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PKA-dependent regulation of Cdc25 RasGEF localization in budding yeast

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ABSTRACT

In *Saccharomyces* cerevisiae the Cdc25/Ras/cAMP pathway is involved in cell growth and proliferation regulation. Ras proteins are regulated by Ira1/2 GTPase activating proteins (GAPs) and Cdc25/Sdc25 guanine nucleotide exchange factors (GEFs).
Most of cytosolic Cdc25 protein was found on internal membranes in exponentially growing cells, while upon incubation in a buffer with no nutrients it is re-localized to plasma membrane. The overexpression of Tpk1 PKA catalytic subunit also induces Cdc25 export from the nucleus, involving two serine residues near the Nuclear Localization Site (NLS): mutation of Ser^{825} and Ser^{826} to glutamate is sufficient to exclude physiologically expressed Cdc25 from the nucleus, mimicking Tpk1 overproduction effect. Mutation of these Ser residues to Ala abolishes the effect of nuclear export induced by Tpk1 overexpression on a Cdc25eGFP fusion. Moreover, mutation of these residues affects PKA-related phenotypes such as heat shock resistance, glycogen content and cell volume.

1. Introduction

In *Saccharomyces cerevisiae*, Ras/cAMP/PKA pathway is involved in cell growth regulation in response to nutrients and namely to glucose availability [1].

Ras proteins activity is modulated by two classes of regulatory proteins: Cdc25 and Sdc25 guanine nucleotide exchange factors (GEFs) [2], stimulating the GDP/GTP exchange on Ras, and Ira1 and Ira2 GTPase activating proteins (GAPs), promoting Ras GTPase activity [3].

The main positive regulator of yeast Ras proteins is Cdc25 protein, a 180 kDa polypeptide with a C-terminal highly conserved Ras-GEF catalytic domain [4]. Cdc25 protein is tightly bound to a crude membrane fraction; treatment with reagents able to release membrane peripheral proteins cannot release it, but it can only be released from membranes in denaturing conditions [4,5]. Several Cdc25 residues within the 114–348 region could influence this membrane attachment, making the protein more soluble when hyper-phosphorylated and less available for association with Ras [6]; however, glucose-induced Tpk1-dependent hyper-phosphorylation of Cdc25 upon glucose addition was recently reported to directly inhibit RasGEF activity instead of affinity for Ras [7–9].

Heavily overproduced Cdc25 showed a particulate localization in distinct patches, while slightly overproduced Cdc25 protein fusion with the green fluorescent protein (eGFP) accumulates in both the cytosol and the nucleus, but not in the plasma membrane [10]. The region responsible for nuclear localization was identified as the central uncharacterized region (residues 353–1100). The endogenous Cdc25 was also found in purified nuclei, and cell stress or unbalanced PKA activity were reported to affect nuclear localization of Cdc25. Here we show that Tpk1 overexpression regulate Cdc25 localization not only in the plasma membrane but also in the nucleus, with a mechanism involving Ser^{825} and Ser^{826} residues.

2. Materials and methods

2.1. Strains and cultures

Top10 *E. coli* strain (Invitrogen) was grown in LB medium (1% NaCl, 1% Bacto-peptone, 0.5% yeast extract, 0.1% glucose) at 37 °C. For Amp^R selection, 50 mg/L ampicillin was added. For solid media, 1.5% agar was added.

Yeast strains are described in Table SI. Yeast cells were grown in YP (1% yeast extract, 2% Bacto-peptone) supplemented with 2% w/v glucose (YPD). Selective medium was synthetic medium (SC) containing 0.67% w/v Yeast nitrogen base w/o amino acids, CSM synthetic amino acid mixture (BIO101, USA) required and the appropriate sugar (2% glucose or 2% galactose with 0.1% glucose). Nitrogen source-free medium was prepared by adding the appropriate sugar to 0.17% Yeast nitrogen base w/o amino acids and ammonium sulphate. Solid medium contained 2–2.5% agar. In all conditions cells were grown at 30 °C. Cell density was determined by measuring optical density (OD_{600}) or by Coulter Counter (Coulter Electronics mod. Z2). Budding index (BI) was determined

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by direct microscopic count of at least 300 cells fixed in 4% formalin and mildly sonicated. Cell volumes distributions were determined with a Coulter Counter Channelyzer 256.

Cells starved for nutrients were grown in YPD or in SC until exponential phase, then collected by filtration, washed with water, resuspended in 0.25 M Mes pH 6.5 and incubated for 4 h.

For glycogen content determination, cells spotted on SD medium and incubated at 30° for 3 days were stained with Lugol's iodine solution.

2.2. Strains and plasmids construction

Strains carrying 4-HA tagged Ira1 and Ira2 were constructed according to Wach et al. [11], by transforming W303-1A strain with a PCR generated cassette amplified with Pfu Turbo enzyme (Promega) using the following oligonucleotides as primers: for *IRA1* cassette, IRA1HA FOR and IRA1HA REV; for *IRA2* cassette, IRA2HA FOR and IRA2HA REV (Table SII), and amplifying the 4-HA tag and a KanMX6 marker using the pDHA plasmid as a template (kind gift of P. Coccetti, University of Milan-Bicocca) [12].

The YEpCDC25^{SASA}eGFP plasmid, carrying *CDC25* gene with mutations in Ser^{825} and Ser^{826} to alanine, was obtained by in vivo recombination of the 2547 bps fragment derived from the pRS413-25-7m plasmid (gently given by Levitzki) [13], digested with *HincII*, with the YEpCDC25eGFP plasmid [10], digested with *BglII*. Mutations were controlled by sequencing. The YEpCDC25^{SESE} plasmid was obtained by site specific mutagenesis from pCDC25-2 [14], performed with the CDC25 SESE FOR and CDC25 SESE REV oligonucleotides (Table SII) and the Phusion enzyme (Euroclone). The product was controlled by sequencing and encodes for a Cdc25 S825E S826E protein, which will be called Cdc25^{SESE}. The plasmid YEpCDC25^{SESE}eGFP was obtained by in vivo recombination of the *PvuII* fragment from YEpCDC25^{SESE} plasmid with YEpCDC25eGFP. YEpCDC25^{SASA} plasmid was obtained by substituting the *SphI* fragment in YEpCDC25^{SASA}eGFP with the *SphI* fragment from pCDC25-2.

The mutant proteins encoding sequences were introduced by gene targeting in *CDC25 locus* by homologous recombination of *PvuII* fragment from either YEpCDC25^{SASA} or YEpCDC25^{SESE} in the WΔhSos1 strain [15] and selecting on 5-FOA, giving respectively RT1180 and RT1190. 4xHA-tag in the C-terminus was inserted as described above for Ira1 and Ira2 tagging.

The YEpTPK1 plasmid was already described [16].

2.3. Cellular membranes fractionation

The method was modified from Sidoux-Walter et al. [17]. Cells were grown until mid-log phase (OD_{600} 0.6–1.0) at 30 °C in YPD medium, harvested by centrifugation at 4 °C for 10–15 min, washed with 20 ml of Cell Wash Buffer (CWB) (10 mM Tris–HCl pH 7.5, 0.5 M sucrose, and either 2.5 mM EDTA or 2 mM magnesium chloride, as indicated) and collected by centrifugation. Cell pellet was weighed and frozen at −80 °C.

Frozen cells were thawed, washed with 20 ml of Homogenization Buffer (HB) (50 mM Tris–HCl pH 7.5, 0.3 M sucrose, 2.5 mg/ml BSA, and either 5 mM EDTA and 1 mM EGTA or 2 mM magnesium chloride, as indicated) and harvested by centrifugation. Pellet was resuspended in HB (supplemented with freshly prepared 2 mM DTT, 1 mM PMSF, 2 μM pepstatin, Roche EDTA-free protease inhibitor cocktail) with 2 ml of buffer per gram of cells wet-weight. About 1 volume of glass beads was added to the suspension and cells were disrupted with Fast-prep (Savant). Extracts were centrifuged at 13 000 rpm for 10 min and the supernatant was collected.

The supernatant was centrifuged at 100 000×*g* for 60 min and stored at −80 °C with 20% glycerol. The pellet (Microsomal Membrane pellet or MMP) was washed with Membrane Wash Buffer (MWB, 10 mM Tris–HCl pH 7, 1 mM DTT, 20%, glycerol and either 1 mM EGTA or 2 mM magnesium chloride, as indicated) and then centrifuged for 60 min at 100 000×*g*. Washed MMP was resuspended with 0.5 ml of MWB and eventually stored at −80 °C or immediately overlayed on a 11 ml-sucrose gradient (1 ml 46%, 2 ml 50%, 2 ml 54%, 2 ml 56%, 2 ml 58%, 1.7 ml 70% sucrose), prepared in sucrose gradient buffer (10 mM Tris·HCl pH 7, 1 mM DTT, and either 1 mM EDTA or 2 mM magnesium chloride, as indicated). Gradients were then centrifuged at 80 000×*g* for 17 h, and 1 ml-fractions were collected from the top and stored at −20 °C.

Protein concentration of fractions was determined with Bio-Rad protein assay (Bio-Rad) and proteins were separated by SDS–PAGE and visualized by Western blot and ECL (Euroclone) detection.

Protein markers were used to follow cellular compartments distribution along the sucrose gradient: Gas1 (post-translationally modified in ER and Golgi compartments and then transported to the plasma membrane) [18]; Anp1 (Golgi apparatus) [19], Tom 40 (mitochondria) [20], Pho8 (vacuole) [21], Nop1 (nucleus) [22] and mature Pma1 (plasma membrane) [23]. As ER marker Sec61-GFP fusion protein was used, expressed in YLR378C strain (Invitrogen). Protein profile was analyzed by Western blot using the following antibodies: goat α-Ras2 (Santa Cruz), mouse α-HA (Roche), goat α-Cyr1 (Santa Cruz), rabbit BD Living Colors antibody against eGFP protein (BD Biosciences, Clontech), rabbit α-Gas1 (kind gift of M. Vai, University of Milano-Bicocca), rabbit α-Tom40 (kind gift of T. Endo, Nagoya University, Japan), mouse α-Pma1 (Abcam), rabbit α-Anp1 (kind gift of S. Munro, MRC Lab of Molecular Biology, Cambridge, UK), mouse α-Pho8 (kind gift of J. Winderickx, K.U. Leuven, Belgium), mouse α-Nop1 (EnCor Biotechnologies). Secondary horseradish peroxidase-conjugated antibodies were from Jackson Immunoresearch.

Proteins profiles in the different fractions were obtained by densitometry analysis of the scanned films, taking at least four replicates for each experiments, and comparing the profiles deriving from different exposure obtained films for each experiment in order to reveal any saturated bands under-estimation. The percentage of signal in each band was calculated on the sum of bands signals.

2.4. Preparation of nuclear extracts

Nuclear extracts were prepared as previously described [10].

2.5. Epifluorescent microscopy

Living cells were observed with a Nikon Eclipse E600 microscope, fitted with a 60× immersion objective and a standard filter set (Nikon, EX 450–490, DM 505, BA 520) for GFP-fluorescence. Images were recorded using a Nikon FDX-35 camera and processed using MetaMorph 6.3r1 (Molecular Devices, Sunnyvale, CA).

Fluorescence measurements were performed by using MetaMorph fluorescence Integrated Intensity tool, and the percentage of nuclear fluorescence was calculated on at least 60 cells per strain as the average ratio of the Integrated Intensity measured in the nucleus over the Integrated Intensity of the whole cell. The significance of the difference among the distributions obtained was evaluated by Student *t* test.

3. Results

3.1. Cdc25. is mainly localized on internal cellular membranes during exponential growth on glucose but incubation in nutrient-free buffer induces Cdc25 accumulation in the plasma membrane

Cdc25 was previously shown to be tightly attached to membranes but also efficiently imported and retained in the nuclear