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Detection of cAMP and of PKA activity in *Saccharomyces cerevisiae* single cells using Fluorescence Resonance Energy Transfer (FRET) probes

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ABSTRACT

In *Saccharomyces cerevisiae* the second messenger cyclic adenosine monophosphate (cAMP) and protein kinase A (PKA) play a central role in metabolism regulation, stress resistance and cell cycle progression. To monitor cAMP levels and PKA activity *in vivo* in single *S. cerevisiae* cells, we expressed an Epac-based FRET probe and a FRET-based A-kinase activity reporter, which were proven to be useful live-cell biosensors for cAMP levels and PKA activity in mammalian cells. Regarding detection of cAMP in single yeast cells, we show that in wild type strains the CFP/YFP fluorescence ratio increased immediately after glucose addition to derepressed cells, while no changes were observed when glucose was added to a strain that is not able to produce cAMP. In addition, we had evidence for damped oscillations in cAMP levels at least in SP1 strain. Regarding detection of PKA activity, we show that in wild type strains the FRET increased after glucose addition to derepressed cells, while no changes were observed when glucose was added to either a strain that is not able to produce cAMP or to a strain with absent PKA activity. Taken together these probes are useful to follow activation of the cAMP/PKA pathway in single yeast cells and for long times (up to one hour).

1. Introduction

In the yeast *Saccharomyces cerevisiae*, the cAMP/PKA pathway plays an important role in the control of metabolism, stress resistance, proliferation and it also affects morphogenesis and development, including pseudohyphal, invasive growth and sporulation [1–5]. The central component of this pathway is adenylate cyclase whose activity is controlled by two G-protein systems, the Ras proteins and the Gα protein Gpa2 [6,7]. Two triggers are known to activate the cAMP/PKA pathway: the addition of glucose to derepressed cells and intracellular acidification.

Cyclic AMP is synthesized by adenylate cyclase, encoded by *CYR1* gene, and induces the activation of the cAMP-dependent protein kinase A (PKA). In turn, PKA phosphorylates a variety of proteins involved in key cellular processes. The whole signaling cascade is tightly regulated and experimental evidences indicate that multiple feedback mechanisms operate within the pathway by the generation of a complex interplay between the cascade components [8–10]. Ras proteins are positively controlled by the activity of Cdc25, that stimulates the GDP-GTP exchange, and negatively regulated by Ira1 and Ira2, that stimulate the GTPase activity of Ras. The inactivation of cAMP is governed by phosphodiesterases that constitute the major feedback mechanism in the pathway [8], although Colombo et al. [9] demonstrated that the feedback inhibition mechanism acts also by changing the Ras2 proteins activation state. A basal level of cAMP is required for growth, while a transient increase of cAMP induced by addition of glucose is required for transition from respiratory to fermentative metabolism. Although the cAMP/PKA pathway has been extensively studied in yeast and both upstream and downstream

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elements are known, the changes in cAMP and in the activity of this pathway were measured in cell populations and usually for a very short time and data on the spatiotemporal variation of cAMP and PKA activity in single cells are till now lacking. Some years ago Nikolaev et al. developed an Epac-based FRET probes for monitoring cAMP levels *in vivo* in single mammalian cells [11]. These sensor consist of part of the cAMP-binding protein Epac1 or Epac2 sandwiched between cyan and yellow fluorescent proteins(CFP and YFP). The construct unfolds upon binding of the second messenger cAMP to the Epac moiety and cAMP increases are thus easily followed as a drop in FRET. A couple of years later, Allen and Zhang developed a FRET-based A-kinase activity reporter (AKAR), AKAR3, for monitoring PKA activity *in vivo* in single mammalian cells [12]. AKAR is a recombinant protein composed of a phosphoamino acid binding domain and a PKA-specific substrate sandwiched between CFP and cp-Venus fluorescent protein. When phosphorylated by PKA, intramolecular binding of the substrate by the phosphoamino acid binding domain drives a conformational reorganization, leading to an increase in FRET between CFP and cp-Venus. Since key cellular processes, including components and mechanisms used for signal transduction, are conserved between human and yeast, methodologies setups and successfully used in mammalian cells might work in yeast as well. In this regard, we recently studied the localization of Ras-GTP *in vivo* in single *S. cerevisiae* cells by expressing a probe consisting of a GFP fusion with a trimeric Ras Binding Domain of Raf1 (eGFP-RBD3) [13], which was proven to be a useful live-cell biosensor for Ras-GTP in mammalian cells [14].

Aim of this work was to develop and test FRET probes for monitoring cAMP levels and PKA activity *in vivo* in single *S. cerevisiae* cells, starting from methodologies setups and successfully used in mammalian cells.

2. Materials and methods

2.1. Yeast strains and media

Strains used in this study: W303-1A (*MATa ade2-1 can1-100 his3-11,15 leu2-3112 trp1-1 ura3-1*) [15]; X4004-3A (*MATa lys5 met2 ura3 trp1*); SP1 (*MATa his3 leu2 ura3 trp1 ade8 can1*) [16]; GG104 (*MATa W303-1A cdc35::KanMX pde2::TRP1 msn2::HIS3 msn4::TRP1*) [17]; ASY62 (*MATa SP1 tpkl::ADE8 tpk2::HIS3 tpk3::TRPl msn2::HIS3 msn4::LEU2)* [18].

Synthetic complete media (SD) contained 2% glucose, 6.7 g/l YNB w/o aminoacids (supplied by ForMedium™, United Kingdom) and the proper selective drop-out CSM (Complete Synthetic Medium, supplied by ForMedium™, United Kingdom). Culture density was measured with a Coulter Counter (Coulter mod. Z2) on mildly sonicated samples.

2.2. Plasmids

To obtain the pYX212-YFP-EPAC2-CFP and the pYX242-YFP-EPAC2-CFP vectors we used the following strategy. The YFP-EPAC2-CFP fragment, obtained digesting the pcDNA3- YFP-EPAC2-CFP construct (kindly provided by Dr. V.O. Nikolaev, University of Wuerzburg, Germany) with XhoI and HindIII, was ligated into the expression vectors, pYX212 and pYX242, digested with the same enzymes. To obtain the pYX212-AKAR3 vector we used the following strategy. The CFP-AKAR3-cpVenus fragment, obtained digesting the pcDNA3- AKAR3 construct (kindly provided by Dr. Jin Zhang, The Johns Hopkins University, Baltimore, USA) with BamHI and XbaI, was ligated into the expression vector pYX212 digested with BamHI and NheI.

2.3. Fluorescence microscopy and FRET determination

Cells were grown in medium containing 2% glucose at 30 °C till exponential phase, collected by centrifugation, resuspended in 25 mM MES buffer, pH 6 (about 5×10^7 cells/ml) and incubated at 30 °C for at least 1 h. Subsequently, 40 μl of glucose-starved cells were seeded on concanavalin A (Sigma-Aldrich, Milano, Italy)-coated cover glass for 10 min [13]. The cover glass was washed four times using 1 ml of 25 mM MES buffer (pH 6), mounted on a custom chamber and covered by 500 μl of the same buffer. Time stacks of images (512 × 512 pixels, typical field of view 150 μm × 150 μm, 400 Hz scanning frequency) were acquired before and after addition of either glucose, cAMP or H_2O by means of a Leica SP5 confocal microscope (Leica BLA Germany) through a 40X oil objective (HCX PL APO CS 1.30). The pinhole was set at 150–170 μm in order to detect a higher signal from each cell and to avoid losing the focal plane in long time acquisitions. CFP was excited at 458 nm, its emission detected in the range 465–495 nm and either YFP or cpVenus emission were detected in the range 514–600 nm. Glucose was added by pipetting, directly into the chamber, 25 μl of 40% glucose dissolved in 25 mM MES buffer, pH 6. cAMP was added by pipetting, directly into the chamber, 75 μl of 20 mM cAMP dissolved in 25 mM MES buffer, pH 6. The acquisition started with non-stimulated cells and continued after the glucose or cAMP addition without interruption for 30–60 min, typically.

For each sample, image time series have been acquired selecting field of view populated with more than 50 cells. After acquisition, data have been analyzed by means of the Leica Application Suite Software (Leica Microsystem, Germany). A ROI has been selected including each cell and the CFP and YFP or cp Venus fluorescence signals in the two acquisition channels have been saved together with their ratio versus time. In this way, both single cell behavior and average values have been calculated for each sample. The raw data were then further elaborated with Excel ™.

Preliminary experiments were performed on a two photon scanning microscope (BX51 equipped with FV300, Olympus, Japan) modified for direct (non descanned) detection of the signal and coupled to a femtosecond Ti:sapphire laser (Mai Tai, Spectra Physics, CA) [19]. The microscope was equipped with a highly efficient objective (N.A. = 0.95, 20X, water immersion, Olympus, Japan). CFP was excited at $\lambda = 820$ nm and the fluorescence was detected through a short-pass 670 nm filter (Chroma Inc. Brattelboro, VT) and selected by a band-pass filter at 485/30 nm (Chroma Inc. Brattelboro, VT) for the CFP channel at 560/50 (Chroma Inc. Brattelboro, VT) for the YFP channel. Time stacks of images 512pixel x 512 pixel were acquired in fast scan mode with a field of view of 250 μm × 250 μm.

3. Results and discussion

3.1. Monitoring cAMP changes in a single Saccharomyces cerevisiae cell

We used a FRET (Fluorescence Resonance Energy Transfer) sensor to monitor the changes in cAMP level in single *S. cerevisiae* cell. To avoid any interference with the cAMP/PKA signaling, we used a sensor based on the mammalian protein EPAC2 (Epac2-camps), originally developed by Nikolaev et al. to monitor cAMP changes in mammalian cells. This sensor exhibits a decrease of FRET in response to cAMP concentration with a SD_{50} of about 1 μM and is therefore suitable for measuring the changes in cAMP in yeast [11]. The sequence coding for the sensor, a fusion between Cyan-fluorescent protein-Epac2 (aa 285–443)-Yellow fluorescent protein (CFP-EPAC2-YFP), was cloned in a yeast expression vector

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