

# Enzymatic and immunological detection of G protein $\alpha$ -subunits in the pathogenic fungus *Candida albicans*

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GTP stimulation of adenylyl cyclase from the dimorphic pathogenic fungus *Candida albicans* is greatly enhanced by preincubation of membrane proteins with cholera toxin, NAD and ATP. In the presence of [ $^{32}$ P]NAD the toxin catalyzes the covalent incorporation of radioactivity into a membrane protein of 40 kDa. Pertussis toxin catalyzes the transference of the radioactivity from [ $^{32}$ P]NAD to a 32 kDa protein. Two major proteins of 40–42 and 30–32 kDa can also be recognized in Western blots by an anti  $G_s$ -common antibody. The results support the idea that G proteins are part of the hormonesensory transduction chain of *Candida* [(1990) Biochem. Biophys. Res. Commun. 167, 1177–1183].

G-protein; Peptide antiserum,  $\alpha$ -commons; Adenylyl cyclase; Hormone response; *Candida albicans*

## 1. INTRODUCTION

Accumulated experimental evidence indicates that certain mammalian hormones directly or indirectly affect the yeast-to-mycelial transformation in the human pathogenic fungus *Candida albicans* [1–3]. Moreover, specific high-affinity binding proteins for human luteinizing hormone (hLH) and human chorionic gonadotrophin (hCG) have been reported by Bramley et al. in microsome fractions of this species [4]. In our laboratory, we have recently demonstrated that the addition of glucagon to yeast cells, under conditions conducive to produce germ tubes, blocked hyphal development [5]. We also showed that glucagon, hLH and hCG acting as first messengers on *C. albicans* cells increased the cAMP endogenous levels and triggered the cAMP activation cascade in a similar way to that found in higher eukaryotic organisms. In the same study we demonstrated that the  $Mg^{2+}$ -dependent, GTP-stimulated adenylyl cyclase was activated by glucagon, hLH and hCG in a dose-dependent fashion [5,6]. Stimulation of *Candida* adenylyl cyclase by steroidogenic hormones has also been reported by Williams et al. [7]. These investigations strongly suggest the functional interaction between surface receptors and effector enzyme via G protein(s). Very recently, Sadhu et al. [8] isolated a gene (CAG1) from *C. albicans* codifying for a putative G

protein  $\alpha$ -subunit which is thought to be involved with components of a specific signal transduction pathway. However until now no functional role between the CAG1 product and any effector system of the pathogen has been found.

The findings described above prompted us to search for G proteins coupled to specific effector molecules. The results presented here clearly establish the existence of a  $G_{\alpha_s}$ -like protein of 40 kDa. Our data support the idea that ADP-ribosylation of this protein is responsible for the toxin-induced changes in adenylyl cyclase activity. We also present chemical and immunological evidence for the existence of a  $G_{\alpha_i}$ -like protein the physiological function of which is still unknown.

## 2. MATERIALS AND METHODS

### 2.1. Chemicals

All reagents were of analytical grade. Radiochemicals were purchased from NEN and toxins from List. A 569 antiserum was a generous gift from Dr. P. Devreotes.

### 2.2. Organism and cultivation

Maintenance and cultivation of *C. albicans* yeast cells (BAFC 1176) were performed as described in a preceding paper [9].

### 2.3. Preparation of crude membranes

Cells from the midlog phase of growth were collected by centrifugation, the pelleted cells were broken with two volumes of alumina in a prechilled mortar and suspended in two volumes of 20 mM Tris-HCl buffer pH 7.5, 5 mM  $\beta$ -mercaptoethanol, 1 mM EDTA, 0.5 mM PMSF, 0.5 mM EGTA, 1 mM  $Na_2S_2O_8$ . The homogenate was centrifuged at  $5,000 \times g$  for 7 min and the supernatant was spun down at  $100,000 \times g$  for 60 min. The pellet (P100) washed once and resuspended in the appropriate buffer was considered the crude membrane preparation. The protein concentration of the membrane preparation was determined by the method of Bradford [10].

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Abbreviations: PMSF, phenyl-methylsulfonyl fluoride; GTP- $\gamma$ -S, guanosine 5'-O-(3-thio-triphosphate).

#### 2.4. Toxin treatment

ADP-ribosylation of crude membranes by cholera or pertussis toxins (CTX or PTX) was performed according to Birnbaumer et al. [11], with slight modifications. CTX (1–6 mg/ml) or PTX (5 mg/ml) were activated in 20 mM DTT, 1 mg/ml BSA and 1% SDS for 30 min at 37°C. Equal volumes of *C. albicans* membrane preparation (2 mg/ml) and preactivated CTX (200 µg/ml) were mixed and incubated for 45 min at 37°C in the presence of 1 mM ATP, and either 1 mM unlabelled NAD or 1 µM [<sup>32</sup>P]NAD (specific activity 5 Ci/mmol). For radiolabelling the mixture was supplemented with 10 mM thymidine, 1 mM GTP, 5 mM MgCl<sub>2</sub>, 0.3 M sodium phosphate buffer pH 7 and 20 µg/ml DNase. For PTX treatment 1% lubrol was added to the membranes and MgCl<sub>2</sub> and sodium phosphate buffer were omitted. The reaction mixture was centrifuged at 178,000 × *g* for 5 min and the resulting pellet was assayed for adenylyl cyclase activity or subjected to 10% SDS-PAGE, stained with Coomassie brilliant blue, destained, dried and autoradiographed.

#### 2.5. Adenylyl cyclase assay

Essentially the enzymatic assay was performed as described in a preceding paper [5]. The reaction was stopped by the addition of 0.12 ml of 0.5 N HCl, and the [<sup>32</sup>P]cAMP produced was determined as described by Alvarez and Daniels [12].

#### 2.6. Immunological detection of G protein $\alpha$ -subunits

Crude membrane preparations (20–60 µg protein) were subjected to 10% SDS-PAGE, transferred to a nitrocellulose membrane and immunoblotted with an affinity-purified antibody raised in rabbit against the  $\alpha$ -common synthetic peptide A-569 (CGAGESGKSTIVKQMK) [13]. Bound antibody was detected with goat anti-rabbit IgG and purified antibody was blocked with the peptide (8 µg/ml) against which it had been raised.

### 3. RESULTS AND DISCUSSION

#### 3.1. Effects of cholera toxin on adenylyl cyclase activity and their relation to covalent modification of membrane proteins from *C. albicans*

Crude membranes treated with CTX in the presence of [<sup>32</sup>P]NAD were analyzed by SDS-PAGE. As shown in Fig. 1 (lane 2), one main protein with an apparent  $M_w$  value of 40 kDa was labelled; no radioactivity was incorporated into membrane proteins incubated either in the absence of CTX or with an excess of non-radioactive NAD (lane 1). In order to correlate the covalent modification of the 40 kDa protein with the CTX induced changes in adenylyl cyclase, the enzymatic activity of membranes treated with CTX and 1 mM NAD was compared with that of untreated controls. As can be seen in Table I pretreatment of membranes with CTX slightly increased the basal activity (1.4-fold); this small activation could be accounted for by endogenous GTP present in the membrane preparation. The activation induced by CTX was enhanced in the presence of GTP (2.4-fold). The enzymatic activity reached under these conditions was similar to that obtained in the presence of the non-hydrolyzable GTP analog GTP- $\gamma$ -S. The response of *C. albicans* adenylyl cyclase to CTX is thus similar to that previously described for mammalian systems [14]. The experiments with CTX reported herein strongly support the involvement of the 40 kDa protein

Table I

Comparison of adenylyl cyclase activity from control and toxin-treated membranes

Additions to the assay	Adenylyl cyclase activity (pmol cAMP/mg per min)	
	Non-treated membranes	Toxin-treated membranes
None	8.5	12.5
GTP	10.4	24.4
GTP- $\gamma$ -S	25.2	28.7

Aliquots of the membrane preparation (0.1 mg protein) were preincubated for 15 min at 30°C with 100-µM NAD<sup>+</sup> in the presence or in the absence of preactivated CTX and assayed for adenylyl cyclase activity with 10 µM GTP or GTP- $\gamma$ -S when indicated. Data are means of triplicate observations.

in the activation of adenylyl cyclase by CTX and more specifically that this protein is indeed the GTP-binding component of the adenylyl cyclase system responsible of the hormone-induced changes of enzyme activity previously reported [5,6].

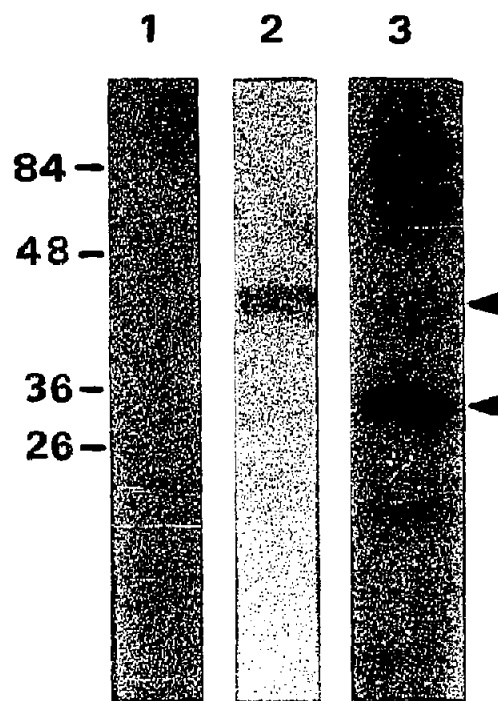


Fig. 1. Cholera and pertussis toxins catalyzed ADP-ribosylation. 20 µg of membrane proteins were treated with [<sup>32</sup>P]NAD and toxin as described in section 2, subjected to analysis by SDS-PAGE, stained with Coomassie blue and autoradiographed. *C. albicans* membranes were incubated with CTX (lane 2), CTX plus 1 mM NAD (lane 1) and PTX (lane 3). In the absence of each toxin no radiolabelling could be observed for the membrane proteins. No cytosolic proteins were ADP-ribosylated with any of the toxins.

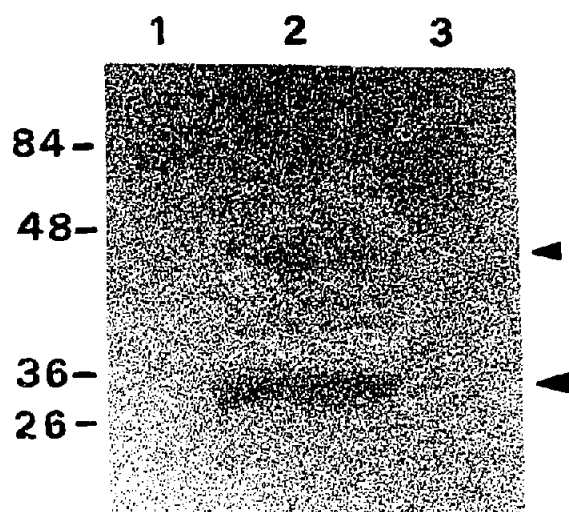


Fig. 2. Immunoblots of *C. albicans* proteins with  $\alpha$ -common G antiserum. Cytosolic (lane 1) and membrane (lanes 2 and 3) proteins were separated by SDS-PAGE, transferred to nitrocellulose and incubated with antiserum A-569 that detects multiple  $\alpha$ -subunits of vertebrate G proteins. Lane 3, membrane proteins incubated with antiserum blocked with the peptide that was used for the generation of the antiserum.

### 3.2. ADP-ribosylation of *C. albicans* membranes by PTX

Crude membrane preparations were subjected to analysis by SDS-PAGE and autoradiographed after treatment with pertussis toxin (PTX) and [ $^{32}$ P]NAD. As shown in Fig. 1 (lane 3), a 32 kDa protein could be revealed indicating the existence of a  $G_i$   $\alpha$ -like protein. Preliminary experiments from our laboratory indicate the existence of a phosphoinositide-specific phospholipase C in crude preparations of the fungus; it is thus possible that the  $G_i$   $\alpha$ -like protein could be involved in this signal transduction pathway.

### 3.3. Immunological detection of G protein $\alpha$ -subunits

An immunoblot of the electrophoretically separated proteins of *C. albicans* membranes with the  $\alpha$ -common antibody A 569 detected two predominant bands with a  $M_w$  of 40–42 and 30–32 kDa, additional faint bands were detectable at 38 kDa and at 36 kDa (Fig. 2, lane 2). The main bands at 40–42 kDa and 30–32 kDa together with the faint bands at 38 and 36 kDa disappeared after the antiserum had been blocked by the peptide that had been used to generate the antibody (lane 3) and can therefore be regarded to be specifically recognized by antibodies against the  $\alpha$ -common peptide. No protein was immunologically detected in the cytosol (lane 1). These results suggest that both the 40–42 and 30–32 kDa proteins are at least in part highly homologous to  $\alpha$ -subunits of vertebrate G proteins and may have a similar GTP-binding site as most eukaryotic G proteins.

Very recently Sadhu et al. [8] reported the cloning and initial characterization of a putative  $G_z$  protein gene, CAG1 from *C. albicans* the deduced amino acid sequence of which exhibits a high degree of homology (62.5% homologous at the coding DNA level) to the product of the *Saccharomyces cerevisiae*  $G_z$  gene SCG1 [15] involved in the mating pheromone signalling pathway [16]. They suggest that this may be an indication of the close evolutionary relationship between *Saccharomyces* and *Candida* species despite the fact that *C. albicans* does not have any mating phase.

Using the classification established by Strathmann and Simon [17] who divided heterotrimeric G proteins into three classes,  $G_s$ ,  $G_q$  and  $G_i$ , based on the primary sequence relationships, Sadhu et al. [8] indicated that the  $G_z$  encoded by *C. albicans* CAG1 gene and the *S. cerevisiae* SCG1 gene both probably belong to a group not included in that classification.

We believe it is worthwhile to mention that despite the close phylogenetic relationships between the two yeast species, *S. cerevisiae* adenylyl cyclase activation is coupled to RAS gene products [18] and not to trimeric G proteins.

In the light of this and the experimental evidence we have presented it is conceivable that the  $G_{zs}$  protein characterized in this paper is different from the putative protein ascribed by Sadhu et al. [8] to the CAG1 gene.

Our data also show that another  $G_z$  subunit, one of the type  $\alpha_i$ , whose physiological role is now under study at our laboratory, is also present in *C. albicans*.

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