Inositol polyphosphate kinase activity of Arg82/ArgRIII is not required for the regulation of the arginine metabolism in yeast

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Abstract Arg82, a nuclear regulator of diverse cellular processes in yeast, is an inositol polyphosphate kinase. Some defects such as the regulation of arginine metabolism observed in an *arg82*Δ, result from a lack of Mcm1 and Arg80 stability. We show here that neither the kinase activity of Arg82 nor inositol phosphates are required for the control of arginine metabolism. Arg82 mutations keeping kinase active affect the expression of arginine genes, whereas mutations in the kinase domain do not impair this metabolic control.

Key words: Arg82; Arginine metabolism; Inositol polyphosphate multikinase; Yeast

1. Introduction

Arg82/ArgRIII was first identified as one of the regulators of the arginine metabolism in *Saccharomyces cerevisiae* [1]. A mutation in *ARG82* led to the loss of anabolic gene repression and catabolic gene induction by arginine, impairing the growth on ornithine as sole nitrogen source. However, deletion of *ARG82* impairs other cellular processes, such as mating, sporulation and growth at 37°C [2]. More recently, two groups showed that Arg82 phosphorylates inositol (1,4,5) trisphosphate (Ins(1,4,5)P_3) to inositol (1,3,4,5) tetrakisphosphate (Ins(1,3,4,5)P_4) and Ins(1,4,5,6)P_4, both of which are then converted by Arg82 to Ins(1,3,4,5,6)P_5 [3–5]. Arg82 is therefore an inositol tris- and tetrakisphosphate 3-/6-kinase that will produce InsP_6, InsP_7 and InsP_8 as end-products of the inositol phosphate metabolism [4]. This is a unique feature of the yeast enzyme, distinct from the mammalian Ins(1,4,5)P_3 3-kinases that only phosphorylate Ins(1,4,5)P_3 in Ins(1,3,4,5)P_4 and share conserved sequences in their primary structures [6].

Besides Arg82, three other regulatory factors Arg80/ArgRI, Arg81/ArgRII and Mcm1 are required to ensure the coordination of arginine anabolic and catabolic gene expression in response to arginine [1,7–9]. Arg80 and Mcm1 are members of the MADS-box transcription factor family, and Arg81 which is a zinc cluster protein, is the sensor of arginine. These three proteins interact and are sufficient to form a complex with DNA (arginine boxes) when arginine is present [10]. Arg82 is not required for the formation of this complex provided that Arg80 and Mcm1 are present in sufficient amounts. We have clearly shown that Arg82 associates with and stabilizes Arg80 and Mcm1 [11]. However, Odom et al. [5] proposed a role for the inositol polyphosphate kinase activity (Ipk2) of Arg82 in arginine regulation. According to these authors, the Arg80–Arg81–Mcm1 transcriptional complex with DNA would be inactive in the absence of Arg82 kinase activity. This assumption was only based on growth data at 30°C on ornithine as sole nitrogen source, obtained with a strain devoid of phospholipase C or impaired in the Arg82 kinase activity [5]. However, in our hands, a *plc1*Δ strain grew poorly on all media, and not only on ornithine. This prompted us to reinvestigate the role of the Ipk2 activity of Arg82 in the regulation of the arginine metabolism.

2. Materials and methods

2.1. Strains and media

Deletions were created in strain 27061b (*ura3, trp1*), derived from the wild type strain Σ1278b. *Escherichia coli* strain XL1-Blue (Stratagene) was used for plasmid amplification.

All yeast strains were grown on minimal medium containing 3% glucose, vitamins and mineral traces [12]. Nitrogen sources were 0.02 M $(NH_4)_2SO_4$ (M. ammonia) or 1 mg per ml ornithine (M. ornithine). Rich medium (YPD) contained 1% yeast extract, 1% bactopeptone and 2% glucose.

2.2. Construction of deletant strains

The long flanking homology strategy was used to perform deletions of *ARG82* and *PLC1* genes [13,14]. A long flanking homology replacement cassette was synthesized using a two-step polymerase chain reaction (PCR) leading to the *kanMX4* cassette flanked by about 500 bp corresponding to the promoter and terminator regions of the target genes. The DNA fragments containing the different cassettes were used to transform yeast strain 27061b (*ura3, trp1*) on rich medium plates containing 200 μg/ml of geneticin. The correct targeting of the deletions in G418^r transformants was verified by PCR, using whole cells as a source of DNA, and appropriate primers.

2.3. Plasmids

The wild type *ARG82* gene was recloned on a pFL38 plasmid (p*UC19, ARS4, CEN6, URA3*) [15]. A 1.85 kb *Bam*HI–*Bam*HI fragment of *ARG82* was synthesized by PCR using two oligonucleotides RIII45 and RIII49 (Table 1) and either genomic wild type DNA, or DNA from plasmid pMJ313 containing the mutated arg82W65R gene

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[16]. These fragments were inserted in the *Bam*HI site of pFL38, yielding plasmids pFV145 and pFV160, respectively. Plasmid pQΔRIII-1 containing the deletion of the C-terminal aspartate-rich region (Δ282–303) was described in Qiu et al. [16]. GST–arg82Δ282–303 fusion was constructed by synthesizing a 1 kb *Bam*HI–*Bam*HI fragment by PCR from plasmid pQΔRIII-1 using oligonucleotides RIII21 and RIII39. GST refers to the glutathione *S*-transferase *E. coli* gene. This fragment was inserted into the *Bam*HI site of plasmid pGEX-5X-3 vector, expressing glutathione *S*-transferase, yielding plasmid pFV182. To express gene *PLC1* under the dependence of the *TPI* promoter (triose phosphate isomerase) we inserted a 2.6 kb *Eco*RI DNA fragment, corresponding to the entire *PLC1* coding sequence, in the *Eco*RI site of plasmid pYX212 (*TPI, URA3, 2μ*), yielding plasmid pFV139. This *Eco*RI fragment was synthesized by PCR, using genomic yeast DNA and oligonucleotides PLC1 and PLC2.

2.4. Creation of mutations in the ARG82 gene

Different oligonucleotides (Table 1) were used to create substitutions by in vitro mutagenesis on double stranded DNA from plasmid pFV145 (*URA3, ARG82*) using the Quick Change site-directed mutagenesis kit from Stratagene. In this plasmid the following amino acid changes, P127A, L129A, D131A, K133A and G135A, were achieved using oligonucleotides RIII56–RIII57, RIII50–RIII51, RIII52–RIII53, RIII47–RIII48 and RIII54–RIII55, respectively. The resulting mutant plasmids were: pFV169 (arg82P127A), pFV147 (arg82L130A), pFV148 (arg82D131A), pFV146 (arg82K133A), pFV149 (arg82-G135A), pFV170 (GST–arg82P127A), pFV157 (GST–arg82L129A), pFV158 (GST–arg82D131A) pFV156 (GST–arg82K133A) and pFV159 (GST–arg82G135A).

All the wild type and mutated *ARG82* genes were completely sequenced to ensure that the gene fusions were in frame and that no error was introduced during the PCR procedure. Denatured double stranded DNA used as template for DNA sequencing was prepared using Qiagen columns. DNA was sequenced by the Sanger method [17], oligonucleotides being used as primers.

2.5. Purification of GST–fusion proteins

Following transformation of *E. coli* XL1-Blue, induction of the fusion proteins was achieved by addition of 500 μM isopropyl-β-D-thiogalactopyranoside for 3 h at 37°C. Bacterial pellets were resuspended in phosphate-buffered saline (140 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 1.8 mM KH_2PO_4) containing a mixture of protease inhibitors, and sonicated on ice for 3 min. After centrifugation (12 000 rpm, 15 min), purification was achieved as in Amar et al. [10].

2.6. Assays of enzyme activities

Ornithine carbamoyltransferase (OTCase) and arginase activities were assayed as described previously [18]. Protein contents were determined by the Folin method.

Ipk2 activity was measured at 10 μM Ins(1,4,5)P_3, in the presence of 1 mM EGTA, as reported in Takazawa et al. [19].

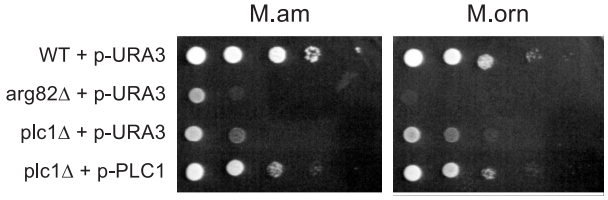

Fig. 1. Effect of deletions in *ARG82* and *PLC1* genes on cell growth. 10-fold serial dilutions of cells were plated and incubated at 30°C for 3 days on M. ammonia and M. ammonia+25 μg/ml of tryptophan, and M. ornithine+25 μg/ml of tryptophan. Strains 27061b (*ura3, trp1*, taken as wild type), 03058c (*ura3, trp1, arg82::kanMX4* = arg82Δ), and 03135a (*ura3, trp1, plc1::kanMX4* = plc1Δ) are isogenic to strain Σ1278b. These strains were transformed with the high copy plasmid pYX212 containing the *URA3* marker and a *TPI* promoter, and strain 03135a was also transformed with the same plasmid in which the *PLC1* gene was expressed from the *TPI* promoter (pFV139).

3. Results

3.1. Deletion of the PLC1 gene impairs cellular growth, but not the regulation of arginine metabolism

PLC1 encodes phospholipase C that makes Ins(1,4,5)P_3 from PtdIns(4,5)P_2. Consequently, deletion of *PLC1* deprives the cells of the substrate of Arg82/Ipk2. To test whether inositol polyphosphates play a direct role in the control of arginine metabolism, we deleted the *PLC1* and the *ARG82* genes by replacing their complete coding sequences by the *kanMX4* cassette, conferring resistance to geneticin. We compared the growth rates of *arg82*Δ (03058c) and *plc1*Δ (03135a) strains on M. ammonia or M. ornithine as sole nitrogen source, to the growth rate of the wild type strain. As shown in Fig. 1, growth of the *plc1*Δ strain was impaired to the same extent on M. ammonia as well as on M. ornithine, and wild type growth was restored when the strain was transformed with the cognate *PLC1* gene. In contrast, the deletion of *ARG82* reduced cellular growth on M. ammonia and led to an absence of growth on M. ornithine. These data indicated that Plc1 was required to ensure a normal growth, and suggested that the loss of Plc1 activity did not affect the regulation of the arginine metabolism. To confirm the growth data, we measured the levels of OTCase, an anabolic enzyme, and of arginase, a

Table 1
Oligonucleotides used for *ARG82* and *PLC1* syntheses by PCR and for in vitro mutagenesis in *ARG82* gene

Oligomer	Length	Sequence		Modification
RIII45	30	CGC**GGATCC**CTTCCGGAAGCTTCTCTCCGC		creation of a *Bam*H1 site at position −310
RIII49	29	CGC**GGATCC**AAGTTTGTAGCTCAAATTGC	CS	creation of a *Bam*H1 site at position +1569
RIII21	30	GAAATTTTTTTC**GGATCC**ATACGGTAAACA		creation of a *Bam*H1 site at position +4
RIII39	28	CGC**GGATCC**CTTCACCTCTCAATATATC	CS	creation of a *Bam*H1 site at position +1086
RIII56	30	GGATTTAGTAAA**GCG**AATATACTTGATATA		substitution of amino acid (aa) P127A
RIII57	30	TATATCAAGTATATT**CGC**TTTACTAAATCC	CS	substitution of aa P127A
RIII50	30	AAACCTAATATA**GCA**GATATAAAATTAGGC		substitution of aa L130A
RIII51	30	GCCTAATTTTATATC**TGC**TATATTAGGTTT	CS	substitution of aa L130A
RIII52	30	CCTAATATACTT**GCA**ATAAAATTAGGCAAA		substitution of aa D131A
RIII53	30	TTTGCCTAATTTTAT**TGC**AAGTATATTAGG	CS	substitution of aa D131A
RIII47	33	AATATACTTGATATA**GCG**TTAGGCAAAACTTTG		substitution of aa K133A
RIII48	33	CAAAGTTTTGCCTAA**CGC**TATATCAAGTATATT	CS	substitution of aa K133A
RIII54	30	GATATAAAATTA**GCG**AAAACTTTGTATGAT		substitution of aa G135A
RIII55	30	ATCATACAAAGTTTT**CGC**TAATTTTATATC	CS	substitution of aa G135A
PLC1	27	CCG**GAATTC**AGGTCATTCACGCAGTGT		creation of an *Eco*RI site at position −24
PLC2	27	CCG**GAATTC**ATATGTGTATTTGGCCGG	CS	creation of an *Eco*RI site at position +2641

The altered nucleotides are indicated in bold. CS means complementary strand.

Geneotype	Plasmid	Repression factor of OTCase	Induction factor of Arginase
*plc1*Δ	p-URA3	12	6
*arg82*Δ	p-URA3	1	1.2
*arg82*Δ	p-ARG82 w.t.	9	10
*arg82*Δ	p-arg82 P127A	6	7
*arg82*Δ	p-arg82 L130A	12	10
*arg82*Δ	p-arg82 D131A	12	13
*arg82*Δ	p-arg82 K133A	9	14
*arg82*Δ	p-arg82 G135A	7	9
*arg82*Δ	p-arg82 W65R	1	1.1
*arg82*Δ	p-arg82 Δ282-303	1	1.2

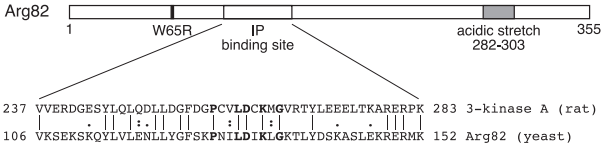

Fig. 2. Effect of *PLC1* deletion and of point mutations in *ARG82* on the control of arginine metabolism. The strain 03135a (*plc1::kanMX4, ura3, trp1*) transformed with plasmid pFL38, and the strain 03058c (*arg82::kanMX4, ura3, trp1*) transformed with the following plasmids, pFL38 (pURA3), pFV145 (p-ARG82 wild type), pFV169 (p-arg82P127A, pFV147 (p-arg82L130A), pFV148 (p-arg82-D131A), pFV146 (p-arg82K133A), pFV149 (p-arg82G135A), pFV160 (p-arg82W65R) and pFV182 (p-arg82Δ282–303), were grown on minimal medium supplemented with 25 μg/ml tryptophan, with or without 1 mg/ml arginine. The repression factor of OTCase corresponds to the ratio between the OTCase specific activity of the *arg82*Δ mutant strain grown on M. ammonium and the OTCase specific activity of the different strains grown on M. ammonium plus arginine. The induction factor of arginase corresponds to the ratio between the arginase specific activity of the different strains grown on M. ammonium plus arginine and the arginase specific activity of the *arg82*Δ strain grown on M. ammonium. The open bar represents the Arg82 protein with the localization of the different mutations analyzed, and amino acid alignment of the Ins(1,4,5)P_3 binding site of Arg82 and rat brain Ins(1,4,5)P_3 3-kinase A. The numbers correspond to the positions of the amino acids in the proteins. The well conserved residues, which were replaced by alanine in Arg82 are indicated in bold.

catabolic enzyme, from cells grown in the presence or absence of arginine.

As shown in Fig. 2, the deletion of *ARG82* abolished repression of OTCase and induction of arginase by arginine, whereas the levels of these enzymes in a *plc1*Δ strain were comparable to those of the wild type strain. Thus the absence of Ins(1,4,5)P_3 did not affect the expression of arginine co-regulated genes, which argues against a direct role of Ins-(1,4,5)P_3 or one of its metabolites in this control.

3.2. Mutations in the putative inositol phosphate binding site of Arg82 do not impair the regulation of the arginine metabolism

As for the *plc1*Δ strain, Odom et al. [5] showed that a mutation in the putative Ins(1,4,5)P_3 binding of Arg82 (D131A) led to an absence of growth on M. ornithine, suggesting that the Ipk2 activity was required to control arginine metabolism. Since our data with the *plc1*Δ strain clearly showed that the slower growth of that strain on M. ornithine resulted from a general growth defect and not from an absence of arginine catabolic gene induction, we created a series of mutations in the putative inositol phosphate binding site. An alignment of the Ins(1,4,5)P_3 binding site of Arg82 and rat brain Ins(1,4,5)P_3 3-kinase A is presented in Fig. 2. The five residues conserved in these kinases (marked in bold) were replaced by alanines. The different plasmids pFV145 (Arg82 wild type), pFV169 (arg82P127A), pFV147 (arg82L130A), pFV148 (arg82D131A), pFV146 (arg82K133A), pFV149 (arg82G135A), pFV160 (arg82W65R) and pFV182 (arg82Δ282–303) were introduced in the strain O3058c (*ura3, trp1, arg82::kanMX4*). The mutation arg82W65R had been selected in vivo for its impairment in arginine regulation and we also tested the effect of the deletion of the aspartate-rich stretch present in the C-terminal end of Arg82 [1,16]. OTCase and arginase activities were measured in extracts from the different transformed strains, grown on M. ammonium with or without arginine. As shown in Fig. 2, none of the mutations created in the Ins(1,4,5)P_3 binding site affected the repression of OTCase and the induction of arginase. In contrast, the mutation arg82W65R, used as a control, led to an absence of repression and of induction. Similarly, the deletion of the acidic-rich region also impaired the regulation of arginine anabolic and catabolic enzymes.

3.3. Determination of the Ins(1,4,5)P_3 kinase activities

Arg82 has been shown to contain a consensus sequence of inositol phosphate kinases [3] which was localized in the Ins(1,4,5)P_3 binding site. Structural identification of the Ins(1,4,5)P_3 binding domain of mammalian Ins(1,4,5)P_3 3-kinase A enabled the identification of a crucial lysine residue (K262) that was essential for Ins(1,4,5)P_3 binding [20]. This residue was conserved in Arg82 (Fig. 2). Arg82 wild type and the different mutants were assayed for Ins(1,4,5)P_3 kinase activity at 10 μM Ins(1,4,5)P_3. All mutants tested, except arg82L130A, had lost partially or completely the Ins(1,4,5)P_3 kinase activity. The deletion of the acidic region in arg82 resulted in a fully active enzyme (Fig. 3). We concluded that

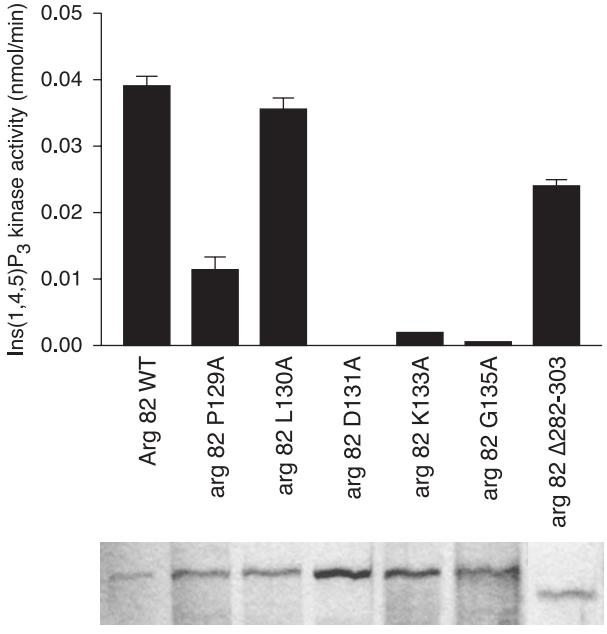

Fig. 3. Effect of point mutations in GST–Arg82 on Ipk2 activity. Since the production of the various GST constructs was not comparable, Ins(1,4,5)P_3 kinase activity was expressed relatively to the amounts of enzyme applied onto the gel and stain by Coomassie blue. Activities are means of triplicates ± S.D.

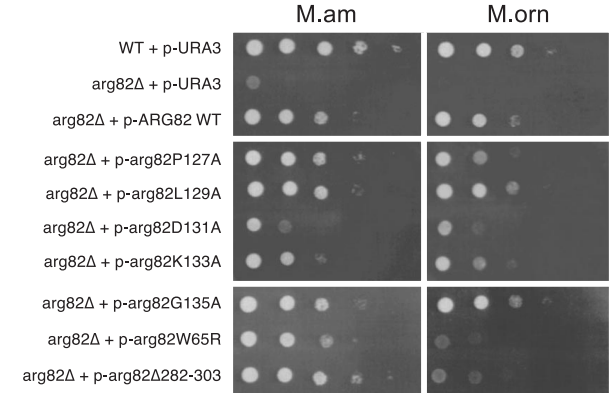

Fig. 4. Effect of point mutations created in *ARG82* gene on cell growth. 10-fold serial dilutions of cells were plated and incubated at 30°C for 3 days on M. ammonia+25 μg/ml of tryptophan, and M. ornithine+25 μg/ml of tryptophan. Strains 27061b (*ura3, trp1*, taken as wild type), 03058c (*ura3, trp1, arg82::kanMX4* = arg82Δ) were transformed with the low copy plasmid pFL38 containing the *URA3* marker, and strain 03058c was also transformed with the same plasmid containing the wild type *ARG82* gene (pFV145), or the mutated *arg82* genes: pFV169 (parg82P127A), pFV147 (parg82L130A), pFV148 (parg82D131A), pFV146 (parg82K133A), pFV149 (parg82G135A), pFV160 (parg82W65R) and pFV182 (parg82Δ282–303).

the regulation of arginine anabolic and catabolic genes is not related to Ins(1,4,5)P_3 kinase activities of the various GST–Arg82 recombinant proteins produced in *E. coli*.

3.4. Effect of point mutations in ARG82 gene on cell growth

Since some of the mutants were impaired in Ins(1,4,5)P_3 kinase activity, we tested whether these strains were affected in growth. This is the major distinction between our study and that of York's group. In their study, both the *arg82*Δ strain transformed with the plasmid expressing arg82D131A and the *plc1*Δ strain were growth-impaired only on ornithine as sole nitrogen source [5]. In our hands, the strain expressing arg82-D131A protein grew very poorly on M. ammonia as well as on M. ornithine (Fig. 4). Two other changes, K133A and W65R, also reduced the growth on M. ammonia, but only the change W65R abolished completely the growth on M. ornithine. In contrast, the deletion of the acidic-rich region reduced the growth on M. ornithine but not on M. ammonia. Three other changes, P127A, L129A and G135A, had no significant effect.

4. Discussion

The recent discovery that Arg82 is an Ipk2 raised the question of a role of inositol phosphates in transcription [4,5,21]. Many of the defects observed in an *arg82* deletant result from a strong decrease in the amount and stability of Mcm1, an essential transcription regulator of mating, G1 and G2/M phase transition, minichromosome maintenance and arginine metabolism. However, the sporulation defect and absence of growth at 37°C of an *arg82* deleted strain, are not linked to a low Mcm1 level. For these cellular processes (Mcm1-independent), Arg82 could associate and stabilize other regulators. Whether the kinase activity of Arg82 is required for all the networks in which this protein is involved is still open. York's group attempted to prove that the kinase activity of Arg82 was required for the regulation of arginine metabolic genes. They showed that the DNA binding activity of the Arg80–Arg81–Mcm1 regulatory complex was unaffected in a strain lacking Plc1 activity or in a strain lacking Arg82 kinase activity. These data argued strongly against the involvement of inositol phosphates in the regulation of the arginine metabolism. However, these two strains showed a growth impairment when ornithine was used as sole nitrogen source, which is a major characteristic of lack of regulation of the arginine catabolic genes. Odom et al. [5] proposed that the regulatory Arg80–Arg81–Mcm1 complex was inactive in the absence of inositol phosphate kinase activity, even bound to the target promoters. We show here that the growth defect observed in the two mutant strains described above is general and not restricted to the nitrogen source requiring expression of arginine catabolic genes. Moreover, repression of the anabolic genes and induction of the catabolic genes by arginine are not impaired in a Δ*plc1* strain, nor in strains which have lost arg82 kinase activity. In contrast, mutations in Arg82, keeping the kinase active, had lost the arginine regulation. The kinase activity of Arg82 is thus dispensable to regulate arginine metabolism, in response to this amino acid. However since Arg82 is pleiotropic, it is likely that other functions could depend on this kinase activity. We observed here that mutations in the Ins(1,4,5)P_3 binding site significantly reduced the growth of these strains, although there was no simple correlation between the growth rates and the kinase activities of recombinant GST–arg82 proteins. To further establish the role of inositol phosphates in the different cellular processes controlled by Arg82 protein, it would be interesting to measure the intracellular variation of these metabolites, under different growth conditions and in different mutant strains. It would also be of interest to measure Arg82 kinase activity in yeast extracts to determine if this activity is regulated by means of protein–protein interactions, as shown for the mammalian Ins(1,4,5)P_3 3-kinases, typical calmodulin sensitive enzymes.

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