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KP4 fungal toxin inhibits growth in *Ustilago maydis* by blocking calcium uptake

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Summary

KP4 is a virally encoded fungal toxin secreted by the P4 killer strain of *Ustilago maydis*. From our previous structural studies, it seemed unlikely that KP4 acts by forming channels in the target cell membrane. Instead, KP4 was proposed to act by blocking fungal calcium channels, as KP4 was shown to inhibit voltage-gated calcium channels in rat neuronal cells, and its effects on fungal cells were abrogated by exogenously added calcium. Here, we extend these studies and demonstrate that KP4 acts in a reversible manner on the cell membrane and does not kill the cells, but rather inhibits cell division. This action is mimicked by EGTA and is abrogated specifically by low concentrations of calcium or non-specifically by high ionic strength buffers. We also demonstrate that KP4 affects ^{45}Ca uptake in *U. maydis*. Finally, we show that cAMP and a cAMP analogue, N 6,2′-O-dibutyryladenosine 3′:5′-cyclic monophosphate, both abrogate KP4 effects. These results suggest that KP4 may inhibit cell growth and division by blocking calcium-regulated signal transduction pathways.

Introduction

Interstrain inhibition in *Ustilago maydis* was discovered by Puhalla (1968) during heterokaryon experiments. Crosses and heterokaryon transfer experiments demonstrated that the inhibitory effect is caused by cytoplasmically inherited factors (Puhalla, 1968; Day and Anagnostakis, 1973) that are now known to be single double-stranded (ds)RNA segments present in some multisegmented dsRNA viral genomes. The inhibitory factors (killer toxins) were shown to be secreted proteins encoded by the single dsRNA segments (Hankin and Puhalla, 1971). A small proportion of *U. maydis* cells can produce killer toxins, to which they are resistant; sensitive cells are the majority in wild populations. Killer toxins have been identified in eight genera of yeast (Young, 1987), but the killer toxins of *Ustilago* are the only ones known in a filamentous fungus. The *U. maydis* killer toxins are effective against species in the family Ustilaginaceae, including those that are known as pathogens to wheat, oats and barley (Koltin and Day, 1975).

There are three killer strains of *U. maydis*: P1, P4 and P6, which secrete the KP1, KP4 and KP6 toxins respectively (Puhalla, 1968; Koltin and Day, 1975; Koltin, 1988). Correspondingly, there are three groups of resistant cells, in which the resistance is determined by three independent recessive nuclear genes, p1r, p4r and p6r (Puhalla, 1968; Koltin and Day, 1976). In KP1, cytoplasmically inherited factors also confer immunity to the KP1 killer toxin (Peery *et al*., 1987), whereas KP4 and KP6 probably do not have cytoplasmically determined immunity (Finkler *et al*., 1992). Cells cannot produce the KP6 toxin unless they have the p6r gene (Finkler *et al*., 1992), and the same is probably true of KP4 (Y. Koltin, personal communication). The p1r, p4r and p6r genes are thought to encode cellular (membrane or membrane cell wall) receptors for their respective toxin. As these putative receptors are different in each case, there is no single resistance allele that confers simultaneous resistance to all three toxins.

KP4 is a single polypeptide of 105 amino acids that is not glycosylated (Park *et al*., 1994). It is the only *Ustilago* toxin not processed by Kex2p, and there is no sequence similarity to other toxins (Ganesa *et al*., 1991; Park *et al*., 1994). Although most of the yeast toxins are acidic (Bussey, 1972) and the KP6 and KP1 toxins have neutral pIs (Levine *et al*., 1979), KP4 is extremely basic with a pI $>$ 9.0 (Ganesa *et al*., 1989). KP4 is an α/β sandwich protein with a relatively compact structure (Gu *et al*., 1995). Based on a tenuous structural relationship to scorpion toxins, it was proposed that the toxin might affect, not create, ion channels. This model was supported by the demonstration that calcium abrogates the KP4 killing effects and that KP4 can block voltage-gated calcium channels in mammalian neuronal cells (Gu *et al*., 1995).

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Calcium is a ubiquitous signalling molecule that has been shown to play an important role in fungal growth. Calcium is involved in such diverse processes as bud formation (Davis, 1995), hyphal elongation (Jackson and Heath, 1993) and cAMP regulation (Iida *et al*., 1990a). Cytosolic calcium levels are normally maintained at between 100 and 200 nM, whereas extracellular calcium concentrations are normally 0.1 μM to 10 mM (Halachmi and Eilam, 1989; Iida *et al*., 1990b). Steady-state calcium levels within the cytosol are maintained by a series of calcium pumps and antiporters in the Golgi, endoplasmic reticulum (ER), and vacuoles. Vacuoles are the major calcium storehouse in fungi. Calcium is sequestered in vacuoles by a $2H^+/Ca^{2+}$ antiporter (Ohsumi and Ankura, 1983; Dunn *et al*., 1994) and by the *PMC1* gene product. This protein is related to mammalian plasma membrane Ca^{2+}-ATPases (Okorokov *et al*., 1993). Sequestered Ca^{2+} is released from vacuoles by IP_3, as was demonstrated in *Neurospora* and *Saccharomyces cerevisiae* (Cornelius *et al*., 1989; Belde *et al*., 1993). This differs from animal cells, in which IP_3 causes the release of sequestered Ca^{2+} from the ER.

The experiments described here further test our hypothesis that KP4 blocks calcium channels. KP4 is shown to interact with the cell membrane in a transient manner. KP4 is shown not to kill the target cells but, rather, it inhibits cell division. KP4 inhibition is abrogated in a specific manner by low concentrations of calcium. KP4 is shown to act in a manner analogous to calcium chelators (EGTA) and calcium channel inhibitors (Cd^{2+}). KP4 inhibits ^{45}Ca uptake in *Ustilago*. Finally, KP4 effects are reversed by cAMP and N 6,2′-O-dibutyryladenosine 3′:5′-cyclic monophosphate (DBcAMP), suggesting that KP4 blocks a cAMP-regulated growth pathway.

Results

Mutagenesis studies

In our previous work, a tenuous structural homology between the KP4 toxin and the channel-blocking scorpion toxin AaHII suggested that KP4 acts by blocking calcium channels (Gu *et al*., 1995). The motif of KP4 that appeared to be similar to the AaHII active site was the β3−β4 loop extending from the end of the β-sheet structure. Like the scorpion toxin, the base of this loop was extremely basic and was stabilized by a disulphide bond with the C-terminus. In the AaHII toxin, modification of lysine 58 at the base of this loop neutralized the toxin (Fontecilla-Camps, 1989). Similarly, KP4 has a lysine (K42) at the base of this loop (Fig. 1A). Therefore, to test this functional homology, we mutated K42 to a glutamine. Similar to AaHII, this mutation decreased KP4 activity by about 90% (Fig. 1B). In KP4, a pseudo twofold axis lies perpendicular to the β-sheet and relates the β3−β4 loop with the β6−α4 loop. As a control, we also mutated R68, which lies at the base of the β6−α4 loop, to glutamine. As predicted by our model, this mutation had no effect on KP4 activity. Similar results were obtained using expressed and purified mutant toxin. Therefore, these results support our contention that

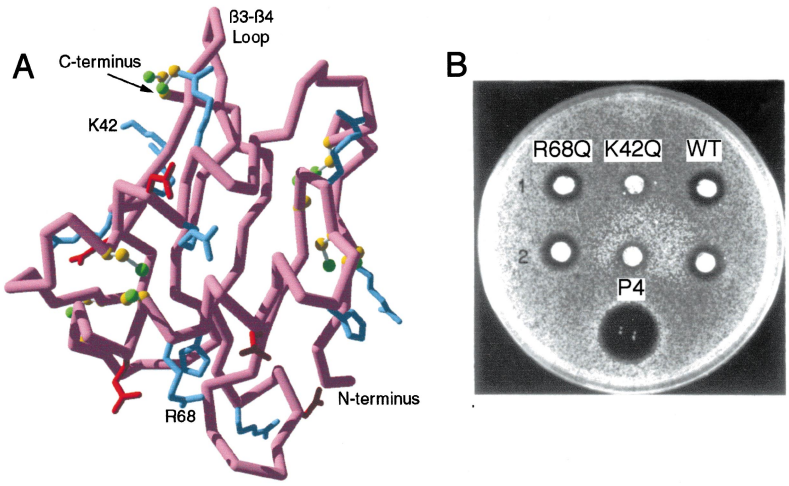

Fig. 1. Mutation of putative active site on KP4.
A. The location of the site-directed mutations in KP4. The mauve cylinders represent the C-α backbone of the toxin. The acidic and basic side-chains are shown in red and blue respectively. The cysteine side-chains are represented by ball and stick models. It should be noted that all neighbouring cysteine residues are involved in disulphide bonds with each other.
B. Killing activity of native and mutant KP4. Native, yeast-expressed KP4 is shown in rows on the right; the yeast expressed K42Q mutant is shown in the middle two rows; and the R68Q mutant is shown in the left two rows. For comparison, UMV4 expressing native KP4 is shown at the bottom of the agar-based killing assay. The K42Q KP4 mutant is clearly less efficacious, whereas the R68Q mutation actually has a slightly higher killing activity. This assay was performed using an equivalent number of cells per blot. Nearly identical results were obtained using toxin purified from each type of cell.

the active site of the toxin lies near the β3–β4 loop and that KP4 may act as a channel blocker like AaHII.

Effects of cations on KP4 activity

Previous studies using the agar-based, killing zone method to measure the effects of cations on KP4 activity clearly showed that calcium was very efficacious in abrogating KP4 activity (Gu *et al.*, 1995). However, this assay cannot be performed over a range of incubation times, is not quantitative and suggested that magnesium might also affect KP4 efficacy (Gu *et al.*, 1995). By growing the cells in 96-well plates, cell division can be continually and accurately monitored optically. Using this method, we re-examined the effects of metals on KP4 killing (Fig. 2). These studies showed that sodium is the most toxic to the cells in the absence of KP4, closely followed by potassium. Potassium appeared to be cytotoxic at ionic strengths > 0.2 M, whereas sodium was cytotoxic at ionic strengths < 0.1 M. In contrast, both magnesium and calcium appear to stimulate cell growth slightly at ionic strengths > 0.2 M.

Of the ions tested, only calcium (Fig. 2D) exhibited a clear, dose-dependent abrogation of KP4 'killing'. At ionic strengths < 0.2 M, sodium exhibited no reversal of KP4 effects. Magnesium exhibited a very slight abrogation of KP4 'killing' at very high concentrations. This may be the result of a non-specific ionic strength effect or from the growth activation observed in the absence of KP4. Potassium, although cytotoxic at very high concentrations, did appear to remove some of the KP4 inhibition, as the growth curves at > 0.8 M were very similar in the presence or absence of KP4. Similar effects were probably not observed in the case of NaCl on account of toxicity and $MgCl_2$ because solubility limited the maximum

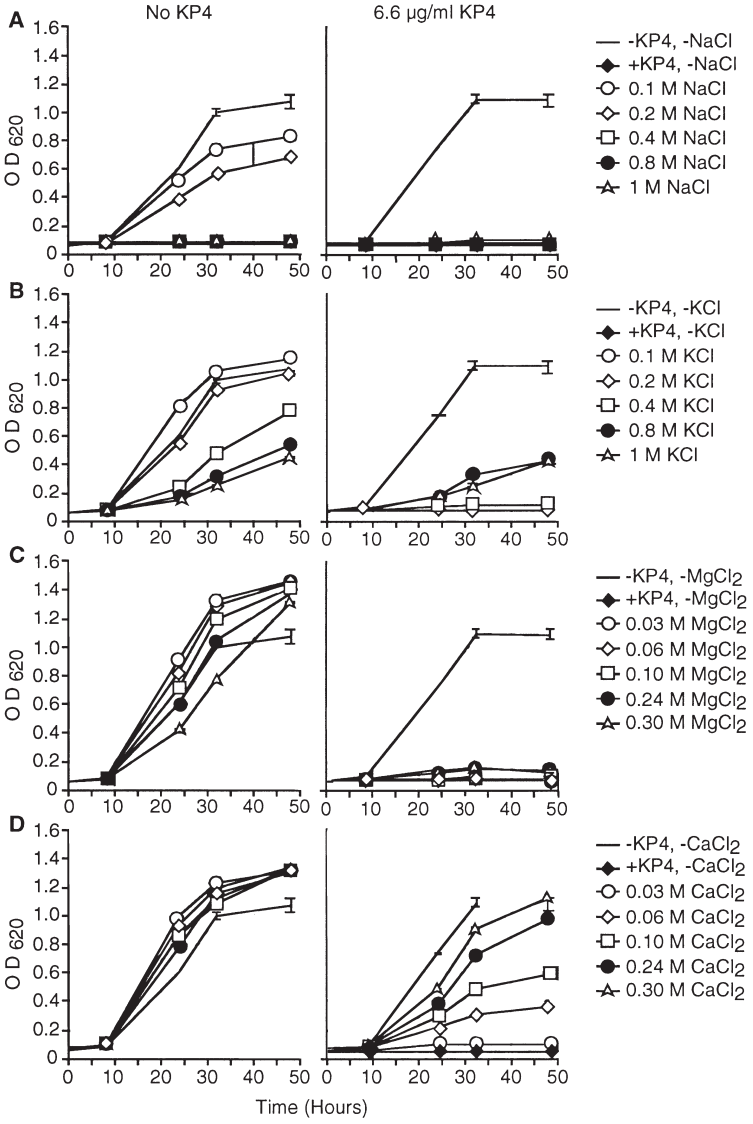

Fig. 2. Effects of metals on P2 growth and KP4 activity. Shown here is the growth of P2 cells (represented by the OD_{620}) in various concentrations of metal salts in the presence (right column) and absence (left column) of KP4. Rows A–D represent growth curves in the presence of sodium, potassium, magnesium and calcium respectively. Each point was measured in triplicate. Note that NaCl and KCl are rather toxic to the P2 cells, and only $CaCl_2$ is able to abrogate KP4 activity at low ionic strengths.

concentrations used for the experiments. These results clearly demonstrate that the calcium is uniquely able to counteract the effects of KP4, but that very high ionic strength buffers may also slightly affect KP4 activity. In the case of the latter, since we propose that the charge of K42 is crucial for toxin binding, abrogation of activity by high ionic strength buffers is expected. However, the fact that calcium is unique in its activity at low concentrations demonstrates cationic specificity.

KP4 reversibility

Some protein toxins are believed to act by creating channels in the cell membrane (Palmer and Merrill, 1994; Song *et al*., 1996). Although the biophysical nature of KP4 makes this unlikely (Gu *et al*., 1995), it has not been directly tested. If KP4 were a channel-forming toxin, one would expect the KP4 effects to be either slowly reversible or irreversible. In contrast, if KP4 were to act via ionic interactions with cell surface proteins, then one might expect it to bind reversibly to the cell membrane. To test for the reversibility of the KP4–cell interaction, P2 cells were incubated with KP4 for varying periods of time, washed, and cell growth was measured (Fig. 3). The longer the P2 cells are treated with toxin, the longer it takes for the cells to reach log-phase growth. As such a high dose of KP4 was used for the incubation period, these results suggest that the toxin does not kill the target cells, but rather inhibits growth during the incubation period. However, there is a lag of 6, 10 and 12 h when the cells are treated with toxin for 1, 2 and 4 h respectively. When 100 mM calcium is added to cell suspensions during the KP4 incubation, the lag phase is nearly eliminated.

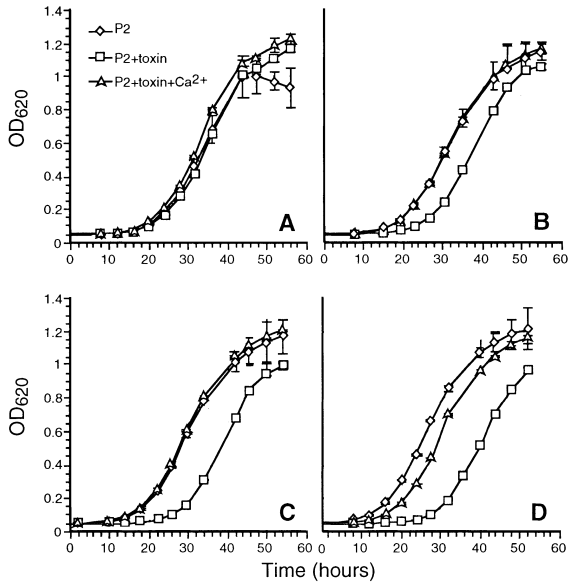

Fig. 3. Growth curves of P2 cells incubated with KP4 and then washed with media. In these experiments, P2 cells were incubated with 6.6 μg ml^{-1} KP4 for 0 (A), 1 (B), 2 (C) and 4 (D) h. After the incubation, the cells were washed three times and aliquoted into 96-well plates, and the OD_{620} was monitored for 52 h. Each point was collected in triplicate, and the error bars are shown in the graphs. Note that, when P2 cells were incubated with KP4 toxin, the growth curves of the cells exhibited a lag phase that was proportional to the incubation time. However, when the cells were incubated with calcium concomitantly with the KP4, the lag phase was eliminated.

One possible reason for this lag phase is that some KP4 may have remained bound to the cell during the washing procedure. Therefore, as calcium appears to compete with KP4, it was speculated that calcium in the wash medium would remove KP4 from the cell surface more effectively. The above experiment was repeated, and the cells were resuspended in media containing 100 mM $CaCl_2$ after washing the cells with media that did not contain calcium. This addition of calcium to the resuspension media eliminates the lag phase (Fig. 4B) as expected, as calcium abrogates KP4 growth inhibition (Figs 2D and 3A). In the next experiment (Fig. 4C and D), cells were washed with media containing 100 mM $CaCl_2$ and resuspended in media that did not contain additional calcium. This wash eliminated the KP4-induced lag time as effectively as when calcium is added to the resuspension media (Fig. 4A and B). These results demonstrate that, even after cells are incubated with toxin for 1 h, KP4 effects can be completely eliminated in the minute it takes to wash the cells. This suggests that the KP4-induced lag phase is caused by residual KP4 trapped on the surface of the cells and not by internalized toxin. When incubations were performed at 0°C, identical results were obtained (data not shown).

As a final test for whether KP4 kills the target cells or merely inhibits growth, the number of viable cells was monitored during an extended incubation with toxin. As shown in Table 1, the number of viable cells (colonies) remains fairly constant during the entire incubation with KP4. Therefore, it seems more than likely that KP4 binds reversibly to the cell surface and acts by inhibiting cell growth.

The role of the cell wall in KP4 activity

Some of the fungal killer toxins appear to require the cell wall for at least initial interactions. The *S. cerevisiae* killer toxin, KT28, requires the presence of the cell wall for activity (Al-Aidroos and Bussey, 1978; Schmitt and Radler, 1988). In the case of the *U. maydis* killer toxin, KP6, spheroplasts are insensitive to the toxin (Steinlauf *et al*., 1987). Therefore, the above experiment was repeated with *U. maydis* spheroplasts to show that the interactions between KP4 and the cell surface did not require the cell wall. The spheroplasts responded to KP4 in an identical manner to whole cells (data not shown). This indicates that KP4 does not require the cell wall for activity.

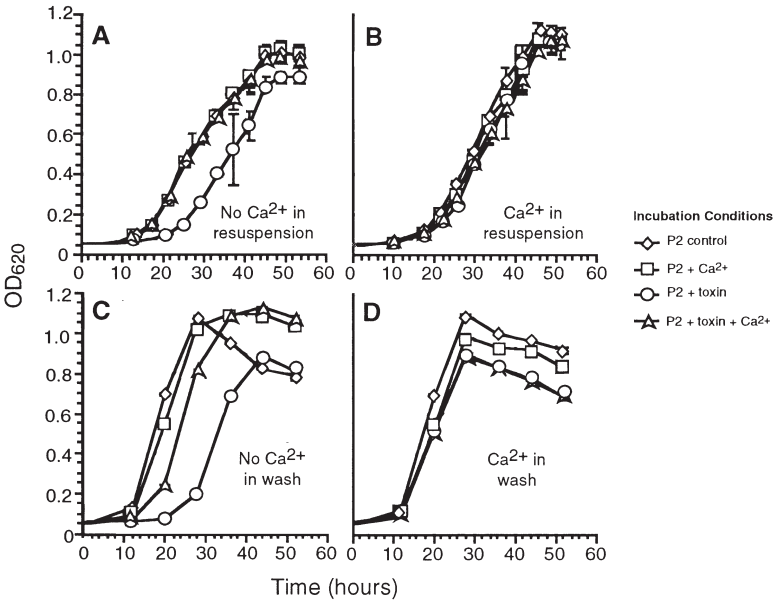

Fig. 4. Growth curves of P2 cells incubated with toxin and washed with calcium or grown in the presence of calcium. P2 cells were incubated for 1 h in media alone, in the presence of 100 mM $CaCl_2$, 6.6 μg ml^{-1} toxin and 6.6 μg ml^{-1} toxin + 100 mM $CaCl_2$.
B. 100 mM calcium was only added to the resuspension media, whereas in (D),10 mM calcium was only added to the washing media. As above, all measurements were made in triplicate.

Effects of EGTA and cadmium on P2 cell growth

If KP4 inhibits cell growth by limiting calcium uptake, then *U. maydis* growth should also be inhibited by EGTA. It has been demonstrated in *S. cerevisiae* that 10 mM EGTA plus 10 μM calcium ionophore A23187 completely blocks cell division in calcium-deficient media (Iida *et al*., 1990a). To determine whether EGTA could block growth, *U. maydis* cells were grown in calcium-deficient media in the presence of EGTA. As shown in Fig. 5B, 10 mM EGTA decreased cell growth by about half, and cell growth is completely blocked by 15 and 20 mM EGTA. Cells treated with 0.33 μM KP4 showed no cell growth for up to 60 h (Fig. 5A). It should be noted that, although KP4 is effective at 0.33 μM, it takes millimolar concentrations of EGTA to inhibit cell growth. This difference in efficacy is probably the result of KP4 specifically targeting Ca^{2+} channels, whereas EGTA needs to chelate all the calcium in the extracellular milieu.

If KP4 and EGTA affected different pathways, it would be expected that their effects would be additive. However, if KP4 and EGTA acted on the same pathway (i.e. calcium uptake), their effects would be expected to be synergistic. Although 0.1 μM KP4 and 5 mM EGTA alone have marginal effects on cell growth, together they block cell growth significantly (Fig. 5C). Therefore, it is very clear that KP4 and EGTA enhance the effects of each other and that their effects are synergistic and not just additive. This experiment was repeated using 0.08 μM KP4 and 10 mM EGTA with similar results (data not shown).

As further evidence that calcium import is essential for *Ustilago* growth, the effects of a Ca^{2+} channel inhibitor, Cd^{2+}, was determined. As shown in Fig. 5D, 1 mM Cd^{2+} is sufficient to block cell growth. However, this inhibition is completely reversed by the addition of 1 mM Ca^{2+}. These results clearly demonstrate that Cd^{2+} effectively blocks cell growth and that this inhibition is probably caused by direct competition with Ca^{2+}. Similar results were obtained using the calcium channel blocker lanthidium, but the dose dependency was difficult to measure because of limited solubility in the media.

These results demonstrate that, if calcium is either removed from the media or if the calcium channels are blocked, *Ustilago* growth is greatly inhibited. This finding is consistent with the observation that calcium channels play an important role in the life cycle of *S. cerevisiae* (Fischer *et al*., 1997). Furthermore, these results also demonstrate that it is entirely plausible that KP4 inhibits cell growth by the inhibition of calcium channels.

Inhibition of ^{45}Ca uptake by KP4

As final evidence that KP4 blocks fungal calcium channels,

Table 1. Effect of KP4 on cell viability.

Time (h)	−KP4 (no. of colonies)	+KP4 (no. of colonies)
0	40.50 ± 9.00	36.25 ± 4.20
7	252.00 ± 25.90	41.75 ± 7.70
16	536.00 ± 74.50	35.25 ± 6.10
24	576.00 ± 334.60	36.50 ± 3.30
32	608.00 ± 320.00	37.50 ± 4.50
40	450.00 ± 251.70	26.50 ± 5.20
48	650.00 ± 378.60	21.75 ± 6.40

P2 cells were incubated with 36 μg ml^{-1} KP4 for 48 h. At each time point, aliquots were removed, diluted and spread onto agar plates. Each time point was collected in quadruplicate and averaged.

sensitive cells were cultured with different concentrations of calcium and treated with $^{45}Ca^{2+}$ in the presence and absence of KP4. As shown in Fig. 6, KP4 clearly causes a decrease in the uptake of $^{45}Ca^{2+}$. $^{45}Ca^{2+}$ uptake by toxin-resistant cells was unaffected by KP4 (data not shown). It should be noted that overall $^{45}Ca^{2+}$ uptake diminishes as the calcium concentration used in the growth media is increased. In *S. cerevisiae*, it has been shown that Na^+ and K^+ transporter activity is regulated by extracellular ion concentration (Serrano *et al*., 1999). This effect has recently been shown to occur with calcium channels in *Saccharomyces* as well (M. Fischer, personal communication). These data demonstrate directly that KP4 blocks calcium uptake and that extracellular calcium levels may regulate *U. maydis* calcium channel activity as well.

cAMP rescue of inhibited cells

Mutations to either adenylyl cyclase (Gold *et al*., 1994) or PKA (Durrenberger *et al*., 1998) can affect the regulation of filamentous growth versus budding growth in fungi. In the case of adenylyl cyclase, it has been shown that exogenously added cAMP can revert the mutant phenotype to a wild-type phenotype (Gold *et al*., 1994). Therefore, KP4 effects may not result from the inhibition of nutritional uptake of calcium but, rather, disruption in the calcium gradients required for budding and filamentous growth (Jackson and Heath, 1993). In order to test for this, P2 cells were treated with exogenous cAMP and KP4 to see whether the secondary messenger, cAMP, could 'short circuit' KP4 inhibition (Fig. 7A). The addition of cAMP causes recovery of cell growth. This recovery exhibits dose dependency, with 15 mM cAMP causing more recovery than 6 mM cAMP (data not shown). The addition of a membrane-permeable cAMP analogue, N 6,2′-O-dibutyryladenosine 3′:5′-cyclic monophosphate (DBcAMP), also demonstrates a similar recovery of growth. What is particularly interesting is that cAMP also abrogates the inhibition caused by EGTA (Fig. 7B). One possible model is that KP4 and EGTA prevent calcium uptake and inhibit the initiation of a calcium-regulated signal transduction pathway. The addition of exogenous cAMP 'short circuits' inhibition by supplanting the downstream secondary messenger.

Discussion

These results strongly suggest that calcium import is essential for fungal growth and that KP4 probably acts at

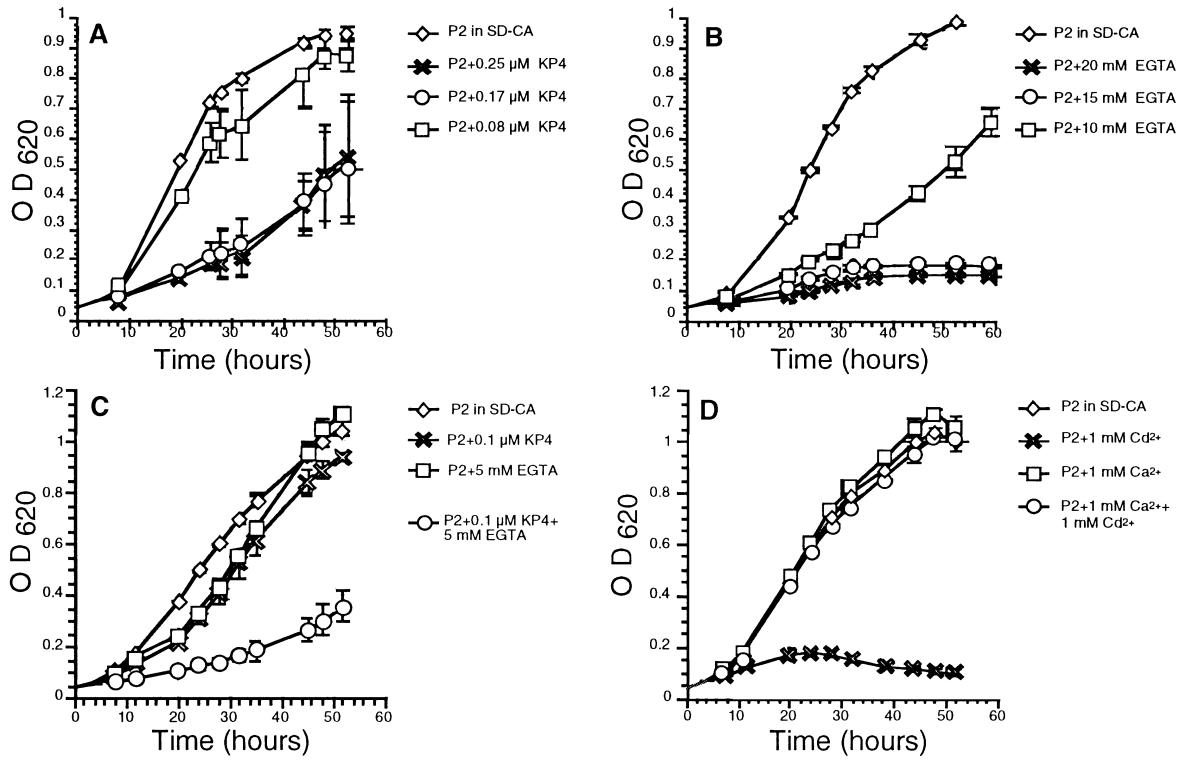

Fig. 5. Effect of EGTA and Cd^{2+} on P2 growth and KP4 effects. Growth curve of P2 cell treated with various concentrations of KP4 (A), EGTA (B) or 0.1 μM KP4 and 5 mM EGTA (C). Each trial was performed in quadruplicate and averaged. Note that EGTA and KP4 effects appear to be synergistic (C). The results shown in (C) are from a separate experiment from those in (A) and (B). They were chosen because they highlight the synergy between KP4 and EGTA.
D. Calcium channel inhibitor, Cd^{2+}, inhibits P2 growth but is competitively eliminated by the addition of exogenous calcium.

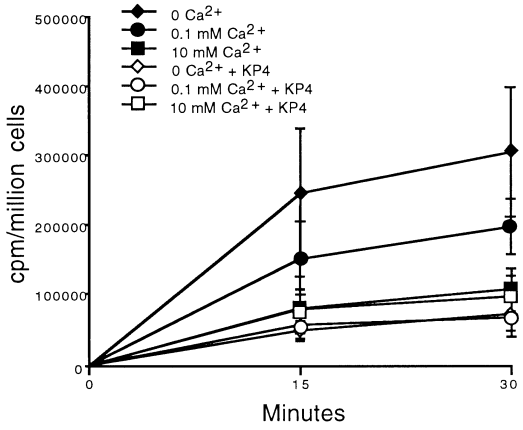

Fig. 6. Effect of KP4 on $^{45}Ca^{2+}$ uptake in sensitive *U. maydis* cells. Uptake of $^{45}Ca^{2+}$ by P2 cells in the presence and absence of KP4 toxin over a 30 min time period. Each trial was carried out five times and averaged. $^{45}Ca^{2+}$ uptake is clearly diminished in cells grown under high calcium concentrations and those treated with KP4.

the plasma membrane to inhibit calcium uptake. We have demonstrated that KP4 blocks calcium import in *U. maydis*. We also demonstrate that this is an inhibitory but non-lethal event. KP4 may prevent progression through the cell cycle by blocking calcium-regulated cell cycle events in *U. maydis.* Calcium has been linked to growth and mating processes in *S. cerevisiae* (Iida *et al*., 1990a; Jackson and Heath, 1993; Hartley *et al*., 1996) although, in *U. maydis*, cAMP has been shown to affect mating pathways (Gold *et al*., 1994; Regenfelder *et al*., 1997; Durrenberger, Wong *et al*., 1998; Kronstad *et al*., 1998; Kruger *et al*., 1998). In *S. cerevisiae*, a calcium influx has been shown to be an important step during the $S \rightarrow G_1$ transition. Once the transition has occurred, this Ca^{2+} spike is dissipated, leaving basal levels of calcium in the cytoplasm.

In *S. cerevisiae*, cells grown in the presence of EGTA and the calcium ionophore, A23187, have decreased intracellular calcium levels and a transient arrest during G_1 followed by block mainly during G_2/M (Iida *et al*., 1990a). Cells treated with EGTA and A23187 also show decreased levels of cAMP production (Iida *et al*., 1990a). However, cells treated with EGTA, A23187 and cAMP analogues simultaneously do not display the transient G_1 arrest. These results imply that calcium is crucial for cell cycle progression and that calcium plays a role in cAMP regulation. Similarly, *Ustilago* growth is inhibited by either chelating external calcium with EGTA or by blocking calcium uptake via KP4 toxin, and both effects can be abrogated by cAMP.

There is considerable evidence that cAMP plays an important role in the dimorphism of *U. maydis*. Gold *et al*. (1994) have proposed a model in which environmental control of *U. maydis* dimorphism occurs through regulation of adenylyl cyclase. When environmental signals upregulate adenylyl cyclase, PKA is activated, causing the phosphorylation of the factors required for budding. When

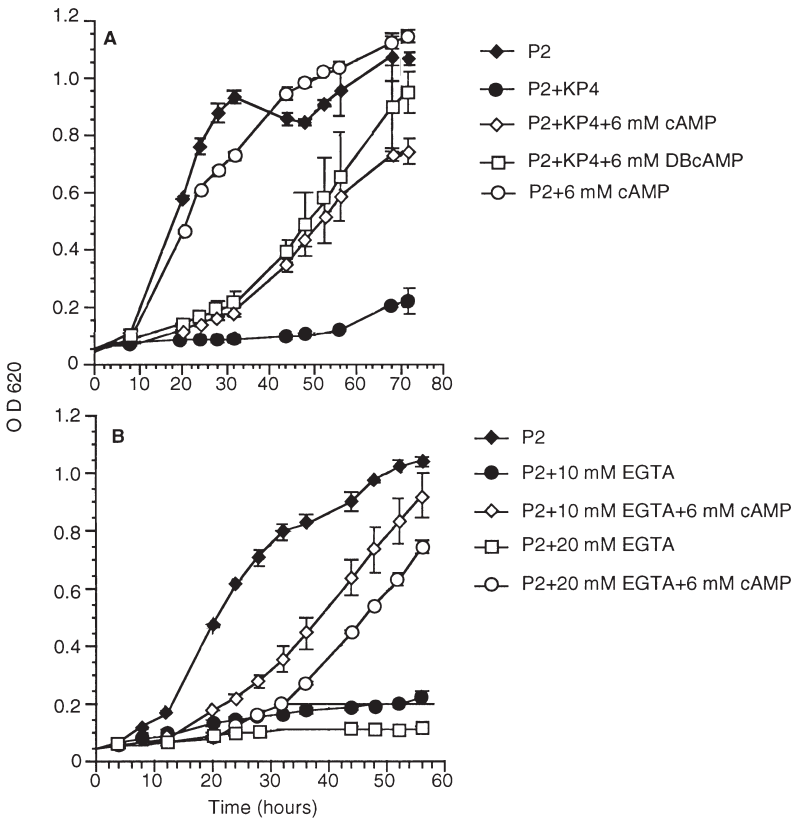

Fig. 7. Effects of cAMP on KP4 and EGTA effects.
A. P2 cell growth was monitored in the presence and absence of 4 $\mu g\,ml^{-1}$ KP4 and cAMP. P2 cells were grown in a calcium-deficient medium alone, in the presence of 6 mM cAMP, KP4, KP4 + 6 mM cAMP or KP4 + 6 mM N 6,2′-O-dibutyryladenosine 3′:5′-cyclic monophosphate (DBcAMP). cAMP and its analogue are clearly effective in removing the growth inhibition caused by KP4.
B. In an analogous way, cAMP also removes the growth inhibition caused by relatively high concentrations of EGTA.

environmental factors downregulate adenylyl cyclase, cAMP levels drop, and PKA becomes inactive, stimulating filamentous growth.

By blocking calcium import, KP4 appears to disrupt the normal signalling pathways involved in budding growth. cAMP appears to play a crucial role in initiating bud formation, as evidenced by the multiple budding phenotype reported. The addition of exogenous cAMP may activate PKA without upregulation of adenylyl cyclase, bypassing the need for calcium uptake to stimulate growth. However, as cAMP and calcium have numerous regulatory roles within the cell, it seems likely that KP4 affects other important growth signalling events as well.

Another possible role for calcium and cAMP might be regulation of the F-actin net. F-actin is found concentrated in the buds of budding yeast (Adams *et al*., 1989). Regulation of the actin net occurs through phosphorylation that might be regulated by cAMP, although PKA and cAMP levels appear to be regulated by cytosolic calcium gradients. Through blocking calcium import, KP4 appears to affect cAMP levels, potentially preventing rearrangement of the actin net and, subsequently, budding.

These results are further evidence of functional similarities between KP4 and the yeast toxin SMKT. SMKT is encoded by the yeast genome and affects *S. cerevisiae* only at low pH and high ionic strength. Even though SMKT is composed of two polypeptides and does not share any significant amino acid homology with KP4, its structure is remarkably similar to that of KP4 (Kashiwagi *et al*., 1997). The killing effect of SMK is enhanced by high NaCl and KCl concentrations (Kashiwagi *et al*., 1997). It has been proposed that increased salt concentration increases the sensitivity of the cell to SMK. The activity of both toxins is related to ion concentration, indicating that a functional as well as a structural relationship may exist. Furthermore, recent studies have shown that SMKT activity is abrogated by calcium at similar concentrations to those observed with KP4 (Suzuki and Shimma, 1999).

There is limited knowledge of the channels that exist in fungi. One potential channel may consist of the CCH1 and MID1 gene products, which have been shown to be involved in calcium import in *S. cerevisiae* (Iida *et al*., 1994; Fischer *et al*., 1997; Locke *et al*., 2000). The CCH1 gene shows homology to the α_1-subunit of animal voltage-gated calcium channels, and it has been proposed that the CCH1 and MID1 gene products interact to regulate calcium import (Fischer *et al*., 1997; Locke *et al*., 2000). It has not currently been determined whether CCH1 is a voltage-gated calcium channel.

Our results provide further evidence in support of our previously published hypothesis that KP4 blocks calcium import into sensitive *U. maydis* cells by binding to a receptor on the cell surface. Based on the results of our previously published patch-clamp experiments, it seems likely that this receptor is a calcium channel. It is also clear that blocking calcium import is not lethal to the cells, but merely inhibitory. Direct measurement of the effects of KP4 on calcium currents in sensitive *U. maydis* cells has been problematic because of the presence of the cell wall. However, our data and the discovery of the CCH1 gene support the use of mammalian voltage-gated calcium channels as a model to study the direct interactions of KP4 with calcium channel proteins.

Experimental procedures

Cloning and mutagenesis of KP4

The cloning of complete and partial cDNAs from P4M2 (the KP4 toxin encoding RNA) has been described previously (Park *et al*., 1994). Mutations in the KP4 cDNA were introduced by site-directed mutagenesis (Kunkel, 1985), sequences verified, and cDNAs encoding the KP4 toxin (bases 1–1006 of the P4M2 plus strand) expressed in *S. cerevisiae* using the pG3 expression vector with a glyceraldehyde-3-phosphate dehydrogenase promoter (Shena *et al*., 1991). The toxin clearly does not kill *S. cerevisiae* expressing KP4. This is more than likely on account of the high specificity that KP4 has for target *U. maydis* cells.

KP4 purification

KP4 was purified as reported previously (Gu *et al*., 1994; 1995). Briefly, the toxin was isolated from the supernatant of the KP4 toxin expressing strains of *U. maydis* (or *S. cerevisiae*) grown in complete *U. maydis* media (2.5% bacto peptone, 1% dextrose, 0.15% ammonium nitrate, 0.1% yeast extract) for 7–10 days. Cells were removed by centrifugation at 10 000 *g* for 30 min. The supernatant was stirred overnight with CM Sephadex-25 beads (Amersham Pharmacia Biotech) that were equilibrated with 25 mM sodium acetate, pH 5.5. The toxin was eluted with 1 M NaCl using a Pharmacia GradiFrac system (Amersham Pharmacia Biotech). The elutant was concentrated using a Millipore Minitan II ultrafiltration system with 1 kDa cut-off membranes and dialysed against a 10 mM malonic acid buffer, pH 6.0. KP4 was then purified using high-resolution cation-exchange chromatography using a Mono-S (Amersham Pharmacia Biotech) matrix attached to a fast protein liquid chromatography (FPLC) system in the same buffer and using NaCl for elution. The toxin was purified further with size exclusion chromatography using a Pharmacia Superdex-75 gel filtration column. Toxin activity was tested throughout the purification using the killing zone activity assay described below, and purity was assessed using Homogenous 20 SDS gels on a Pharmacia Phastgel system (Amersham Pharmacia Biotech).

Killing zone activity assay

KP4-sensitive P2 cells were grown overnight in complete *U. maydis* media. P2 cells ($\approx$ 1 ml 100 ml^{-1}) were added to warm complete *U. maydis* media containing 2% bacto agar

and poured into 100 mm × 20 mm culture dishes. Once the agar solidified, wells were cut into the agar, and 0.01 ml of the test solutions was added to each well. The plates were then incubated at 30°C for ≈ 36 h. KP4 activity presents a clear zone around the point of application.

Effects of metals on KP4 activity

NaCl and KCl were dissolved in *U. maydis* complete media to generate 4 M stock solutions of each of the monovalent cations. $MgCl_2$ and $CaCl_2$ were dissolved in complete *U. maydis* media to generate 1 M stock solutions of divalent cations. Freshly prepared P2 cells were diluted with complete media to a final OD_{620} of 0.05. Aliquots (0.1 ml) of this solution were placed in the wells of a 96-well culture plate. An aliquot of 0.01 ml of a 0.1 mg ml^{-1} KP4 solution was added to the wells along with NaCl, KCl, $MgCl_2$ and $CaCl_2$ from the stock solutions. Additional media were added to each of the wells to generate a total well volume of 0.15 ml. Optical density (OD) measurements were made using a Spectra Shell microplate reader.

Reversibility of KP4 effects

Freshly prepared P2 cells were diluted with complete media to an OD_{620} of 0.05. An aliquot of 2 ml of these cells was mixed with 0.2 ml of 0.1 mg ml^{-1} KP4 and 0.8 ml of complete media ± 100 mM $CaCl_2$. The solutions were incubated at 30°C, and 0.5 ml samples were collected at various time points. The aliquots were microcentrifuged at ≈ 14 000 *g* for 30 s, the supernatant was discarded, and the cell pellet was washed and resuspended in complete media. Aliquots (0.1 ml) of this solution were then placed in each well of a 96-well plate. Each condition was assayed in triplicate. Initial densities were measured and then at 8 h intervals during incubation at room temperature. The experiment was terminated when the cell density reached a plateau (≈ 1.0–1.5 OD_{620}).

Colony count assay

Fresh P2 cells were diluted with fresh media to an OD_{620} of 0.05. Diluted cells (7 ml) were mixed with 0.755 ml of 0.5 mg ml^{-1} KP4 and 2.745 ml of complete media. The sample was incubated at 25°C and shaken at 120 strokes min^{-1}. Aliquots (0.5 ml) were removed at 8 h intervals and washed in the same manner as described above. The samples were diluted before plating in order to yield < 100 colonies per plate. The plates were incubated at 30°C for 32 h, and the number of colonies on each plate was counted.

Spheroplast generation

Freshly prepared P2 cells (1 ml) were pelleted and resuspended in 1 M sorbitol with 2 mg of lysing enzymes from *Trichoderma harzianum* (Sigma-Aldrich Fine Chemicals). After incubation at 30°C for 1 h, the cell wall is digested, causing the cells to adopt a spherical rather than their normal bacillus shape.

^{45}Ca uptake in U. maydis

Calcium-deficient media (SD-Ca) was prepared according to the formula given in the Difco Manual, eliminating $CaCl_2$ or calcium pantothenate (Difco Laboratories, 1984). KP4-sensitive P2 were cultured overnight in SD-Ca minimal media with 0 mM, 0.1 mM and 10 mM $CaCl_2$ added to the media. Fresh P2 cells were diluted 10-fold with fresh media and grown for 3–4 h to log phase. Cells were washed twice and resuspended in 10 ml of 5 mM HEPES–choline, pH 7.0, 0.1 mM $CaCl_2$, 2% glucose. The cells were treated with 0.1 ml of 3 mg ml^{-1} KP4 and 8 kBq of ^{45}Ca. For each time point, 1 ml of cells was filtered onto 0.45 μm nitrocellulose filter paper. Filters were washed twice with 20 ml of 5 mM $CaCl_2$, and ^{45}Ca concentration was determined by scintillation counting. Each trial was performed five times.

EGTA and cAMP effects on cell growth and KP4 killing

KP4-sensitive P2 cells were grown overnight in SD-Ca minimal media. Fresh P2 cells were diluted with SD-Ca media to a final OD_{620} of 0.05. Aliquots (0.2 ml) of the diluted cells were placed in the wells of a 96-well culture plate. KP4 and varying amounts of a 0.5 M EGTA stock solution were added. Distilled water was added to make a well volume of 0.25 ml. For the cAMP studies, 0.8 ml of P2 cells, 0.16 ml of dH_2O and 0.04 ml of 0.1 mg ml^{-1} KP4 were added to varying amounts of dry cAMP. Aliquots of 0.25 ml of these solutions were added to the wells of a 96-well plate. Cell density was measured as described above.

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