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Interacting proteins of protein kinase A regulatory subunit in *Saccharomyces cerevisiae*

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ABSTRACT

cAMP-dependent protein kinase mediates many extracellular signals in eukaryotes. The compartmentalization of PKA is an important level of control of the specificity of signal transduction mediated by cAMP. Unlike mammalian PKA for which proof insights in the mechanism that controls its localization through anchoring proteins (AKAPs) has been obtained, in the case of *Saccharomyces cerevisiae* PKA there was little information available. In this work, we present results that demonstrate the isolation and identification of yeast PKA regulatory subunit (Bcy1) associated proteins using a MS-based proteomic analysis and a bioinformatic approach. The verification of some of these interactions was assessed by immunoprecipitation, pull down and co-localization by subcellular fractionation. The key role of positively charged residues present in the interaction domain of the identified proteins was demonstrated. The defined interaction domain has therefore different molecular characteristics than conventional AKAP domains. Finally we assess initial experiments to visualize the physiological relevance of the interaction of both Ira2 and Hsp60 with Bcy1. Bcy1 interacts with Ira2 tethering PKA to the Ras complex and Hsp60 chaperone localizes PKA to mitochondria and has a role in the kinase stability.

Biological significance
Our work has an important impact in the field of signal transduction especially of protein kinase A. Components of the cAMP signaling cascade are localized in the cell via scaffold proteins named AKAPs that contribute to the high level specific regulation of the cAMP–PKA-signaling pathway. In the unicellular eukaryote *Saccharomyces cerevisiae* PKA has a pleiotropic role in the cell and the compartmentalization therefore is key to achieve the specificity in the response. At present all AKAPs have been described in mammals and it is unknown whether functional homologs of mammalian AKAPs exist in yeast. Therefore, it is unknown which molecular features of the mammalian anchoring proteins are general and which are distinctive. We have identified and characterized interacting proteins of protein kinase A regulatory subunit in *Saccharomyces cerevisiae*, through a proteomic and bioinformatic approach. Bcy1 tethering proteins have a domain in which charged positives residues are key for the interaction with regulatory subunit of PKA and Bcy1 N-terminus is important in the interaction. In mammalian AKAPs a hydrophobic amino acid face of an amphipathic α-helix is essential for the high affinity of the binding interaction. The results

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obtained in this work seem to indicate that the domains identified in the interacting Bcy1 proteins have a structural nature of the interaction different than those defined for mammalian AKAPs-R interaction. Not only positive charged residues are involved as distinctive molecular determinants but also the hydrophobic face of the helix in which they are included was not relevant in the interaction with Bcy1.
Even though generally the use of very well characterized models is essential to answer questions, as would be in this case AKAPs from mammals, the study of other alternative models contributes to the building of more universal concepts.

1. Introduction

The cells often respond to its environment by signaling through the action of enzyme cascades. The organization of these enzymes into multiprotein complexes allows a high degree of fidelity, efficiency and spatial precision in signaling responses. Spatial and temporal control of signal transduction events is frequently achieved by compartmentalization of intracellular effectors through adaptors or anchoring proteins [1].

Components of the cAMP signaling cascade are localized in the cell via scaffold proteins named A kinase anchoring proteins (AKAPs). The tetrameric protein kinase A (PKA) holoenzyme from mammals is composed of two regulatory R subunits and two catalytic C subunits, which are encoded by multiple genes: RIα, RIβ, RIIα, RIIβ, Cα and Cβ. There are two types of holoenzymes depending on the R type I or II that conform the tetramer. The differential interaction of the R isoforms with AKAPS [2] contributes to the high level specific regulation of the cAMP–PKA-signaling pathway. Type II PKA cell distribution is typically particulated through binding to subcellular compartments. The majority of the identified AKAPs are specific for type II PKA. However, although the type I PKA is assumed to be mainly cytoplasmic, several RI-specific AKAPs have been identified and characterized. A third class of AKAPs defined as dual-specificity AKAPs (D-AKAPs), such as Ezrin, Merlin, PAP7, D-AKAP1 and D-AKAP2 bind to both types of R subunits (reviewed in [3] and references therein).

Almost all the AKAP proteins bind the R subunit of PKA through an amphipathic helix of 14–18 amino acids in which large aliphatic side chains aligned create an extended hydrophobic surface [4]. The protein–protein interaction with R subunit involves the hydrophobic face of the α-helical region in the AKAP that binds tightly to a complementary hydrophobic groove preformed in the docking/dimerization (D/D) domain formed by the N-terminal α-helices of both protomers of the R subunit dimer. D/D domain (1–45 aa in RII and 12–61 aa in RI) folds into an antiparallel X type four-helix bundle that creates both a dimerization domain between R subunits and a docking site for AKAPs [5–10].

In the unicellular eukaryote *Saccharomyces cerevisiae*, the cAMP–PKA pathway controls a variety of essential cellular processes associated with fermentative growth, the entrance into stationary phase, stress responses and development [11–13]. This pleiotropic role of PKA is exerted through phosphorylation of many different proteins as transcription regulators, kinases, phosphatases and metabolic enzymes [14]. An interesting point, not well studied, is how PKA recognizes a great diversity of substrates, all with very similar phosphorylation consensus sequence, located at different subcellular sites. Different regulatory mechanisms must exist to ensure the phosphorylation of the correct substrate under the proper stimulus. PKA from *S. cerevisiae* is a tetrameric holoenzyme conformed by a dimer of regulatory subunit (Bcy1), and three different isoforms of catalytic subunits (Tpk1, Tpk2, Tpk3). Specific physiological roles have been reported for each Tpk isoform [15,16]. However, the substrate specificity for the isoforms Tpk1, Tpk2 and Tpk3 in yeast does not seem to rely on the sequence determinants around the phosphorylation sequence nor on a difference in K_{cat} for each isoform [17]. Other points of regulation that could contribute to isoform selectivity, and therefore to specificity of PKA-signaling, are the participation of the substrate in the activation of PKA by cAMP [17] and the subcellular localization of Bcy1 and hence of the holoenzyme.

Bcy1 presents a dynamic pattern of localization dependent on carbon source [18]. It has been demonstrated that residues 1 to 48 of Bcy1 are required for its localization [18]. Two α-helices can be predicted at the Bcy1 N-terminus; structure modeling of this region suggests an N-terminal structure similar to the one of mammals ([19] and N. González Bardeci, personal communication).

At present it is unknown whether functional homologs of mammalian AKAPs exist in yeast. The protein Zds2 was identified as an interacting protein in a two-hybrid screen, using the N-terminal domain of Bcy1 as bait, and its homolog Zds1 was shown to participate in PKA localization [20]. An interesting feature of Bcy1 N-terminus is its phosphorylation at multiple serine residues distributed in two clusters; cluster I within the first 10 aa and cluster II from 68 to 84. Both clusters are needed for Bcy1 cytoplasmic localization in the absence of glucose [20]. Bcy1 phosphorylation is dependent to some extent, on the protein kinase Yak1 [20,21], and the phosphorylation of serines in cluster II are suggested as possible targets to increase the affinity of Bcy1 for Zds1 [19]. Since its discovery, Zds1 has been proposed as a putative functional homolog of mammalian AKAPs but its interaction with Bcy1 has not been characterized at molecular or structural levels.

To get insight into the mechanism of PKA localization in *S. cerevisiae* we have isolated and identified Bcy-1-associated proteins using a computational approach and an MS-based proteomic analysis, verified some of these interactions, determined the residues on the interacting proteins that are important for the interaction with Bcy1 N-terminus and finally conducted some initial experiments to visualize the physiological relevance of the interaction of two of these targets, Ira2 and Hsp60 with Bcy1.

2. Experimental procedures

2.1. Growth media, plasmids, bacteria and yeast strains

Yeast media were prepared as described [22]. Yeast strains were grown in rich medium containing 2% bactopeptone, 1% yeast extract and 2% glucose (YPD), 2% galactose (YPGal) or 2% glycerol (YPGly). Synthetic medium containing 0.67% yeast nitrogen base without amino acid, 2% glucose, plus the necessary addition to fulfill auxotrophic requirements (SD) were used to maintain the selectable plasmids. Solid medium contained 2% agar. Bacterial cultures were grown in Luria–Bertani medium (LB) containing 1% peptone, 0.5% yeast extract and 0.5% NaCl. YEp51-BCY1 plasmid was constructed previously in our laboratory [23]. For construction of YEp51-Δ85BCY1 plasmid, the DNA fragment containing the sequence comprising amino acids 86–416 of Bcy1 was cloned in Yep51 vector. The primers used to amplify this sequence were: *Forward:* 5′GTCCATGGGTATGTTCAAATCCCCCTT3′, *Reverse:* 5′GTAAGCTTT TAATGTCTTGTAGGATCAT3′. The restriction sites used were NcoI and HindIII. pRSETb-Δ168BCY1 and pRSETb-Δ138BCY1 were constructed previously in our laboratory [24]. *Escherichia coli* BL21-CodonPlus (DE3)-RIPL cells were transformed with pRSETb-Δ168BCY1 or pRSETb-Δ138BCY1. Strains containing plasmids BCY1 WT and cluster I and II mutated were prepared as follows: 33pBGHBwt expresses a GFP-HA–BCY1 fusion, encoding wild-type Bcy1 fused to GFP-HA, under the control of BCY1 promoter in vector YCplac3333pBGHB; 33pBGHB (Ser cluster I Ala) is a 33pBGHBwt derivative encoding Bcy1 bearing S3A, S4A, and S9A substitutions; 33pBGHB (Ser cluster II Ala) is a 33pBGHBwt derivative encoding Bcy1 bearing S74A, S77A, S79A, S81A, S83A, and S84A substitutions; 33pBGHB (Ser cluster I + II Ala) is a 33pBGHBwt derivative encoding Bcy1 bearing S3A, S4A, S9A, S74A, S77A, S79A, S81A, S83A, and S84A substitutions. Yeast strains used in this study are listed in Table 1

Table 1 – Yeast strains.

Strain	Genotype	Reference or source
KT 1115	*Mata leu2 ura3 his3 pep4Δ*	[25]
1115-BCY1	KT1115 + YEp51-BCY1	
133 (*TPK1bcy1Δ*)	(SP1) *tpk2::HIS3, tpk3::TRP1, bcy1::LEU2*	[26]
THY428a	*MATa IRA2-3HA-G418 ura3-52 leu2Δ::hisG*	[27]
IRA2-TAP	*S288C (ATCC 201388) MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 IRA2-TAP*	Open Biosystems
BCY1-TAP	*S288C (ATCC 201388) MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 BCY1-TAP*	Open Biosystems
ENO2-TAP	*S288C (ATCC 201388) MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 ENO2-TAP*	Open Biosystems
COP1-TAP	*S288C (ATCC 201388) MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 COP1-TAP*	Open Biosystems
MYO2-HA-His	*KT1115 + pBG1805-MYO2-HA-His*	Open Biosystems
HSP60-HA-His	*KT1115 + pBG1805-HSP60-HA-His*	Open Biosystems
YHSP60-JK9-3d (Hsp60-WT)	*ura3 leu2 his4 trp1 rme1 HMLa hsp60::TRP1 + pYCPlacHSP60*	[28]
YMIF-JK9-3d (Hsp60-ts)	*ura3 leu2 his4 trp1 rme1 HMLa hsp60::TRP1 + pYCPlac MIF4*	[28]

2.2. Protein purifications

2.2.1. Bcy1 and deletion mutants expression and purification

Bcy1 and Δ85BCY1 proteins were overexpressed in *S. cerevisiae* 1115 strain containing the plasmid YEP51-BCY1 or YEp51-Δ85BCY1 under galactose promoter. The deletion mutants, Δ138Bcy1 and Δ168Bcy1 proteins were expressed in *E. coli* BL21-CodonPlus (DE3)-RIPL cells containing the plasmids pRSETb-Δ138BCY1 or pRSET-Δ168BCY1. Bcy1 full length and deletions mutants were purified on cAMP-agarose beads.

2.2.2. TAP purification

The standard TAP purification protocol was used for protein complex purification [29].

2.2.3. cAMP-agarose purification

Yeast strain 1115 harboring the plasmid YEP51-Bcy1 (1115-BCY1) grown in YPGly or YPD medium o.n at 30 °C (OD_{600nm} = 1.5) or during 7 days (stationary phase) were used to purify the Bcy1 interacting proteins. Cells were harvested by centrifugation and extracts were prepared by vortexing the pellets with glass beads in lysis buffer (10 mM NaH_2PO_4, 15 mM Na_2HPO_4, 0.1% NP-40, 150 mM NaCl, 2 mM EDTA, pH 7.2) plus protease inhibitors. Equal amounts of the clarified extracts were incubated o.n. at 4 °C with 50 μl of N6-cAMP-agarose (BioLog) in the absence or presence of 50 mM cAMP (nonspecific control). The resin was exhaustively washed with lysis buffer and the proteins eluted by addition of 50 μl of 4x sample buffer. The eluted proteins were electrophoresed on 10% SDS-PAGE and the gel was stained with colloidal Coomassie Blue. The bands chosen to be excised from the gel, were those that diminished or were absent when the extract was loaded in the presence of cAMP. Extracts prepared from the strain expressing Δ85Bcy1, and from the strain *bcy1Δ* were subjected to the same cAMP-agarose chromatography procedure, and SDS-PAGE analysis.

2.3. MALDI-MS and protein identification

2.3.1. In-gel tryptic digestion

Gel bands were cut in equal slices, distained in ACN: 20 mM NH_4HCO_3 pH 8.5 (1:1), dried at room temperature, rehydrated with 25 mM NH_4HCO_3, reduced with 10 mM DTT, and alkylated with 55 mM iodoacetamide in 25 mM NH_4HCO_3. Tryptic digestion was performed incubating each slice with 120 ng of trypsin (Promega sequencing grade modified) in 20 mM NH_4HCO_3 pH 8.5 o.n. at 37 °C. Peptides were extracted by incubating each slice with 50% ACN 0.5% TFA.

2.3.2. MALDI-MS and protein identification

Aliquots of the extracted peptides were mixed 1:1 with matrix (3 mg/ml 4-hydroxy-α-cyano-cinnamic acid in 50% ACN 0.5% TFA), loaded onto MTP Anchor Chip target (Bruker Daltonics) for co-crystallization and analyzed in an Ultraflex II MALDI TOF–TOF mass spectrometer for MS and MS/MS analysis. Data analysis was performed using the MASCOT search tool embedded in the BioTools software package (Bruker Daltonics) using the UniProtKB/Swiss-Prot release 15.3 database, taxonomy *S.*

cerevisiae, up to two allowed trypsin miscleavages, fixed cysteine carbamidomethylation, variable methionine oxidation, and mass accuracy of 150 ppm and 0.7 Da for precursor and fragment ions respectively. The protein identifications were considered confident according to a significant Mascot score ($p < 0.05$).

2.4. Peptide Array

Cellulose-bound peptide arrays were prepared automatically at Susan Taylor's laboratory, Department of Chemistry and Biochemistry, University of California San Diego, according to standard spot synthesis protocols by using a spot synthesizer (Abimed Analysen-Technik, Langenfeld, Germany). The membranes were first wet in ethanol, sonicated 20 min, washed three times with Tris-buffered saline (TBS), blocked in blocking solution (5% BSA, 0.1% Tween 20 in TBS buffer) overnight at 4 °C, and incubated with purified Bcy1 or amino-terminal deletion mutants of Bcy1 at a final concentration of 20 μg/ml in blocking solution for 2 h at room temperature. For the Ht31 competition assay, Ht31 or scramble peptide were added at a final concentration of 100 μM together with purified Bcy1. Membranes were rinsed three times with TBS 0.1% Tween 20 and incubated with *Mucor rouxii* anti-regulatory subunit rabbit polyclonal antiserum at a dilution of 1/5000 for 1.5 h at room temperature followed by incubation with anti rabbit IgG peroxidase. The *M. rouxii* antibody cross-reacts and specifically recognizes Bcy1 (see Supplementary Fig. 1) and the signal obtained with this antibody was markedly stronger than that obtained with commercial anti-Bcy1 antibody.

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2.5. Immunoprecipitation

Yeast strain IRA2-HA was grown in YPD medium to mid-exponential phase (OD_{600nm} = 0.7–0.8). Crude extracts were prepared using glass beads and buffer containing 10 mM NaH_2PO_4-H_2O, 15 mM Na_2HPO_4, 150 mM NaCl, 2 mM EDTA, 0.3% CHAPS and protease inhibitors and incubated with anti-HA (HA-probe Antibody (Y-11): sc-805) 1 h at 4 °C, followed by the addition of A/G PLUS agarose (Santa Cruz Biotechnology) and incubation for 16 hs at 4 °C. Immunoprecipitates were washed with 25 mM Tris–HCl pH 8, 150 mM NaCl, 0.1% NP-40 buffer and TBS buffer. The immunoprecipitates were subjected to Western blot analysis with the indicated antibodies.

2.6. Pull-down assays

Bcy1 protein was immobilized on 50 μl of cAMP-agarose resin. The resin was equilibrated with lysis buffer (20 mM MES pH 6.5, 100 mM NaCl, 2 mM DTT and protein inhibitors). Crude extracts from IRA2-TAP, PTP1-TAP, ENO2-HA, MYO2-HA or HSP60-HA containing strains were prepared using lysis buffer (50 mM Tris–HCl pH 7, 5 mM EDTA, 3 mM EGTA, 2 mM 2-β-mercaptoethanol, 5 mM glycerol-phosphate, 100 mM NaCl, 0.3% CHAPS, and protein inhibitors). The clarified lysates were added to the Bcy1-cAMP agarose resin, incubated for 2 h at 4 °C, washed with lysis buffer containing 150 mM NaCl plus 0.2% NP-40 and 0.3% SDS, and proteins eluted with 50 μl of 4 x sample buffer. As a control, an equal amount of crude extract was incubated with cAMP-agarose beads in presence of 50 mM cAMP. The samples were electrophoresed on 10% or 6% SDS-PAGE, the proteins visualized by western blot with anti-Bcy1 (Santa Cruz Biotechnology Inc), anti-TAP (Open Biosystems) or anti-HA antibodies (Santa Cruz Biotechnology Inc).

2.7. Subcellular fractionation, membrane isolation

2.7.1. Spheroplast preparation

Yeast strains were grown in YPD medium to mid-exponential phase. Cells were harvested by centrifugation at 3000x*g* for 5 min. The cellular pellet was washed with distilled water, resuspended in 100 mM Tris–HCl pH 9.4, 10 mM DTT buffer, shaken slowly for 20 min at 30 °C, washed with zymolyase buffer (1.2 M sorbitol, 20 mM potassium phosphate pH 7.4) and incubated with lyticase (Sigma-L2524) for 20 min at 30 °C with gentle agitation until conversion into spheroplasts. Spheroplasts were washed, resuspended in homogenization buffer (0.6 M sorbitol, 10 mM Tris–HCl pH 7.4, 1 mM EDTA, 1 mM PMSF) and the homogenate prepared with 15 strokes of a glass potter.

2.7.2. Subcellular fractionation, membrane isolation

Yeast IRA2-HA was grown in YPD medium to mid-exponential phase (OD_{600nm} = 0.7–0.8). Cells were harvested by centrifugation and spheroplasts were prepared as detailed above. Organelles and membranes enrichment was performed according to the protocol for differential centrifugation in yeast cells described in [30]. The crude extract was clarified by centrifugation for 10 min at 300x*g*. The supernatant (S3) was centrifuged 10 min at 13,000x*g* . This supernatant (S13) was centrifuged 60 min at 100,000x*g* resulting in S100 and P100. The S100 was precipitated with 25% of trichloroacetic acid; the S100 pellet and the P3, P13 and P100 fractions were resuspended in 8 M urea 5% SDS and heated for 10 min at 65 °C. The aliquots were saved for immunoblot analysis. The same fractionation procedure was performed to assess Bcy1 localization, but the strains used were transformed with the different plasmids containing the construct GFP-Bcy1 wt, or CI, CII or CI plus CII mutations. The Western blots were developed using anti-GFP (GFP Antibody (B-2): sc-9996 Santa Cruz Biotechnology).

2.7.3. Preparation of crude mitochondrial fraction

Yeast strains HSP60-HA, YHSP60-JK9-3d or YMIF-JK9-3d were grown in the appropriate medium to OD_{600nm} = 0.7–0.8 at 24 °C. Half of the culture was harvested by centrifugation and the rest was incubated for 2 h at 37 °C. Spheroplasts were prepared as detailed above. The homogenate was centrifuged for 5 min at 1500x*g* resulting in S1500 (S1) and P1500 (P1). The S1 fraction was centrifuged for 5 min at 3000x*g* obtaining S2 and P2 fractions. The S2 supernatant was centrifuged for 15 min at 13000x*g* resulting in the crude mitochondrial fraction, S3 and P3. The S1, S2 and S3 fractions were precipitated with 25% trichloroacetic acid and the resulting pellets, plus the P1, P2 and P3 fractions were resuspended in 8 M urea and 5% SDS and heated for 10 min at 65 °C. The aliquots were saved for immunoblot analysis.

2.8. Protein kinase A assay

Yeast Tpks were used as source of enzyme to determine the phosphorylation of Ira2 derived peptides. The catalytic subunits purification and the determination of kinetic parameters were performed as described previously [17]. PKA activity was determined by assay of its phosphotransferase activity using Nth1-1 (control), Ira2-S1018 or Ira2-S1725 peptides as substrates. The assay was initiated by mixing different amounts of catalytic subunits (Tpks mixture) with peptide substrates at the concentration indicated in each experiment plus buffer containing 15 mM Tris–HCl pH 7.5, 0.1 mM EGTA, 0.1 mM EDTA, 10 mM 2-mercaptoethanol, 15 mM $MgCl_2$, 100 μM [γ-^{32}P]ATP (1000 dpm/pmol). The reaction mix was incubated for 10 min at 30 °C. Aliquots of the reaction mix were processed according to the phosphocellulose paper method (P81; Whatman) [31].

For PKA kinase assays in Hsp60 WT and ts mutant, yeast strains YHSP60-JK93d (Hsp60-WT) or YMIF-JK93d (Hsp60-ts) were grown overnight at 24 °C. The cultures were transferred to 37 °C and aliquots were collected at 0, 15, 30 and 60 min. cAMP-dependent kinase activity (in the presence of 100 μM cAMP) and endogenous levels of Tpk1 were assayed in crude extracts of each aliquot.

3. Results

3.1. Identification of Bcy1 interacting proteins

The search and identification of Bcy1-interacting proteins was addressed using two strategies: an experimental assay based on cAMP-agarose affinity purification of Bcy1 interacting complexes followed by proteomic analysis, and a computational prediction of PKA binding domains on the protein sequences. We purified interacting proteins derived from cultures of two yeast strains, grown to exponential stage using glucose as carbon source; a strain with endogenous levels of Bcy1 and another one in which Bcy1 was overexpressed in order to increase the probability of interaction among the bait and the interacting proteins. The crude extracts were incubated with cAMP-agarose beads, in the absence or presence of cAMP (50 mM). Following incubation and affinity purification, the enriched proteins were separated using one-dimensional gel electrophoresis and stained with colloidal Coomassie Brilliant Blue (Fig. 1A). We used two controls: a strain lacking Bcy1 (TPK1*bcy1*Δ) and a strain overexpressing a monomeric Bcy1 protein lacking the first 85 amino acids (Fig. 1A and B). Both control samples were subjected to the same treatment. The protein bands chosen to be analyzed by MALDI-TOF-TOF were those with a high decrease in intensity or totally absent in the extracts that had been incubated in the presence of cAMP. The rationale was the following: in the presence of cAMP during the incubation of the extract containing either endogenous or increased levels of Bcy1 with cAMP-agarose, a high decrease in Bcy1 and Bcy1 binding proteins was expected due to a competition between the cAMP in the mobile phase and the agarose-bound cAMP. As we hypothesidzed that the proteins that decreased or disappeared were bound through the N-terminus of Bcy1, then a control performed with an extract from a strain deleted for Bcy1, or from a strain expressing a mutant of Bcy1 with its N-terminus deleted (Δ85 Bcy1), should not have retained any protein. Therefore both controls should

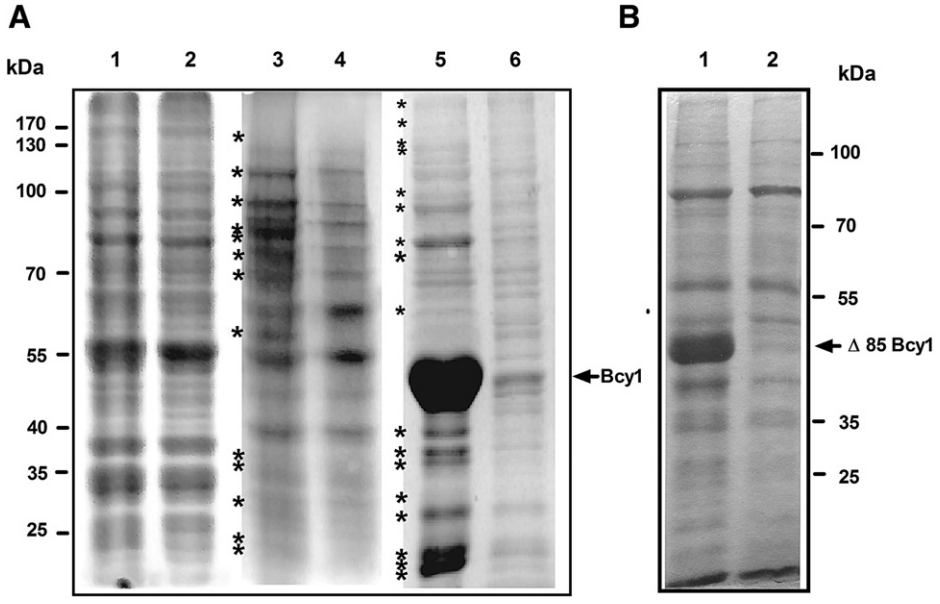


Fig. 1 – Purification of Bcy1 interacting proteins. **Extracts from the following strains of *S. cerevisiae* were loaded onto cAMP-agarose in the absence (panel A, lanes 1, 3, 5 and panel B, lane 1) or in the presence of 50 mM cAMP (panel A, lanes 2, 4, 6 and Panel B, lane 2): 1115-BCY1 (Bcy1 overexpression, lanes 5–6, panel A), KT1115 (Bcy1 endogenous level, lanes 3–4, panel A), control TPK1*bcy1*Δ (lanes 1–2, panel A) and control Δ85-Bcy1 (lanes 1–2, panel B). Proteins bound to the resin were analyzed by SDS-PAGE and stained with Colloidal Coomassie Blue. The arrows indicate the bands corresponding to Bcy1 and Δ85-Bcy1. Gels are representative experiments from at least three biological replicates from each strain. Asterisks indicate excised bands selected for mass spectrometric identification.**

have produced similar protein profiles in the cAMP-agarose affinity chromatography independently of the addition of cAMP to the incubation buffer, as in fact occurred (Fig. 1A (lanes 1 and 2) and 1B (lanes 1 and 2)). From these purification experiments we could identify an important number of proteins, summarized in Supplementary Table 1.A. Even though under the experimental condition using overexpression of Bcy1 the bait expression is extremely high, most of the identified proteins were shared with those isolated when using a physiological condition with endogenous levels of Bcy1. Besides the identification of these common proteins, singular proteins were identified in each case. Among the identified proteins, there were chaperones and heat shock proteins. Heat shock proteins are sometimes considered as artifacts derived from the interaction with misfolded bait proteins [32]. However, we demonstrate below that Hsp60 is a *bona fide* Bcy1 interactor.

Since interacting PKA proteins have not been described in *S. cerevisiae*, we used the computational method described by McLaughlin *et al* [33] to predict a Bcy1 interacting domain within yeast proteins. In this method a hidden Markov model (HMM) of sites that interact with the RIIα isoform was generated from the alignment of 22 known interaction sequences of mammalian AKAPs. The chosen sequences, based on three dimensional structural analysis were strings of 19 amino acids that directly interact with R subunit. The HMM search was applied to the proteins identified by affinity purification and MS-based proteomic analysis and to the entire *S. cerevisiae* proteome (SGD). Putative false positives were removed based on a low likelihood of adopting an alpha helical conformation and of being embedded within a region with low evolutionary conservation.

Potential PKA R subunit binding sites were ranked according to their similarity to the HMM and from this analysis only six candidate peptides were defined as putative interactors of PKA. These peptides arose with low probability but a high rank above the threshold. We found putative interacting domains in the following proteins identified by affinity purification: ENO2 Enolase II (YHR174W); MYO2 Type V myosin motor (YOR326W); COP1 alpha subunit of COPI vesicle coatomer complex (YDL145C) and HSP60 mitochondrial chaperonin (YLR259C). We also identified putative interaction domains in two proteins derived from the whole genome analysis: IRA2, a Ras GTPase-activating protein (YOL081W) and PTP1, phosphotyrosine-specific protein phosphatase (YDL230W). The 25 amino acid strings of these predicted domains were summarized in Table 2.

Purification of Bcy1 interacting proteins was also performed in extracts from yeast glucose cultures grown up to stationary phase, as well as in exponentially growing cells in which glycerol was the carbon source. Under these conditions we could identify some proteins in common with those identified from cultures grown in glucose, but also some new ones. Purification from stationary phase cells is shown in Supplementary Fig. 1, lanes 1 and 2, while those of glycerol grown cells is shown in Supplementary Fig. 1, lanes 3 and 4. The identified specific proteins from lanes 1 and 4 are summarized in Supplementary Table 1 B.1 and B.2 respectively.

3.2. *In vitro binding of Bcy1 with interacting proteins peptide domains*

The interaction between the proteins and Bcy1 was assessed in vitro by pull down assays and western blot analysis. Bcy1 bound to cAMP-agarose was incubated with extracts from yeast strains expressing the following fusion proteins: Ira2-TAP, Ptp1-TAP, Hsp60-HA, Myo2-HA, Eno2-HA or Cop1-HA. As shown in Fig. 2, Ira2, Ptp1, Hsp60 and Myo2 proteins showed interaction with Bcy1 and no direct interaction with cAMP-agarose control resin. Eno2-HA showed a very slight interaction and Cop1 showed no detectable interaction (not shown). These results indicate that both Hsp60 and Myo2 that were isolated from in vivo complexes with Bcy1 also interacted in vitro. The interaction with Bcy1 of the candidate binding site motives obtained from the bioinformatic analysis was tested by experimental peptide array. Known binding sequences from mammalian AKAPs were used as controls: AKAP7, an AKAP that binds RIIα subunit with high affinity; AKAP2, also named D-AKAP2 because it can interact with type I or type II R subunits, and the peptide Ht31 from AKAP-Lbc which binds RII, and is currently used as an in vivo and in vitro disruptor of PKA–AKAP interactions [34]. Peptide arrays were incubated with Bcy1 (20 μg/ml) and developed with polyclonal anti-R from *Mucor rouxii*. The specificity and sensibility of *M. rouxii* anti-R toward Bcy1 was assessed and shown to recognize Bcy1 wt protein and mutant constructions (see below and Supplementary Fig. 2). As shown in Fig. 3, the peptides Ira2, Myo2, Hsp60 and Ptp1 showed interaction with Bcy1 while no interaction was detected for Eno2 or Cop1. Eno 2 had shown a slight interaction in the pull-down assay and therefore the lack of interaction in the array suggests that either Eno2 is not a specific interacting protein or that the chosen domain was not the real interaction domain or that it could be incomplete. Peptides derived from mammalian AKAP proteins, known to interact with RI/RII when assayed on peptide arrays similar to the one of Fig. 3, showed almost no interaction with Bcy1. Ht31 was assayed as a competitor peptide by addition of the peptide to the incubation with Bcy1 but no inhibition of binding of the yeast proteins was observed (data not shown).

Table 2 – Peptide interacting domains.

Systematic Name	Standard Name	Residue Range	Peptide sequence
YOL081W	**Ira2**	2507–2531	TVLPTTEVANNIIQKILAKIRSFLP
YHR174W	**Eno2**	222–246	AEEALDLIVDAIKAAGHGDKVKIGL
YOR326W	**Myo2**	943–968	LENKVIELTQNASKVKENKEMTERI
YDL145C	**Cop1**	618–642	LIKDSNLVGQNIISYLQKSGYPEIA
YLR259C	**Hsp60**	137–161	LRRGSQVAVEKVIEFLSANKKEITT
YDL230W	**Ptp1**	285–309	SDRATEEYTRDLIEQIVLQLRSQRM

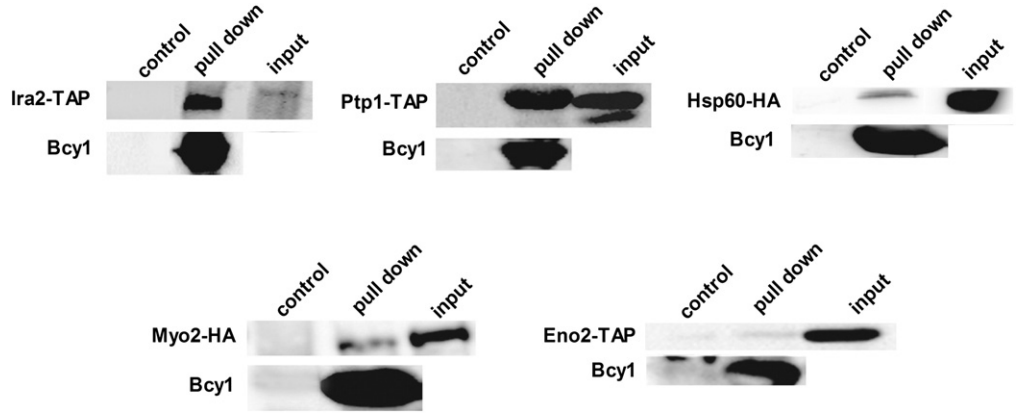


Fig. 2 – Ira2, Ptp1, Myo2 and Hsp60 bind Bcy1 in vitro. Pull-down assays performed using Bcy1 bound to cAMP-agarose and crude extracts from strains expressing IRA2-TAP, PTP1-TAP, HSP60-HA, MYO2-HA or ENO2-TAP. Western blots were performed with antibodies anti-TAP, anti-HA or anti-Bcy1. Extracts from the same strains were incubated with cAMP-agarose in the presence of 50 mM cAMP as controls. Inputs correspond to 0.5% from total extract loaded onto the resin.

The peptide arrays were also developed with N-terminal deletion mutants of Bcy1: Δ85 Bcy1 in which the D/D domain, plus part of linker I is being deleted, Δ138 Bcy1, which contains the deletion of the D/D domain and the whole of linker I domain but preserves the inhibitory site of the R subunit and Δ168 Bcy1 which contains almost exclusively the two cAMP binding domains. The results, shown in Fig. 3, indicated that the amino terminus of Bcy1 was necessary for the observed interaction with peptides derived from Ira2, Myo2 and Hsp60.

These results suggested that the interaction of the yeast proteins, through the selected interacting peptides might be through the D/D domain of Bcy1; however the difference in behavior of the interacting yeast peptides as compared to the selected mammalian AKAP peptides, not interacting with Bcy1, suggested that the structural nature of the interaction might be different.

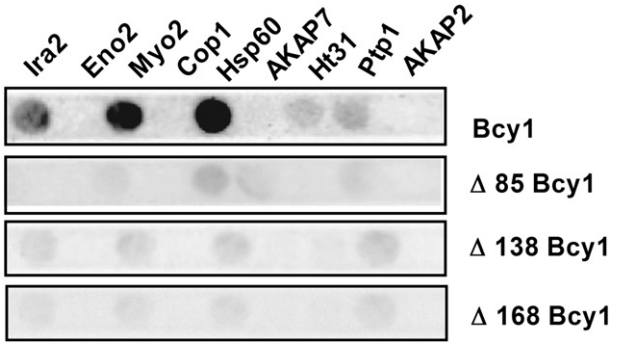


Fig. 3 – N-terminus of Bcy1 is necessary to bind the interacting proteins binding domains. Peptide array of the Bcy1-binding peptide-derivatives (as 25-mers) and peptides derived from AKAP7, Ht31 and AKAP2 (mammalian AKAPs) were synthesized in quadruplicate on membranes and incubated with equal amounts of Bcy1 or the indicated N-terminally truncated Bcy1 proteins (20 μg/ml). Binding of the Bcy1 proteins was detected using antibody anti R subunit from *M. rouxii*. The image corresponds to a representative experiment of three independent experiments.

3.3. Charged amino acids in interacting proteins are required for their interaction with Bcy1

To assess the requirement of individual side chains in the binding of the peptides to the regulatory subunit, we chose to study the behavior of the 25-mer Ira 2 peptide. This peptide was predicted to fold into an α-helix that contains hydrophobic and hydrophilic surfaces (Fig. 4). Peptide arrays were synthesized containing substitutions to alanine at 13 positions in the 25-mer Ira2 peptide, as shown in Fig. 4, top. The results of Ala scanning showed that the only residues that when mutated abolished the binding of Bcy1 were the positively charged residues Lys and Arg. This result was completely different from the one shown by RII AKAP peptides that abolish the binding when hydrophobic residues from the hydrophobic face of the helix are mutated to Ala. In the case of Ira2 peptide, although the presence of an α-helix with amphipathic characteristics was predicted, with the classical spacing of hydrophobic amino acids (see Supplementary Fig. 3 bottom), the replacement of branched chain hydrophobic amino acids did not significantly affect the interaction with Bcy1. As control the Ala scanning was carried out on the peptide derived from AKAP7, which showed an almost undetectable interaction with Bcy1 in the peptide array (Fig. 3). The replacement of some hydrophobic or acidic residues in the proximity of the positively charged residues Lys and Arg improved the binding of Bcy1 to the peptide (Fig. 4 bottom). These results indicated that specific electrostatic interactions were involved in the stability of Ira2-Bcy1 interaction. To corroborate these results Ala scanning for Hsp60 and Myo2 were assessed, together with Ira2 (Supplementary Fig. 3). As occurred with Ira2, the only replacements that decreased significantly the interaction with Bcy1 were those of Arg and Lys residues.

The Bcy1 N-terminal domain contains two clusters (I and II) of amino acids which are particularly serine-rich. Cluster I is located near the N terminus (1–10), and cluster II is located between residues 68 to 84. There are some evidences indicating that cluster II and cluster I serines can be phosphorylated. Mutants containing the two serine clusters substituted by Ala have been shown to enhance nuclear accumulation of Bcy1 in

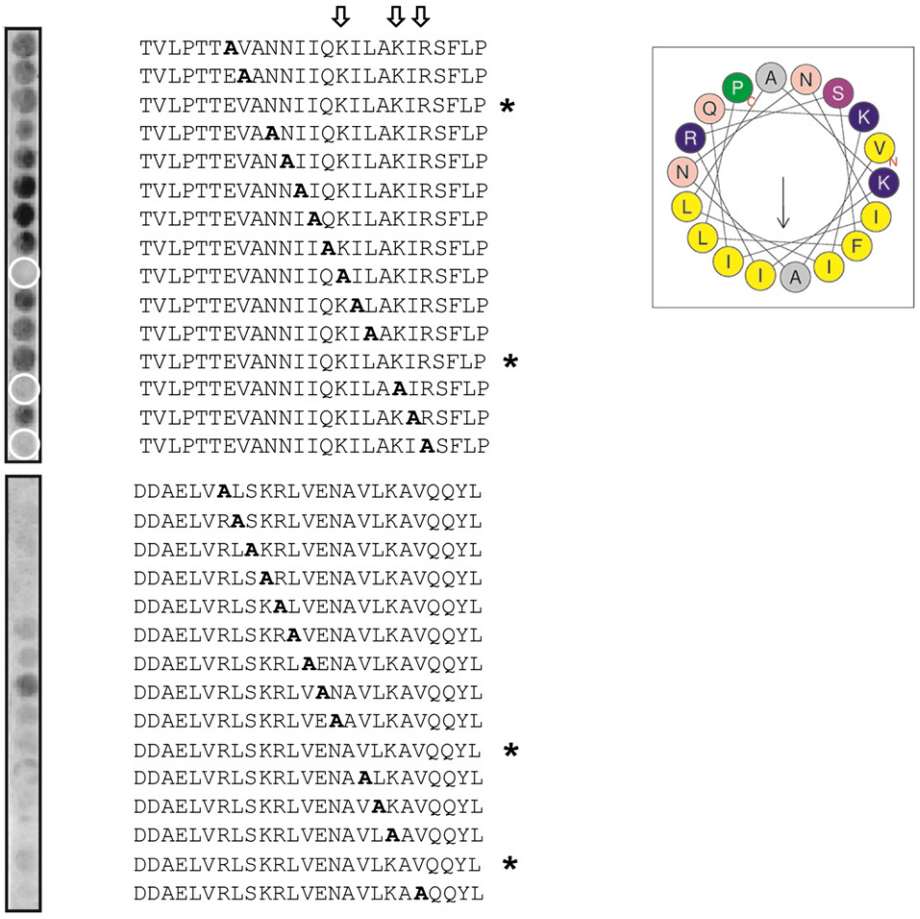


Fig. 4 – Positively charged residues in interacting protein binding domains are key in the interaction with Bcy1. Peptide substitution arrays of the 25-residue domain of Ira2, and AKAP7 were prepared by SPOT synthesis. Amino acids were substituted by Alanine into 13 positions along the binding domains (right side of blot). The sequences with an asterisk are identical to the wild type unsubstituted peptide. Binding was evaluated by using an antibody anti-*M. rouxii* R subunit. White circles on the array indicate those positions that disrupt binding to Bcy1; these positions correspond to those indicated with an arrow on top of the sequences. The top right panel shows the predicted amphipathic α-helix for the Ira2 peptide using the HeliQuest web server [35]. The arrow indicates the helical hydrophobic moment; amino acids are colored according to the chemical nature of the amino acid and the program standards.

ethanol-grown cells. In contrast, substitutions to Asp produce an increase of cytoplasmic localization in glucose-grown cells [20]. This result seems to indicate that a negatively charged amino acid regulates localization of Bcy1. Trying to get further insight into this non-canonical interaction between the interacting proteins and Bcy1, we assessed the importance of the CI and CII in the localization of R subunit in different subcellular fractions. Differential centrifugation fractionation of extracts of yeast transformed with plasmids containing the CI, CII, or CI and CII Bcy1 mutants (Ser to Ala) as fusion proteins with GFP were used to analyze Bcy1 localization in different membrane or soluble fractions (P3, S3; P13, S13 and P100, S100) (Fig. 5). The ratio of Bcy1 amount between P13/S13 and particularly between P100/S100, when both clusters CI and CII were mutated was lower than the corresponding ratio in the strain containing the WT version of Bcy1. According to the differential centrifugation protocol [30] P100 contains mainly transport vesicles, Golgi complex and endosomal membranes, while P13 is enriched in vacuolar membranes, nuclear membranes, endoplasmic reticulum, plasma membranes, endosomal membranes, mitochondrias, and Golgi complex in contrast to S100 fraction containing soluble cytoplasmic proteins (Fig. 5 panel A and C). No differences were observed in the Bcy1 distribution when the fractionations were performed with cells expressing versions of Bcy1 with only CI or only CII mutated (Fig. 5 panel B, only P100 and S100 are shown). The results suggested that Bcy1 N-terminus phosphorylation is important for Bcy1 association to membranes and vesicles and allow us to speculate that the correlation between the positive charges present in the interaction domain of the proteins and the negative charges of the serine residues present in the N-terminus of Bcy1 has a structural/physiological significance.

3.4. *Hsp60 and Ira2 co-localize in vivo with Bcy1: possible physiological relevance of the interaction with Bcy1*

To get further insight in the characterization of the interaction between Bcy1 and the identified proteins, we selected two of them, Ira2 and Hsp60 to assess the in vivo interaction by protein colocalization and immunoprecipitation analysis. Ira2 is a GTPase-activating protein; it negatively regulates RAS by

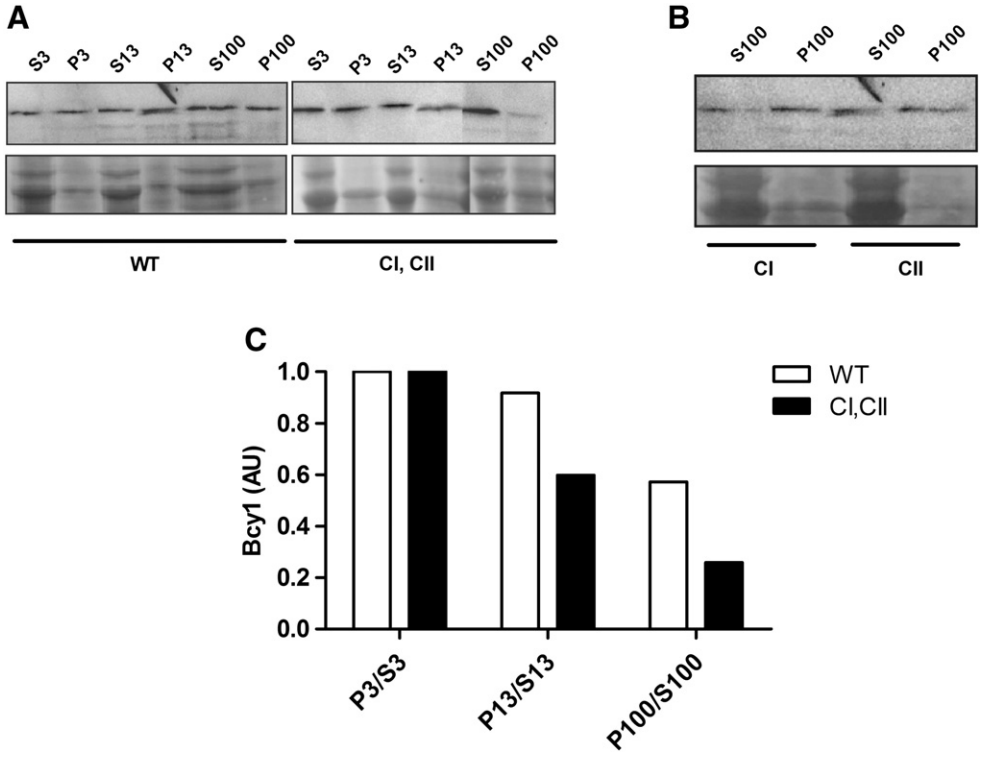
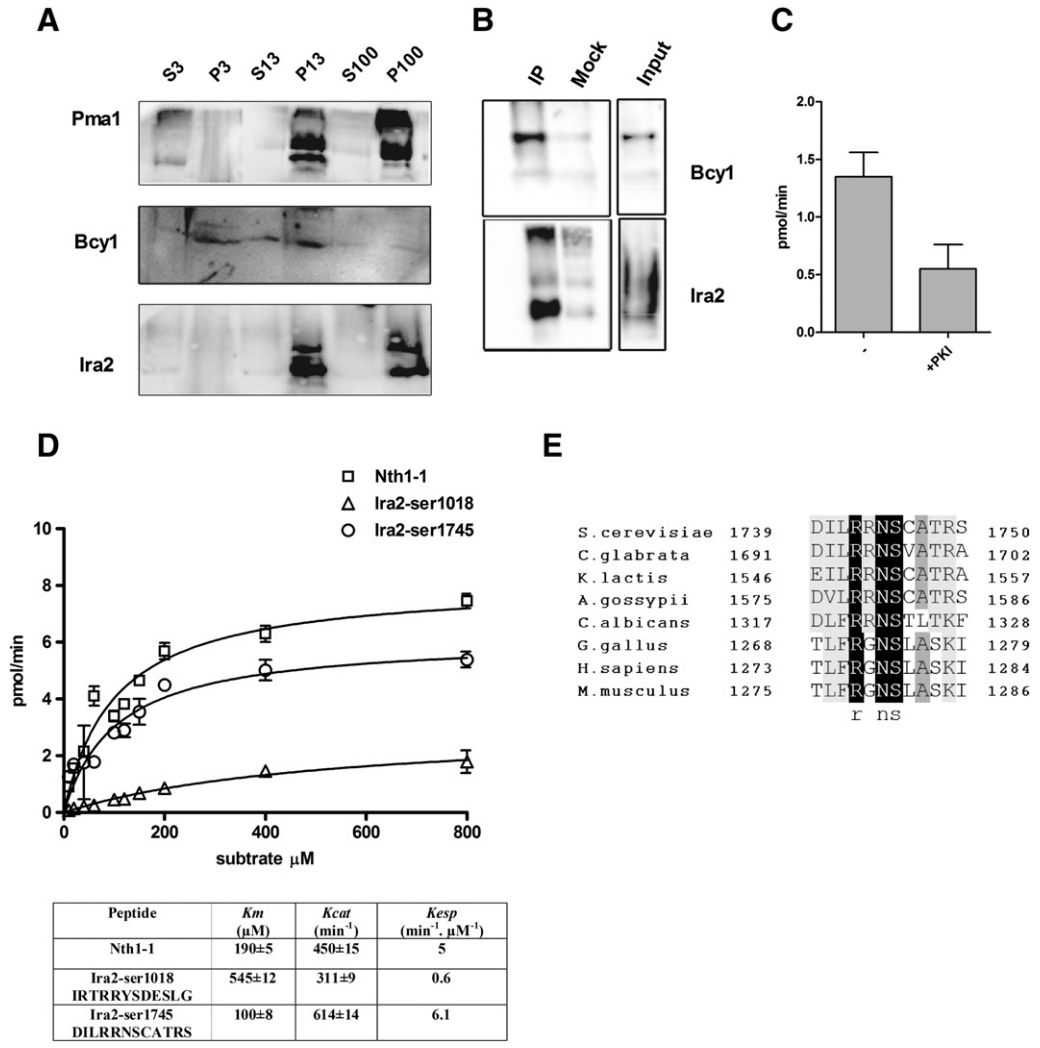


Fig. 5 – Bcy1 localization in membrane fractions. **Extracts of yeast transformed with plasmids containing Bcy1 WT, or Bcy1 with CI, CII, or CI and CII mutated (Ser to Ala) as fusion proteins with GFP were analyzed by differential centrifugation fractionation. Bcy1 in membrane or soluble fractions P3, S3; P13, S13 and P100, S100 was visualized by Western-blot with anti-GFP . A) Bcy1 WT and Bcy1 with clusters CI and CII mutated from Ser to Ala; top panel Bcy1, bottom panel total proteins in each fraction stained with Ponceau Red; B) S100 and P100 fractions of strains containing either Bcy1 CI or Bcy1 CII mutant plasmids; C) Band quantification of panel A Western blot; Bcy1 amount is expressed as the ratio between the amount in Pellet vs Soluble fractions, relative to the ratio P3/S3 taken as 1. AU: arbitrary units.**

converting it from the GTP- to the GDP-bound inactive form, required for reducing cAMP levels under nutrient limiting conditions [14]. Hsp60 is a tetradecameric mitochondrial chaperonin; required for ATP-dependent folding of precursor polypeptides and complex assembly; prevents aggregation and mediates protein refolding after heat shock [36] Crude cellular membranes were prepared and followed by the analysis of the distribution of endogenously expressed Ira2-HA protein by Western blot. The results shown in Fig. 6A indicate that Ira2 was mainly localized in membrane fractions (P13 and P100) as expected [37] while Bcy1, although detected in S13, co-localized with Ira2 mainly in P13 and slightly in P100 fractions. The subcellular fractions containing plasma membranes were identified by visualizing Pma1 (Plasma membrane ATPase 1) (Fig. 6A), indicating the correct fractionation process. We assessed and confirmed the in vivo interaction of Bcy1 with Ira2 by co-immunoprecipitation of a cell lysate from a yeast strain expressing Ira2-HA, with anti-HA antibody followed by Western blot analysis (Fig. 6B). It has been reported that the intracellular localization of Ras signaling complex, the bulk of Cyr1 (adenylate cyclase), Ras (GTP binding protein), Cdc25 (Ras-GEF) and Ira2 (Ras-GAP) proteins, co-localize with internal membranes, while a minor amount of these proteins are found associated with the plasma membrane fractions in exponentially growing cells [37]. The interaction of Bcy1, and thus PKA holoenzyme, with Ira2 suggested that PKA might form part of this signaling complex.

As a preliminary study of the physiological relevance of this interaction, the possibility that Ira2 was a substrate of PKA was analyzed assessing the phosphorylation of this protein obtained from yeast strains expressing Ira2-HA by immunoprecipitation with anti-HA and incubation of the immunoprecipitate with [γ^{32}P]ATP and further separation through SDS-PAGE and image analysis. Unfortunately, the huge protein Ira2, of 350 kDa, was proteolyzed during kinase assay incubation, and no intact protein band could be recovered and its in vivo phosphorylation could not be demonstrated. However in the same anti-HA immunoprecipitate, PKA activity, measured in the presence of cAMP, and specifically inhibited by the protein kinase inhibitor PKI, could be detected, suggesting that the PKA holoenzyme had co-immunoprecipitated, most probably via the interaction Ira2-Bcy1 (Fig. 6C).

Using prediction programs such as NetPhos2.0 and Scan site, to analyze the Ira2 primary sequence looking for consensus PKA phosphorylation motifs, two potential phosphorylation sites (Ser 1018 and Ser 1745) were identified. Two peptides IRT**RRYS**^{1018}DESLG and DIL**RRNS**^{1745}CATRS containing the potential phosphorylation consensus sequence (in bold) were synthesized. The ability of these peptides to behave as substrates for PKA was examined and compared with that



Peptide	*Km* (μM)	*Kcat* (min^{-1})	*Kesp* ($min^{-1}.\mu M^{-1}$)
Nth1-1	190±5	450±15	5
Ira2-ser1018 IRTRRYSDESLG	545±12	311±9	0.6
Ira2-ser1745 DILRRNSCATRS	100±8	614±14	6.1

Fig. 6 – Ira2 in vivo interaction with PKA. **A) Extracts of a yeast strain expressing Ira2-HA were centrifuged to obtain different subcellular fractions as described under methods. Bcy1, Ira2 and Pma1 (plasma membrane marker) proteins were detected by Western-blot; B) Extracts from a yeast strain expressing Ira2-HA were immunoprecipitated with anti-HA antibody (IP) or normal rabbit IgG (mock), Bcy1 and Ira2 were detected by Western Blot of the immunoprecipitate; C) Protein kinase A activity was measured in the immunoprecipitate, in the presence of cAMP; specificity was tested by inhibition with the specific PKA inhibitor, PKI. A representative experiment is shown from duplicate experiment, D) In vitro phosphorylation of Ira2 peptides was assayed using a purified preparation of Tpks. Phosphorylation of Nth1-1 peptide was included as a control. The inset table below summarizes the kinetic parameters obtained and correspond to the mean ± S.D. from n = 4.; E) Homology of the PKA consensus phosphorylation sequence around Ser 1745 in fungi and mammals.**

of a peptide substrate Nth1-1, derived from neutral trehalase, which we have already demonstrated to be an excellent substrate [17]. Fig. 6D shows a classical kinetics of phosphorylation at variable substrate (peptides) concentration; the kinetic parameters K_m, K_{cat} and K_{esp}, were calculated and summarized in the inset of Fig. 6D. The results show that the peptide containing Ser 1745 was an excellent substrate, almost similar to Nth1-1, and that the one containing Ser 1018 was a poorer one, particularly due to its higher K_m. According to our own results on yeast PKA substrates characterization [17] acidic residues in positions P + 1 and P + 2 are strongly inhibitory to the phosphorylation at the Ser (P0) of a peptide substrate; and this is the case for the phosphorylation in Ser 1018, which has an Asp and a Glu in P + 1 and P + 2 positions. Although preliminary, these results were a clue to a possible behavior of Ira2 as a PKA substrate in yeast.

A search in the PhosphoGrid database (an online database of experimentally verified in vivo protein phosphorylation sites in *S. cerevisisae*) was performed. The tryptic peptide RYSDESLGK (1 miscleavage), containing Ser 1018 was found in the phosphopeptide database; as an additional and interesting information its phosphorylation was decreased in a *tpk3*Δ strain. The tryptic peptide NSCATR (or RNSCATR with 1 miscleavage) containing phosphorylated Ser 1745 was not reported in the database; however the m/z size of this peptide is outside the range of sizes usually selected to do an MSMS scan in the LC-MSMS mass spectrometers in which peptides are usually multicharged. Ira 2 is an homolog protein to human tumor suppressor neurofibromin NF1, a protein of around 300 kDa [38].

Both proteins share a GAP domain. It is interesting that the sequence surrounding Ser 1745, containing the PKA consensus phosphorylation motif, lies in the core of the GTPase activating domain of Ira2 and is highly conserved among fungi and mammals, pointing to an evolutionary conserved significance (Fig. 6E).

The Hsp60 chaperone family of proteins is very important in the mitochondrial biogenesis and in polypeptide folding, assembly and prevention of aggregation within the mitochondrial matrix [39]. Hsp60 is a protein of nuclear origin, mainly defined as a mitochondrial chaperone, although some cytoplasmic roles have been proposed [40]. In mammals there is a subgroup of AKAPs that anchor PKA to the mitochondrial outer membrane (mtAKAPs) that efficiently transmit cAMP signal to mitochondria [40]. In yeast, the coupling of mitochondria to growth and survival and therefore energy metabolism is controlled by the cAMP–PKA pathway [41]. Yeast mitochondria have been described to have associated PKA activity [42,43]. There are also evidences for participation of PKA in the regulation of mitochondrial gene expression, and cAMP responsive elements have been described in cis-acting sequences [44]. PKA has also been shown to be involved in the maintenance of mitochondrial DNA through phosphorylation of the DNA binding protein Abf2p, belonging to the HMG family of architectural proteins [45]. These results suggested that in yeast, a pool of PKA might be also localized on or within mitochondria by direct interaction with proteins behaving similarly to mtAKAPs.

We chose to investigate whether the interaction between Hsp60 and Bcy1 could be related to PKA localization in mitochondria. It has been reported that the level of Hsp60, is increased about two- to three-fold in cells shifted to 37 °C [46]. On the other hand we have demonstrated that the expression of Tpk1, but not of Bcy1, nor of Tpk2, is up-regulated by heat shock [47]. We therefore chose this stress condition to assess the co-localization of Hsp60 with PKA, following the rationale that Hsp60 could be localizing Tpks to mitochondrias through its interaction with Bcy1. Fig. 7 shows the results of this experiment. We first verified that after submitting yeast cells expressing Hsp60-HA to a heat shock of 30 min at 37 °C, the levels of Hsp60 increased, mainly in the P3 fraction containing the bulk of mitochondria (13,000 *x g*) (Fig. 7C, bottom). The fractionation process was evaluated by the membrane marker Pma1 (Fig. 7 C, top). The localization of Tpk1 and Bcy1 associated to mitochondria was evaluated in a WT strain before and after a 60 min heat shock at 37 °C. There was an increase in both Bcy1 and Tpk1 in the mitochondrial fraction P3 upon heat stress as shown in Fig. 7A. This increase could not be observed when measured in a strain Hsp60-ts (with no levels of Hsp60 at the restrictive temperature) (Fig. 7B). Under these conditions the levels of Tpk1 were much lower than in the WT strain and the levels of Bcy1 were undetectable (not shown). The results indicated that the increase of Tpk1 and Bcy1 in the mitochondrial fraction after heat shock correlated with the concomitant increase of Hsp60 in the same fraction and were compatible with the proposal that Hsp60 might be responsible for the localization of PKA to mitochondria via its interaction with Bcy1. Hsp60 also showed to be involved in the stability of Tpk1 and Bcy1 proteins, as in the strain Hsp60 at restrictive temperature the protein amounts were not increased (Tpk1) or were not detected (Bcy1). To evaluate whether besides this effect on protein amount there could also be an effect on Tpk1 enzymatic activity, we measured total kinase activity in crude extracts of the WT and Hsp60-ts strains at different times after a shift to 37 °C (Fig. 7D). Protein kinase activity in the extracts is contributed mainly by Tpk1 and Tpk2, in approximately equal amounts; the contribution of Tpk3 is almost negligible [17,48]. PKA activity in Fig. 7D was represented as the ratio of the total activity *vs* the amount of Tpk1 in the crude extracts detected by Western blot at each time point. This ratio of activity/Tpk1 protein in the WT strain remains constant after the heat shock; the increase of Tpk1 protein occurring after the heat shock was concomitant with a corresponding equivalent increase in its activity; therefore its contribution to the relative total activity remained constant. Since the relative total activity is constant we have to assume that the activity and the amount of Tpk2 also remained constant after the heat shock (Tpk2 levels can not be measured, since there is no specific antibody). However, in the Hsp60-ts strain, the ratio of total activity/Tpk1 protein decreased almost to 20% of the activity at time 0. This result indicated that the activity decreased more than the protein level; therefore there was not only a decrease in protein stability but also a decrease in kinase activity of both Tpk1 and Tpk2 isoforms. These results suggest a dual role of Hsp60 on the protein stability and on its catalytic activity.

4. Discussion

In this report we have identified in *S. cerevisiae* proteins that interact with Bcy1, the regulatory subunit of PKA, through the use of affinity purification and computational prediction. These proteins add to the list of interactors of Bcy1 recollected in the Saccharomyces Genome Database (www.yeastgenome.org). The interactors summarized in SGD come from different approaches performed to identify the global landscape of protein complexes within the yeast cell, but none of them was performed with the specific aim of finding Bcy1 interactors. The list of physical interactions is reduced and in each experimental approach the identified interactors are different and not overlapping. In our work, besides the identification of interacting proteins, interacting peptides for these proteins were predicted (Table 2 and Supplementary Fig. 3) and validated. The following proteins: Ira2, Hsp60, Myo2 and Ptp1 were confirmed to be Bcy1-interacting proteins by biochemical interaction studies and/or co-localization by differential centrifugation (Figs. 2, 3 and data not shown for Rim15). By bioinformatic approach, an interaction domain was predicted in the affinity purified proteins or in the whole yeast genome. This analysis was based on the characteristics of the known mammalian AKAP interaction domains. Conventional interaction domains in AKAPs consist of an array of pairs of hydrophobic and hydrophilic residues forming an amphipathic α-helix. Therefore, as expected, all the interacting peptides predicted share, with more or less homology, this amino acid organization (see Supplementary Fig. 3, bottom). By peptide array substitution of the predicted interacting peptides from these proteins, we demonstrate that the mutation of hydrophobic residues had no evident effect on the interaction with Bcy1; however, mutation of the positively charged residues Lys and Arg, highly decreased the interaction (Fig. 4 and

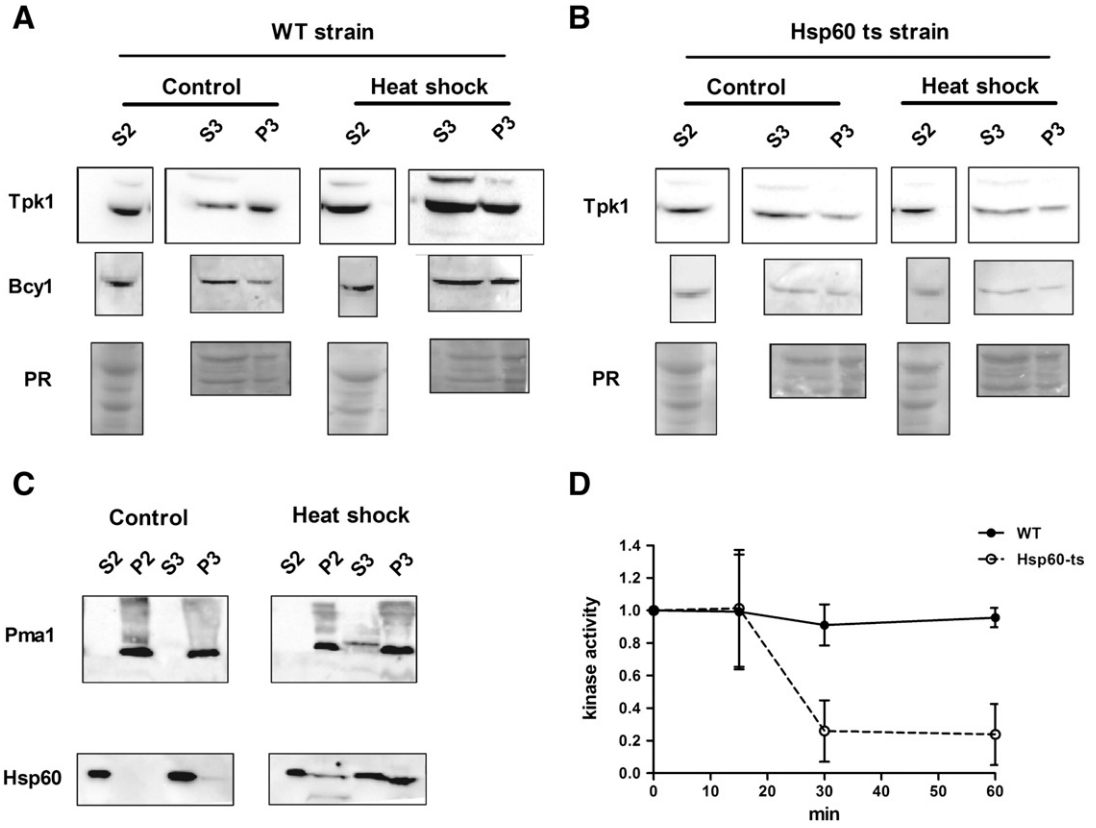


Fig. 7 – Hsp60 affects Bcy1 localization. **Mitochondrial fractions obtained from extracts of yeast strains WT and Hsp60-ts were analyzed by Western blot before and after heat shock (60 min 37 °C), to detect endogenous Tpk1 and Bcy1 (panel A and B, fractions S2, S3 and P3), in WT and Hsp60-ts strains; Ponceau Red (PR) staining is shown for WT and Hsp60-ts strains in both control and heat shock conditions. Bcy1 and Tpk1 were detected on the same stripped blot membrane. Mitochondrial fractions obtained from extracts of a yeast strain overexpressing Hsp60-HA were analyzed by Western blot before and after heat shock (60 min 37 °C) (panel C); top, Pma1 as a marker of membrane fraction, and bottom, Hsp60 (with anti HA antibody)., panel D) Tpk1 levels and kinase activity were measured in crude extracts of WT and Hsp60-ts strains at different time points after a heat shock at 37 °C. Tpk1 levels and kinase activity were measured in aliquots corresponding to the times indicated. Kinase activity is expressed relative to t = 0 of heat shock for each strain and to Tpk1 protein level at each incubation time. The graphic corresponds to a representative experiment.**

Supplementary Fig. 3). The array experiment also demonstrated that conventional AKAP domains, such as those from AKAP7 or from the typical anchoring disruptor peptide Ht31 (derived from AKAP-Lbc) [49], did not bind Bcy1. Ira2 25 aa peptide, in particular, has been demonstrated by circular dichroism to form a very stable α-helix as predicted *in silico* (N. González Bardeci, S. Rossi and S. Moreno, to be published elsewhere). The results, however, showed that the hydrophobic face of this helix was not relevant in the interaction with Bcy1.

The interaction of Bcy1 with the described interacting proteins depends on its amino terminus, since a mutant of Bcy1 lacking the first 85 amino acids had no interaction (Fig. 3). The amino terminus of Bcy1 is predicted to be responsible for its dimeric structure, and is essential in the dynamic localization of Bcy1 within the cell [18, 19 and unpublished work from our group]. The phosphorylation of two clusters of Ser within this domain (cluster I aa 4–10 and cluster II aa 68–84) participate in the localization of Bcy1 and are important for the interaction of Bcy1 with Zds1, a protein which has been identified in a two-hybrid screening as interacting with Bcy1 [20]; this protein has been proposed to behave as a putative AKAP, although the molecular and structural determinants have not been studied. The fact that Bcy1 N-terminus phosphorylation is important for its localization could be somehow related with the observation that this interaction is highly dependent on positively charged residues in the interacting peptide. We propose that Ira2, Hsp60 and Myo2 bind to the amino terminus of Bcy1 through electrostatic interactions, quite different from the interaction of mammalian R subunit which interact with AKAPs mainly through hydrophobic interactions [50]. The importance of the phosphorylation of Clusters I and II in this interaction is currently under study. Electrostatic interactions have been also shown to be important in the case of RI specific AKAPs, in which a an extra α-helix domain termed RI Specifier Region (RISR) has been demonstrated to enhance binding to RI through positively charged residues, besides the classic amphipathic helix [51]. As stated in the introduction, only a few non canonical AKAPs have been described; two are the reasons we can envisage for losing potential AKAPs. One is the fact that the search is biased towards interactions competed with the canonical Ht31 disruptor [49], and the second that search of interactors are mainly performed using the isolated D/D domain and not the whole R subunit. In

the case of Bcy1, the prediction of interacting proteins and domains through a bioinformatics approach was biased toward the amino acid distribution characteristic of the amphipathic helix shown by most of the described mammalian AKAPs; however these peptides, although sharing these characteristics (Supplementary Fig. 3), show a non canonical interaction with positive charged amino acids involved and its interaction could not be competed by Ht31.

In order to get further insight into the physiological relevance of the interaction between the interacting proteins and Bcy1 we performed some preliminary experiments with Ira2 and Hsp60. Ira2 is a Ras-GAP protein involved in the cAMP signaling pathway in *S.cerevisiae*. In yeast, two distinct Gpr1–Gpa2 (G protein-coupled receptor Gpr, and Gpa, G protein α-subunit) and Ras-mediated pathways converge on Cyr1 (adenylyl cyclase) [14]. Gpr1–Gpa2 and Ras2 play a shared role in glucose-induced cAMP production. Gpa2 forms a protein complex with the kelch Gpb1/2 proteins; these proteins negatively control cAMP–PKA signaling via the conserved C-terminal domain of Ira1/2 [52–54]. Here we show that Bcy1 interacts with Ira2, and has been found to co-fractionate together with Ira2 in membrane fractions (Fig. 6), besides being also located in soluble fractions. We demonstrate that a peptide of Ira2 containing Ser1745 is an excellent substrate for in vitro PKA phosphorylation; more relevant still is the fact that Ser1745 belongs to the so called "arginine finger loop" a conserved domain, shared by GAP proteins (Fig. 6D), determinant in the specificity and activity of GAPs [55,56]. Another peptide, containing Ser1018, was also shown to be an in vitro substrate of PKA, although less efficient than Ser1745 containing peptide; it is interesting to add that Ser1018 has been found additionally as an in vivo phosphopetide in databases.

Ira2 is therefore an excellent candidate to accomplish an AKAP function, by localizing PKA, via its interaction with Bcy1, to the cAMP signaling complex [37,57]. It has been speculated and even included in models [58] that Ira2, a negative regulator of Ras, could be a target of the negative feedback loop initiated by the active catalytic subunit of PKA to shut down the cAMP signaling pathway [59]. In support of this there are results that showed that the percentage of active GTP-Ras was increased in a strain containing a catalytically weak version of Tpk1 [60]. Our own results on the in vitro phosphorylation of Ser 1745 in Ira2, within a very strong consensus PKA sequence, together with the in vitro and in vivo phosphorylation of Ser 1018, gives additional support to this model, and let us speculate that the consequences of this phosphorylation could impact positively on Ira2 Ras-GAP activity.

Hsp60 is one of the heat shock proteins classically involved in mitochondrial biogenesis in yeast [61,62]. However its role in cytoplasmic processes is not to be disregarded since a good number of the physical interactors of Hsp60 analyzed by affinity-capture MS and deposited in the Saccharomyces Genome Database are cytoplasmic proteins. Specifically it was found in complex with Pfk1 in cytoplasm, and it was proposed as a potential candidate for the stabilization of Pfk-1 subunits in vivo [40] PKA activity has been demonstrated to be associated with mitochondria in yeast, although the presence of PKA subunits inside the mitochondria was not demonstrated [42,43]. In this work we have demonstrated that upon a heat stress there is an increase in Tpk1 and Bcy1 associated to mitochondrial fraction (Fig. 7B) concomitantly with the expected increase of Hsp60 (Fig. 7C) in the same fraction. The participation of Hsp60 in this increase of PKA in mitochondria is made evident when we show that in strains containing a thermosensitive Hsp60 (Hsp60-ts) the increase of Tpk1 and Bcy1 in mitochondria after heat shock did not occur (Fig. 7B). In addition to the decrease of Tpk1 protein level, we demonstrated that there was also a severe decrease in its activity (Fig. 7D). Molecular chaperones, such as Hsp70 and its cochaperones Cdc37 or Hsp40, have been demonstrated to be involved in folding and activity of protein kinases [63]. It was reported that Ydj1, a *S. cerevisiae* Hsp40 molecular chaperone that functions with Hsp70 to promote polypeptide folding, has two specific functions in protein kinase folding. One involves protecting the nascent kinase chain from degradation, and the other promoting efficient folding and maturation. Ydj1 maintains the steady state levels of several protein kinases including Tpk2 [63]. Our results suggest that Hsp60 could also be a kinase chaperone for protein kinase A. On the other hand the following evidences suggest that Hsp60 is a physiological substrate of protein kinase A. Phosphoproteomic analysis of yeast proteins (PhosphoPep database www.phosphopep.org) has clearly shown the existence of a tryptic phosphopetide (R.RGS*QVAVEK.V) in which the phosphorylated Ser is within a strong PKA consensus sequence. Hsp60 has been described as substrate of Tpk1 in a global phosphorylation analysis [64] and finally found as an interacting hit of both Tpk1 and Tpk2 in an affinity capture MS study [65]. Taking all these evidences as a whole it is tempting to speculate that there is a strong interrelationship between Hsp60 and PKA.

5. Conclusion6

We have contributed to the knowledge of the mechanism of PKA localization, by the identification of Bcy-1-associated proteins in *Saccharomyces cerevisiae*. We isolated and identified interacting proteins of Bcy1, verified some of these interactions, and characterized the interaction with the N-terminus of Bcy1. Bcy1 tethering proteins have a domain in which charged positives residues are key for the interaction with the regulatory subunit of PKA. Ira2 and Hsp60 are two examples of the identified Bcy1 interacting proteins. Bcy1 interacts with Ira2 tethering PKA to the Ras complex and Hsp60 chaperone localizes PKA to mitochondria and has a role in the kinase stability. The mechanism of PKA localization in budding yeast seems different to the already described for other organisms. Bcy1 localization in yeast is dynamic, and the interaction with the proteins which could contribute to its localization involves electrostatic interactions, in clear difference to the conventional mammalian AKAP-R interaction domains.

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.jprot.2014.07.008.

Transparency document

The Transparency document associated with this article can be found, in the online version.

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