Mutants affected in the putative diacylglycerol binding site of yeast protein kinase C

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Abstract In an attempt to study the functional similarities between protein kinase C from the yeast *Saccharomyces cerevisiae* and its human homologues we have started in vitro mutagenesis to alter specific domains. Here we report on the exchange of four cysteine residues by serines in yeast Pkc1p that have been shown to be essential for diacylglycerol (DAG) binding and activation by this compound in humans. The mutant yeast protein leads to sensitivity to caffeine and low concentrations of SDS when expressed in a *pkc1* deletion strain. However, sensitivity to staurosporine was not affected. Our data indicate that the conserved DAG binding domain serves an important function in yeast Pkc1p.

Key words: Protein kinase C; Signal transduction; In vitro mutagenesis; *Saccharomyces cerevisiae*

1. Introduction

Protein kinase C (Pkc) is an important component of signal transduction pathways from yeast to man [1]. Its medical importance is exemplified by the fact that phorbol esters, which activate Pkc in analogy to the natural activator diacylglycerol (DAG), act as tumor promoters in humans [2].

In yeast, protein kinase C has been shown to be part of a regulatory MAP kinase cascade essential for cellular integrity and response to low osmolarity environments [3]. Earlier reports on kinetic parameters of the yeast enzyme [4,5] have been questioned after cloning, sequencing and deletion of the encoding gene [6]. Deletions in *pkc1* are lethal in the absence of osmotic stabilization [7,8]. A lytic phenotype in the absence of osmotic stabilizers, at least at higher temperatures, is also characteristic for deletion mutants affecting most of the downstream components of the pathway, which by themselves are activated by a phosphorylation cascade [9]. As a direct target of Pkc1p, Bck1p protein has been identified by isolation of suppressor mutants [10]. It in turn phosphorylates Mkk1p/Mkk2p (the MAP kinase kinases [11]) that act on Mpk1p (= Slt2p), the actual MAP kinase of the pathway [12]. Recently, the MADS box protein Rlm1p and the SBF transcription complex (composed of Swi4p and Swi6p) have been shown to be targets of the latter [13,14].

The structure and function of Pkc1p in yeast have been studied by both biochemical and genetic means. Thus, four functional domains have been postulated in the primary sequence (compare also Fig. 1). The C-terminal part of the protein contains the actual kinase domain, responsible for phosphorylation of the target protein Bck1p. This function can be blocked by another domain of Pkc1p that is localized in the N-terminal part and described to be a pseudosubstrate site [15]. Between these, two motifs, called C1 and C2, presumably binding DAG and calcium, can be found, with distinct homologies to the mammalian Pkc protein [6]. Finally, a long N-terminal, yeast-specific, extension of about 390 amino acids in Pkc1p as compared to the mammalian counterpart could be involved in specific regulatory functions. Similar sequences can also be found in the homologous proteins from *Schizosaccharomyces pombe* [16,17] and *Candida albicans* [18].

Pkc1p *RHO1* [19,20], a small G-protein, was identified as an upstream component for the activation of yeast. This in turn is controlled by a variety of proteins, with their encoding genes discovered in genetic screens, such as *TOR2* [21], *ROM1* and *ROM2* [22]. Despite this progress, the role of DAG in the activation of yeast Pkc1p remains a point of ambiguity. Thus, in contrast to the mammalian system, biochemical data indicate that the enzyme is activated neither by DAG itself, nor by phorbol esters [15]. On the other hand, sequence analyses clearly show a conservation in the DAG binding site of yeast Pkc1p as compared to the human enzyme (see above). In addition, increased DAG levels have been found in a yeast *cdc28* mutant, where Cdc28p is thought to be involved in Pkc1p activation [23]. These data suggest a role of DAG in activation of yeast Pkc1p similar to the function in human cells. To address this question, we started in vitro mutagenesis, exchanging conserved amino acids in the putative DAG binding site of the yeast enzymes.

2. Materials and methods

2.1. Media and culture conditions

Rich media were based on 1% yeast extract and 2% bacto peptone (Difco) and supplemented with 2% glucose (YEPD) or 2% galactose. Yeast transformants were selected on minimal medium (0.67% yeast nitrogen base, 2% glucose) supplemented with amino acids and bases, lacking leucine or tryptophan as described [24]. For preparation of screening media, SDS from a 10% stock solution, sterile calcofluor white solution (final concentration 1.5 mg/ml) or sterile caffeine from a 100 mM stock solution was added to YEPD as indicated.

For growth of *Escherichia coli*, standard LB media with addition of the appropriate antibiotics were employed [25].

2.2. Strains

Saccharomyces cerevisiae strain HD56-5A (*MATα ura3-52 leu2-3,112 his3-11,15 MAL SUC GAL* [26]), and its isogenic diploid derivative DHD5 were used for most of the work described. VW1A (*MATa ura3-52 leu2-3,112 his3D1 trp1-289 MAL2-8^C SUC2 GAL* [27]) was used as a recipient strain for determination of stabilities of the tagged Pkc1p proteins (see below). DHD5Δpkc (*MATa/α Δpkc1::HIS3/PKC1 ura3-52/ura3-52 leu2-3,112/leu2-3,112 his3-11,15/his3-11,15 MAL/MAL SUC/SUC GAL/GAL*) was constructed in this work as a diploid recipient strain. It contains a substitution of one of

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the *PKC1* alleles by the *HIS3* marker, where bases 723–3170 relative to the ATG translation start codon have been deleted.

For work with *E. coli*, strain DH5αF′ (Gibco BRL) was used throughout.

2.3. Plasmids used

To construct a yeast vector carrying the complete *PKC1* coding region and its flanking sequences, a 4.2 kbp genomic *Sph*I fragment from strain VW1A was cloned into YCplac111 [28]. The fragment was obtained by PCR using the primers 5′-GGCTTCGCATCG-CATCGC-3′ and 5′-ATGGGACGCAATCACGC-3′. The resulting vector was called YCpPKC1.

For in vitro mutagenesis of *PKC1*, the *Xba*I/*Pst*I fragment from YCpPKC1 was cloned into pUC18 and pUC19 ([29], as a second *Xba*I site at position 2157 of the *PKC1* coding sequence is protected by methylation in *E. coli*). The resulting plasmids were called pHPS10 and pHPS11, respectively. For recloning of the mutagenized fragments, YCpPKC1 was digested with *Asp*718/*Sal*I, filled in with Klenow fragment of DNA polymerase I and religated to remove the *Xba*I site from the polylinker. This resulted in plasmid pHPS13. The products of the PCR mutagenesis were then cloned as *Xba*I fragments into pUC19 [29], to generate plasmids pHPS17 (mutation in the second DAG binding site) and pHPS18 (mutation in the first DAG binding site).

The mutation in the first binding site was liberated as an *Xba*I/*Nco*I fragment from pHPS18, the mutation in the second binding site as a *Bam*HI/*Nco*I fragment from pHPS17. Each of the fragments subcloned was sequenced in the vectors they originated from. Both fragments were ligated together with the large fragment of pHPS13 digested with *Bam*HI/*Xba*I. This resulted in pHPS23.

For epitope tagging of wild-type and mutant *PKC1*, PCR reactions with either pHPS13 or pHPS23 as templates and the primers 5′-GCGAGATCTGGGAGCAGTTTACAG-3′ and 5′-AACAGCTAT-GACCATG-3′ (M13 reverse sequencing primer) were performed. The first primer introduced a *Bgl*II site (underlined) directly in front of the translational start codon of *PKC1*. Products were then digested with *Bgl*II/*Sph*I and inserted into the polylinker region of pUK21 [30]. From these plasmids, called pHPS27 and pHPS28, respectively, the *Bam*HI/*Not*I fragment was cloned into the *Bgl*II/*Not*I digested vector YCpIF17 [31] which resulted in an N-terminal fusion of Pkc1p to the hemagglutinin epitope of hemophilus influenza.

2.4. Genetic manipulations, transformations and DNA preparations

E. coli was transformed by the method of Hanahan and Glover [32]. Yeasts were transformed by a variation of the freeze method [33]. Plasmid DNA was prepared from *E. coli* either by alkaline lysis [34] or with the kit of Qiagen (Düsseldorf), according to the instructions of the manufacturer.

Other genetic engineering techniques followed standard procedures [25].

2.5. Sequence analysis

Sequencing was performed by the dideoxy-chain termination method [35], using the Sequenase version 2.0 from USB in conjunction with [^{35}S]dATP. The universal and reverse sequencing primers as well as custom-made oligonucleotides (from MWG, Munich) were used.

2.6. In vitro mutagenesis of PKC1

For in vitro mutagenesis of *PKC1* we followed the PCR-based method of Boles and Miosga [36] using the primers 5′-GGTATACT-GGGTTCCAATcTCAAGATTcTAAATTTTTATGTCAC-3′ and 5′-TACCAgATTCAGAAgATTTGCGTACTTTATGTC-3′ (bases differing from the wild-type sequence are depicted in lower case letters) and pHPS10 or pHPS11 as templates.

2.7. Immunoblot analyses

Preparation of crude extracts was done as described in [13]. Total protein concentration was measured by the method of Zamenhoff [37]. Samples containing 50 μg of crude extract were loaded onto 7.5% SDS polyacrylamide gels (SDS-PAGE). All following procedures have been described in [38]. Alternatively, crossreacting proteins were detected by fluorescence using the kit of Tropix (Serva/Heidelberg) according to the recommendations of the manufacturer. Monoclonal antibodies against the hemagglutinin epitope used were obtained from BAbCO (Berkeley, CA, USA).

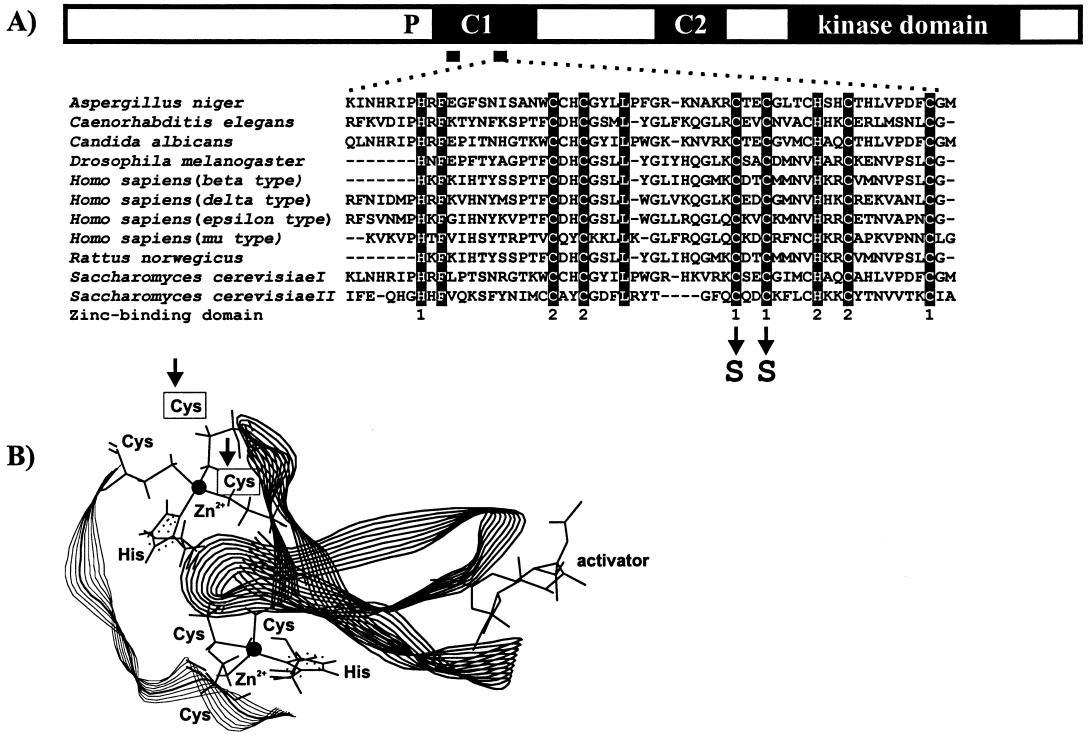

Fig. 1. A: Schematic representation of the domain structure of Pkc1p. 'P' indicates the pseudosubstrate site. The nomenclature for the other domains is explained in the text. Bars below indicate the two DAG binding domains in the C1 region. A comparison of the yeast Pkc1p sequence with the homologous proteins from other organisms in the putative DAG binding domains is depicted below. B: Model of the DAG binding domain adapted from the mammalian homologue according to [39]. As an activator, phorbol ester is placed in the binding groove where DAG binding is supposed to take place. Cysteine residues exchanged for serines in this work are boxed and marked by arrows.

3. Results and discussion

3.1. Mutagenesis of putative DAG binding sites

Alignments of the deduced amino acid sequences of Pkc1p from yeast to a variety of other organisms shows a conservation of key residues that have been demonstrated to take part in the formation of zinc binding. This in turn is necessary for the correct folding of a DAG binding groove [39] (see also Fig. 1B). In fact, two of these DAG binding motifs are located adjacent to each other in the yeast enzyme, a region which in analogy to its mammalian counterparts is designated 'C1' (Fig. 1A). By in vitro mutagenesis, we exchanged two critical cysteine residues, essential for the coordination of Zn^{2+}, in each of these regions for serines in yeast Pkc1p (i.e. residues 434, 437, 514 and 517, according to the numbering of the yeast enzyme, counting the initiating methionine). As the PCR-based method of Boles and Miosga [40] was used for mutagenesis, the entire *Xba*I fragment used to replace the wild-type sequence was sequenced to confirm that no other exchanges were introduced (see Section 2 for cloning details). The wild-type and mutant *PKC1* alleles were introduced on *CEN/ARS* plasmids (pHPS13 and pHPS23, respectively; see Section 2) into a diploid strain heterozygous for a deletion in *pkc1*. Transformants were selected for leucine prototrophy, sporulated, tetrads dissected and segregants were again allowed to grow on medium lacking leucine. In both cases viable segregants were obtained, indicating that the mutations introduced did not lead to a complete lack of Pkc1p function. Strains not carrying a *PKC1* allele on a plasmid segregated 2:2 for viability even in the presence of osmotic stabilizers. Thus, *PKC1* is essential for viability in the yeast strains employed here. A haploid deletion construct with a *GAL1/10* promoter fusion of *PKC1* integrated at the *URA3* locus was not very useful in our hands, as after transfer from galactose to glucose media the strain continued to grow on solid media even after replica-plating twice on glucose [13]. Thus, a very low Pkc1p activity seems to be sufficient to retain viability.

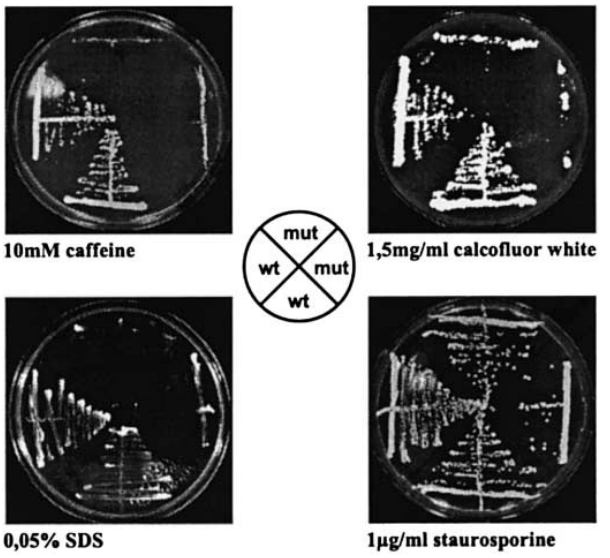

Fig. 2. Phenotypes of a *pkc1* deletion mutant transformed with the *pkc1* allele affected in a putative DAG binding site. Cells of transformants with *CEN/ARS* plasmids carrying either the wild-type allele of *PKC1* (wt; pHPS13) or the allelele with the cysteine residues exchanged for serines in both DAG binding domains (mut; pHPS23; compare Fig. 1) were plated on the indicated media and allowed to grow for 3 days at 30°C.

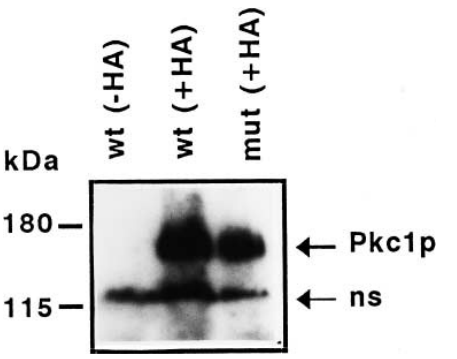

Fig. 3. Western blot detecting the HA-tagged Pkc1p. As a recipient strain, VW1A was used, which was either not transformed (wt −HA), transformed with the wild-type *PKC1* version carrying an HA tag at the N-terminal end (wt +HA; pHPS30), or transformed with the mutant *pkc1* containing the cysteine to serine exchanges in the two putative DAG binding domains and an HA tag at the N-terminal end (mut +HA; pHPS29). Monoclonal antibodies against hemagglutinin were used for detection by fluorescence. The filter was exposed to a Fuji RX-film for 20 min. The band corresponding to Pkc1p is indicated by an arrow. The second arrow points to a band reacting non-specifically (ns) in all extracts.

3.2. Growth characteristics of the mutant alleles in a pkc1 deletion mutant

As the haploid transformants carrying the mutated *PKC1* allele were viable on our standard media, we tested growth on plates containing several compounds known to affect the Pkc1p pathway in yeast (Fig. 2). As transformants carrying the wild-type allele, the ones with the mutant allele were capable of growth in the presence of staurosporine. However, the latter were sensitive for growth in the presence of caffeine, calcofluor white and SDS. Thus, several phenotypes typical for defects in the Pkc1p signal transduction pathway could be observed in the mutants. This could be due either directly to a lack of function introduced by the amino acid exchanges or to an increased instability of the mutant protein. To exclude the latter possibility, we constructed fusions of Pkc1p with an N-terminal hemagglutinin tag. Again, the same fusion was constructed with a wild-type *PKC1* allele. Western analysis confirmed that the stability of the mutant protein does not notably differ from that of the wild-type (Fig. 3). Thus, the phenotypes observed can be attributed to a functional difference in the protein introduced by the amino acid exchanges.

3.3. Conclusions

Although biochemical data indicated that yeast Pkc1p is activated neither by DAG nor by phorbol esters, our data suggest that the conserved DAG binding sites serve a critical function. Thus, distortion of these sites leads to sensitivity to a variety of compounds known to affect the signal transduction pathway mediated by Pkc1p. However, it is noteworthy that the sensitivity to staurosporine was not affected. Together with the fact that the mutant allele restored viability to the *pkc1* deletion strain this points to a dual role of Pkc1p in yeast, where only one of the functions is dependent on DAG binding. This further supports similar conclusions drawn from genetic studies aimed at the interaction of Pkc1p with other yeast proteins [1]. Whether the binding do-

main really interacts with DAG, an analogue of it or even with other yeast proteins, as suggested for Rho1p [20], remains to be elucidated.

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