see Materials and Methods for coordinates). Restriction enzymes important for the discussion are shown below the line, with abbreviations as follows: P, PstI; A, AccI; M, MunI; and F, AflII. DNA fragments important for the results are diagrammed below the line, including a PstI fragment (1,277 bp), a PstI/AccI fragment (1,252 bp), a PstI/AflII fragment (1,287 bp), and a PstI/MunI fragment (1,544 bp) that does not contain the first MunI restriction enzyme site (at position 1252 from the PstI site). (B) Diagram of loss of allelic variation of the ERG16 gene region. Allelic differences were detected in the ERG16 and the THR1 gene sequences of isolate 1 (shown as different shadings in the top panel [labeled sensitive]). No allelic differences were detected in the two gene sequences in the resistant isolate (uniform shadings, bottom panel [labeled resistant]). Both alleles in the resistant isolate contain the R467K amino acid change (black box near the 3’ end of gene). The hypothetical intermediate (center panel) would contain the R467K amino acid change in only one of two alleles. Cells in which the loss of allelic variation occurred, selected for by an increased resistance to fluconazole as the result of the point mutation, would have the mutation in both alleles and coincidentally eliminate adjacent allelic differences.
The most significant changes are in the amount of ergosterol produced in the extracts (Fig. 2D). Ergosterol production in extracts from isolates 1 and 12 are decreased substantially with 0.1 \mu g/ml of fluconazole per ml (50% inhibition at 0.06 to 0.08 \mu g/ml), while ergosterol production in extracts from isolates 13 and 17 remains active with fluconazole at concentrations of >1 \mu g/ml (50% inhibition at 1.0 to 1.12 \mu g/ml). This demonstrates that the enzyme in isolates 13 and 17 is at least 12-fold more resistant to fluconazole than the enzyme from isolates 1 and 12. No changes in enzyme susceptibility were observed in the series other than the change observed between isolates 12 and 13 (37).

**Cloning of the promoter and termination regions of the ERG16 gene.** The regions 5′ and 3′ of the gene were cloned by inverted PCR (see Materials and Methods). The cloned promoter region extends for 1,285 nucleotides upstream of the start of the ERG16 coding region. Several potential TATA boxes that might function as part of a promoter were present within 200 nucleotides upstream of the ATG. The promoter

**RESULTS**

**Biochemical analysis of the Erg16p enzyme.** The target enzyme of the azole drugs, Erg16p, was tested biochemically for indications that it became less susceptible to fluconazole at some point in the series of 17 isolates. The enzymatic assay (3, 20) used cell extracts and labeled mevalonic acid, which is an early precursor of ergosterol (see Materials and Methods), to produce labeled ergosterol as well as intermediates, including squalene, squalene epoxide, and lanosterol.

All extracts behaved identically in the absence of fluconazole, producing roughly equivalent amounts of ergosterol per milligram of protein (37). At increasing doses of fluconazole (0.1 to 1.0 \mu g/ml), differences between the isolates were observed (Fig. 2). Increasing concentrations of fluconazole did not affect the production of squalene or squalene epoxide (Fig. 2A and B). The concentrations of lanosterol were increased slightly, most likely as the result of the inhibition of Erg16p and the accumulation of precursors including lanosterol (Fig. 2C).

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PCR-SSCP analysis. Four sequence differences were identified. A single nucleotide change occurred at position 1547; a G in isolate 1 was changed to an A in isolate 17. This transition resulted in an Arg-to-Lys amino acid substitution at position 467 in the Erg16p protein (denoted R467K). Two sequence positions were identified where a heterogeneity was identified in isolate 1, most likely the result of differences between the alleles of the ERG16 gene in the diploid genome. Both heterogeneities were resolved in isolate 17 and neither of these two heterogeneities had any effect on the amino acid sequence. Finally, a single base change was identified in the 3′ untranslated region of the ERG16 gene. Since the same nucleotide differs from the published sequence in both isolates 1 and 17, this difference is most likely the result of strain differences between the published sequence and the series under analysis.

The only difference in the DNA sequence that had an effect on the amino acid sequence was the single amino acid substitution R467K. This amino acid change occurred in a conserved region of the protein (Fig. 4). This region of the Erg16p protein was highly conserved even between mammals and yeasts. Two residues surrounding R467K were noteworthy: the Cys three amino acids downstream of R467K that coordinates the fifth position of the iron atom in the heme (24) and the Phe four amino acids upstream of R467K that is important for interactions with the heme (23). Therefore, R467K was closely linked to amino acids that interact with the heme cofactor, an essential part of the active site of the Erg16p enzyme.

To confirm the sequence analysis of PCR fragments, restriction enzyme digestion of genomic DNA was used to monitor

| Position* | Nucleotide(s) | Amino acid |
|-----------|--------------|------------|
| 1547      | G            | Arg        |
| 1587      | A            | Leu        |
| 1617      | T            | Asn        |
| 1767      | T            | Untranslated |

*Positions (in nucleotides) are based on the published sequence (16). Standard numbering based on the A of the ATG start codon is lower by 146 nucleotides. 

**—**, no change from published sequence.

**Homo sapiens**

Rattus sp.

Ustilago maydis

S. cerevisiae

C. tropicalis

C. glabrata

C. krusei

C. albicans

**Resistant mutant**

**FIG. 4.** Alignment of Erg16 amino acid sequences from yeasts and mammals. The region surrounding the R467K amino acid substitution is presented for known Erg16 sequences and the resistant mutant. Residues in boldface type include F463 (thought to be important in interaction with the heme [23]), R467K, C470 (which coordinates the fifth position of the iron atom [24]), and I471T (a position at which a Thr is found in the two intrinsically resistant species, C. glabrata and C. krusei [5]). The amino acid residues are translations of cloned genes. The sequence sources and GenBank reference numbers are as follows: Homo sapiens, D55653; Rattus sp., D55681; U. maydis, Z48186; S. cerevisiae, M15109; C. tropicalis, M23673; C. glabrata, S75389; C. krusei, S75391; and C. albicans, X13296.
specific nucleotides. Fortuitously, the R467K mutation destroyed an AccI restriction enzyme recognition site. Similarly, restriction enzyme sites were present at the two heterogeneities, positions 1587 and 1617, such that only one of the two sequences at each position was digested with the enzyme (MunI at position 1587 and AflII at position 1617). Genomic DNA from the series was digested with PstI and with AccI, MunI, or AflII (Fig. 1A). Southern blots of these digests were probed with fragment 6. In each case, the restriction enzymes confirmed the sequence analysis and demonstrated that the change in pattern occurred between isolates 12 and 13 (37). Fragment PE (see Fig. 1A) was selected for further analysis since it is most likely to include regions of the promoter for the ERG16 gene. The PCR fragment was sequenced from both directions. A portion of this sequence (136 nucleotides) that was identical to that of the cloned promoter fragment, which was from a different strain of C. albicans (see Materials and Methods). Partial sequence analysis of fragments PA and TC showed similar losses of allelic variation (37).

**DISCUSSION**

In the present study, the target enzyme of the azole drugs (Erg16p) has been investigated by both biochemical and molecular techniques on a series of clinical isolates that developed azole resistance. The Erg16p enzymatic activity became more azole resistant, the Erg16p gene (ERG16) acquired an amino acid substitution, and allelic variation in the vicinity of the ERG16 gene was lost. All of these changes occurred between isolates 12 and 13 in the series, such that isolates 13 to 17 exhibit all of these characteristics. The R467K mutation is the first point mutation to be described in a clinical isolate of C. albicans that modifies the enzyme activity of Erg16p. Its contribution to the development of resistance in this series is currently being evaluated.

Cell extracts clearly showed that there is a 12-fold-reduced susceptibility of the enzyme in isolate 13 compared to that of the enzyme from isolate 12. The equivalent amounts of squalene, squalene epoxide, and lanosterol produced in the extracts indicate that other enzymes in the pathway were not appreciably affected by fluconazole (Fig. 2). Since the extracts used in this assay were cell free and unlikely to contain intact membranes, the azole-resistant activity seen in extract 13 (Fig. 2) cannot be attributed to efflux pumps in cell membranes.

Sequence of the ERG16 gene from isolates 1 and 17 identified a single point mutation (G to A, a transition) that resulted in a single amino acid substitution (R467K). This amino acid substitution resides in an important region of the ERG16 gene, between two residues known to be involved in interactions with the heme moiety in the active site of the enzyme. The conservative amino acid substitution (Arg to Lys) may have been sufficient to alter the azole-susceptibility of the active site, while not affecting the interactions of the enzyme with its normal cellular substrates. The R467K point mutation is unrelated to T315A, a mutation in C. albicans ERG16 that alters enzyme susceptibility and was created by site-directed mutagenesis based on knowledge of the active site of the enzyme (17). R467K in C. albicans is also unrelated to the well-characterized point mutation D310G in S. cerevisiae SG (11, 39). R467K is in a different region of the protein from D310G and T315A, although all of these mutations are in close proximity to the active site of the enzyme.

Allelic variation is observed for isolate 1 in all regions of the ERG16 gene, including the putative promoter and terminator, and even in the adjacent THR1 gene (Fig. 5 and 6; reference 37), as expected for a clinical isolate. These allelic variations are eliminated in isolate 13 in this series, indicating that a
major alteration occurred in this genomic region. The simplest explanation for this change is a mitotic recombination or gene conversion as diagrammed in Fig. 1B. In this model, the point mutation that causes the R467K substitution occurred in one of the two alleles of ERG16. This would most likely increase the azole resistance of that cell. A mitotic recombination or a gene conversion event would then create a cell with two copies of the point mutation in ERG16. The alteration would not be restricted to the R467K point mutation. Potentially, long regions of one allele could be transferred to the other allele, and this would account for the loss of genetic variation surrounding the ERG16 gene. Constant azole drug pressure in the patient would not cause the alteration but would select for the outgrowth of cells in which the event occurred. This hypothesis is based on the supposition that a cell with two copies of ERG16 containing R467K mutations is more resistant than a cell with only one copy of ERG16.

Mitotic recombination and gene conversion have been described previously for *C. albicans* (33–35) associated with protoplast fusions of two diploid cells, and subsequent UV-induced gene segregation. However, the current analysis is the first description of this type of event in a normal, nonirradiated diploid cell. From the limited data, it is impossible to predict which process was involved.

Several other possible, though unlikely, explanations for the loss of allelic variation can be suggested. A gene or chromosome deletion may have eliminated one copy of ERG16, or mating between two cells carrying one copy of the R467K allele could have occurred (both are highly unlikely). The possibility of a gene or chromosome deletion can be addressed using a genetic system with a dominant selectable marker such as IMP dehydrogenase as has recently been described (15).

The changes in the target enzyme that are detailed above are first detected in isolate 13 and persist in the five highly resistant isolates (13 to 17). Isolate 13 was obtained from the patient only 17 days after isolate 12 (26). It is unlikely that all of these changes occurred in a cell line that became the dominant strain in just 17 days. Unfortunately, only one isolate was stored from each patient sample, so it is impossible to determine if a subpopulation with these alterations was a significant fraction of cells at the time that isolate 12 was obtained. It is likely that the changes that have been identified accumulated over time in a subpopulation and that this subpopulation was not the dominant population until isolate 13.

The timing of all three changes is noteworthy. At the same time that the enzyme becomes more resistant to fluconazole, the R467K mutation appears in both alleles of the ERG16 gene and differences between the alleles are eliminated. This strongly suggests a correlation between the azole-resistant enzyme activity and the R467K mutation. Proof that the R467K mutation causes resistance will depend on a genetic analysis of the mutation in a defined laboratory strain in which other factors can be carefully controlled.

In addition to the genetic alterations associated with ERG16 described above, overexpression of the ERG16 gene has been recently documented by Northern blot analysis (36). This fourfold increase in mRNA levels also occurs between isolates 12 and 13. The increase in mRNA levels is unlikely to account for the resistant enzyme activity for several reasons. First, the 4-fold increase in mRNA levels is unlikely to account for a 12-fold increase in enzyme resistance. Second, studies with *Saccharomyces* show only modest increases in resistance associated with increased expression (6, 13). Finally, preliminary biochemical data shows that the protein levels and enzyme activity levels in the cell are the same in both susceptible and resistant isolates (12).

Elegant molecular and genetic studies have shown that two types of efflux pumps are important in azole-resistant clinical isolates of *Candida* (1, 31, 32). Recent Northern blot analysis has shown that these two types of pump are overexpressed in this series but at points in the series different from those of the changes described above (36).

The R467K mutation and the loss of allelic variation described above clearly result in an azole-resistant enzyme. This azole-resistant enzyme is likely to contribute to the increased MICs that are observed for isolates 13 to 17. However, an exact correlation is not likely, since many other factors may contribute to the overall MICs. The overexpression of two types of efflux pumps and ERG16 itself has been shown to correlate with the MICs in the series (36). It is unlikely that the effects of overexpression of an efflux pump and the development of an azole-resistant enzyme are significant for the MIC for the yeast. In addition, it is possible that other factors not yet identified also contribute to resistance in these isolates.

As identified in this study, the R467K substitution and the loss of allelic variation in the ERG16 gene locus are the first molecular characterizations of naturally occurring point mutations in the *C. albicans* genome that are associated with azole resistance, and the R467K substitution is only the second mutation identified in the ERG16 gene of *C. albicans* that alters enzyme susceptibility. It will be useful to identify other mutations in ERG16 and in other genes that may be important in antifungal resistance. With an inventory of possible resistance genotypes, it will be possible to assess the overall importance of gene mutations and overexpression in the azole-resistant strains of *C. albicans* that are arising in the HIV-infected population.

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