

Characterization of a Human Chorionic Gonadotropin-Like Protein from *Candida albicans*

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ABSTRACT

Studies from our laboratory have demonstrated that human CG (hCG), human LH, (hLH), and an hCG-like protein extracted from *Xanthomonas maltophilia* were able to induce *Candida albicans* transition from the blastospore to the germ tube stage. In the present study, we describe the characterization of an hCG-like material extracted from *Candida albicans* blastospores (CaCGLP), which is potent in inducing transition and presumably represents the endogenous transition-inducing substance.

This material was extracted from *Candida albicans* blastospores with glacial acetic acid and purified by affinity chromatography using a polyclonal rabbit anti-hCG antibody. The product obtained is a 68-kilodalton single band protein, as analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and Western blot analysis. Under reduced conditions a protein smear is seen. Amino acid analysis showed a predominance of glycine (22%), followed by serine (12%), and glutamate (12%).

This protein reacted in the following hCG immunoassays: 1) a polyclonal rabbit anti-hCG equilibrium assay, 2) a carboxyl-tail hCG equilibrium assay, 3) two hCG equilibrium assays using monoclonal antibodies (CG no. 4 and CG no. 9), 4) a free α -subunit equilibrium assay using a monoclonal antibody, and 5) an ultrasensitive immunoradiometric assay for hCG which does not cross-react with hLH, nor the free β -subunit of hCG. The CaCGLP showed no reaction in a specific hLH immunoradiometric assay.

When CaCGLP was tested in the transition assay, in the presence of 4% rat serum, it was found that this protein was 100 times more potent than hCG in producing *Candida albicans* transition.

We conclude that *Candida albicans* produces a protein that has certain tertiary structure similarities to hCG and that this material is able to induce germ tube formation. We postulate that CaCGLP has an autocrine/paracrine effect in *Candida albicans* as a transition factor to control its own pathogenicity. (*Endocrinology* 132: 667-673, 1993)

HYPHAL forms of *Candida albicans* are known to be more associated with pathogenicity in humans and animal models than the yeast forms (1). Little is known about the biochemical and molecular basis of the *Candida albicans* transition from yeast to hyphae, and genes unique to either of the forms have not yet been isolated (2). However, environmental changes are well known to influence *Candida albicans* transition, and several enhancers and inhibitors of transition have already been described (1). Of particular interest is the description by Hazen and Cutler (3) of an autoregulatory substance produced by *Candida albicans*, called by these authors, morphogenic autoregulatory substance. This substance, produced by cultures of high concentrations of yeast (10^8 yeast/ml), was reported to inhibit *Candida albicans* transition. To our knowledge, only the activity of this substance, measured in culture media, was described; no further biochemical characterization has been published.

Recently we described (4) the effects of human CG (hCG), human LH (hLH) and an hCG-like material isolated from *Xanthomonas maltophilia* on *Candida albicans* transition. All three proteins stimulated transition with a minimal dose of 100 ng/ml, and also bound with high affinity nanomolar Kds to *Candida albicans* blastospores. Specific hCG and hLH binding, as well as the effects of hLH and steroids in the transition of *Candida albicans*, have also been previously published (5, 6). *Pseudomonas* CG is a protein of 48.5 kilo-

dalton (kDa) and exhibits immunologic cross-reaction to the β -subunit of hCG (7). This protein, when added to cultured *Xanthomonas maltophilia*, causes a dose-dependent change in both the morphology of the bacteria and the concentration of bacteria reached at the stationary phase of the growth cycle (Carrell, D. T., personal communication). The same bacteria possess a surface binding site which binds hCG, but not hLH (8), and this site is the only known high affinity binding site in nature which distinguishes hCG from hLH.

Other authors (9-14) reported, using immunological methods, the presence of CG-like substances on the surface of strains of *Streptococcus* species [American Type Culture Collection (ATCC) 12818], *Staphylococcus* species (ATCC 19433, 27848), *Enterococcus coli* (ATCC 25922), as well as *Pseudomonas maltophilia* transferred to the genus *Xanthomonas* as *Xanthomonas maltophilia* [ATCC (13637)]. These authors attempted to establish a link between human cancers and the production of CG-like substances by bacteria, since these bacteria were obtained from patients with cancer.

The studies reported herein show the characterization of a protein that exhibits immunological cross-reactivity with hCG and is a potent stimulator of *Candida albicans* transition. We have named this substance *Candida albicans* CG-like protein (CaCGLP). These findings lead us to conclude that *Candida albicans* produces a protein that modulates its own pathogenicity.

Materials and Methods

Hormone preparations

The purified preparations of the hormones used, all obtained from the National Hormone and Pituitary Program of the NIH, were as

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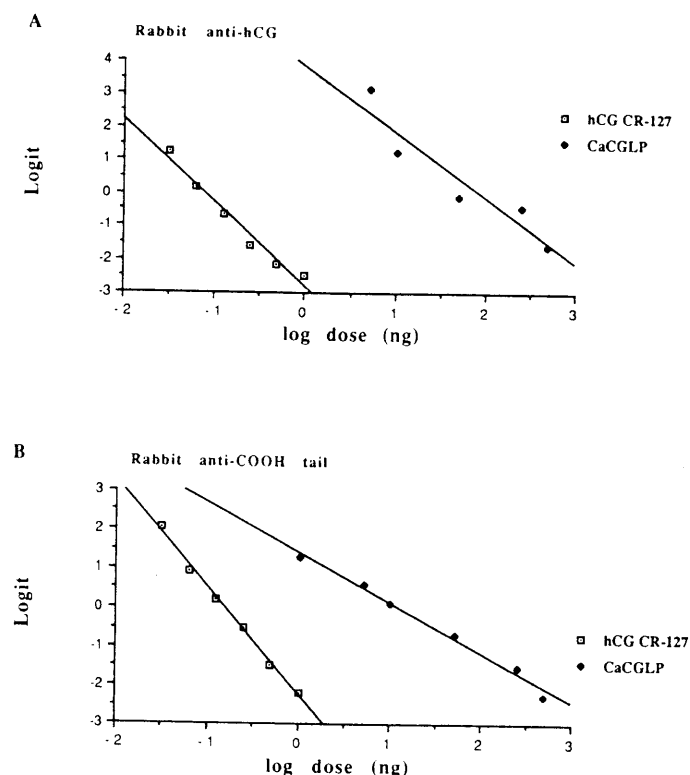


FIG. 1. Dose-response curves for purified hCG (CR 127) and for CaCGLP. Different concentrations of the CaCGLP preparation were used (1–500 ng/tube). A, Polyclonal antibody rabbit anti-hCG; B, polyclonal antibody rabbit anti-hCG carboxyl tail. The logit/log dose graphs are shown. The slopes of the lines and the cross-reactivity data are described in the text.

follows: hCG (CR 127); hLH-B-1 (AFP 0642-B); and free α -subunit of hCG (CR 123).

Assays

Two polyclonal and six monoclonal antibodies were used in our studies. The polyclonal antibodies were: 1) rabbit anti-hCG (1:500,000 final dilution), which has been used for many years in our laboratory and reacts equally well with intact hCG, hLH, and the isolated β -subunit of hCG; and 2) rabbit anti-hCG carboxyl-tail [1:10,000 final dilution; the carboxyl-tail RIA has been previously described by Matsuura *et al.* (15)].

The mouse monoclonal antibodies (mAb), all developed in our laboratory, were: 1) mAb anti-CG no. 4 (1:10,000 final dilution), which is an immunoglobulin G₁ (IgG₁) that reacts with both intact hCG and isolated β -subunit, but does not react with the free α -subunit of hCG, intact hLH, or the free carboxyl-tail of hCG and appears to be directed toward hCG-specific epitopes on the body of β hCG subunit [this antibody has been described previously (16)]; 2) mAb anti-CG no. 9 (1:10,000 final dilution), which is also an IgG₁ antibody and does not react with the free α - or β -subunit of hCG, but does react with the intact hCG molecule and intact hLH; we hypothesize that this antibody is directed against epitopes of intact hCG shared by hLH [this antibody has also been previously described (16)]; 3) mAb anti-CG no. 21 (1:10,000 final dilution) shows reaction with the β -subunit of hCG and intact hCG, but no reaction with hLH, hFSH, hTSH, or the α -subunits of the pituitary glycoproteins [this antibody has been partially described previously (16)]; 4) mAb anti-CG α , which was used at a final dilution of 1:10,000 and shows 1.6% cross-reaction with intact hCG, about 1% cross-reaction with intact hFSH, hLH, and hTSH, and no cross-reaction with the free β -subunit of hCG [previously described (17)]; 5) mAb anti-hLH no. 1, which reacts with intact hLH and hCG and with the free α -subunit [previously described (18)]; and 6) mAb anti-hLH no. 12, which

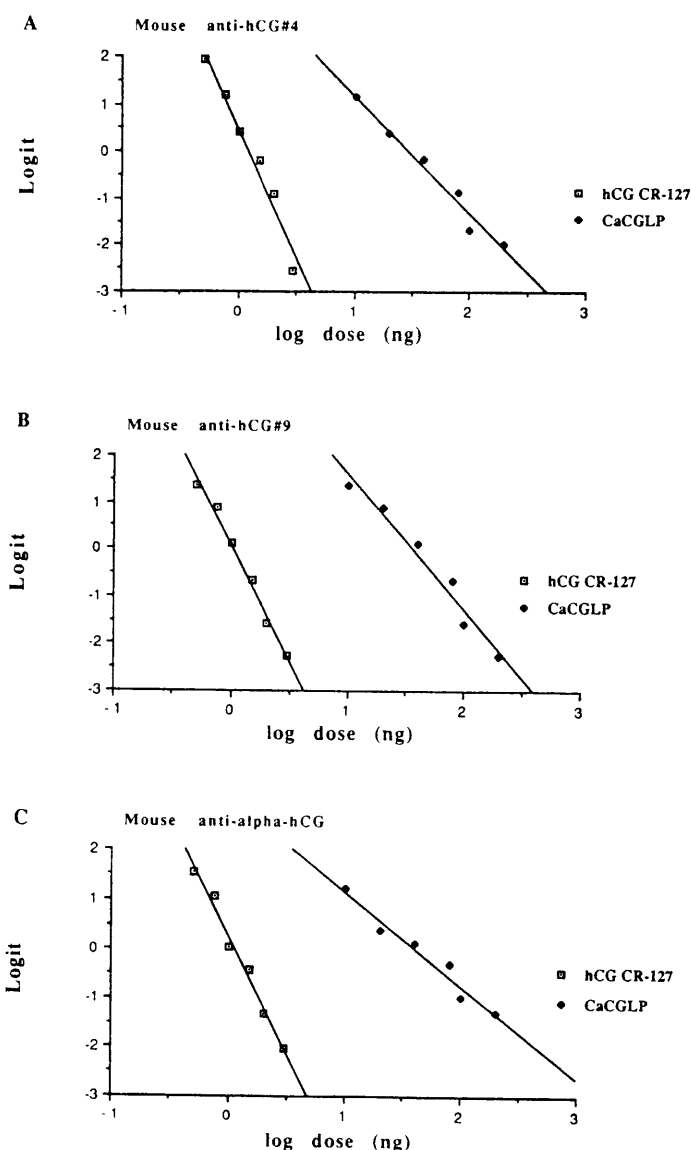


FIG. 2. Equilibrium assays with monoclonal antibodies. Dose-response curves made using hCG (CR 127) and different amounts of CaCGLP (10–200 ng/tube). A, Monoclonal mouse anti-CG no. 4; B, monoclonal mouse anti-CG no. 9; C, monoclonal mouse anti-CG α -subunit. The results using the antibody mouse anti-CG no. 21 are not shown. This antibody did not react with CaCGLP. Values of the slopes and cross-reactivity are described in the text.

reacts with hLH and α -subunit [also previously described (18)].

The mAb anti-hLH no. 1 and mAb anti-hLH no. 12 were only used for the specific hLH immunoradiometric assay (IRMA).

All equilibrium-type RIAs were conventional (19) and used ¹²⁵I-hCG (CR 127) with a specific activity of 80–100 μ Ci/ μ g. Radioiodinated hCG was prepared using the chloramine-T method, as modified in our laboratory (20).

The IRMA for hCG has been described in detail previously (16). In summary, two antibodies were used in a sandwich assay, using an avidin-biotin-polystyrene separation technique. The antibodies react in solution with the hCG to be quantified, and the sandwich containing the two antibodies and hCG is removed by means of an avidin bridge joining the biotin antibody to the avidin conjugated to polystyrene. The assay was performed in 12 by 75-mm glass test tubes with the following conditions: 100 μ l sample to be assayed, an 8-mm polystyrene ball coated with avidin, and 100 μ l buffer containing 1% normal mouse

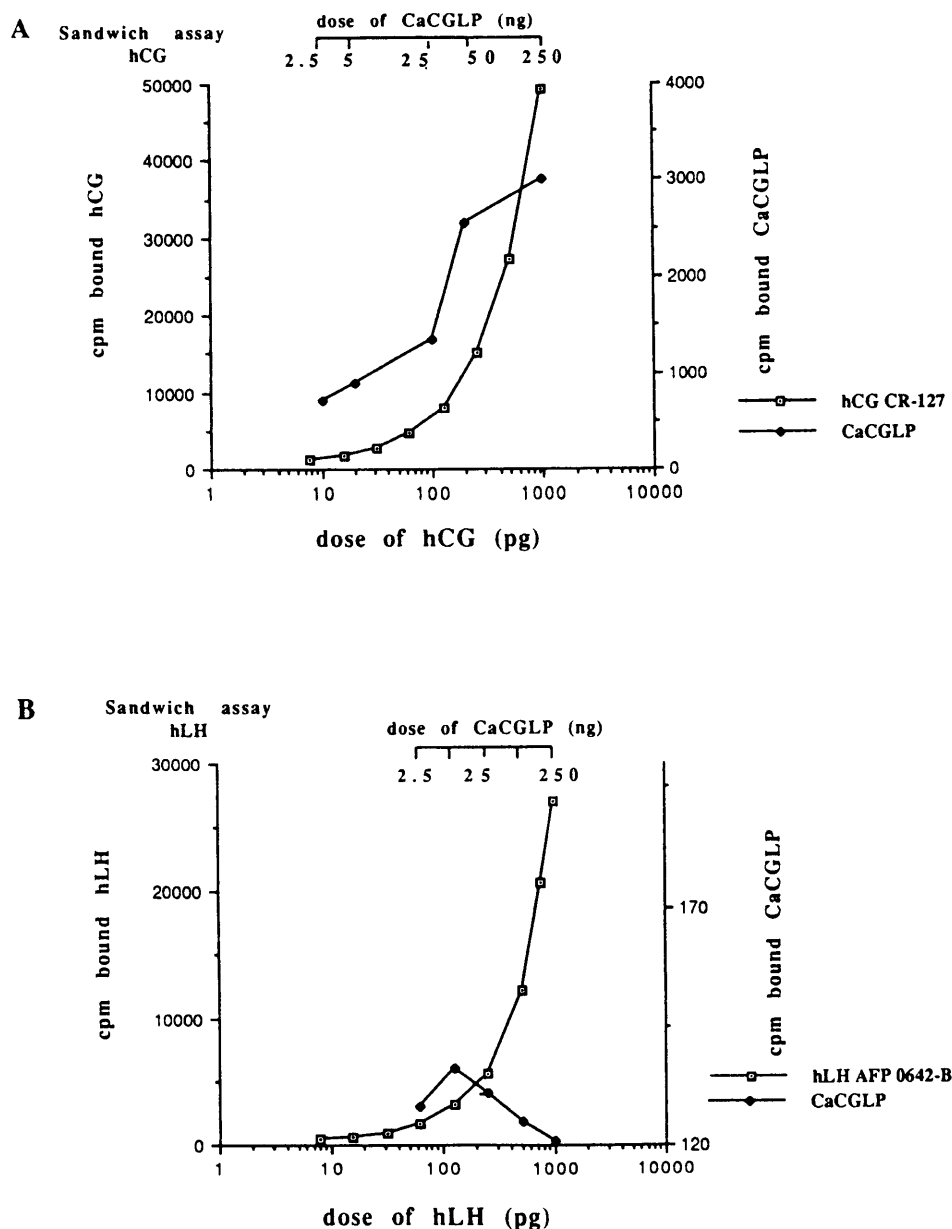


FIG. 3. IRMA. A, hCG; B, hLH. In these ultraspecific double-antibody sandwich assays, hCG (CR 127), hLH (AFP 0642-B), and immunopurified CaCGLP were used. Different amounts of CaCGLP were used, ranging from 2.5–250 ng. The *Candida albicans* CG material was only recognized by the hCG assay (A). The cross-reactivity is small (0.024%), but the sensitivity of the assay is enough to measure CaCGLP in a dose-response manner.

serum and 0.5% BSA containing radioiodinated mAb no. 9 (300,000 cpm) and 0.5 μ g biotinylated mAb no. 4. The tubes were incubated overnight at room temperature and the polystyrene ball was washed four times and counted for radioactivity in a γ -spectrophotometer (Packard, Cobra). This is an ultrasensitive IRMA for whole hCG, which does not cross-react with hLH or the free β -subunit of hCG.

The specific IRMA for hLH was also described elsewhere (18). This assay uses mAb anti-hLH no. 1 and mAb anti-hLH no. 12, and the conditions are the same as described above.

Yeast cultures

Two strains of *Candida albicans* were used: 1) β 311-a, ATCC 32354, and 2) F-16, from a patient with acquired immunodeficiency syndrome obtained from the Associated Regional University Pathologists, a commercial laboratory. Yeast were subcultured from the stock culture in liquid media enriched with 12.6 g synthetic complete powder [uracil, 597 mg; L-histidine-HCl, 597 mg; L-arginine, 597 mg; L-methionine, 597 mg; L-leucine, 896 mg; L-isoleucine, 896 mg; L-lysine-HCl, 896 mg; L-phenylalanine, 1.493 g; L-glutamic acid, 2.985 g; L-valine, 4.478 g; L-

serine, 11.194 g; tryptophan, 19.98 mg; adenine, 19.98 mg; tyrosine, 29.97 mg; threonine, 199.15 mg; aspartate, 99.97 mg; and glucose, 19.99 g (all obtained from Sigma Chemical Co., St. Louis, MO)], 200 g yeast nitrogen base without amino acids (Difco Laboratories, Detroit, MI)/1 liter double distilled water.

Protease activity of the *Candida albicans* extracted material

Since proteases may cause degradation of ^{125} I-labeled hormones to produce interference in equilibrium-type RIAs, the extracted material was assessed for the presence of proteases in two ways: 1) Protease-casein substrate gel tablets (Bio-Rad, Richmond, CA) were used to assess for the presence of proteolysis. The protocol of Bio-Rad was followed and no proteolysis was detected; 2) The extracted material was incubated under assay conditions with ^{125} I-labeled hCG. Total immunoprecipitable ^{125} I-hCG was assessed at the beginning and the end of the incubation to determine the difference in immunoactivity. For these studies, polyclonal rabbit anti-hCG was used in excess (1:1,000 final dilution) and ^{125} I-hCG showed no loss of immunoactivity at the end of the incubation time.

TABLE 1. Amino acid composition of the *Candida albicans* CaCGLP and hCG

	CaCGLP (%)	hCG (%)
Aspartate	10.21	8.0
Threonine	2.96	7.4
Serine	12.28	8.9
Glutamate	12.82	7.9
Proline	4.73	12.2
Glycine	22.6	5.6
Alanine	6.22	5.7
Valine	3.5	7.4
Methionine	1.01	1.4
Isoleucine	2.45	2.5
Leucine	4.57	6.6
Tyrosine	2.03	3.0
Phenylalanine	1.97	2.8
Histidine	3.18	1.9
Lysine	5.99	4.4
Arginine	3.46	6.6

hCG amino acid analysis as published elsewhere (24). The CaCGLP was resolved on a Beckman 6500 analyzer.

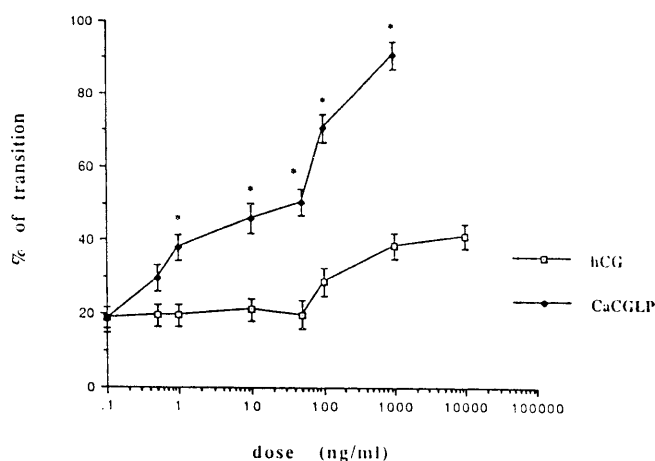


FIG. 4. Dose response of hCG and *Candida albicans* CG on *Candida albicans* transition. Human CG induced transition at a minimal dose of 100 ng/ml. The smallest effective dose of CaCGLP is 1 ng/ml. Asterisks show significant differences ($P < 0.05$) between groups. Bars represent SD of the triplicates analyzed.

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) studies

Approximately 2 μ g purified CaCGLP (method below) were analyzed by 12.5% SDS-PAGE in a Pharmacia LKB-Phast System. Markers with a range of 14,300–200,000 kDa (purchased from Bio-Rad) were also run in each gel. Reduced and nonreduced samples were analyzed. The gels were stained with Coomassie blue R-350, and if the protein was not visible, further staining was done with an ultrasensitive silver technique for the LKB-Phast System (21).

For the Western blot studies, 10 μ g CaCGLP and unstained markers were run in a 12.5% polyacrylamide gel under nonreduced and reduced conditions with SDS. The gel was transblotted onto a nitrocellulose membrane (Amersham, UK) using the LKB-Phast System blotting apparatus. The antibodies used were mAb anti-CG no. 4 (final dilution 1:1,000), and ^{125} I-rabbit antimouse antibody (4×10^5 cpm/ml). The membrane was exposed to an x-ray film (Kodak, OMAT AR) for 24 h.

Amino acid analysis

Amino acid analysis was performed after hydrolysis of 10 μ g protein in 5.7 N HCl containing 0.1% phenol for 24 h *in vacuo*. The resulting

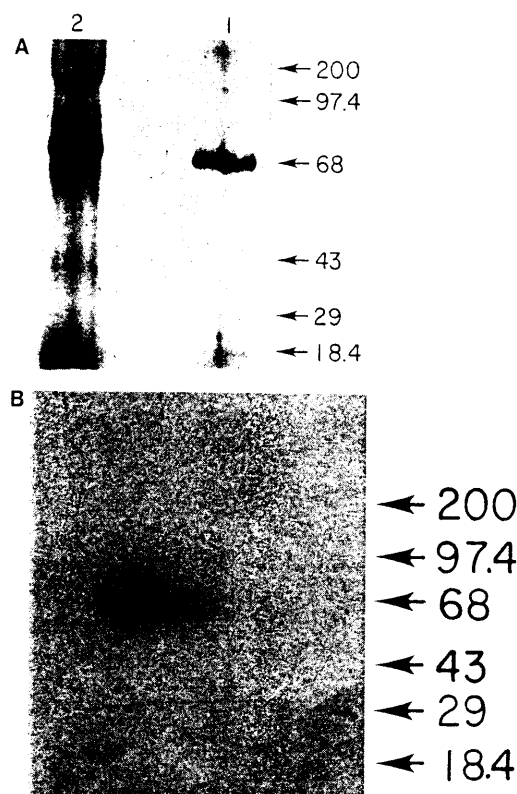


FIG. 5. Gel electrophoresis and Western blotting of the *Candida albicans* CG. A, SDS-PAGE 12.5% silver stained. The purified CaCGLP without mercaptoethanol (nonreduced) shows a band at about 68 kDa (lane 1). In the presence of mercaptoethanol (reduced), a smear is seen (lane 2). Both lanes were loaded with the same amount of CaCGLP (2 μ g); B, Western blot analysis of the CaCGLP under nonreduced conditions shows a single reactive band at 68 kDa. The primary antibody was monoclonal anti-CG no. 4 and the second antibody was ^{125} I-rabbit antimouse. The markers were run alongside and transblotted together with the CaCGLP to the nitrocellulose membrane and used as negative controls. The reduced material did not react in the Western blot.

amino acids were resolved on a Beckman 6500 analyzer with Ninhydrin detection.

Candida albicans transition

The transition studies were performed using the F-16 strain from the Associated Regional University Pathologists using previously described techniques of Shepherd *et al.* (22) with minor modifications. Briefly, 5×10^5 aerated *Candida albicans* blastospores were resuspended in 20 ml 0.02 M imidazole-HCl (pH = 6.6) containing 4% rat serum and 0.002 M MnCl_2 , plus the hormone or substance to be tested. The percentages of germ tubes and blastospores were determined using a hemocytometer under optical microscopy. Germ tubes were counted when they were as long as the width of the blastospore and the percentage determined counting 100 yeast elements. The various hormone concentrations were all studied in triplicate, with four hemocytometer readings per tube. The four hemocytometer readings were averaged, and the three numbers obtained were used for statistical analysis.

To compare the effects of different hormones on *Candida albicans* transition, data were subjected to nonpaired *t* test and analysis of variance.

Purification of the CaCGLP

Twenty grams of *Candida albicans* yeast (two strains done in separate experiments) were homogenized in glacial acetic acid 1 ml/g yeast using

a Brinkman homogenizer. After about 3 min, the crude yeast preparation was centrifuged at 2,000 rpm for 20 min, and the supernatant was snap frozen and lyophilized (Freeze dryer 18, Labconco). The powder obtained was resuspended in 50 ml 5% acetic acid, pH adjusted to 7, and centrifuged at 2,000 rpm for 20 min to remove proteins which had precipitated out. This supernatant was assayed for protein concentration by the Bradford technique (23), and also further purified using affinity chromatography. Rabbit anti-hCG antisera were purified on a recombinant protein A column (Beckman) and conjugated to CNBr-activated sepharose (Pharmacia, Piscataway, NJ) for further purification of the CG-like material. The supernatant was incubated overnight with the beads at room temperature mixing end-to-end. After incubation, the beads were collected in a column, washed with 300 ml PBS, pH 7.4, and eluted with 3.8 M MgCl₂, pH 6.8. One-milliliter fractions were collected and measured for protein concentration by a UV spectrometer at an OD of 280 nm. Six tubes containing protein by UV absorption were pooled and immediately dialyzed against four changes of 4 liters deionized water. The protein was subsequently lyophilized and the powder obtained incubated in chloroform overnight at 4°C to increase solubility. The chloroform/protein preparation was mixed with acetone (1:4) and centrifuged at 14,000 rpm. The protein pellet obtained was air-dried and stored at -20°C. Approximately 1 µg CaCGLP/g wet yeast was obtained by this procedure.

Results

CG-Like activity of the CaCGLP

Figure 1, A and B, show dose-response curves for purified hCG (CR 127) and CaCGLP in RIAs using: A) a polyclonal antibody directed against whole hCG, and B) a polyclonal antibody directed against β hCG carboxyl-tail. CaCGLP was used in concentrations ranging from 1–500 ng/tube in the RIAs. Both equilibrium assays show that an hCG-like reactivity is seen in the CaCGLP preparation. Analysis of the slopes of the lines obtained in a logit/log dose graph was done. The assay using the polyclonal antibody against whole hCG had slopes (-2.794 for hCG and -1.983 for CaCGLP), not significantly different in the 95% confidence limits. On the other hand, in the carboxyl-tail assay, the slopes were significantly different (-2.761 for hCG and -1.283 for CaCGLP). Cross-reactivity of the two proteins was calculated at a B/B₀ of 50%, and the values found were 0.125% (whole hCG assay) and 1.25% (carboxyl-tail assay). The reaction with the anti-carboxyl-tail polyclonal antibody suggests that there is a region in CaCGLP that exhibits certain homology with β hCG carboxyl-tail, not present in the hLH molecule.

Figure 2, A B, and C, show RIAs using mAbs: A) mAb anti-CG no. 4, B) mAb anti-CG no. 9, C) mAb anti- α -chain. Data related to the use of the mAb anti-CG no. 21 are not shown; this antibody showed no reaction with CaCGLP. In the three assays shown, the slope of the dose response lines for hCG and CaCGLP were significantly different (-5.48 and -2.48, for mAb no. 4; -5.57 and -2.88, for mAb no. 9; and -4.73 and -1.89, for mAb anti- α -chain). The percentages of cross-reactivity calculated at a 50% B/B₀ were: 6.9%, 2.5%, and 4.4%, for mAb no. 4, mAb no. 9, and mAb anti- α -chain, respectively.

Figure 3, A and B, show the IRMAs using the purified preparation of CaCGLP: A) in the ultrasensitive hCG sandwich assay, and B) in the ultrasensitive hLH sandwich assay. The pure CaCGLP reacted in a dose-response manner in the specific hCG IRMA, even though the cross-reactivity was

small (0.024%). On the other hand, no reaction was seen in the specific hLH IRMA (<0.002%).

Amino acid analysis

Table 1 shows the amino acid composition of the CaCGLP. The table also shows the amino acid composition of hCG, as published elsewhere (24). A high concentration of glycine (22.6%), glutamate (12.82%), and serine (12.28%) is seen in the CaCGLP.

Transition studies

The comparative potencies of hCG and CaCGLP in inducing germ tube formation are shown in Fig. 4. Both proteins induced significant increase in the transition rate. The minimal effective doses were 100 ng/ml for hCG, and 1 ng/ml for CaCGLP. The minimal effective dose was defined as the first dose that induced a significant ($P < 0.05$) increase in the number of germ tubes, compared to the effect of rat serum alone (about 20%). Both CaCGLP and hCG were not able to induce transition in the absence of rat serum (not shown), suggesting that other unknown serum substances are needed in order to permit induction of the hyphae/germ tube transition. We have previously reported that the following hormones, hFSH, hTSH, rat and bovine LH, hGH, and hPRL, did not induce transition when tested in the same assay (4). The present results were reproducible in two other experiments not shown.

SDS-PAGE studies

Figure 5A shows the SDS-PAGE of the silver stained purified material extracted from *Candida albicans*. A single band corresponding to a mol wt of 68 kDa is seen under nonreduced conditions. Under reduced conditions, a smear is seen, demonstrating that reducing conditions break the protein in several products. The results obtained were the same for the CaCGLP purified from the two different strains of *Candida albicans* studied.

The band seen under nonreduced conditions was proven to be CaCGLP, since it reacted with the monoclonal antibody anti-CG no. 4, as shown by Western blot analysis (Fig. 5B). The reduced material did not react in the Western blot, probably because of the intense breakage induced by the reducing conditions. The nonstained markers were also trans-blotted to the same membrane, and also did not react in the Western blot, thus serving as additional negative controls.

Discussion

The ability of *Candida albicans* blastospores and membranes to bind hCG and LH and the effect of these glycoproteins in the *Candida albicans* transition has been described by us (4) and others (5, 6). A natural protein produced by *Candida albicans* with structural and immunological similarities to a human glycoprotein has not been previously described. The present paper shows that *Candida albicans* produces an hCG-like material with a mol wt of 68 kDa that is

able to induce transition and consequently transform these yeast into more pathogenic organisms. The reduced form of this protein did not allow us to conclude that a subunit structure exists. This form was not immunologically recognized, whereas the nonreduced protein reacted in a Western blot, showing a band in the same position seen in the SDS-PAGE.

The possibility of this protein being the natural ligand of the CG/LH receptor in *Candida albicans* remains to be studied, but these results suggest that *Candida albicans* can control its own pathogenicity. Establishing the importance of the production and expression of CaCGLP in *Candida albicans* infections could be a promising and interesting line of research. Also, further characterization of the protein and determination of the gene sequence could open the possibility of better understanding of *Candida albicans* pathogenicity, as well as offering valuable information concerning evolution of the hCG genes.

The purified CaCGLP reacted in two polyclonal and three monoclonal equilibrium assays developed for the measurement of hCG. The reactivity shown implicates the presence of some epitope similarities between CaCGLP and hCG. The unexpected reaction of the pure form of this protein on the ultrasensitive hCG specific sandwich assay, which does not react with hLH, could also indicate that a slight tertiary structure homology with hCG is present. It is interesting to point out that it would appear that CaCGLP is an hCG and not an hLH-like protein since: 1) it reacts with an antibody directed against the COOH-tail, not present in hLH; 2) it reacts in an ultrasensitive hCG IRMA, which is specific for intact hCG; and 3) it does not react in a specific hLH IRMA.

Studies from our laboratory show that *Xanthomonas maltophilia* produce an hCG-like protein which is immunologically similar to the β -subunit of hCG (7). The physiological action of this protein is not completely understood, but further studies show that when added to cultured *Xanthomonas maltophilia*, both morphology and the concentration of bacteria reached at the stationary phase of the growth cycle were changed (Carrell, D. T., personal communication). Other microorganisms have been reported to produce hCG-like materials, but physiological actions of these materials have not been described (9–14).

In summary, we describe the characterization of a CG-like protein present in *Candida albicans* that is able to induce transition from yeast to hyphae forms. We postulate that *Candida albicans* can modify its own pathogenicity.

Acknowledgments

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Twelfth North American Testis Workshop: Function of Somatic Cells in the Testis Tampa, Florida, April 13-17, 1993

This program will present and discuss recent developments in the study of the male gonad with particular emphasis on testicular somatic cells, their regulation, functions, and interactions. Several lectures on peripheral actions of male sex hormones and on male germ cells will also be included.

Invited speakers include: Terry Brown, Gloria Callard, Marco Conti, Jennifer Dorrington, Maria Dufau, E. M. Eddy, James Griffin, Michael Griswold, Ilpo Huhtaniemi, Fernand Labrie, Hennie Mather, Joanne Orth, Martti Parvin, Anita Payne, Dirk de Rooij, Lonnie Russell, Barbara Sanborn, Anna Steinberger, David Wildt, and William Wright. Chairman: Andrezej Bartke, Ph.D.

For information regarding brochure, registration, and poster abstract forms, please contact Bruce K. Burnett, Ph.D., Serono Symposia, USA, 100 Longwater Circle, Norwell, Massachusetts 02061. Tel 800-283-8088, 617-982-9000, or Fax 617-982-9481.
