Mol Gen Genet (1997) 256: 104–114

ORIGINAL PAPER

H. Prokisch · O. Yarden · M. Dieminger · M. Tropschug
I. B. Barthelmess

Impairment of calcineurin function in *Neurospora crassa* reveals its essential role in hyphal growth, morphology and maintenance of the apical Ca^{2+} gradient

Received: 18 February 1997 / Accepted: 20 April 1997

Abstract The function of *Neurospora crassa* calcineurin was investigated in *N. crassa* strains transformed with a construct that provides for the inducible expression of antisense RNA for the catalytic subunit of calcineurin (*cna-1*). Induction of antisense RNA expression was associated with reduced levels of *cna-1* mRNA and of immunodetectable CNA1 protein and decreased calcineurin enzyme activity, indicating that a conditional reduction of the target function had been achieved in antisense transformants with multiple construct integrations. Induction conditions caused growth arrest which indicated that the *cna*-1 gene is essential for growth of *N. crassa*. Growth arrest was preceded by an increase in hyphal branching, changes in hyphal morphology and concomitant loss of the distinctive tip-high Ca^{2+} gradient typical for growing wild-type hyphae. This demonstrates a novel and specific role for calcineurin in the precise regulation of apical growth, a common form of cellular proliferation. In vitro inhibition of *N. crassa* calcineurin by the complex of cyclosporin A (CsA) and cyclophilin20, and increased sensitivity of the induced transformants to the calcineurin-specific drugs CsA and FK506 imply that the drugs act in *N. crassa*, as in T-cells and *Saccharomyces cerevisiae*, by inactivating calcineurin. The finding that exposure of growing wild-type mycelium to these drugs leads to a phenotype very similar to that of the *cna-1* antisense mutants is consistent with this idea.

Key words Calcineurin · Apical Ca^{2+} gradient · Cyclosporin A · FK506 · *Neurospora crassa*

Communicated by C. A. M. J. J. van den Hondel

H. Prokisch · I. B. Barthelmess (✉)
Institut für Angewandte Genetik, Universität Hannover,
Herrenhäuserstr. 2, D-30419 Hannover, Germany
Fax: +49-511-762-3608
e-mail: Holger.Prokisch@mbox.genetik.uni-hannover.de

O. Yarden
Department of Plant Pathology and Microbiology,
and Otto Warburg Center for Agricultural Biotechnology,
Faculty of Agriculture, The Hebrew University of Jerusalem,
Rehovot 76100, Israel

M. Dieminger · M. Tropschug
Institut für Biochemie und Molekularbiologie,
Universität Freiburg, D-79104 Freiburg, Germany

Introduction

In filamentous fungi growth proceeds by both polarised linear extension of hyphal tips and branching. Tip growth is characterised by a dynamic equilibrium between the synthesis and expansion of cell wall and plasmalemma and the application of an expansive force derived from turgor pressure or the cytoskeleton. Regulation of this equilibrium is very precise and generates a tube of constant diameter. Growing hyphae contain a tip-high Ca^{2+} gradient thought to be crucial for establishing and maintaining apical organisation, morphogenesis and growth (Jackson and Heath 1993).

Ca^{2+} homeostasis is tightly controlled in all eukaryotic cells since intracellular Ca^{2+} plays important roles in signal transduction and in the regulation of many proteins. The Ca^{2+}-binding protein calmodulin is ubiquitous, and functions to regulate various membrane proteins and enzymes. One of these enzymes, calcineurin, is a highly conserved Ca^{2+}/calmodulin-regulated serine/threonine phosphoprotein phosphatase (PP2B, Klee and Cohen 1988). The functional enzyme is a heterotrimer composed of a catalytic subunit (calcineurin A, CNA), a regulatory subunit (calcineurin B), and calmodulin. Calcineurin B and calmodulin are structurally similar Ca^{2+}-binding proteins responsible for the synergistic Ca^{2+}-dependent activation of calcineurin (Stemmer and Klee 1994).

Calcineurin was identified as a key component in the T-cell activation pathway of the immune response (Liu et al. 1991a). In T-cells either of the immunosuppressive/fungicidal drugs cyclosporin A (CsA) or FK506 can selectively inhibit calcineurin when complexed with

specific proteins – the immunophilins cyclophilin and FK506 binding protein (FKBP), respectively – thereby preventing Ca^{2+}-dependent intermediate steps in the signal transduction cascade leading to T-cell activation (for reviews, see Schreiber and Crabtree 1992; Crabtree and Clipstone 1994).

Studies of CsA-resistant mutants of *Neurospora crassa* and *Saccharomyces cerevisiae* first revealed that a cyclophilin is required for sensitivity to CsA but dispensable for viability (Tropschug et al. 1989). Today, the roles of the immunophilins as the mediators of CsA or FK506 action, and of calcineurin as their joint target are well established, not only in T-cells but in *S. cerevisiae* as well (Foor et al. 1992; Parent et al. 1993).

Here we present a study of the function of calcineurin of *N. crassa* using a combined genetic and biochemical approach. Since disruption mutations in the *Aspergillus nidulans* CNA-encoding gene are lethal (Rasmussen et al. 1994), we anticipated that the same would apply for the *cna-1* gene of *N. crassa*. Therefore we chose to inhibit the target function via the regulated expression of *cna-1* antisense RNA in transgenic *N. crassa* strains, i.e. to create conditional mutations. Detailed investigations confirmed that conditional calcineurin depletion could be achieved: only on induction medium did transformants develop a complex mutant phenotype that facilitated the analysis of calcineurin function in the hyphal growth process, as well as its role as the target for CsA and FK506 in *N. crassa*. Consequently the drugs could be used as an additional means to investigate calcineurin function.

Materials and methods

N. crassa strains, growth and transformation conditions

The *N. crassa* wild-type strain (St. Lawrence 74*A*, obtained from the Fungal Genetics Stock Center, Kansas City, KS), the Δ*am* (allele 132) strain (from W. Fecke, Düsseldorf), and the drug-resistant mutant strains *csr-1* (allele B12; Tropschug et al. 1989) and *fkr-2* (Barthelmess and Tropschug 1993) were used. Strains were cultivated on Vogel's standard (non-induction) medium (2% glucose; Davis and de Serres 1970), on induction medium, where glucose was replaced by 0.3% quinic acid, or on submaximal induction medium (2% glucose plus 0.3% quinic acid; Giles et al. 1985). The mycelial extension rate of *N. crassa* was measured on petri dishes or in 30 cm 'race tubes' containing 14 ml of solid standard or induction medium. Where appropriate, the medium was supplemented with either CsA (Sandoz), FK506 (Fujisawa), benomyl, hygromycin B or cycloheximide (for concentrations see Fig. 9). The first three were dissolved in methyl alcohol and cycloheximide in doubly distilled water; all drugs were added to sterile media at ~55° C.

Protoplasts from germinated conidia of the $\Delta am_{132}a$ strain were obtained using the modified Novozyme 234 technique and transformed with pHPcna1 according to the protocol of Vollmer and Yanofsky (1986). The selection medium for *am* prototrophic colonies contained 0.19% glycine in bottom and top agar layers.

Isolation and analysis of nucleic acids

Plasmid DNA was isolated from *E. coli* using the Plasmid Midi Kit (Qiagen). For PCR amplification of the *cna-1* cDNA, primers HP1 (5′-ATGGAGGATGGCTCCCAA-3′) and HP2 (5′-TTAA-GAAGTGCTGAGCCT-3′) were used with pNORF (Higuchi et al. 1991) as template.

For *N. crassa* DNA or RNA isolation, mycelium was grown at 29° C overnight in exponential culture (in 100 ml of standard medium with agitation). Genomic DNA was isolated according to Lee et al. (1988), total RNA was obtained using the RNeasy Plant Mini Kit (Qiagen). For Southern blots the non-radioactive DIG DNA Labelling and Detection Kit (Boehringer) was used. Primer HP1 or HP2, respectively, was used in a linear PCR to synthesize [α-^{32}P]dCTP labeled strand-specific *cna-1* DNA for use as hybridization probe in Northern blots (Stürzl and Roth 1990). pACTIN (kindly provided by M. Plamann, Texas A&M University), a *N. crassa* actin cDNA clone, was used to generate a [α-^{32}P]dCTP labeled probe using the Random Primers DNA Labeling System (Gibco BRL).

Mapping of *cna-1* was carried out by taking advantage of a restriction fragment length polymorphism detected with the *cna-1* cDNA, using the mapping population and procedure described by Metzenberg et al. (1985).

Preparation of recombinant *N. crassa* CNA1 from *E. coli*, generation of polyclonal antibodies and Western blot analysis

The *cna-1* cDNA was cloned into pET19b (Novagen), which provided a stretch of 10 consecutive histidine residues at the N-terminus. Recombinant CNA1 was purified using the Ni-NTA Protein Purification System (Qiagen). 200 μg of CNA1 (in 500 μl of buffer D) were emulsified with an equal volume of complete Freund's adjuvant and injected into a rabbit; two booster injections (200 μg each) were administered at 2-week intervals. Thereafter serum was collected via an ear vein at 8- to 10-day intervals (stored at −20° C) and tested for immunoreactivity by Western blot analysis.

Western blotting methods followed standard procedures. Samples (20 μg) of crude protein extract, derived from homogenised mycelium grown at 29° C in exponential culture, were fractionated by 12% SDS-PAGE and transferred to nitrocellulose. Membranes were incubated with a mixture of rabbit polyclonal antibodies specific for either CNA1 (1:1000) or FKBP29 (1:1000; Tropschug 1997b). Immunoreactive proteins were detected using alkaline phosphatase-conjugated secondary antibodies (1:1000) and BCIP/NBT (Sigma).

Calcineurin enzyme assays

Calcineurin assay. Calcineurin specific activity in crude extracts was measured according to Higuchi et al. (1991), as adapted for crude extracts by Szöór et al. (1994). Rabbit muscle inhibitor-1, which had been phosphorylated with [γ-^{32}P]ATP, and bovine cAMP-dependent kinase was kindly provided by V. Dombradi, Debrecen. Average phosphatase activity was calculated on the basis of duplicate estimates from independent cultures.

Estimation of the influence of CsA on the in vitro *activity of calcineurin*. To obtain purified calcineurin, mycelium was grown and disrupted in a Moulinex blender (type 320) for 2 min and resuspended in buffer A (100 mM NaCl, 2 mM $CaCl_2$, 2 mM $MgCl_2$, 10 mM MOPS-KOH pH 7.2, 5 mM DTT and 1 mM PMSF from fresh stock in ethanol). The homogenate was centrifuged at 30 000 g for 30 min, the supernatant again centrifuged at 100 000 g for 90 min and the supernatant applied to a Cibacron blue-Sepharose column (Bio-Rad Laboratories, 100–200 mesh, 70–100 μm, equilibrated with buffer A) at a rate of 5 ml/min. The column was washed with 1 l of buffer A, and 15-ml fractions were collected. Absorbance was monitored at 280 nm and aliquots were assayed by Western blot analysis for CNA1. Calcineurin-containing fractions were pooled and immediately applied to an anion exchange chromatography column (Resource Q; Pharmacia) equilibrated with buffer A. The column was washed with 50 ml of buffer A until absorbance at 280 nm was < 0.01. Bound proteins were eluted with a linear 0–1 M NaCl gradient in 120 ml of buffer

A. Calcineurin-containing fractions (1 ml each) were pooled and 50 μg protein was used for the phosphatase assay according to Enz et al. (1994). The reaction (200 μl volume) was started by adding the calcineurin-specific synthetic peptide substrate, a fragment of the RII subunit of the cAMP-dependent protein kinase (kindly provided by A. Enz, Sandoz), at 15 μM final concentration.

Microscopy

For the observation of chlortetracycline (CTC, Sigma) fluorescence (Jackson and Heath 1993), hyphae were grown on dialysis membranes laid on solid minimal or induction medium. After 12 h the dialysis membranes were lifted from the agar surface and transferred to liquid medium. After about 4 h, the freshly grown mycelium was transferred to a microscope slide and excess medium removed. CTC (100 μM final concentration) dissolved in the incubation medium was applied directly to the culture. After approximately 5 min, a coverslip was placed over the culture, which was immediately inspected (for up to 60 min) using a Zeiss Axioscope-20 microscope with filter combination Zeiss 487709. Photographs were taken with Kodak TriX pan film (sensitivity enhanced to 800 ASA using Acufine developer).

Results

Construction of an inducible *cna-1* antisense RNA expression vector

The cDNA coding for the catalytic subunit of *N. crassa* calcineurin, CNA1, was kindly supplied by R. Kincaid (plasmid pNORF; Higuchi et al. 1991). The corresponding gene, designated *cna-1*, was mapped by a restriction fragment length polymorphism to linkage group V, close to *cyh-2* and about 10 cM away from *fkr-1* (data not shown).

An inducible *cna-1* antisense RNA expression vector, pHPcna1 (Fig. 1), was constructed by inserting the PCR-amplified full-length *cna-1* cDNA, in reverse orientation, downstream of a promoter fragment of the *qa-2* gene and upstream of the polyadenylation and termination sequences of the *qa-4* gene in the vector pWF1 (Fecke et al. 1993). A 2.7-kb *Bam*HI fragment harbouring the *N. crassa am* gene (Kinsey and Rambosek 1984) was inserted in the *Bam*HI site of pWF1, providing a selectable marker for screening of transformants.

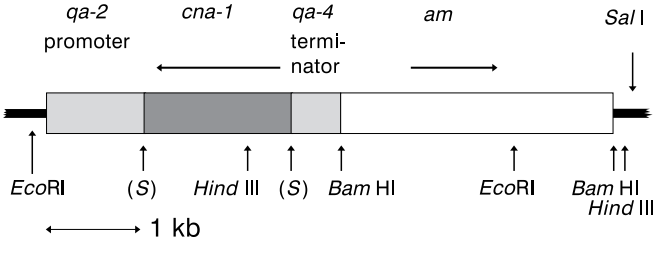

Fig. 1 Expression vector pHPcna1. The entire *N. crassa cna-1* cDNA fragment was cloned into the *Sma*I (*S*, lost on integration) restriction site and is flanked by the *N. crassa qa-2* promoter and *qa-4* terminator sequences of the vector pWF1 (Fecke et al. 1993). The *N. crassa am* gene, used as a selectable marker, was cloned into the *Bam*HI site of pWF1. The direction of sense transcription is indicated by *arrows*

The *qa* genes belong to the *N. crassa* quinic acid catabolic pathway. The inducer, quinic acid, is not a very efficient carbon source for growth of *N. crassa* and its catabolism is governed by glucose repression. The *qa-2* promoter can be induced up to 500-fold by growth on quinic acid as the sole carbon source, or submaximally (25- to 50-fold) when both glucose and quinic acid are present simultaneously (Giles et al. 1985).

Isolation of *N. crassa* transformants harbouring the *cna-1* antisense cassette

Protoplasts of the Δam_{132} strain were transformed with pHPcna1. From stable transformants (complemented for the *am* deletion) microconidial isolates (Ebbole and Sachs 1990) were derived. Homokaryotic transformants which grew on solid standard medium at about the wild-type rate and with wild-type morphology were further investigated. On induction medium (quinic acid as sole carbon source) six transformants showed a novel growth phenotype: once they had grown a certain distance from the inoculation point, growth was arrested. No inducible growth arrest was observed in over 50 control transformants harbouring the expression vector with a *cna-1* cDNA insert in sense orientation (data not shown). Growth of the wild-type on induction medium was continuous but less dense.

Southern blot analysis verified the integration and approximate number of additional *cna-1* sequences in the genomes of these transformants. All contained multiple (5–8) ectopically integrated copies, in addition to the native *cna-1* sequence. This is documented in Fig. 2 for the transformants (T) relevant for this analysis: T3, T5 and T11, transformants which arrested growth, and T14, a transformant that showed no alteration in phenotype on induction medium. A hybridising fragment of about 5.0 kb (comprising the entire *cna-1* antisense cassette and the *am* gene) was expected after *Eco*RI digestion if the whole plasmid had integrated. The corresponding signal was found in all transformants that manifested a growth arrest on induction medium. The lack of additional *cna-1* copies in T14 could be explained by integration of the functional *am* gene, but loss of the adjacent *cna-1* sequence.

Since multiple integration events appeared undesirable, transformants with only one to three ectopic integrations were selected: among eight such transformants, no mutant phenotype was detected (data not shown). To confirm that the growth arrest was caused by *cna-1* antisense expression, transformant T3 was once more transformed with a *cna-1* cDNA sense expression construct based on pWF1. Restoration of continuous growth on induction medium, albeit with subnormal growth rate, was observed for one antisense/sense transformant, T3s, out of a total of nine examined.

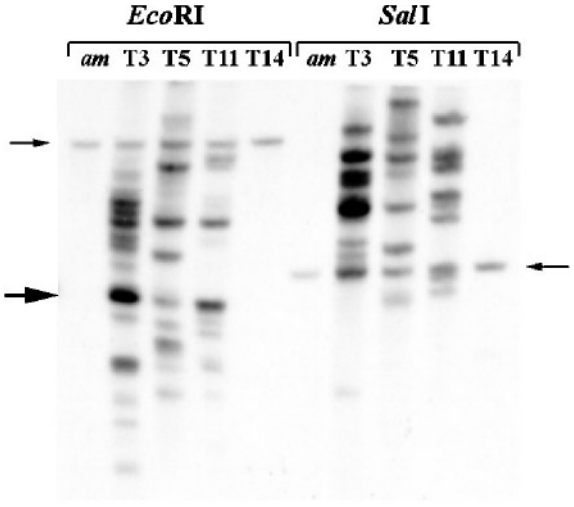

Fig. 2 Southern blot analysis of *Eco*RI and *Sal*I digests of genomic DNA isolated from the *N. crassa* recipient strain Δam_{132} (*am*) and the transformants T3, T5, T11 and T14. *Eco*RI cuts twice in pHPcna1, generating a 5.2-kb fragment (*thick arrow*); *Sal*I cuts once in the vector (Fig. 1). The probe was the random-primed, DIG-labelled *cna-1* cDNA. *Thin arrows* indicate the native *cna-1* gene on a fragment of about 15 kb after *Eco*RI digestion and about 6 kb after cleavage with *Sal*I

Induction of *cna-1* antisense RNA expression correlates with reduction in *cna-1* mRNA levels

Expression of the *cna-1* antisense cassette and its effect on the level of *cna-1* mRNA was investigated by Northern blot analysis. Probes specific for either *cna-1* antisense or sense transcripts were obtained via linear PCR, using primers annealing to either the 5′ or 3′ end of the *cna-1* cDNA, respectively. Antisense RNA was detected in transformants T11, T5 and T3 on induction medium only, while the level of *cna-1* sense transcripts was found to be much reduced under this condition (Fig. 3A, B). Relative to T3, the antisense/sense-transformant T3s was characterised by a reduction in detectable antisense RNA, and an increase in the level of the native *cna-1* mRNA, in addition to the expression of the ectopic sense-transcript (recognisable by its smaller size) on induction medium. On standard medium no antisense transcript was detected in any of the strains and the observed level of *cna-1* mRNA was in the range of the wild-type and T14 controls.

These data provided evidence that the *cna-1* antisense cassette was indeed conditionally transcribed in response to quinic acid in the medium, and that this correlated with a significant reduction in the level of *cna-1* mRNA.

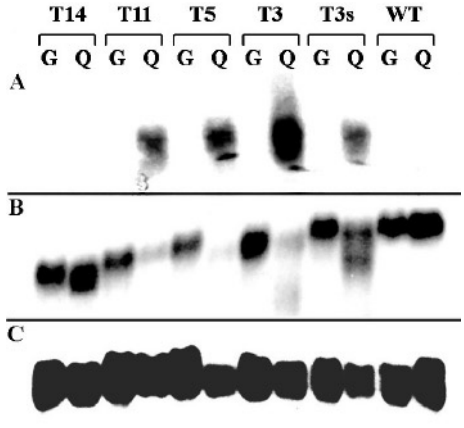

Fig. 3A–C Northern blot analysis of *cna-1* antisense and sense transcripts in *N. crassa* transformants. Total RNA was prepared from the transformants T14, T11, T5, T3, T3s and the wild-type (three filters). Strains were grown for 15 h on glucose- (G) or quinic acid (Q)-containing medium. The filters were hybridised with (**A**) antisense or (**B**) sense-specific *cna-1* probes obtained by linear PCR (see Materials and methods) or with (C) an *N. crassa* actin probe as an internal control. (Based on the position of the ribosomal bands, the size of the *cna-1* band was about 2.4 kb and the antisense RNA about 1.3 kb long

CNA1 protein level and calcineurin specific activity are reduced in antisense RNA- expressing strains

To determine what effect antisense transcription had on the synthesis of CNA1 protein, the level of immunodetectable CNA1 was investigated. Crude extracts from mycelia of the transformants T3, T11 and T14, and wild-type (grown in liquid medium containing either glucose or quinic acid) were probed on Western blots, simultaneously with polyclonal antibodies specific either for the CNA1 protein or, as an internal control, for the FKBP29 protein of *N. crassa* (Tropschug 1997b). Noticeably reduced levels of CNA1 were found reproducibly in T3 and T11 after growth for 12 h on induction medium (Fig. 4). Western blot analysis repeatedly confirmed that the level of the FKBP29 control protein was not affected by growth on quinic acid.

The specific activity of the calcineurin Ca^{2+}/calmodulin-dependent phosphoprotein phosphatase was also assayed. Biochemical studies of the antisense transformants encountered the difficulty that very little biomass was produced under induction conditions, due to early growth arrest. In order to obtain more mycelium, quinic acid was added to the glucose-containing medium of the growing cultures, i.e. induction was submaximal in this experiment. Under these conditions a 40% reduction in relative specific activity of calcineurin (ratio of antisense-induced over non-induced) was found in T3 and T11 compared with the T14 control (Fig. 5).

Together, these data provide evidence for a specific and quinic acid-inducible reduction in the level of the calcineurin catalytic subunit and the phosphoprotein phosphatase activity of the holoenzyme in the transformants.

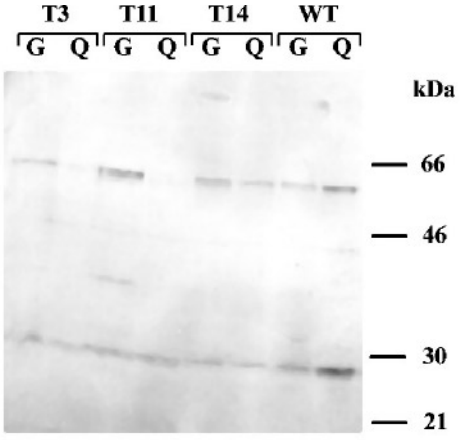

Fig. 4 Western blot analysis of *N. crassa* CNA1 (60 kDa). Crude protein extracts of the *cna-1* antisense RNA transformants T3, T11, the control transformant T14 and the wild type, each grown for 12 h on either glucose- (G) or quinic acid (Q)-containing medium, were fractionated on SDS-polyacrylamide gels and transferred to a nitrocellulose filter. The filter was probed simultaneously with polyclonal antibodies against CNA1 and FKBP29 (29kDa), as internal control. (Previous experiments had shown that probing with each antibody separately resulted in one signal only)

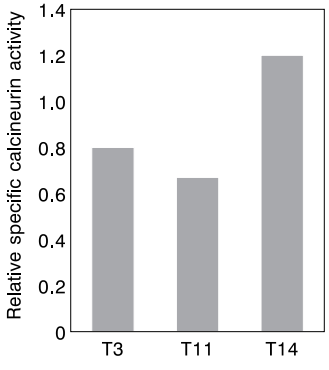

Fig. 5 Relative specific activities of calcineurin (ratio of antisense-inhibited over non-inhibited) in extracts of the *N. crassa cna-1* antisense RNA transformants T3 and T11 and the T14 control. Strains were cultured in duplicate on glucose medium for 24 h, at which time 0.3% quinic acid (final concentration) was added to one sample of each strain to achieve submaximal antisense induction. All mycelia were harvested after another 24 h of growth. Phosphatase activity was measured in cell-free extracts by assaying the amounts of ^{32}P released from ^{32}P-labelled rabbit muscle inhibitor-1, a calcineurin-specific substrate (see Materials and methods)

Calcineurin depletion causes extensive hyphal branching, altered hyphal morphology and growth arrest

Arrest of growth was the most obvious phenotype when transformants T3, T11 or T5 were grown at 29° C on plates with induction medium; arrest was observed once the mycelium had grown a limited distance (17–40 mm). The area covered before arrest was least in T3, followed by T11 and then T5. In each strain growth arrest was preceded by an increase in hyphal branching and loss of the apparent dominance of the main hypha (see Fig.10A), resulting in formation of a very dense mycelial mat (Fig. 6E versus B). Approaching the point of growth arrest, hyphae acquired an irregular shape and a reduced diameter in comparison with the wild type (Fig. 6F versus C), ultimately failing to elongate any further. Arrest was almost complete, transformants requiring 4–8 days for a further extension by 1 cm which the wild-type covered within 4 h. In contrast to the wild-type, very little conidiation occurred at the surface of the mycelial layer of the transformants, except at the densely branching growth front near the point of growth arrest.

Inhibition of growth on quinic acid was found to be temperature dependent: At 37° C the growth arrest of each transformant was overcome after about 1 day; typically growth resumed from a small sector of the arrested hyphal front. Renewed hyphal extension occurred until excessive hyphal branching and conidiation, once again, preceded the arrest of the growing front. After a few such growth cycles, the zones of temporary mycelial arrest were reflected on the plate by essentially regular concentric rings of dense conidiation. Figure 7 illustrates this growth behaviour at 37° C for transformant T3 and wild-type on glucose and quinic acid medium, respectively, when growing through 30-cm long 'race tubes'. For T3 growth and arrest phases alternated at fairly regular intervals of about 20 h (the decreased area covered in later growth cycles probably being due to a drying out of the medium). Similar results were obtained with T11 and T5, except that growth and arrest phases occured with different rhythms (not shown).

When mycelial samples from transformants grown at either 29° C or 37° C on quinic acid medium were taken from various points, i.e. from the arrested growth front, areas behind the front or near the inoculation point, and reinoculated on fresh induction medium they were viable and gave rise to the typical phenotype, as described above, independent of the site of origin of the inoculum.

In conclusion, unconventional conditional *cna-1* mutants have been created, insofar as the genetic alteration maps at various sites in the genome and not at the gene whose function is affected. The data argue that *cna-1* of *N. crassa* codes for a vital function, in that a certain calcineurin activity is essential for normal hyphal proliferation and morphology.

CsA (and FK506) inhibit *N. crassa* calcineurin in vitro and in vivo

Since studies in various organisms have shown that calcineurin is the target of the immunosuppressive/fungicidal drugs CsA and FK506, the drugs could provide an additional means to study calcineurin function. In vitro proof for such an interaction between *N. crassa* calcineurin and the drug-immunophilin complexes was obtained when the phosphatase activity of partially purified *N. crassa* calcineurin was investigated in the

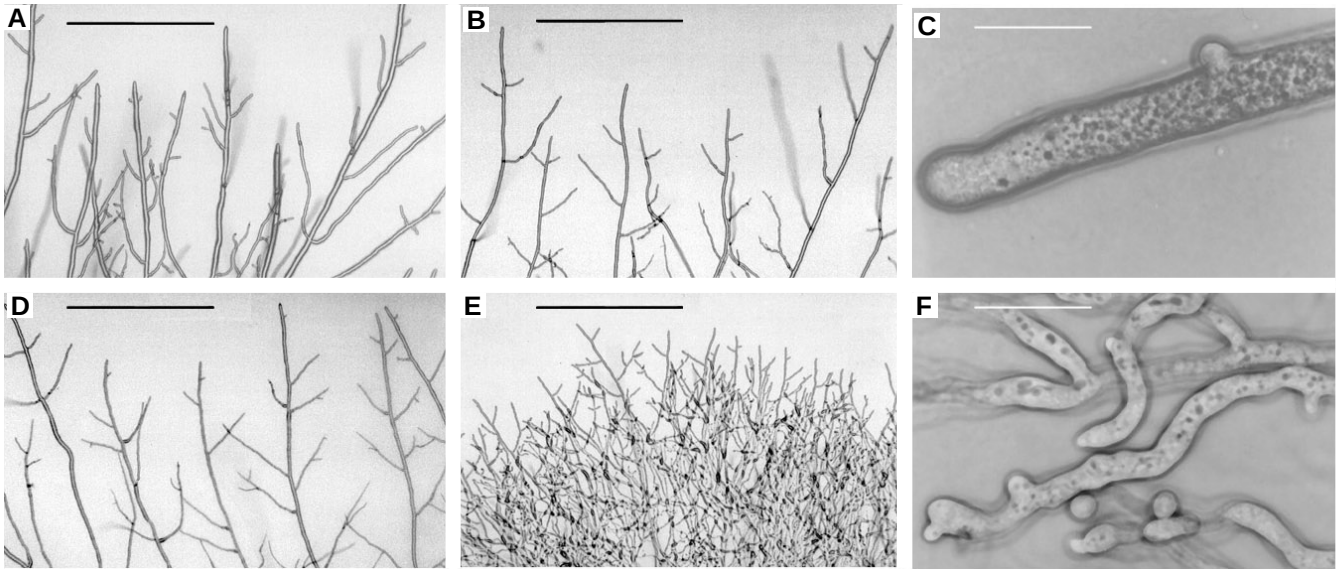

Fig. 6A–F Hyphal morphology of *N. crassa* wild-type (**A–C**) and transformant T3 (**D–F**) grown either on solid standard (**A** and **D**) or induction medium (**B** and **E**) for 16 h, or on induction medium (**C** and **F**) for 24 h, i.e. up to the time when growth arrest occurred in T3. *Dark bars* 400 μm, *light bars* 40 μm

presence and absence of cyclophilin CyP20 (Tropschug et al. 1988, 1997a) and CsA: a severe inhibition (85–90%) was observed when both (but not when either alone) were added to the assay system (Fig. 8).

Given this inhibition, growth of induced transformants was expected to display increased sensitivity to the drugs. In order to quantify drug sensitivity, the effect of the drugs on growth rates was measured. To allow for some linear growth prior to arrest, submaximal induction conditions (2% glucose and 0.3% quinic acid) and a low drug concentration (that would still effect growth of wild-type) were chosen. *N. crassa* was found about 100-fold more sensitive to the immunosuppressive drugs on solid than on liquid medium (Tropschug et al. 1989, Barthelmess and Tropschug 1993), i.e. in the same nanomolar concentration range that suppresses T-cell activation (Dumont et al. 1990). To confirm the specificity of the drugs for the target affected by antisense expression, calcineurin, the analysis included unrelated growth inhibitors, i.e. benomyl, hygromycin B and cycloheximide, which act on targets other than calcineurin.

The relative linear growth rates of the transformants T3 and T11 were found to be significantly and specifically more sensitive to CsA and FK506 during growth

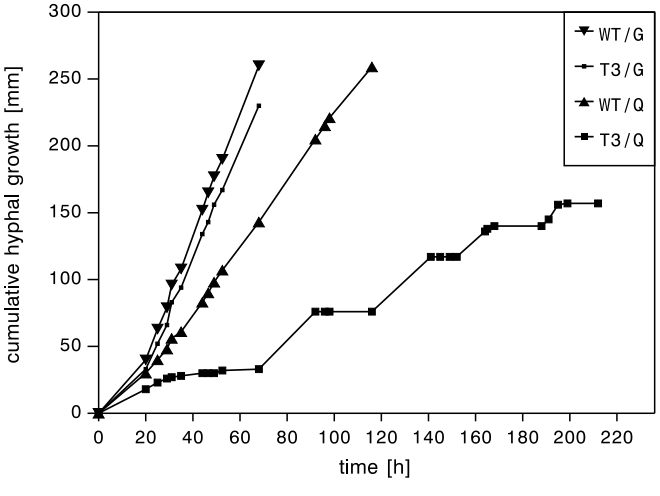

Fig. 7 Cumulative hyphal extension of the *N. crassa* transformant T3 and wild-type at 37° C in 'race tubes' containing solid medium with glucose (G) or quinic acid (Q) as carbon source

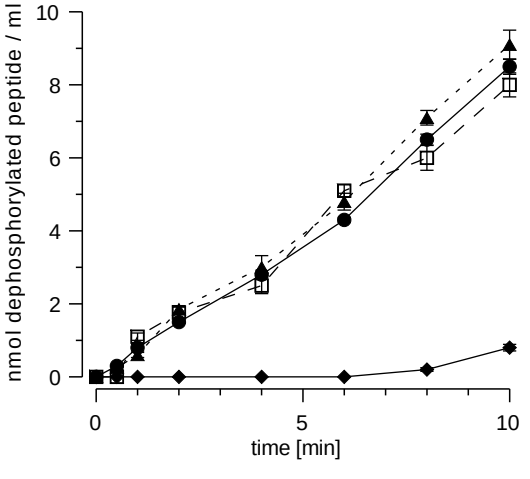

Fig. 8 Specific phosphatase activity of partially purified *N. crassa* wild-type calcineurin incubated with a calcineurin-specific phosphorylated peptide substrate (a fragment of the RII subunit of the cAMP-dependent protein kinase) alone (*filled circles*), or in the presence of either 4 μM CsA (*open squares*), or 200 nM CyP20 (*filled triangles*), or both, CsA + CyP20 (preincubated at 25° C for 10–15 min) (*filled diamonds*) (see Materials and methods)

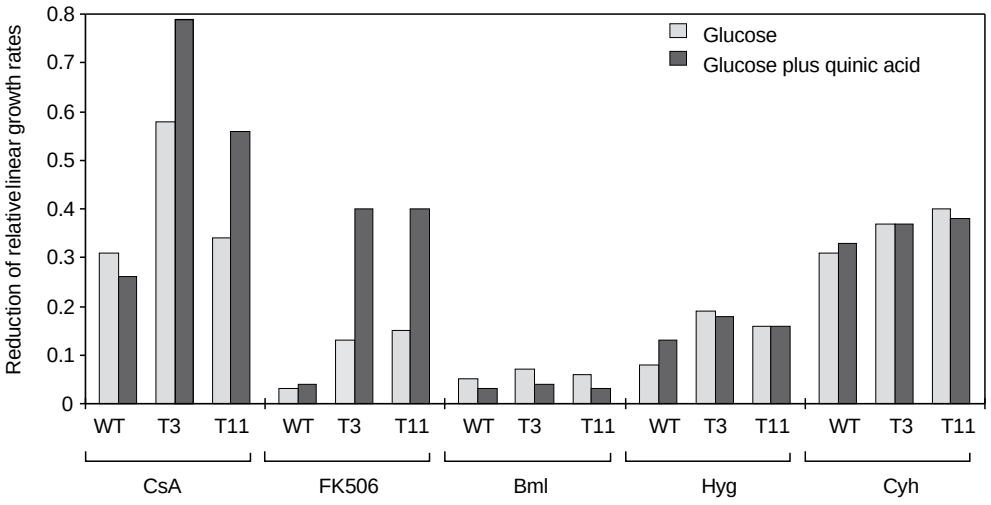

Fig. 9 Reduction of relative linear growth rates of *N. crassa* transformants T3 and T11, and the wild-type control grown on solid medium in the presence of various drugs at 29° C. Linear growth rates were calculated for all strains and conditions between 17 and 27 h after inoculation. In order not to confuse the drug effects with that exerted by quinic acid, relative growth rates were calculated by dividing the growth rate on each drug (b_{drug}) by the growth rate on the respective medium without drug (b), e.g. $b_{glucose+CsA}/b_{glucose}$. For graphical representation $1\text{-}b_{drug}/b$ was calculated, to show the 'reduction of relative linear growth rates'. The ranking and magnitude of the effects were similar in three independent experiments. The general level of the relative growth rates, however, varied with each fresh drug preparation. The results were therefore not pooled and an overall error was not estimated. Drug concentrations (final): CsA, 8.3 nM; FK506, 2.4 nM; benomyl (Bml), 35 nM; hygromycin B (Hyg), 2.9 μM and cycloheximide (Cyh) 72 nM

on induction medium than on standard medium (Fig. 9). In comparison with the wild-type, the transformants were also more sensitive to the immunosuppressive drugs on standard medium. No differential sensitivity of the different strains was observed on benomyl, hygromycin B or cycloheximide, regardless of the induction status.

The differential response of the transformants to the calcineurin-specific, but not to other unrelated, inhibitors represents in vivo evidence that the drugs affect hyphal growth of *N. crassa* by inactivating calcineurin.

Loss of the apical Ca^{2+} gradient in hyphae deprived of calcineurin

Published evidence (Schmid and Harold 1988; Dicker and Turian 1990; Jackson and Heath 1993; Levina et al. 1995) points to the importance for polarised growth of a steep, tip-high gradient of free Ca^{2+} observed in the most apical portion of growing *N. crassa* hyphae. Chlortetracycline (CTC) was used to investigate this gradient. Ca^{2+} ions form a fluorescent complex with CTC, which associates subsequently with adjacent membranes. Assuming that Ca^{2+} ions in the complex are in equilibrium with those of the cytosol, CTC fluorescence can be taken as an indication of the spatial distribution of the total intracellular Ca^{2+} (Schmid and Harold 1988). This interpretation of CTC fluorescence is supported by the results of a radiometric dye technique (Levina et al. 1995).

The gradient was found to be disturbed in induced transformants, as well as in the wild-type, after drug application. Since drug action occurs rapidly and interferes with calcineurin activity directly, the timing and efficiency of this inhibition can be monitored more precisely than the delayed effect of antisense RNA expression. Therefore the analysis concentrated on the effects that CsA (and FK506) have on the Ca^{2+} gradient in the wild-type.

Figure 11B shows the typical apical gradient of CTC fluorescence in growing wild-type hyphae. Upon addition of CsA, the gradient started to disperse rapidly: after 15 min, fluorescence was much reduced, except at the very tips of the hyphae, and after 20 min only a weak uniform signal could be detected (Fig. 11D). Simultaneously CsA affected hyphal extension and morphology: after about 10 min multiple branches emerged as small swellings ('buds') unusually close to, or even at the tip of, the main hypha, causing after about 30 min the loss of the dominance of the main tip, followed by growth arrest (Figs. 10B and 11C), i.e. CsA treatment of the wild-type results in a phenocopy of the induced *cna-1* antisense RNA mutants (Fig. 10A), which is consistent with its proposed mode of action. The effects of FK506 were very similar (not shown). In contrast inhibition of growth by hygromycin B or benomyl led to a comparatively slow (1 h) dissipation of the gradient and manifested quite different disturbances of growth morphology (not shown), indicating that the phenotypes described are specific for calcineurin deprivation.

The Ca^{2+} gradient was investigated in the CsA- and FK506-resistant strains *csr-1* and *fkr-2*, respectively, which are mutant for the genes coding for CyP20 (Rassow et al. 1995) and FKBP13 (Tropschug et al. 1990, H. Prokisch, unpublished results), respectively,

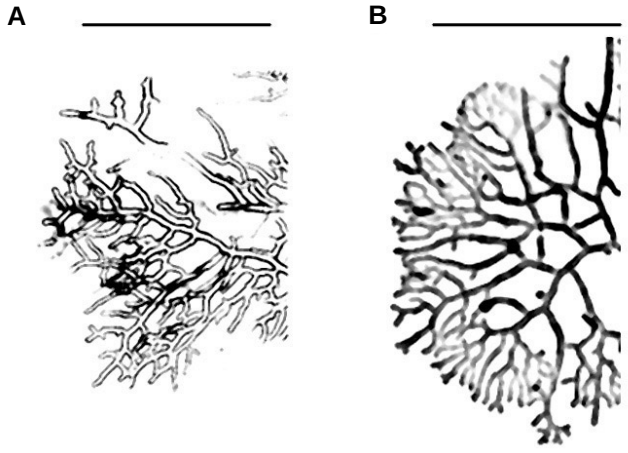

Fig. 10A, B Branching behaviour of transformant T3 and the wild-type strain. The mycelial fronts of transformant T3 at the point of growth arrest on induction medium (**A**) and of wild-type hyphae grown on standard medium supplemented with CsA (50 nM final). (**B**) *Bar* 200 μm

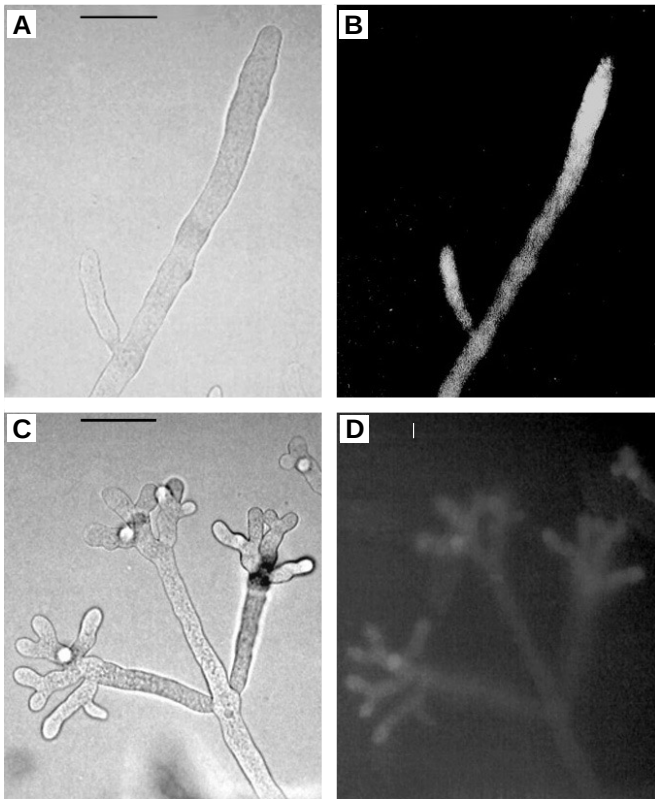

Fig. 11A–D Hyphal tips of *N. crassa* wild-type grown on CTC-containing liquid standard medium either without CsA (**A** and **B**) or 20 min after the addition of CsA to a final concentration of 1 μM (**C** and **D**). **A** and **C** show hyphae in visible light, **B** and **D** the Ca^{2+} gradient revealed by CTC fluorescence (see Materials and methods). *Bar* 40 μm

and have been found not to contain the corresponding products (Tropschug et al. 1989, Barthelmess and Tropschug 1993). Neither the Ca^{2+} gradient nor the growth morphology were affected by CsA in *csr-1* strains, in contrast to *fkr-2* strains. Conversely, FK506 affected these features only in *csr-1* but not *fkr-2* mutants (not shown). This confirmed the high specificity of drug action, which depends exclusively on the presence of the corresponding immunophilin.

Direct observation of the Ca^{2+} gradient in the hyphal tips of the induced transformants T3 and T11 indicated a similar sequence of events: well before growth arrest, the tip-high CTC fluorescence gradient disappeared, concomitant with the onset of hyperbranching (not shown).

To differentiate between an effect of reduced calcineurin activity on Ca^{2+} uptake or gradient maintenance, the Ca^{2+} concentration in the medium was increased to boost uptake. Neither growth nor gradient could be corrected by Ca^{2+} concentrations of up to 0.5 M (not shown).

In conclusion, the data point to a vital role for calcineurin in the maintenance of the Ca^{2+} gradient and the regulation of hyphal branching, hyphal morphology and hyphal elongation, calcineurin depletion leading ultimately to growth arrest.

Discussion

The mechanism of antisense RNA-mediated mutations is not well understood, and no single model can account for all the results obtained in different systems (Nellen and Lichtenstein 1993). Hybridisation between complementary RNAs is generally taken as a fact, even though RNA hybrids are usually not detected in antisense-expressing cells. They are believed to be rapidly degraded by non-specific dsRNases. This would explain the strongly reduced level of *cna-1* mRNA found in the transformants grown on induction medium.

It is considered to be a common feature of antisense action that only a minor fraction of transgenic organisms that harbour antisense cassettes show a complete inhibition of the target gene (Van der Krol et al. 1988); this may be due to position effects. In plants, Kuipers et al. (1995) found that the probability of complete inhibition of the expression of specific genes is highest in transgenic clones with three or more copies of an antisense cassette based on full-length cDNA. The multiple integration of the antisense construct in the transformants of the present study was therefore not unexpected and was probably instrumental in generating an obvious phenotype.

Multiple integration raises the possibility of target-independent disturbances. However, we consider the phenotype described here to be a genuine consequence of the experimentally demonstrated reduction in calcineurin levels, for several reasons: (i) the inducible, temperature-sensitive growth arrest of all three transformants was accompanied by the same array of cellular disturbances; (ii) in the control group of over 50 sense transformants with multiple integrations, no conditional

growth arrest was found; (iii) overexpression of the sense transcript in the antisense/sense transformant T3s restored continuous growth on induction medium; and (iv) a very similar phenotype is caused by the immunosuppressive drugs that interact with calcineurin.

The biochemical characterisation of the *cna-1* antisense transformants did not document a complete loss of *cna-1* mRNA, protein or calcineurin activity; however, the growth arrest at full induction does indicate that the equivalent of a conditional loss-of-function mutant had been generated via antisense RNA expression. The limited initial growth on induction medium at 29° C is explainable by the presence of calcineurin synthesized prior to antisense RNA-mediated inhibition becoming effective. Since no temperature effect was observed for the inhibition of wild-type growth by CsA or FK506 (not shown), it appears unlikely that here we deal with the kind of temperature effect found in calcineurin A mutants of *Schizosacchaomyces pombe* (Yoshida et al. 1994). We suggest that the intermittent growth at 37° C, observed for all three transformants, results from a less effective sense-antisense RNA hybridisation at this temperature, leading to the gradual accumulation of a threshold level of calcineurin and subsequent growth of some hyphae.

The analysis indicates that *N. crassa cna-1* is an essential gene, justifying the use of the technique of regulated antisense expression as an alternative to gene disruption or destruction by RIP (repeat-induced point mutation; Selker 1990).

These studies can account for the complete sequence of events involved in drug toxicity in *N. crassa*. Immunophilin-deficient *N. crassa* mutants with unrestricted viability had led to the postulate that the drug-immunophilin complexes target a protein that is essential for growth of *N. crassa* (Tropschug et al. 1989, Barthelmess and Tropschug 1993). Here we show that in *N. crassa*, as in other organisms, CsA inhibits calcineurin with high specificity, and calcineurin is essential for growth.

To our knowledge it has not been reported that calcineurin is involved in controlling tip growth and hyphal branching. However, the observation in *N. crassa* of high concentrations of calcineurin at the hyphal tip (Kincaid 1993) and the involvement of the counteracting process, protein phosphorylation, in hyphal elongation and branching (Yarden et al. 1992) is compatible with such a function.

Even though calcineurin has proved to be essential for growth of *A. nidulans*, the consequences of calcineurin depletion appear different from those in *Neurospora*: in *cnaA* disruption mutants of *A. nidulans* the nuclei are found to be blocked early in the cell cycle (Rasmussen et al. 1994). Microscopic inspection of DAPI-stained wild-type *N. crassa* hyphae grown on CsA or FK506, or induced *cna-1* antisense transformants, showed no abnormalities in nuclear size or distribution (data not shown).

In fission yeast CNA is not essential, but is also involved in cytokinesis, as well as nuclear and spindle pole body positioning and mating (Yoshida et al. 1994). In *S. cerevisiae* two genes encode calcineurin catalytic subunits and one gene the regulatory subunit. Mutants defective in the former, the regulatory or all of these genes display normal vegetative growth, but are unable to recover from pheromone-induced G_1 arrest (Liu et al. 1991b; Cyert et al. 1991; Cyert and Thorner 1992; Foor et al. 1992). The same is found when wild-type cells are deprived of Ca^{2+} (Iida et al. 1990). An impressive number of recent investigations argues that calcineurin, though not essential for yeast, is involved in a wide range of cellular processes:

Analysis of yeast calcineurin mutants (Nakamura et al. 1993; Breuder et al. 1994; Mendoza et al. 1994; Marquez and Serrano 1996) shows that the loss of calcineurin causes decreased tolerance to Li^+ or Na^+ salts and OH^- ions and a defect in the K^+ transport system. Therefore yeast calcineurin appears to be involved in the cellular adaptation response to stress conditions, i.e. the maintenance of intracellular cation homeostasis and thereby turgor pressure. Since turgor pressure is part of the expansive force behind tip growth this idea indicates a possible function for calcineurin in this process.

The counterpart of turgor pressure is the synthesis, and modulation of the rigidity, of the cell wall. If these latter were disturbed, turgor pressure might lead to apical hyperbranching. In yeast, it is known that calcineurin plays a role in cell wall synthesis (Mazur et al. 1995). We investigated the antisense transformants for indications of differences in septum formation or gross changes in chitin deposition using Calcofluor staining, but observed no significant changes in cell wall structure. Furthermore osmotic stabilisation of the cells (by addition of up to 1 M sorbitol to the medium) did not prevent growth arrest (H. Prokisch, unpublished results).

Another set of data from yeast (Cunningham and Fink 1994, 1996; Garrett-Engele et al. 1995; Hemenway et al. 1995; Tanida et al. 1995, 1996) shows an immediate influence of calcineurin on Ca^{2+} homeostasis via the regulation of, or interaction with, a number of ion transport systems (e.g. vacuolar membrane Ca^{2+}-ATPase, vacuolar H^+/Ca^{2+} exchanger and a Ca^{2+}-ATPase localised to the Golgi complex).

It is not known which mechanism maintains the tip-high Ca^{2+} gradient in growing hyphae. The effects of CsA or FK506 on the CTC gradient and on growth of *N. crassa* are similar to those found following treatment of wild-type *N. crassa* hyphae with the Ca^{2+} channel blocker verapamil (Dicker and Turian 1990). However, verapamil-treated hyphae can resume normal branching and re-establish an observable CTC gradient after application of exogenous Ca^{2+}. The finding that exogenous Ca^{2+} cannot restore the Ca^{2+} gradient in hyphae with inactivated CNA1 (this investigation) indicates that the loss of the CTC gradient may be due to a failure to maintain the gradient rather than changes in the availability of exogenous Ca^{2+}. This notion is supported by the in vivo patch clamp recordings using plasma membranes of *N. crassa* by Levina et al. (1995). They suggest

that ion transport across the plasma membrane of the growing tip is not essential for growth and that therefore the gradient must be generated from internal stores in an unknown way.

Here we have shown that functional calcineurin is required to maintain the steep Ca^{2+} gradient as measured by CTC fluorescence. The loss of the gradient and the succession of events observed under calcineurin depletion argues that *N. crassa* calcineurin is involved in a positive feedback loop that establishes the hyphal gradient and affects, either subsequently or in parallel, hyphal morphology and growth.

These genetic and biochemical investigations of *N. crassa* calcineurin emphasize its central role in the physiology of filamentous growth. Kincaid (1993) pointed out that fungal hyphae share many morphological and molecular elements with the growing tips of axons in the nervous system, which are characterised by similar features with respect to growth and branching. Indeed, Chang et al. (1995) have shown that calcineurin is involved in neurite outgrowth and directed filopodial motility. Future research will show what parallels exist between the molecular functions of calcineurin in filamentous fungi and nerve cells or the immune response.

Acknowledgements We thank R. Kincaid for the calcineurin cDNA, M. Plamann for the actin cDNA and V. Dombradi for providing O. Y. with the opportunity to perform the radioactive phosphatase assay in his lab. This work was supported, in part, by grants from the Volkswagen Foundation and the Ministry of Science of Lower Saxony (Collaborative projects between The Hebrew University of Jerusalem and universities in Lower Saxony, to O. Y. and I. B. B.), BARD (United States-Israel Binational Agricultural Research and Development Fund, to O. Y.), the Deutsche Forschungs gemeinschaft (SFB 388, to M. T.) and the Fond der Chemischen Industrie (to M. T.).

References

Barthelmess IB, Tropschug M (1993) FK506-binding protein of *Neurospora crassa* (*Nc*FKBP) mediates sensitivity to the immunosuppressant FK506; resistant mutants identify two loci. Curr Genet 23:54–58

Breuder T, Hemenway CS, Movva NR, Cardenas ME, Heitman J (1994) Calcineurin is essential in cyclosporin A- and FK506-sensitive yeast strains. Proc Natl Acad Sci USA 91:5372–5376

Chang HY, Takel K, Sydor AM, Born T, Rusnak F, Jay DG (1995) Asymmetric retraction of growth cone filopodia following focal inactivation of calcineurin. Nature 376:686–690

Crabtree GR, Clipstone NA (1994) Signal transmission between the plasma membrane and nucleus of T lymphocytes. Annu Rev Biochem 63:1045–1083

Cunningham KW, Fink GR (1994) Calcineurin-dependent growth control in *Saccharomyces cerevisiae* mutants lacking PMC1, a homolog of plasma membrane Ca^{2+} ATPases. J Cell Biol 124:351–363

Cunningham KW, Fink GR (1996) Calcineurin inhibits *VCX-1*-dependent H^+/Ca^{2+} exchange and induces Ca^{2+} ATPases in *Saccharomyces cerevisiae*. Mol Cell Biol 16:2226–2237

Cyert M, Thorner J (1992) Regulatory subunit (*CNB1* gene product) of yeast Ca^{2+}/calmodulin-dependent phosphoprotein phosphatases is required for adaptation to pheromone. Mol Cell Biol 12:3460–3469

Cyert M, Kunisawa R, Kaim D, Thorner J (1991) Yeast has homologs (*CNA1* and *CNA2* gene products) of mammalian calcineurin, a calmodulin-regulated phosphoprotein phosphatase. Proc Natl Acad Sci USA 88:7376–7380

Davis RH, de Serres FJ (1970) Genetic and microbiological research techniques for *Neurospora crassa*. Methods Enzymol 17A:79–143

Dicker W, Turian G (1990) Calcium deficiencies and apical hyperbranching in wild-type and the 'frost' and 'spray' morphological mutants of *Neurospora crassa*. J Gen Microbiol 136:1413–1420

Dumont FJ, Melino MR, Staruch MJ, Koprak SL, Fischer PA, Sigal NH (1990) The immunosuppressive macrolides FK506 and rapamycin act as reciprocal antagonists in murine T cells. J Immunol 144:1418–1424

Ebbole DJ, Sachs MS (1990) A rapid and simple method for isolation of *Neurospora crassa* homokaryons using microconidia. Fungal Genet Newsl 37:17–18

Enz A, Shapiro G, Chappius A, Dattler A (1994) Nonradioactive assay for protein phosphatase 2B (calcineurin) using a partial sequence of the subunit of cAMP-dependent protein kinase as substrate. Anal Biochem 214:147–153

Fecke W, Nehlsand U, Weiss H (1993) An antisense-RNA expression vector for *Neurospora crassa*. Fungal Genet Newsl 40:34–35

Foor F, Parent SA, Morin N, Dahl AM, Ramadan N, Chrebet G, Bostian KA, Nielsen J (1992) Calcineurin mediates inhibition by FK506 and CsA of recovery from alfa-factor arrest in yeast. Nature 360:682–684

Garrett-Engele P, Moilanen B, Cyert M (1995) Calcineurin, the Ca^{2+}/calmodulin-dependent protein phosphatase, is essential in yeast mutants with cell integrity defects and in mutants that lack a functional vacuolar H^+-ATPase. Mol Cell Biol 15:4103–4114

Giles NH, Case ME, Baum J, Geever R, Huiet L, Patel V, Tyler B (1985) Gene organisation and regulation in the *qa* (quinic acid) gene cluster of *Neurospora crassa*. Microbiol Rev 49:338–358

Hemenway CS, Dolinski K, Cardenas ME, Hiller MA, Jones EW, Heitman J (1995) *vph6* mutants of *Saccharomyces cerevisiae* require calcineurin for growth and are defective in vacuolar H^+-ATPase assembly. Genetics 141:833–844

Higuchi S, Tamura J, Giri PR, Polli JW and Kincaid R (1991) Calmodulin-dependent protein phosphatase from *Neurospora crassa*. J Biol Chem 266:18104–18112

Iida H, Yagawa Y, Anraku Y (1990) Essential role for induced Ca^{2+} influx followed by $[Ca^{2+}]_i$ rise in maintaining viability of yeast cells late in the mating pheromone response pathway. J Biol Chem 265:13391–13399

Jackson SL, Heath IB (1993) Roles of calcium ions in hyphal tip growth. Microbiol Rev 57:367–382

Kincaid R (1993) Calmodulin-dependent protein phosphatases from microorganisms to man. In: Shenolikar S, Nairn AC (eds) Advances in Second Messenger and Phosphoprotein Research, vol 27. Raven Press, New York, pp 1–23

Kinsey JA, Rambosek JA (1984) Transformation of *Neurospora crassa* with the cloned *am* (glutamate dehydrogenase) gene. Mol Cell Biol 4:117–122

Klee CB, Cohen P (1988) The calmodulin-regulated protein phosphatase. In: Cohen P, Klee CB (eds) Calmodulin: molecular aspects of cellular regulation. Elsevier, Amsterdam, pp 225–248

Kuipers AGJ, Soppe WJJ, Jacobsen E, Visser RGF (1995) Factors affecting the inhibition by antisense RNA of granule-bound starch synthase gene expression in potato. Mol Gen Genet 246:745–755

Lee SB, Milgroom MG, Taylor JW (1988) A rapid, high yield miniprep method for isolation of total genomic DNA from fungi. Neurospora Newsl 35:23–24

Levina NN, Lew RR, Hyde GJ, Heath IB (1995) The roles of Ca^{2+} and plasma membrane ion channels in hyphal tip growth of *Neurospora crassa*. J Cell Sci 108:3405–3417

Liu J, Farmer JD, Lane WL, Friedman J, Weissman I, Schreiber SL (1991a) Calcineurin is a common target of cyclophilin-cyclosporin A and FKBP-FK506 complexes. Cell 66:807–815

Liu Y, Ishii S, Tokai M, Tsutsumi H, Ohki O, Akada R, Tanaka K, Tsuchya E, Fukui S, Miyakawa T (1991b) The *Saccharomyces cerevisiae* genes (*CMP1* and *CMP2*) encoding calmodulin-binding proteins homologous to the catalytic subunit of mammalian protein phosphatase 2B. Mol Gen Genet 227:52–59

Marquez JA, Serrano R (1996) Multiple transduction pathways regulate the sodium-extrusion gene *PMR2/ENA1* during salt stress in yeast. FEBS Lett 382:89–92

Mazur P, Morin N, Baginsky W, El-Sherbeini M, Clemas JA, Nielsen JB, F Foor (1995) Differential expression and function of two homologous subunits of yeast 1,3-β-D-glucan synthase. Mol Cell Biol 15:5671–5681

Mendoza I, Rubio F, Rodriguez-Navarro A, Pardo JM (1994) The protein phosphatase calcineurin is essential for NaCl tolerance of *Saccharomyces cerevisiae*. J Biol Chem 269:8792–8796

Metzenberg RL, Stevens JN, Selker EU, Morzycka-Wroblewska E (1985) Identification and chromosomal distribution of 5S rRNA genes in *Neurospora crassa*. Proc Natl Acad Sci USA 82:2067–2071

Nakamura T, Liu Y, Hirata D, Namba H, Harada S, Hirokawa T, Miyakawa T (1993) Protein phosphatase type 2B (calcineurin)-mediated, FK506-sensitive regulation of intracellular ions in yeast is an important determinant for adaptation to high salt stress conditions. EMBO J 12:4063–4071

Nellen W, Lichtenstein C (1993) What makes an mRNA anti-sense-itive? Trends Biochem Sci18:419–423

Parent SA, Nielsen JB, Morin N, Chrebet G, Ramadan N, Dahl AM, Hsu M-J, Bostian K, Foor F (1993) Calcineurin-dependent growth of an FK506- and CsA-hypersensitive mutant of *Saccharomyces cerevisiae*. J Gen Microbiol 139:2973–2984

Rasmussen C, Garen C, Brining S, Kincaid RL, Means RL, Means AR (1994) The calmodulin-dependent protein phosphatase catalytic subunit (calcineurin A) is an essential gene in *Aspergillus nidulans*. EMBO J 13:2545–2552

Rassow J, Mohrs K, Koidl S, Barthelmess IB, Pfanner N, Tropschug M (1995) Cyclophilin 20 is involved in mitochondrial protein folding in cooperation with molecular chaperones Hsp70 and Hsp60. Mol Cell Biol 15:2654–2662

Schmid J, Harold FM (1988) Dual roles for calcium ions in apical growth of *Neurospora crassa*. J Gen Microbiol 134:2623–2631

Schreiber SL, Crabtree GR (1992) The mechanism of action of cyclosporin A and FK506. Immun Today 13:136–142

Selker EU (1990) Premeiotic instability of repeated sequences in *Neurospora crassa*. Annu Rev Genet 24:579–613

Stemmer PM, Klee CB (1994) Dual calcium ion regulation of calcineurin by calmodulin and calcineurin B. Biochemistry 33:6859–6866

Stürzl M, Roth WK (1990) Run off synthesis and application of defined single stranded DNA hybridisation probes. Anal Biochem 185:164–169

Szöör B, Fehèr Z, Szabò G, Gergely P, Dombrádi V (1994) Detection of Ser/Thr protein phosphatases in *Neurospora crassa*. Fungal Genet Newsl 41:82–84

Tanida I, Hasegawa A, Iida H, Ohya Y, Anraku Y (1995) Cooperation of calcineurin and vacuolar H^+-ATPase in intracellular Ca^{2+} homeostasis of yeast cells. J Biol Chem 270:10113–10119

Tanida I, Takita Y, Hasegawa A, Ohya Y, Anraku Y (1996) Yeast CLS2p/Csg2p localized on the endoplasmic reticulum membrane regulates a non-exchangeable intracellular Ca^{2+} pool cooperatively with calcineurin. FEBS Lett 379:38–42

Tropschug M (1997a) *N. crassa* cyclophilins. In: Gething M-J (ed) Guidebook to molecular chaperones and protein folding factors. Sambrook and Tooze at Oxford University Press, Oxford, in press

Tropschug M (1997b) *N. crassa* FKBPs. In: Gething M-J (ed) Guidebook to molecular chaperones and protein folding factors. Sambrook and Tooze at Oxford University Press, Oxford, in press

Tropschug M, Nicholson DW, Hartl F-U, Köhler H, Pfanner N, Wachter E, Neupert W (1988) Cyclosporin A-binding protein (cyclophilin) of *Neurospora crassa*. J Biol Chem 263:14433–14440

Tropschug M, Barthelmess IB, Neupert W (1989) Sensitivity to cyclosporin A is mediated by cyclophilin in *Neurospora crassa* and *Saccharomyces cerevisiae*. Nature 342:953–955

Tropschug M, Wachter E, Mayer S, Schönbrunner ER, Schmid FX (1990) Isolation and sequence of an FK506-binding protein from N. *crassa* which catalyses protein folding. Nature 346:674–677

Van der Krol AR, Mol JNM, Stuitje AR (1988) Modulation of eukaryotic gene expression by complementary RNA or DNA sequences. Biotechniques 6:958–976

Vollmer SJ, Yanofsky C (1986) Efficient cloning of genes of *Neurospora crassa*. Proc Natl Acad Sci USA 83:4867–4873

Yarden O, Plamann M, Ebbole DJ, Yanofsky C (1992) *cot-1*, a gene required for hyphal elongation in *Neurospora crassa*, encodes a protein kinase. EMBO J 11:2159–2166

Yoshida T, Toda T, Yanagida M (1994) A calcineurin-like gene $ppb1^+$ in fission yeast: mutant defects in cytokinesis, cell polarity, mating and spindle pole body positioning. J Cell Sci 107:1725–1735