Induction of extracellular proteinase in *Candida albicans*

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Pulse-chase experiments indicated that the extracellular proteinase (EPR) of *Candida albicans* originates as a 45 kDa precursor protein which is processed to a 43 kDa protein prior to secretion. Secretion was routinely stimulated in EPR induction medium which contains bovine serum albumin (BSA) and glucose. Although EPR was not induced without glucose as a carbon source, EPR secretion was induced without the addition of BSA or other nitrogen sources. Furthermore, it was shown that EPR production was not induced at pH > 6.0, irrespective of the presence of a nitrogen source. This suggests that medium pH may act directly upon EPR induction, and not as a secondary effect of the nitrogen supply from EPR-mediated protein digestion, which exhibited a pH optimum of around pH 3.5. When germ tube induced cells were transferred to EPR induction medium, EPR was not induced. Thus, EPR production and germ tube formation may not be induced by the same conditions. We speculate that EPR production and germ tube formation do not co-operate in the invasive process but play different and separate roles.

Introduction

The medically important *Candida* yeasts, *C. albicans*, *C. tropicalis* and *C. parapsilosis*, secrete an extracellular aspartic proteinase (EPR). *C. albicans* is most frequently isolated as a yeast pathogen in humans (Odds, 1988b). Titres of antibody against EPR occur more often in patients who are *C. albicans*-positive than in negative patients and healthy people (Ishiguro *et al.*, 1992a; Macdonald & Odds, 1980). However, EPR antibody titres are not useful in the diagnosis of candidiasis (actual infections of *C. albicans*), probably because a fairly high frequency of healthy people also have anti-EPR antibodies and *C. albicans* is commensal in the human mouth, vaginal mucosae and gastrointestinal tract. EPR is thought to be a virulence factor (Odds, 1988c; Cutler, 1991). Although its pathological role is not completely clear, it is believed to play an important role during invasive hyphal growth of *C. albicans*. When the fungal cells invade host tissue, they must overcome surface barriers such as skin or mucosa. It has been suggested that EPR secreted by yeast forms of *C. albicans* may degrade surface barrier proteins prior to hyphal form-

ation and the deeper invasion into host tissues (Borg & Ruchel, 1988; Ray & Payne, 1988; Wingard *et al.*, 1982). EPR may be secreted at hyphal tips and thus assist in tissue invasion, but there is no direct evidence supporting this suggestion.

C. albicans is able to grow in media containing protein as a sole nitrogen source because EPR hydrolyses the protein. EPR digests various substrates, such as bovine haemoglobin, BSA, human stratum corneum and others (Negi *et al.*, 1984). It is induced in minimal medium containing protein as a sole nitrogen source (EPR induction medium); however, low molecular mass nitrogen sources, such as amino acids or ammonium sulphate, repress EPR production (Banerjee *et al.*, 1991; Homma *et al.*, 1992; Ross *et al.*, 1990). Glucose also affects the degree of EPR production (Crandall & Edwards, 1987; Samaranayake *et al.*, 1984).

The pH optimum for EPR activity is between 3.5 and 4.0 (Germaine & Tellefson, 1981; Hattori *et al.*, 1984; Remold *et al.*, 1968; Shimizu *et al.*, 1987). Although *C. albicans* can grow at pH 3–7 in Sabouraud broth, it cannot grow at pH 6 or higher in buffered EPR induction medium (Germaine & Tellefson, 1981; Matsuda, 1986). The lack of EPR production in media at neutral or basic pH results in a growth defect because nitrogen cannot be supplied by protein degradation. However, it is not clear whether pH directly affects EPR production. During cell growth, the medium pH changes from an initial pH of

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about 3.0 to 7.0 to about pH 3.5 at late exponential phase and then rises to about pH 7.0 in the stationary phase in a weakly-buffered medium (Matsuda, 1986).

C. albicans EPR is a 43 kDa protein (Remold *et al.*, 1968; Shimizu *et al.*, 1987). The 43 kDa protein and a 45 kDa form, which cross-reacts with anti-EPR antibody, are detected in the intracellular space and can be recovered in different membrane fractions (Homma *et al.*, 1992). The 43 kDa protein does not appear to be anchored to the membrane structure and is easily released from such fractions, as would be expected for a secreted enzyme. The intracellular 43 kDa protein was assumed to be the same as the extracellular EPR by the identity of their molecular masses. The 45 kDa protein, called CRM-EPR, is assumed to be a precursor of EPR (Homma *et al.*, 1992). A 54 kDa protein, immunologically detected in translation products of RNA prepared from EPR-induced cells (Banerjee *et al.*, 1991), may also be a precursor protein.

In this study, by using a system developed previously to detect intracellular forms of EPR (Homma *et al.*, 1992), we demonstrate that EPR is produced as a 45 kDa precursor protein, is processed to the 43 kDa form, and excreted into the extracellular space. Furthermore, we examine conditions required for EPR induction by monitoring both extracellular and intracellular EPRs.

Methods

Strains. The strains of *C. albicans* used were C9 (Homma *et al.*, 1992; Iwaguchi *et al.*, 1990) and FC18 (Iwaguchi *et al.*, 1990). FC18 strain forms germ tubes efficiently.

Materials. Proteins were radiolabelled using Tran³⁵S-label (ICN Biomedicals).

Media. The media used were YPD broth, YNB medium, EPR induction medium, and EPR non-induction medium. EPR induction medium was composed of 2% (w/v) glucose and 0.2% BSA (Sigma) in YNB medium which is a basic medium without carbon and nitrogen sources. The EPR non-induction medium was used as a control medium. It contained 0.1 M-ammonium tartrate in the EPR induction medium to suppress EPR production. All the media have been described previously (Homma *et al.*, 1992).

Electrophoresis and immunoblotting. SDS-PAGE and immunoblotting were performed as described by Ishiguro *et al.* (1992b). Samples for SDS-PAGE were prepared by mixing 50 µl protein solution with 10 µl 6 × TDG buffer [0.4 M-Tris/HCl, pH 6.8, 6% (w/v) SDS, 38% (w/v) glycerol, 0.006% bromophenol blue] and 5 µl 2-mercaptoethanol. The sample solutions were heated at 100 °C for 5 min before being loaded on to the gels.

Anti-EPR serum. Anti-EPR rabbit serum was prepared as described previously (Homma *et al.*, 1992) and was stored at -80 °C.

Immunoprecipitation of proteins reacting with antibody. Cells were boiled in 1% (w/v) SDS and the suspension was diluted 10-fold with TNET buffer (50 mM Tris/HCl, pH 7.8, 0.15 M-NaCl, 5 mM-EDTA, 1% (w/v) Triton X-100). Immunoprecipitation using protein A-Sepharose CL-4B was done as described previously (Homma *et al.*, 1985).

Induction of EPR. Induction of EPR was done as described previously (Homma *et al.*, 1992) with the following modifications. Cells were grown to exponential phase ($OD_{660} \approx 1.0$) in YPD broth containing 1.1% (w/v) Casamino acids (Difco) with shaking at 37 °C. They were washed with distilled water, suspended in EPR induction medium (or in control medium) to give an OD_{660} of 1 or 5, and incubated with shaking at 37 °C. After further incubation, the cells were sedimented by centrifugation, and both the resulting supernatant and the pellet (suspended in 10 mM-NaN₃ to give $OD_{660} = 30$ or 50) were analysed by immunoblotting for extracellular and intracellular fractions, respectively.

Induction of germ tube formation. Cells were grown to exponential phase as above for the induction of EPR and were collected by centrifugation. Cells were starved for 2 h in EPR induction medium without BSA and glucose or in distilled water. This treatment potentiates germ tube formation. The starved cells were collected by centrifugation, and resuspended in distilled water to $OD_{660} = 30$. The cell suspension was diluted 50-fold into EPR non-induction medium containing 10% (v/v) bovine calf serum and incubated at 37 °C for 1 h.

Results

Pulse-chase experiment

We detected two intracellular forms of EPR in cells induced to produce it: a 43 kDa protein (EPR) and a 45 kDa protein (cross-reacting material of EPR; CRM-EPR) (Homma *et al.*, 1992). To test whether CRM-EPR could be a precursor of EPR, we carried out a pulse-chase experiment (Fig. 1). Radiolabelled intracellular CRM-EPR (pre-EPR) was detected first. Intracellular

Fig. 1. Pulse-chase experiment. Cells (strain C9) were grown to exponential phase prior to EPR induction and were then suspended in EPR induction medium, incubated at 37 °C for 1 h 45 min with shaking. A portion (10 µl) of ³⁵S protein labelling mix (4 MBq) was mixed with 500 µl cell suspension and incubated for 1 min. Chase solution (10 µl, 8 mM-methionine, 2 mM-cysteine) was then added. After 0.5, 1, 2, 4 and 8 min, the medium was directly analysed by SDS-PAGE (b). Cellular proteins were immunoprecipitated using anti-EPR antiserum, and then analysed by SDS-PAGE (a). Arrowheads show the position of EPR (43 kDa) and of pre-EPR (45 kDa).

Fig. 2. SDS-PAGE to detect EPR in culture medium with various concentrations of Casamino acids. Stationary cultures (20 μ l) of strain C9 were inoculated into EPR induction media (2 ml) containing a series of dilutions of 1.1% Casamino acids or no Casamino acids (none). After incubation at 37 °C for 2 d, cells were removed by centrifugation, the culture media were analysed by SDS-PAGE, and proteins were stained by CBB-R250. Arrowheads on the right and left sides show the positions of EPR and BSA, respectively.

EPR was detected only after 2 min incubation, and by 4 min the radiolabel had shifted to extracellular EPR. Almost all the radiolabel was finally recovered as extracellular EPR after 8 min. This shows that CRM-EPR was a precursor form of EPR. CRM-EPR was therefore renamed pre-EPR.

Effect of a low molecular mass nitrogen source upon EPR secretion

This was determined by varying the concentration of additional Casamino acids in the EPR induction medium. At all concentrations used, the cells achieved stationary phase within 2 d at 37 °C. Culture medium, analysed by SDS-PAGE (Fig. 2), revealed that EPR was not detected and BSA was not degraded in media containing 1.1% (w/v) or 0.55% Casamino acids. At 0.275% Casamino acids, EPR secretion was detectable and BSA was degraded, but the amount of the EPR secretion was maximal in media containing 0.138% Casamino acids or less. If 110 Da is used as the average molecular mass of amino acids, EPR induction is prevented by the addition of greater than 12.5 mM-amino acids (equivalent to 0.138%). Other low molecular mass nitrogen compounds, such as glycine, glutamic acid,

urea, ammonium tartrate, and ammonium sulphate also gave EPR repression at similar concentrations (data not shown).

Nitrogen and carbon sources required for EPR induction

To investigate the metabolic requirements of EPR induction, exponential phase cells were transferred into EPR induction medium without BSA, without glucose, or lacking both, and incubated for 2 h. The cells and culture medium were then separated by centrifugation. Samples of the cell suspension and the medium were analysed by electrophoresis and immunoblotting, using an anti-EPR antibody. EPR was detected at reduced levels without BSA (Fig. 3, lane 2), but little was detected when either glucose or both BSA and glucose were omitted (Fig. 3, lanes 1 and 3). This suggests that the presence of glucose in the media is essential for EPR induction, whereas proteins are not required. We did not examine whether other carbon sources act like glucose. The two bands above pre-EPR represent non-specific reaction against anti-EPR antibody (Homma *et al.*, 1992).

The effect of a low molecular mass nitrogen supply was examined further. After cells were incubated in EPR induction medium without BSA for 1 h, various concentrations of Casamino acids were added to the medium. After an additional 1 h incubation, EPR was detected (Fig. 4). At approximately 3.1 mM-amino acids final concentration, which is equivalent to 0.034%, maximal intracellular EPR and pre-EPR were detected from the band intensities. On the other hand, extracellular EPR levels were elevated at higher concentrations of Casamino acids.

Growth phase and pH effects of EPR induction

After cells (strain FC18) were transferred to EPR induction medium, EPR was detected within 30 min, and the pH of the culture medium gradually decreased with the cell growth (Fig. 5c, d). Intracellular amounts of EPR and pre-EPR reached their maximum per unit cell density after 5–7 h incubation as extracellular EPR accumulated (Fig. 5a, b). When maximal levels of EPR were produced, the pH of the medium was about 3.5–4.0, and OD₆₆₀ of the culture had reached about 6–8. The immunoreactive bands appearing later are probably degradation products of EPR. Another *C. albicans* strain, C9, produced more EPR than FC18 and maximal EPR levels were detected 2–3 h earlier (data not shown).

The effect of the starting pH of the EPR induction media was determined by adjusting the pH of the induction medium by addition of NaOH or HCl (Fig. 6a). Incubation was initially at OD₆₆₀ = 1 and pH values

Fig. 3. Immunoblotting to investigate culture nutrient requirements for EPR induction. Cells (strain C9) of an exponential phase culture were suspended in EPR induction medium without both BSA and glucose (lane 1), without BSA (lane 2), without glucose (lane 3) or lacking neither (lane 4). After 2 h incubation, cellular proteins (a) and culture medium (b) were solubilized by boiling in TDG buffer, analysed by electrophoresis, and immunoblotted using anti-EPR antiserum.

Fig. 4. Effect of Casamino acids concentration on EPR production. Cells (strain C9) of exponential phase culture were suspended in EPR induction medium without BSA. After 1 h incubation, supplements were added to a series of dilutions of 1.1% Casamino acids, no Casamino acids (none), and 0.2% BSA (+BSA). After an additional 1 h, cells and culture medium were analysed by immunoblotting as described in the legend to Fig. 3.

of 2, 3, 4, 5 and 6. After 2 h incubation, OD_{660} had increased to 2.5, 2.5, 3.2, 3.7 and 3.1, respectively. Maximal EPR levels were seen when the starting pH was 4.0, whilst little EPR was detected when the starting pH was 6.0. This repression may be directly or indirectly due to the pH, as EPR is inactive at pH 6 (Germaine & Tellefson, 1981; Hattori *et al.*, 1984; Remold *et al.*, 1968; Shimizu *et al.*, 1987), so the available nitrogen source for protein synthesis is not supplied. To resolve this issue, the pH effect was investigated under EPR induction conditions in the absence of BSA (Fig. 6b). Although maximal levels of EPR were detected at pH 5.0 or 5.5,

Fig. 5. pH changes and EPR induction during growth. Cells (strain FC18) of an exponential phase culture were suspended in EPR induction medium. At various times, cells (a) and culture medium (b) were analysed by immunoblotting as described in the legend to Fig. 3 and the OD_{660} (c) and pH of the medium (d) were measured.

little EPR was produced above pH 6.0. This suggests that high pH may directly repress EPR induction.

EPR induction during germ tube formation

It has been suggested that EPR could assist germ tube invasion by digesting the surrounding tissues. Germ tubes form preferentially above neutral pH (around pH 6.0) (Odds, 1985, 1988a); however, we have shown that EPR induction requires low pH. We therefore wanted to determine whether EPR is actually produced by germ tube cells. Starved cells (strain FC18) were

Fig. 6. Effects of medium pH on EPR induction. Cells (strain C9) of an exponential phase culture were suspended in EPR induction medium (a) and EPR induction medium without BSA (b), whose pH was adjusted by HCl or NaOH to the indicated value, at a cell density of $OD_{660} = 1.0$. The medium used in (b) was buffered with 25 mM-sodium citrate. After 2 h incubation at 37 °C, cells and culture medium were analysed by immunoblotting as described in the legend to Fig. 3.

incubated in EPR non-induction medium containing 10% bovine calf serum at 37 °C. After 1 h incubation, at least 80% of cells formed germ tubes and were transferred to EPR induction medium. When comparable amounts of germ tube and yeast cells were analysed by immunoblotting, EPR was not detected in germ tubes until yeast cells started to propagate (data not shown).

Discussion

The detection of intracellular EPR as well as extracellular EPR is essential to clarify the mechanisms underlying EPR induction. Without this information, it is impossible to distinguish between defects in transport and synthesis when EPR is not detected in the culture medium. By immunological detection we found two intracellular forms of EPR; a 43 kDa and a 45 kDa protein (Homma *et al.*, 1992). These were previously assumed to represent the mature extracellular EPR and a precursor form, respectively. In the studies reported here, we used a pulse-chase experiment to confirm this prediction. In this experiment, radiolabel of the intracellular 45 kDa protein clearly shifted to the intracellular 43 kDa protein, and

then to the extracellular 43 kDa protein. In addition to the 45 kDa precursor form, 54 kDa and 47 kDa immunologically related proteins have been reported. The 54 kDa protein, which was not detected in our previous study (Homma *et al.*, 1992) or the present report, was detected as a translation product from *C. albicans* mRNA *in vitro* (Banerjee *et al.*, 1991). The 47 kDa protein was copurified with 45 kDa pre-EPR in our previous study (Homma *et al.*, 1992). As secretory proteins are usually processed to a mature form by way of several intermediate precursor forms (Reid, 1991), the 54 kDa or the 47 kDa protein might be precursors for the 45 kDa pre-EPR. From the amino acid sequence deduced from the DNA sequence data (Hube *et al.*, 1991), the molecular masses of the unprocessed and mature proteins are estimated to be 41.7 and 36.1 kDa, respectively, provided that the proteins are not modified post-translationally by additions such as sugar residue addition. The difference between protein sizes estimated by SDS-PAGE and from DNA sequence data have not been clarified.

EPR production was repressed when the cells were grown in a medium containing more than about 12.5 mM-amino acids (Fig. 2). The actual concentration required for repression is probably lower than 12.5 mM, because they are catabolized and their concentrations thus decrease during cell growth. Other low molecular mass nitrogen sources also repress EPR production. This may show that its production is controlled by certain key compounds of nitrogen metabolism.

It has been supposed to date that proteins in the EPR induction medium are essential for EPR production. However, we found that EPR was produced and secreted into a medium that lacks protein and that EPR was not induced in a medium lacking glucose even in the presence of a protein source (Fig. 3). On the other hand, Crandall & Edwards (1987) reported that nitrogen starvation did not induce *C. albicans* EPR, leading them to suggest that proteins acted as both a substrate and an inducer of EPR. Our results do not support this possibility. Their conclusion was derived from evidence that EPR was not detected in a medium with glucose and without a nitrogen source after 4 d incubation. In that experiment, cells could not grow because of a lack of alternative nitrogen source; thus all protein synthesis decreased or stopped, and EPR production was inhibited. This interpretation is supported by the following arguments. When EPR-induced cells which had been incubated in a medium without nitrogen source or protein were transferred into a medium containing varying concentrations of Casamino acids, intracellular EPR and pre-EPR levels were maximal with 3.1 mM-amino acids, but extracellular levels were maximal at higher concentrations which repressed EPR production (Fig. 4). These results suggest that a high concentration of a low molecular mass

nitrogen source may activate protein synthesis but that excess nitrogen source may repress EPR induction. On the other hand, at lower concentrations of the nitrogen source (around 3.1 mM), protein synthesis was activated to some extent without the repression of EPR induction. EPR induction and repression seem to be regulated by a very delicate balance of nitrogen supply.

Nitrogen and carbon metabolism are interrelated and regulated in a complex manner (Marzluf, 1981; Wiame *et al.*, 1985). Glutamate and glutamine are amino donors in the flow of nitrogen into organic compounds, and glutamate dehydrogenase (GDH), which functions in the biosynthesis of glutamate from ammonia, is thought to play a pivotal role in nitrogen metabolism. Increasing amounts of inorganic nitrogen compounds repress yeast NADP-GDH activity (Bogonez *et al.*, 1985; Holmes *et al.*, 1989; Uno *et al.*, 1984) and there is a loss of this activity in medium lacking glucose (Mazon, 1978; Mazon & Hemmings, 1979). It will be interesting to determine whether the nitrogen-sensing system for GDH regulation is similar to that underlying EPR regulation.

During *C. albicans* culture, the pH of the medium falls in late exponential or early stationary phase, before returning to neutral values in late stationary phase (Mazon, 1978). Similar pH changes were observed in our EPR induction system with extracellular EPR accumulating during the cultivation. The highest intracellular EPR levels were detected at late exponential phase when the pH of the medium was around 3.5 (Fig. 5). The increase in intracellular EPR levels, normalized with respect to cell density, can be explained by either increased synthesis or decreased EPR secretion.

We examined the effect of medium pH on EPR synthesis. No induction was found in medium of neutral pH either with or without BSA, suggesting that EPR induction is directly affected by the pH of the medium (Fig. 6). The optimal initial pH was 4.0 for EPR induction medium with BSA, but 5.0 or 5.5 for EPR induction medium without BSA. This difference in pH optima between medium with and without BSA may be explained as follows. The optimal pH for EPR induction is probably around 5.0 or 5.5; however, cells grow more actively at around pH 4.0 in the presence of BSA because a nitrogen source is being supplied by the degradation of BSA by EPR, whose optimal pH is 3.5–4.0 (Germaine & Tellefson, 1981; Hattori *et al.*, 1984; Remold *et al.*, 1968; Shimizu *et al.*, 1987).

Transformation conditions of yeast and hyphal forms have been examined extensively (for review see Odds, 1985, 1988a). Medium pH is one of the most important factors in the dimorphic switch. Neutral pH is usually necessary for hyphal formation, but inhibits EPR induction. We therefore examined EPR production in germ-tube induced cells transferred to EPR induction

medium. No EPR was detected. This may show that EPR production and germ tube formation are not induced under the same conditions and that EPR is not always secreted at hyphal tips. Therefore, EPR secretion by hyphal cells may not be necessary for tissue invasion if EPR can be secreted prior to the formation of hyphae. This suggestion is consistent with the evidence that EPR-deficient strains cannot usually invade chick chorioallantoic membranes but are able to do so if the chorioallantoic membrane is treated with purified EPR (Kobayashi *et al.*, 1989; Shimizu *et al.*, 1987).

Based on our results and other information, a speculative model for *C. albicans* invasion is presented, although the real *in vivo* process is probably more complicated. First, a cell attaches to an epithelial layer. EPR is induced at an acidic pH, and environmental pH is changed to the optimal pH for EPR. Second, the secreted EPR digests the epithelial layer, and the environmental pH is changed to a more neutral pH. Finally, the cell germinates to give hyphal growth and invades beyond the epithelial layer.

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