The *Saccharomyces cerevisiae* CCH1 gene is involved in calcium influx and mating

Marc Fischer[a,*], Norbert Schnell[b], Jayne Chattaway[a], Paul Davies[b], Graham Dixon[b], Dale Sanders[a]

[a] *Biology Department, University of York, P.O. Box 373, York YO1 5YW, UK*
[b] *Zeneca Pharmaceuticals, Mereside, Alderley Park, Macclesfield SK10 4TG, UK*

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Abstract The yeast *Saccharomyces cerevisiae* gene CCH1 (ORF YGR217w) shows high homology with animal calcium channel α1-subunit genes. Knock-out mutants were constructed of Cch1 and of Mid1 which is known to mediate Ca^{2+} influx in response to the α-mating pheromone. Cch1 is involved in calcium influx and the late stage of the mating process. The *cch1* mutant sensitivity against the α-mating pheromone can be reduced by the addition of extra calcium. The product of this gene is likely to interact with the MID1 gene product in Ca influx or its control.

Key words: Calcium channel; CCH1; Mating; *Saccharomyces cerevisiae*

1. Introduction

In eukaryotic cells, Ca^{2+} signals usually arise due to opening of Ca^{2+} channels in the plasma membrane or in organellar membranes. Channel opening drives a transient and rapid increase of cytosolic free Ca^{2+} from around 0.1 μM to 1 μM. These modifications in the Ca^{2+} concentration are linked to a wide range of effects. In yeast, as in other organisms, Ca^{2+} plays an important role as a metabolic regulator and especially in the control of gene expression and of the cell cycle [1–7]. Thus, in *Saccharomyces cerevisiae*, bud emergence and initiation of S phase are coincident with and are linked to an increase of Ca^{2+} uptake, and external Ca^{2+} is required for mitotic cells to traverse the G1 and G2/M phases [1].

Calcium has also been demonstrated to be a major component in the mating process in yeast [8,9] which is triggered by the mating pheromone a- and α-factors (respectively synthesized by strains with MATα and MATa mating type). The mating pheromone of one cell type induces responses in the other cell type which, in the late steps of the mating process, involves differentiation of the yeast cells into shmoos. Iida et al. [10] have identified a gene, MID1, which is required for Ca^{2+} influx and mating. Thus, in the absence of Ca^{2+}, *mid1* mutations are lethal after exposure to α-factor and differentiation of cells into shmoos. In Ca^{2+} depleted medium *mid1* mutants also exhibit remarkably reduced Ca^{2+} influx after presentation of α-factor [10].

The sequence of MID1 indicates a transmembrane protein with some homology to cyclic nucleotide-gated cation channels [10]. Surprisingly, given the prominence of Ca^{2+} signalling events in both yeast and plants [11], channels with high selectivity to Ca^{2+} have not been firmly identified at a molecular level outside the animal kingdom. It is therefore of considerable interest that the yeast genome sequencing project has led to the identification of a gene, CCH1 [12] which shows similarity to bona fide Ca^{2+} channels from higher eukaryotes. We explore here the consequences of deletion of this gene, and demonstrate its involvement in Ca^{2+} uptake in response to α-mating factor.

2. Materials and methods

2.1. Strains

The yeast strains used in this study are based on the parental strains JK9-3da (*MAT*a, *leu2-3*, *112*, *his4*, *trp1*, *ura3-52*, *rme1*) and JK9-3daα (*MAT*a/α, *leu2-3*, *112*, *his4*, *trp1*, *ura3-52*, *rme1*) [13]. They were maintained in a synthetic medium, SD, prepared as described by Sherman et al. [14], and supplemented with the appropriate amino acids.

2.2. Disruption of loci

2.2.1. Single knock-out mutants construction. The MID1 and CCH1 reading frames were completely replaced by the kanMX knock-out cassette [15]. All primer sequences used for PCR of kanMX were homologous to the target gene sequence immediately downstream of the start codon and upstream of the stop codon. PCR reactions were set up containing 30 pmol kanMX primers (Table 1), 100 ng pFA6-kanMX plasmid, 0.2 mM of each nucleotide, 1.5 mM $MgCl_2$, 10 mM Tris-HCl pH 8.8 and 2.5 U Taq polymerase. Yeast cells were transformed with 10 μl of the kanMX PCR reaction (without any purification of PCR product) using the lithium acetate method as described by Wach et al. [15]. Diagnostic primers annealed approximately 100 bp upstream of the start codon of the target gene and internal to the kanMX cassette to give PCR products of 1.15 kb if correct integration had occurred. Transformants were

Table 1
PCR oligonucleotides

1	GGATGATAGTGTGGCAAGCACTATTCGTGGTTTACTGCCTATT-TACCCCAGCTGAAGCTTCGTACGC
2	CGGTCTCTTCTACGTATCGTCCAATGGATGAATTACCATCAAG-GATGAGTTGCATAGGCCACTAGTGGATCTG
3	CTATGCAGGGGAGAAAAAGGACGCTTACGGAACCATTTGAGC-CAAATACCCCAGCTGAAGCTTCGTACGC
4	TCTATCAATTAGATCATTTGCGCTATCGTCATTATTATC-TATTGGTTCGCGCATAGGCCACTAGTGGATCTG
5	CAAATACTTGACTGGCTTAACTGC
6	ATGAATCAATGGTTGGTGACAAGC
7	AGTGAGAAATCACCATGAGTGACG

Oligonucleotides used for kanMX deletion cassette construction: 1: (5′ kanMX) and 2: (3′ kanMX) for disruption of MID1; 3: (5′ kanMX) and 4: (3′ kanMX) for disruption of CCH1. Oligonucleotides used for diagnostic PCR: 5: 5′ diagnostic oligonucleotide for MID1, 6: 5′ diagnostic oligonucleotide for CCH1, and 7: primer internal to the kanMX sequence.

*Corresponding author. Fax: +44 (1904) 434317.
E-mail: mff1@york.ac.uk

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picked and re-streaked on YPD plates containing 200 μg/ml G418. Large colonies were picked and resuspended in 50 μl of water, boiled for 5 min, cooled briefly on ice and then centrifuged at 13000×*g* for 1 min. Five μl of the supernatant was added to a PCR reaction containing 10 mM Tris-HCl pH 8.8, 2 mM $MgCl_2$, 0.2 mM of each nucleotide and 30 pmol of diagnostic primers (Table 1). After overlaying with mineral oil the mixture was heated to 80°C and 2.5 U of Taq polymerase was added. The mixture was then immediately used for PCR: 35 cycles of 1 min at 94°C, 1 min at 55°C and 2 min at 72°C.

2.2.2. Double knock-out mutant construction. All gene deletions were carried out in both diploid JK9-3daα and JK9-3da yeast strains. Diploid cells with the heterozygous gene::kanMX deletion were sporulated and tetrad dissected. The kanMX marker was observed to segregate in a 2:2 ratio in the haploids as expected. Kan resistant haploids of opposite mating type to JK9-3da were identified via cross with a tester strain (*MAT*a, *his*3) or (*MAT*α, *his*3). Single colonies of MATa MID1::KanMX and MATα CCH1::KanMX were picked and mated on a YPD plate at 30°C for 3–4 h. The doubly heterozygous diploids were sporulated and tetrads dissected. Since there are two unlinked kan markers present in each diploid cell these segregate to yield either: 4 kan resistant, or 3 kan resistant and 1 sensitive, or 2 kan resistant and 2 sensitive spores. Therefore, haploid yeast cells arising from tetrads showing ratios of kanamycin resistance to sensitivity of 3:1 or 2:2 were analysed by colony PCR for the presence of the two Kan in two different locations. The presence of the kanMX cassette in the two different genes was also confirmed by Southern blotting [16].

2.3. Calcium uptake and accumulation experiments

The SD medium used for these experiments was modified as described by Iida et al. [10] to have a concentration of 100 μM Ca by replacing the calcium pantothenate with sodium pantothenate and reducing the concentration of $CaCl_2$ from 680.2 μM to 100 μM. Exponentially growing cells grown at 30°C with shaking were harvested and resuspended at a density of 10^8 cells/ml in SD 100 μM Ca medium. For the α-factor experiment they were then exposed to 15 μM α-factor for 1 h with shaking at 30°C. The cells were incubated at room temperature with 185 kBq of $^{45}CaCl_2$ per ml (1.8 kBq/nmol). At various incubation times, samples were taken, filtered through Millipore filters (type HA; 0.45 μm) presoaked in 5 mM $CaCl_2$ and washed with 25 ml of the same solution. The filters were dried and radioactivity was measured by placing the filters in Ultima-GoldXR liquid cocktail (Packard) and counting in a Packard spectrometer.

The calcium accumulation experiments were performed at 30°C with SD 100 μM Ca medium in a shaking incubator at 30°C.

2.4. Yeast growth

The growth of the strains used in this study was determined by shaking cultures at 30°C in SD 100 μM Ca medium supplemented with the appropriate amino acids [14], measuring optical density at 600 nm using a Shimadzu spectrophotometer.

2.5. Cell viability

The viability of the cells after an overnight incubation with shaking at 30°C in a medium with or without α-factor (15 μM) was determined under a differential interference-contrast microscope with a 40× objective by staining the cells with 0.01% methylene blue. The media used for this experiment were SD with 100 μM or 10 mM Ca supplemented with the appropriate amino acids.

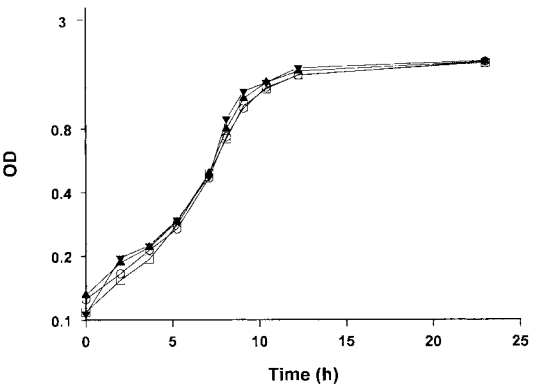

Fig. 1. Growth curves at 30°C of the parental strain JK9-3da (□), the *mid1* mutant (○), the *cch1* mutant (▼) and the double mutant *mid1-cch1* strain (▲) in SD medium supplemented with 100 μM Ca and the appropriate amino acids. Results are the means of three separate experiments.

3. Results

The identity of the constructs was verified by Southern blotting with the kan gene and by PCR (first primer inside the kan gene, the other 5′ of the disrupted gene). The PCR products were also sequenced and their junctions confirmed to be gene::kan-MX (data not shown). All the transformed strains proved to have the insertion of a single copy of the transformation vector at the expected target locus.

The viability of the mutated strains was tested with two media different in their calcium concentration (Table 2). At the lower Ca^{2+} concentration, the viability of all three mutated strains is lower than that of the parental strain. However, this difference of viability is considerably reduced at elevated Ca^{2+} levels. It is also noteworthy that the viability of the single mutated strains and the double mutated strain are similar to each other, regardless of the growth medium and sampling time.

Fig. 1 shows growth curves on low calcium medium (SD 100 μM Ca) at 30°C for the three mutated strains *mid1*, *cch1* and the double mutant *mid1-cch1* and for the parental strain JK9-3da. Clearly, there is no difference in growth rate and the mutated strains seem to have the same ability to grow in these conditions.

The sensitivity of the four different strains to α-factor was tested with the two SD media, at low and high calcium concentration (Table 3). At low Ca^{2+} concentration (100 μM) the sensitivities of the mutated and the parental strains are dramatically different. The three mutated strains have the same phenotype and die after incubation with α-factor, whereas the majority of the parental strain cells remains viable. Signifi-

Table 2
Viability of mutated yeast strains

Medium	Time	Survival (%)			
		JK9-3da	*mid1*	*cch1*	*mid1-cch1*
SD 100 μM Ca	24 h	85	53	63	56
	1 week	59	33	29	30
SD 10 mM Ca	24 h	82	74	81	77
	1 week	56	49	49	51

Difference of viability between yeast strains was assessed in SD 100 μM Ca or SD 10 mM Ca media supplemented with the appropriate amino acids. Viability was determined by methylene blue staining after the growth time indicated. Results are the means of three separate experiments. The standard errors did not exceed 5% of the mean.

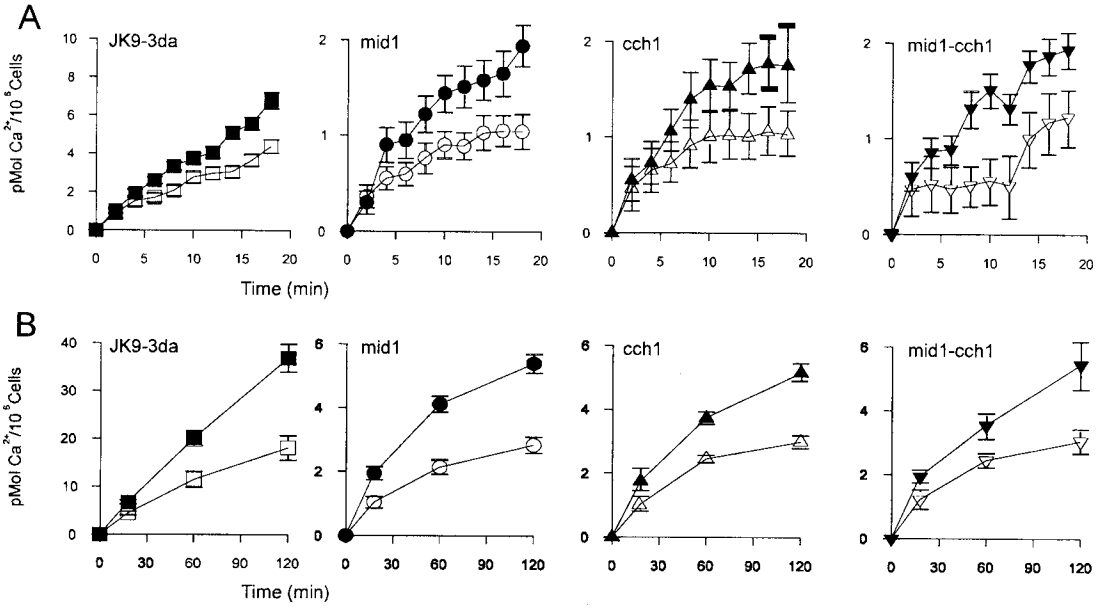

Fig. 2. Initial rate of Ca^{2+} uptake (A) and longer term accumulation (B) for the parental strain JK9-3da, the *mid1* and *cch1* mutants and the double mutant *mid-cch1* in SD medium supplemented with 100 μM Ca and the appropriate amino acids. Exponentially growing cells were assayed for $^{45}Ca^{2+}$ uptake either in the absence (open symbols) or after 1 h exposure to 15 μM α-factor (solid symbols). Cells were incubated with 185 kBq of $^{45}CaCl_2$ per ml and at indicated times samples were taken, filtered and counted. Results are the means of ten separate experiments.

cantly, elevation of calcium to 10 mM rescues the mutated cells and restores viability to a level which is comparable to that of the parental strain. There is no apparent difference in sensitivity to α-factor concentration or in the kinetics of the viability loss between the single mutant strains (*mid1*, *cch1*) and the double mutated *mid1-cch1* strain (data not shown).

These survival experiments were also performed with the *mid1* mutant used by Iida et al. [10] (data not shown). Comparison of the sensitivity to α-factor of the two mid1 mutant strains showed that the strain used in the present study was less sensitive. The α-factor concentration required to elicit a clear phenotype (15 μM) was higher than that used by Iida et al. (6 μM). This difference is probably due to the fact that the parental strain we used is not defective for the protease Sst1 responsible for the degradation of the α-factor, and that some degradation takes place during the overnight incubation.

Fig. 2A and B show the Ca^{2+} uptake activity of exponentially growing cells for each of the strains in the absence and the presence of α-factor. Mutated strains are severely compromised both with respect to initial uptake rate and to longer term accumulation in comparison with the parental strain JK9-3da. In all the strains used for this study, the exposure to α-factor for 1 h increases the calcium uptake and accumulation. Nevertheless, in all cases, Ca^{2+} uptake was stimulated by α-factor by similar proportional amounts, suggesting that the α-factor response is unaffected by these mutations. Its is particularly noteworthy that the calcium uptake activity of the single mutated strain is similar to that of the double mutant strain. The results obtained with the *mid1* mutant derived from the parental strain JK9-3da described in the present study are very similar to those obtained by Iida et al. [10] with a *mid1* mutant derived from the parental strain H207.

4. Discussion

Until now, only two genes potentially involved in the formation of calcium permeable channels have been identified in non-animal cells. Iida et al. [10] first showed that the gene MID1 which encodes a 548 residue and 61.5 kDa transmembrane polypeptide is involved in calcium uptake at a low calcium concentration of 100 μM. In addition, the yeast genome project has identified the gene CCH1 [12] which encodes a 2039 residue and 234.6 kDa polypeptide. Sequence analysis shows that it is a transmembrane protein sharing high homology with animal voltage-gated calcium channel α1-subunits. By constructing single mutants for the MID1 and CCH1

Table 3
Impact of α-factor on survival rate

Medium	α-factor	Survival (%)			
		JK9-3da	*mid1*	*cch1*	*mid1-cch1*
SD 100 μM Ca	−	84	62	68	65
	+	70	< 1	< 1	< 1
SD 10 mM Ca	−	80	75	74	77
	+	82	61	54	62

Survival rates of the parental strain JK9-3da, the *mid1* and *cch1* mutants and the double mutant *mid1-cch1* in SD modified medium supplemented with 100 μM or 10 mM Ca and the appropriate amino acids after an overnight exposure to α-factor (15 μM) added at the beginning of growth. Viability was determined by methylene blue staining; results are the means of four separate experiments.

genes and the double mutant strain, we aimed to determine whether the phenotype of the *cch1* mutant resembled that of *mid1*, and whether there appeared to be any interaction between the two genes which would appear specifically in the double mutant.

Our data show that inactivation of MID1 or CCH1 or the double inactivation of MID1 and CCH1 give similar phenotypes with respect to the lethality of α-factor and to the depression of Ca^{2+} uptake. These results suggest that the two gene products interact in the same pathway. The α-factor sensitivity experiments with the different strains show that the conditional phenotypes obtained with the MID1 and CCH1 deletants are the same and identical to the phenotype of the double deletant MID1-CCH1 strain. The results demonstrate that at a low concentration of calcium (100 μM), the α-factor induces the death of the mutated cells in a few hours and with the same kinetics that have been observed for the deletion of the MID1 gene by Iida et al. [10]. The cells do differentiate into shmoos, proving that the early events of the mating process are possible but death occurred in the late stages of the mating phenomena. In the case of the MID1-deleted strains, the mating pheromone induced death has been shown to be associated with a diminution in calcium influx and not to a defect in the influx of any other ion. The similarity of the reactions of the three single and doubly mutated strains is an indication that the CCH1 gene is certainly also involved in calcium influx or signalling during the late events of the mating process.

Iida et al. [10] have also shown that Mid1 does not function alone because overexpression of the MID1 gene on a multicopy vector does not affect Ca^{2+} influx. At the time of their work, the CCH1 sequence had not been determined. It now appears that the CCH1 gene product could be a good candidate for interacting with the Mid1 protein. The results from the calcium uptake and accumulation experiments performed here show quite clearly that the mutated strains have far lower activities than the parental strain. The comparison of longer term calcium accumulation detected in the chosen experimental conditions shows that for any of the single or the double mutants, calcium accumulation represents less than 20% that of the parental strain JK9-3da. This is another indication that Cch1 and Mid1 are probably not working as two independent systems but are more likely to be part of the same pathway for calcium uptake.

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