

Calmodulin controls organization of the actin cytoskeleton via regulation of phosphatidylinositol (4,5)-bisphosphate synthesis in *Saccharomyces cerevisiae*

Sylvane DESRIVIÈRES^{*1}, Frank T. COOKE[†], Helena MORALES-JOHANSSON^{*}, Peter J. PARKER[†] and Michael N. HALL^{*2}

^{*}Division of Biochemistry, Biozentrum, University of Basel, Klingelbergstrasse 70, CH-4056 Basel, Switzerland, and [†]Protein Phosphorylation Laboratory, Cancer Research UK London Research Institute, Lincoln's Inn Fields Laboratories, 44 Lincoln's Inn Fields, London WC2A 3PX, U.K.

Phosphoinositides regulate a wide range of cellular processes, including proliferation, survival, cytoskeleton remodelling and membrane trafficking, yet the mechanisms controlling the kinases, phosphatases and lipases that modulate phosphoinositide levels are poorly understood. In the present study, we describe a mechanism controlling *MSS4*, the sole phosphatidylinositol (4)-phosphate 5-kinase in *Saccharomyces cerevisiae*. Mutations in *MSS4* and *CMD1*, encoding the small Ca^{2+} -binding protein calmodulin, confer similar phenotypes, including loss of viability and defects in endocytosis and in organization of

the actin cytoskeleton. Overexpression of *MSS4* suppresses the growth and actin defects of *cmd1-226*, a temperature-sensitive calmodulin mutant which is defective in the organization of the actin cytoskeleton. Finally, the *cmd1-226* mutant exhibits reduced levels of phosphatidylinositol (4,5)-bisphosphate. These findings suggest that calmodulin positively controls *MSS4* activity and thereby the actin cytoskeleton.

Key words: *CMD1*, *MSS4*, PtdIns(4)*P* 5-kinase, PtdIns(4,5)*P*₂, *Saccharomyces cerevisiae*.

INTRODUCTION

Phosphatidylinositol 4,5-bisphosphate [PtdIns(4,5)*P*₂] plays a role in a remarkable number of cellular processes. PtdIns(4,5)*P*₂ is cleaved by phospholipase C (PLC) to generate the second messengers diacylglycerol and Ins(1,4,5)*P*₃ that activate protein kinase C (PKC) and increase intracellular Ca^{2+} respectively [1]. Uncleaved PtdIns(4,5)*P*₂ directly binds and regulates several target proteins (for review see [2]). For example, PtdIns(4,5)*P*₂ (i) binds pleckstrin homology domains present in a variety of proteins involved in signal transduction [3–5]; (ii) binds actin-binding proteins and thereby regulates organization of the actin cytoskeleton [2]; (iii) regulates the small GTPase ADP-ribosylation factor (ARF) by recruiting both the guanine nucleotide exchange factor and the GTPase-activating protein for ARF [6–8], but see [7a]); and (iv) binds several proteins involved in the formation of clathrin-coated vesicles during endocytosis [9–11].

In mammalian cells, PtdIns(4,5)*P*₂ is synthesized via two independent reactions. It is the product of PtdIns(4)*P* 5-kinases, which phosphorylate PtdIns4*P* at the D-5 position of the inositol ring. It is also produced by phosphorylation of PtdIns5*P* at the D-4 position of the inositol ring by PtdIns(5)*P* 4-kinases [12]. Little is known about the regulation of PtdIns(4,5)*P*₂ synthesis. The activation of PtdIns(4)*P* 5-kinases by phosphatidic acid, a product of phospholipase D, is well documented [13–16], but two recent studies [17,18] demonstrate that PtdIns(4)*P* 5-kinases are also activated by ARF to control membrane ruffling and structural integrity of the Golgi complex. The investigation of the physiological function of PtdIns(4,5)*P*₂ and the mechanisms regulating its synthesis in mammalian cells is complicated not only due to the existence of both PtdIns(4)*P* 5-kinases and PtdIns(5)*P* 4-kinases, but also because of the presence of different isoforms of these kinases. In the yeast *Saccharomyces cerevisiae*,

PtdIns(4,5)*P*₂ is produced solely via *MSS4*-mediated phosphorylation of PtdIns4*P* [19]. *MSS4* is encoded by the essential gene *MSS4* which when mutated causes disorganization of the actin cytoskeleton, aberrant cell morphology and loss of cell integrity [19,20].

Calmodulin is a Ca^{2+} -binding protein also implicated in the regulation of many proteins, including metabolic enzymes, protein kinases, a protein phosphatase, transcription factors, ions transporters, receptors, motor proteins and cytoskeletal components (for reviews see [21,22]). Studies using temperature-sensitive (ts) calmodulin mutations (*cmd1^{ts}*) in *S. cerevisiae* have demonstrated that calmodulin is an essential protein required for organization of the actin cytoskeleton, endocytosis, nuclear division and bud emergence [23,24]. Interaction of calmodulin with some target proteins has been reported [25,26]; however, the exact mechanisms by which calmodulin controls these cellular processes is largely unknown. Our findings reveal a new mode of calmodulin action which operates via regulation of PtdIns(4,5)*P*₂ synthesis.

EXPERIMENTAL

Strains, plasmids and media

The *S. cerevisiae* strains used in this study are listed in Table 1. All strains were isogenic derivatives of JK9-3d. Plasmids used in this work are listed in Table 2. Rich media, YPD [1% (w/v) yeast extract, 2% (w/v) peptone and 2% (w/v) dextrose], and synthetic defined minimal media, SD [0.17% (w/v) yeast nitrogen base, 0.5% (w/v) ammonium sulphate and 2% (w/v) dextrose], supplemented with the appropriate nutrients for plasmid maintenance were as described previously [27].

Abbreviations used: ARF, ADP-ribosylation factor; MAPK, mitogen-activated protein kinase; PKC, protein kinase C; PLC, phospholipase C; SD, yeast nitrogen base/ammonium sulphate/dextrose media; TRITC, tetramethylrhodamine β -isothiocyanate; ts, temperature-sensitive; YPD, yeast extract/peptone/dextrose media.

¹ Present address: Georg Speyer Haus, Paul-Ehrlich-Strasse 42–44, D-60596 Frankfurt am Main, Germany.

² To whom correspondence should be addressed (e-mail M.Hall@unibas.ch).

Table 1 Yeast strains

Strain	Genotype
JK9-3da	MATa <i>leu2-3,112 ura3-52 trp1 his4 rme1 HMLa</i>
JK9-3dα	MATα <i>leu2-3,112 ura3-52 trp1 his4 rme1 HMLa</i>
TB50a	JK9-3da <i>his3 HIS4</i>
SD18-1d	TB50a <i>mss4::HIS3MX6/Ycplac111::MSS4</i>
SD19-3a	TB50a <i>mss4::HIS3MX6/Ycplac111::mss4-2^{ts}</i>
SD54-11d	SD18-1d <i>bar1::kanMX4</i>
SD55-1b	SD19-3a <i>bar1::kanMX4</i>
TS45-1a	TB50a <i>mpk1::TRP1</i>
SH121	JK9-3da <i>ade2 tor2::ADE2-3/Ycplac111::tor2-21^{ts}</i>
SH27-1b	JK9-3dα <i>ade2 mpk1::TRP1 tor2::ADE2-3/Ycplac111::tor2-21^{ts}</i>
DBY7462	MATa <i>leu2-3,112 ura3-52 trp1 his3 lys2</i>
DBY7446	DBY7462 <i>cmd1-226</i>
DBY7445	DBY7462 <i>cmd1-228</i>
DBY7449	DBY7462 <i>cmd1-239</i>

Determination of cellular phosphatidylinositol levels

Cells were grown at 24 °C in the presence of 50 μCi/ml [³H]inositol (Amersham International, Little Chalfont, Bucks., U.K.) to a D_{600} of 0.7 (D is attenuation), harvested by centrifugation, and resuspended in one-fifth the original volume. After control samples had been removed, cells were incubated at 37 °C and aliquots (200 μl) were collected at different times. Cells were arrested by the addition of 2.5 vols. of methanol, and disrupted by vortexing for 5 min with glass beads. Lipids were extracted and analysed as described previously [28]. The radioactivity associated with each phospholipid was quantified and corrected to the level of total phosphatidylinositol found in the control samples.

Endocytosis assays

Lucifer Yellow (Fluka, Buchs, Switzerland) accumulation in vacuoles was assayed as described previously [29]. Cells were grown in YPD at 24 °C to mid-log phase, shifted to 37 °C and incubated for 1 h in the presence of 4 mg/ml Lucifer Yellow. Cells were then washed three times in 1 ml of ice-cold buffer (50 mM sodium phosphate, 20 mM sodium azide and 20 mM sodium fluoride, pH 7.0). [³⁵S]α-Factor-uptake assays were performed using the continuous-presence protocol as described previously [29]. Cells were grown in YPD at 24 °C to exponential phase, and the α-factor-uptake assay was carried out at 24 °C or 37 °C. Cells assayed at the restrictive temperature (37 °C) were preincubated at this temperature for 15 min.

Rhodamine-phalloidin staining of actin

Cells were grown to early logarithmic phase, fixed in formaldehyde, and stained with phalloidin-tetramethylrhodamine β-isothiocyanate (TRITC) conjugate (Sigma) to visualize actin as described previously [30].

RESULTS

Calmodulin and MSS4 are functionally related

We have shown previously that the yeast PtdIns(4) P 5-kinase MSS4 is an essential protein required for proper organization of the actin cytoskeleton [19]. The actin cytoskeleton plays a fundamental role in endocytic uptake in yeast [31] and recent studies [32,33] in mammalian cells suggest a role for PtdIns(4,5) P_2 in receptor-mediated endocytosis. To investigate further the role

Table 2 Plasmids

Plasmid	Description
pROM2	pAS30, <i>ROM2</i> (2 μ, <i>URA3</i>) [53]
pRHO2	pC-186, <i>RHO2</i> (2 μ, <i>URA3</i>) [54]
pCMD1	pSD14, <i>CMD1</i> in YEplac195 (2 μ, <i>URA3</i>)
pBCK1-20	<i>BCK1-20</i> in pRS352 (<i>CEN. URA3</i>) [55]
pMKK1	<i>MKK1</i> in YEp352 (2 μ, <i>URA3</i>) [55]
pMPK1	<i>MPK1</i> in YEp352 (2 μ, <i>URA3</i>) [55]
pMSS4	pSH22, <i>MSS4</i> in pSEY18 (2 μ, <i>URA3</i>) [56]
pPKC1	pSH24, <i>PKC1</i> in pSEY18 (2 μ, <i>URA3</i>) [56]

of PtdIns(4,5) P_2 in endocytosis, we examined fluid-phase and receptor-mediated endocytosis in a temperature-sensitive (*ts*) *mss4* mutant. *mss4^{ts}* cells shifted to non-permissive temperature (37 °C) were defective in fluid-phase endocytosis, as evidenced by a lack of vacuolar accumulation of the dye Lucifer Yellow, and in receptor-mediated uptake, as evidenced by a reduction in internalization of the α-factor pheromone (Figure 1). These results suggest that PtdIns(4,5) P_2 is required for the internalization step of endocytosis.

The above finding revealed that the phenotype of a *mss4^{ts}* mutant is remarkably similar to that of a *cmd1* mutant defective in the Ca²⁺-binding protein calmodulin. Calmodulin, like MSS4, is an essential protein required for proper organization of the actin cytoskeleton [23] and for the internalization step of endocytosis [24,25]. Furthermore, like a *mss4^{ts}* mutant, the temperature-sensitive *cmd1-226* mutant loses viability after prolonged incubation at restrictive temperature; approx. 30% of *cmd1-226* cells and 80% of *mss4^{ts}* cells were non-viable after 4 h at 37 °C, as evidenced by staining with the vital dye Methylene Blue. No loss of viability was detected with a similarly treated wild-type strain. The growth defect of both the *mss4^{ts}* and *cmd1-226* mutants was at least partly suppressed by osmotic stabilization of the cells, i.e. by the addition of 1 M sorbitol to the growth medium (results not shown), indicating that both mutants are impaired in cell integrity. Finally, both *mss4* [20] and *cmd1-226* (Figure 2) mutants display aberrant morphology at 37 °C. Altogether, these findings suggest that MSS4 and calmodulin have related functions.

To investigate further whether calmodulin and MSS4 are functionally related, we examined whether *CMD1* and *MSS4* interact genetically. Temperature-sensitive *CMD1* mutations fall into different intragenic complementation groups, indicating a role for calmodulin in different, essential cellular processes [23,34]. Representative mutations from the different complementation groups were examined for suppression by a multicopy *MSS4* gene. Overexpression of MSS4 restored growth (Figure 3) in a *cmd1* mutant defective in organization of the actin cytoskeleton (*cmd1-226*), but did not restore growth of mutants defective in calmodulin localization (*cmd1-228*) or in nuclear division (*cmd1-239*) (results not shown). The suppression of the *cmd1-226* growth defect by overexpression of MSS4 suggested that *MSS4* can restore proper organization of the actin cytoskeleton in *cmd1-226* cells. To test this, the actin cytoskeleton was visualized in *cmd1-226* mutant cells overexpressing MSS4 (Figure 2). Cells were grown at permissive temperature (24 °C) and then shifted to restrictive temperature (37 °C) for 4 h. *cmd1-226* cells exhibited a severe defect in the organization of the actin cytoskeleton; actin cables were undetectable and cortical actin patches were randomly distributed in mother and daughter cells, instead of being concentrated at the site of growth. Conversely, *cmd1-226* cells overexpressing

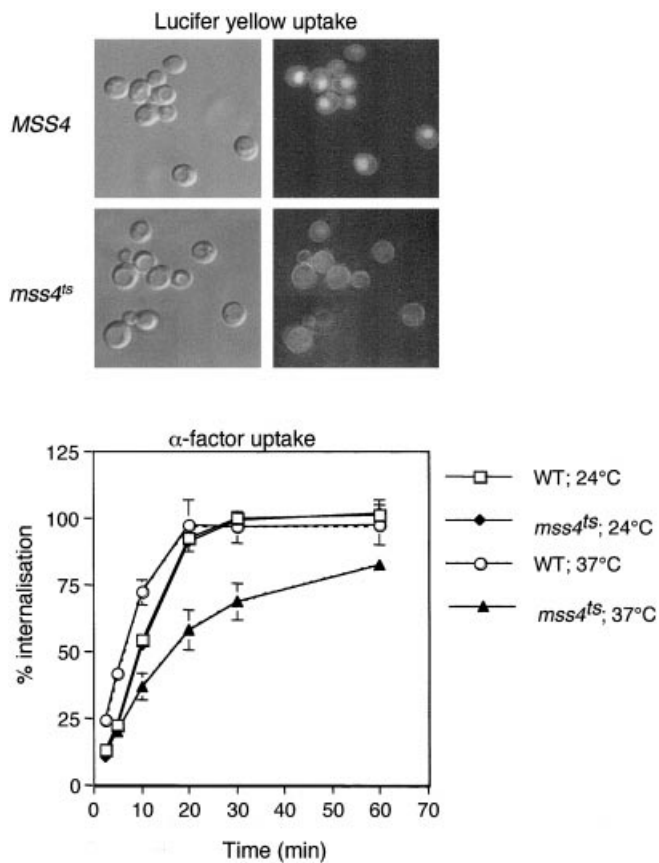


Figure 1 MSS4 is required for the internalization step of endocytosis

Wild-type (WT; *MSS4*; SD54-11d) and *mss4^{ts}* (SD55-1b) cells were assayed for Lucifer Yellow accumulation in the vacuole at 37 °C (upper panel) or α -factor uptake at 24 °C and 37 °C (lower panel). For the Lucifer Yellow uptake, the same field of cells is viewed by fluorescence (right-hand panels) and Nomarski (left-hand panels) microscopy. The results shown for α -factor uptake represent the mean of two independent experiments \pm S.E.M.

MSS4 (*cmd1-226* + *pMSS4*) exhibited a polarized actin cytoskeleton, as observed in wild-type *CMD1* cells (Figure 2). Overexpression of *MSS4* also suppressed the abnormal morphology of medium-sized buds on *cmd1-226* mutant cells (Figure 2). Thus, consistent with the suppression of the *cmd1-226* growth defect by *MSS4*, overexpression of *MSS4* suppresses the actin defect of *cmd1-226* cells.

These findings indicate that calmodulin and *MSS4* are functionally related, particularly in the regulation of the organization of the actin cytoskeleton. Furthermore, the above findings, combined with the observation that multicopy *CMD1* failed to restore growth in a *mss4^{ts}* mutant (results not shown), suggest that calmodulin may act upstream of *MSS4*.

Calmodulin is required for synthesis of PtdIns(4,5) P_2 *in vivo*

To determine if calmodulin acts upstream of *MSS4*, we examined phosphoinositide levels in the *cmd1-226* mutant. Cells were labelled with [3 H]inositol at 24 °C, shifted to 37 °C for various times, and assayed for phosphorylated phosphatidylinositol levels (Figure 4). As observed previously [19], a temperature up-shift induced production of PtdIns(4,5) P_2 in a wild-type strain (*CMD1*), due to activation of *MSS4*. PtdIns(4,5) P_2 levels increased approx. 2-fold within 1 h of the temperature shift and

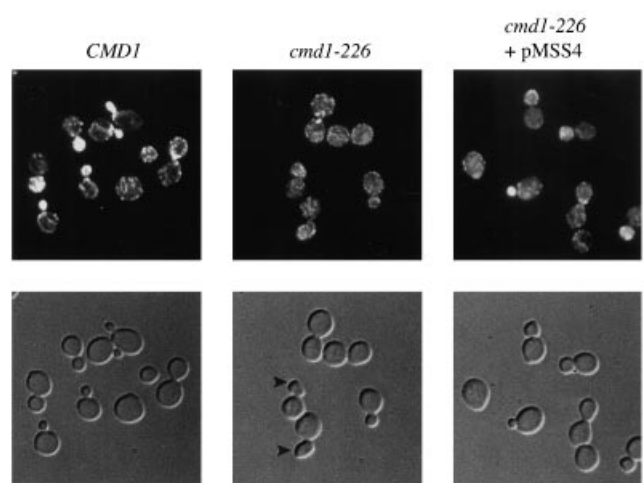


Figure 2 Multicopy *MSS4* suppresses a *cmd1* mutation

Logarithmic cultures of *cmd1-226* (DBY7446) cells carrying an empty vector (*cmd1-226*), *pCMD1* (*CMD1*) or *pMSS4* (*cmd1-226* + *pMSS4*) were grown at 24 °C, shifted to 37 °C for 4 h, fixed, stained with TRITC-phalloidin, and observed by Nomarski (lower panel) and fluorescence (actin staining; upper panel) microscopy. Arrowheads indicate the abnormally shaped, medium-sized buds of *cmd1-226* cells.

remained at this level for at least 2 h. In contrast, *cmd1-226* cells failed to induce PtdIns(4,5) P_2 synthesis upon temperature up-shift, indicating that calmodulin regulates PtdIns(4,5) P_2 levels. The *cmd1-226* mutation had little to no effect on the production of PtdIns3 P , PtdIns4 P and PtdIns(3,5) P_2 , indicating that calmodulin regulates specifically PtdIns(4,5) P_2 levels. These findings provide further evidence that calmodulin acts upstream of *MSS4*, in particular, as an activator of *MSS4*.

Calmodulin and *MSS4* signal independently of the PKC1-activated mitogen-activated protein kinase (MAPK) cascade

Organization of the actin cytoskeleton and cell integrity, both of which are affected in *mss4* and *cmd1* mutants, are controlled in part by the PKC1-dependent MAPK cascade [35]. The PKC1-dependent MAPK cascade comprises BCK1/SLK1 (MAPK kinase kinase; 'MAPKKK'), MKK1 and MKK2 (MAPK kinases; 'MAPKKs') and MPK1/SLT2 (MAPK) [36]. To investigate if calmodulin and *MSS4* signal to the actin cytoskeleton via activation of this pathway, we examined whether overexpression of PKC1 or components of the MAPK cascade restore growth in *cmd1-226* and *mss4^{ts}* mutants. As shown in Figure 3, the growth defect of the *cmd1-226* mutants is weakly suppressed by overexpression of PKC1. Overexpression of components upstream of PKC1, such as the guanine nucleotide exchange factor ROM2 or the small GTPase RHO2, suppressed very weakly, if at all, the *cmd1-226* mutation. Expression of a constitutively active allele of *BCK1* (*BCK1-20*) or overexpression of MKK1 or MPK1 failed to suppress *cmd1-226* (Figure 3). Overexpression of PKC1 or components of the MAPK cascade failed to suppress the *mss4^{ts}* mutation (results not shown). These results suggest that the growth defects of the *cmd1-226* and *mss4^{ts}* mutants are not due solely to a lack of activation of the MAPK pathway downstream of PKC1. To determine if calmodulin and *MSS4* are required for the activation of the PKC1-dependent MAPK pathway, we assayed activation of MPK1 in *cmd1-226* and *mss4^{ts}* cells. Shifting

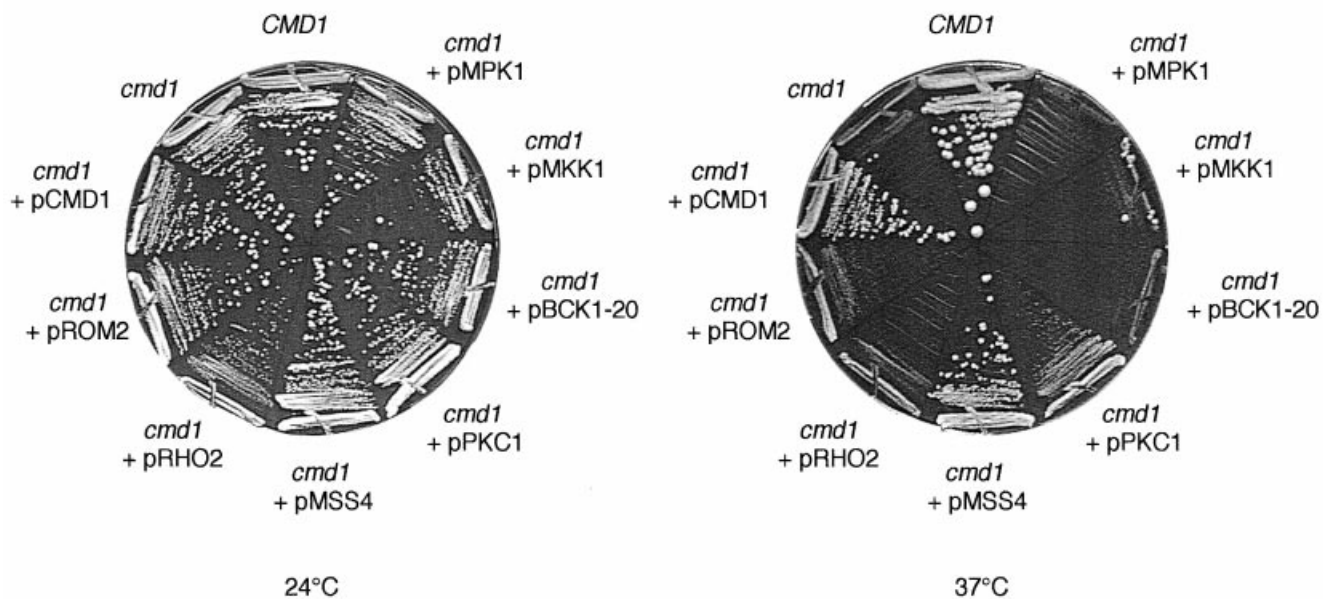


Figure 3 Overexpression of PKC1, but not other members of the PKC1-activated MAPK pathway, suppresses a *cmd1* mutation

Wild-type (*CMD1*; DBY7462) cells and *cmd1-226* (*cmd1*; DBY7446) cells carrying an empty vector or p*CMD1*, p*ROM2*, p*RHO2*, p*MSS4*, p*PKC1*, p*BCK1-20* (containing an activated allele of *BCK1*), p*MKK1* or p*MPPK1* were streaked out on YPD and incubated at 24 °C or at 37 °C.

wild-type cells to 39 °C for 1 h resulted in activation of the cascade, as determined by Western blotting using an antibody specific for the activated form of MPK1 [37–39] (results not shown). Elevated temperature induced activation of MPK1 in *mss4^{ts}* cells to a level similar to that observed in wild-type cells. The *cmd1-226* mutation resulted in an even stronger and longer activation of MPK1 (results not shown). These results indicate that calmodulin and MSS4 are not required for activation of the PKC1-dependent MAPK pathway. However, calmodulin and possibly MSS4 may still signal via PKC1, but independently of the MAPK cascade, as suggested by the suppression of *cmd1-226* by multicopy *PKC1*.

DISCUSSION

We have shown previously [19] that MSS4 is the only PtdIns(4)*P* 5-kinase in *S. cerevisiae*, and that it is required for proper organization of the actin cytoskeleton. In the present study, we provide evidence that calmodulin activates MSS4-mediated synthesis of PtdIns(4,5)*P*₂, and that it is via MSS4 that calmodulin controls the actin cytoskeleton. First, cells lacking functional calmodulin or MSS4 proteins have similar phenotypes. Secondly, overexpression of MSS4 restores both growth and actin organization in the *cmd1-226* mutant. Finally, PtdIns(4,5)*P*₂ levels are reduced in cells lacking calmodulin.

How does calmodulin activate MSS4? Two models could explain how calmodulin regulates PtdIns(4,5)*P*₂ synthesis. First, calmodulin could activate a PtdIns 4-kinase that would, in turn, yield more substrate for MSS4. Alternatively, calmodulin could activate MSS4 independently of a PtdIns 4-kinase. Several lines of evidence suggest that calmodulin does not activate a PtdIns 4-kinase. First, unlike PtdIns(4,5)*P*₂, total cellular levels of PtdIns4*P* do not increase upon shift of wild-type cells to

37 °C. Secondly, a calmodulin deficiency prevents heat-induced PtdIns(4,5)*P*₂ accumulation, but does not cause a significant change in PtdIns4*P* levels. Finally, overexpression of either one of the two *S. cerevisiae* PtdIns 4-kinases, PIK1 and STT4, failed to suppress the growth defect of the *cmd1-226* mutant (results not shown). Thus calmodulin may activate MSS4 independently of the PtdIns 4-kinases. Frequentin, another member of the calmodulin superfamily, has been shown to bind and stimulate PIK1 [40]. This suggests that activation of PtdIns-kinases by Ca²⁺-binding proteins may be a common theme. However, we could not detect a direct interaction between calmodulin and MSS4 using the yeast two-hybrid system (results not shown), suggesting that calmodulin may not activate MSS4 directly.

How do calmodulin and MSS4 control the organization of the actin cytoskeleton? One possibility is that they regulate the interactions between actin and the actin-binding proteins. Cell motility and changes in morphology initiated by binding of extracellular ligands to transmembrane receptors, for example during the activation of platelets or neutrophils, require rearrangement of the actin cytoskeleton. This is generally achieved by a transient increase in cytoplasmic Ca²⁺ concentration and by synthesis of PtdIns(4,5)*P*₂. Ca²⁺ leads to solubilization of the cytoskeleton via activation of proteins that disrupt the actin network and inhibition of actin cross-linking proteins. Conversely, PtdIns(4,5)*P*₂ promotes actin polymerization by inhibiting actin-filament severing, capping and monomer-sequestering proteins (for review see [41]). The opposite effects of Ca²⁺ and PtdIns(4,5)*P*₂ have led to a two-step model for the reorganization of the actin cytoskeleton during cell spreading or crawling [42]. First, Ca²⁺ induces the release of actin filaments from the membrane and depolymerization of the cytoskeleton. The second phase, characterized by repolymerization of actin, is triggered by synthesis and rearrangement of phosphoinositides. Our previous observations in yeast cells undergoing thermal stress support this model [19,43].

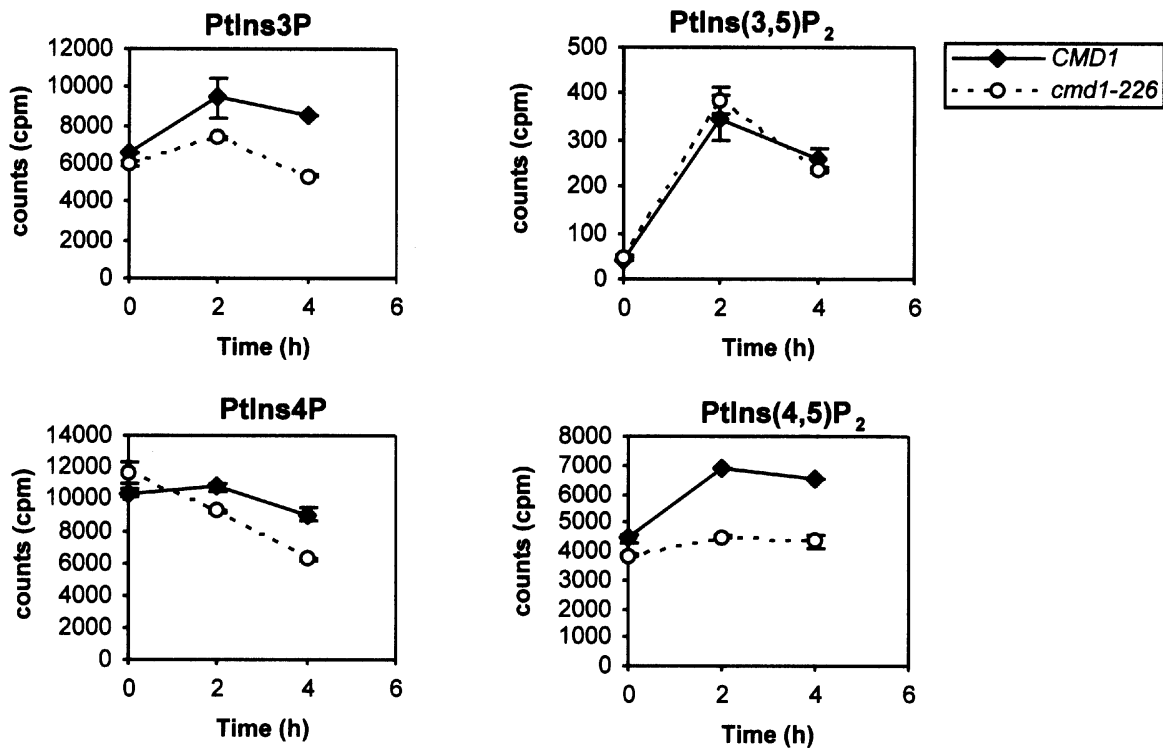


Figure 4 Calmodulin regulates PtdIns(4,5) P_2 levels *in vivo*

Wild-type (*CMD1*; DBY7462) and *cmd1-226* (DBY7446) strains were labelled at 24 °C with [3 H]inositol and transferred to 37 °C. Aliquots were collected at the indicated times, and the [3 H]inositol-labelled lipids from cell extracts were deacylated and separated by HPLC. Radioactivity associated with each lipid was quantified and corrected to total levels of phosphatidylinositol found in the control samples. Total phosphatidylinositol levels (c.p.m.) were as follows: *CMD1*, 0 h, 663 147; 2 h, 693 733; 4 h, 687 418; *cmd1-226*, 0 h, 753 572; 2 h, 902 835; 4 h, 617 489. Data represent means \pm S.E.M. of two independent experiments.

Rearrangement of the actin cytoskeleton in response to heat is biphasic. A rapid disappearance of actin cables and depolarization of the actin cytoskeleton is followed by an MSS4-dependent phase in which actin cables reappear and the cytoskeleton repolarizes. Our finding that calmodulin is required for heat-induced activation of MSS4 suggests that calmodulin is involved in the second phase of actin rearrangement and might act as a negative feedback regulator of Ca^{2+} -induced actin depolarization and as a switch between actin depolarization and repolarization.

The suppression, albeit weak, of *cmd1-226* by multicopy *PKC1* suggests that calmodulin and MSS4 may also signal to the actin cytoskeleton via PKC1. PKC1 is activated by the GTP-bound form of the GTPase RHO1 or its homologue RHO2 [44,45]. Surprisingly, we found that overexpression of either RHO2 or its GDP/GTP exchange factor ROM2 did not suppress the *cmd1-226* mutation. This may suggest that there are different pools of PKC1 regulated by different mechanisms. This notion is consistent with the observations that PKC1 displays punctate cytoplasmic localization and is also found associated with the plasma membrane at the site of bud growth, and that shifting cells to 37 °C induces nuclear translocation only of PKC1 fractions initially localized at the plasma membrane (results not shown). Thus MSS4 and RHO1/2 might activate different pools of PKC1. However, if MSS4 does signal to the actin cytoskeleton via PKC1, this signalling appears to be independent of the PKC1-effector MAPK cascade, as MPK1 activation is not affected in *cmd1* or *mss4* mutants. The existence of a calmodulin-

activated PKC1-dependent pathway parallel to the MAPK cascade is consistent with an earlier suggestion that there is functional redundancy of the Ca^{2+} - and MPK1-regulated pathways [46]. The Ca^{2+} -activated protein calcineurin and the PKC1-activated MAPK pathway act in concert to regulate transcription of *FKS2*, which encodes a subunit of the 1,3- β -glucan synthase required for cell-wall synthesis [47,48]. Calcineurin and MPK1 also act in synergy to control the onset of mitosis by activating the SWI1 transcription factor at the transcriptional and post-translational levels respectively [49]. In addition, the phenotypes of *gle7* cells, carrying a mutation in the catalytic subunit of type 1 protein serine/threonine phosphatase, suggests a role for this phosphatase in maintenance of cell-wall integrity, possibly via a PKC1-related pathway [50].

We also found that cells lacking functional MSS4 are defective in endocytosis. This provides additional support for the idea that PtdIns(4,5) P_2 is required for endocytosis. An involvement of PtdIns(4,5) P_2 in endocytosis was suggested previously by the finding that a dominant-negative isoform of PtdIns(4)P 5-kinase *I β* inhibits endocytosis of human colony-stimulating factor 1 receptor [32]. Furthermore, key components of the clathrin-mediated endocytic machinery are PtdIns(4,5) P_2 -binding proteins [31,33]. Deletion of members of the synaptojanin inositol 5-phosphatases family in yeast also leads to reduced endocytosis [51], suggesting a role for PtdIns(4,5) P_2 turnover in endocytosis. Targeted disruption of the *synaptojanin 1* gene in mice leads to death shortly after birth [52]. Neurons of mutant animals have elevated levels of PtdIns(4,5) P_2 and accumulate clathrin-coated

vesicles at the synaptic vesicle cluster, the site where clathrin-mediated internalization of the synaptic vesicle membrane occurs. Interestingly, cells lacking calmodulin, like *mss4^{ts}* mutants, are defective in endocytosis [24,25]. It remains to be determined if calmodulin also regulates endocytosis via activation of MSS4.

We thank D. Botstein for strains, T. Schmelzle for plasmids, strains and valuable discussion, and members of the Riezman laboratory for advice. S.D. was the recipient of a long-term European Molecular Biology Organization Fellowship. This work was supported by Cancer Research UK (P.J.P. and F.T.C.), and by grants from the Swiss National Science Foundation and the Canton of Basel (M.N.H.).

REFERENCES

- Berridge, M. J. and Irvine, R. F. (1989) Inositol phosphates and cell signalling. *Nature (London)* **341**, 197–205
- Martin, T. F. J. (1998) Phosphoinositide lipids as signaling molecules: common themes for signal transduction, cytoskeletal regulation, and membrane trafficking. *Annu. Rev. Cell Dev. Biol.* **14**, 231–264
- Salim, K., Bottomley, M. J., Querfurth, E., Zvebil, M. J., Gout, I., Scaife, R., Margolis, R. L., Gigg, R., Smith, C. I., Driscoll, P. C. et al. (1996) Distinct specificity in the recognition of phosphoinositides by the pleckstrin homology domains of dynamin and Bruton's tyrosine kinase. *EMBO J.* **15**, 6241–6250
- Harlan, J. E., Hajduk, P. J., Yoon, H. S. and Fesik, S. W. (1994) Pleckstrin homology domains bind to phosphatidylinositol-4,5-bisphosphate. *Nature (London)* **371**, 168–170
- Rameh, L. E., Arvidsson, A., Carraway, III, K. L., Couvillon, A. D., Rathbun, G., Crompton, A., VanRenterghem, B., Czech, M. P., Ravichandran, K. S., Burakoff, S. J. et al. (1997) A comparative analysis of the phosphoinositide binding specificity of pleckstrin homology domains. *J. Biol. Chem.* **272**, 22059–22066
- Randazzo, P. A. (1997) Functional interaction of ADP-ribosylation factor 1 with phosphatidylinositol 4,5-bisphosphate. *J. Biol. Chem.* **272**, 7688–7692
- Randazzo, P. A. and Kahn, R. A. (1994) GTP hydrolysis by ADP-ribosylation factor is dependent on both an ADP-ribosylation factor GTPase-activating protein and acid phospholipids. *J. Biol. Chem.* **269**, 10758–10763
- Erratum (1994) *J. Biol. Chem.* **269**, 16519
- Terui, T., Kahn, R. A. and Randazzo, P. A. (1994) Effects of acid phospholipids on nucleotide exchange properties of ADP-ribosylation factor 1. Evidence for specific interaction with phosphatidylinositol 4,5-bisphosphate. *J. Biol. Chem.* **269**, 28130–28135
- Gaidarov, I. and Keen, J. H. (1999) Phosphoinositide-AP-2 interactions required for targeting to plasma membrane clathrin-coated pits. *J. Cell. Biol.* **146**, 755–764
- Hao, W., Tan, Z., Prasad, K., Reddy, K. K., Chen, J., Prestwich, G. D., Falck, J. R., Shears, S. B. and Lafer, E. M. (1997) Regulation of AP-3 function by inositides. Identification of phosphatidylinositol 3,4,5-trisphosphate as a potent ligand. *J. Biol. Chem.* **272**, 6393–6398
- Zheng, J., Cahill, S. M., Lemmon, M. A., Fushman, D., Schlessinger, J. and Cowburn, D. (1996) Identification of the binding site for acidic phospholipids on the pH domain of dynamin: implications for stimulation of GTPase activity. *J. Mol. Biol.* **255**, 14–21
- Rameh, L. E., Tolia, K. F., Duckworth, B. C. and Cantley, L. C. (1997) A new pathway for synthesis of phosphatidylinositol-4,5-bisphosphate. *Nature (London)* **390**, 192–196
- Moritz, A., De Graan, P. N., Gispen, W. H. and Wirtz, K. W. (1992) Phosphatidic acid is a specific activator of phosphatidylinositol-4-phosphate kinase. *J. Biol. Chem.* **267**, 7207–7210
- Jenkins, G. H., Fiset, P. L. and Anderson, R. A. (1994) Type I phosphatidylinositol 4-phosphate 5-kinase isoforms are specifically stimulated by phosphatidic acid. *J. Biol. Chem.* **269**, 11547–11554
- Ishihara, H., Shibasaki, Y., Kizuki, N., Katagiri, H., Yazaki, Y., Asano, T. and Oka, Y. (1996) Cloning of cDNAs encoding two isoforms of 68-kDa type I phosphatidylinositol-4-phosphate 5-kinase. *J. Biol. Chem.* **271**, 23611–23614
- Ishihara, H., Shibasaki, Y., Kizuki, N., Wada, T., Yazaki, Y., Asano, T. and Oka, Y. (1998) Type I phosphatidylinositol-4-phosphate 5-kinases. Cloning of the third isoform and deletion/substitution analysis of members of this novel lipid kinase family. *J. Biol. Chem.* **273**, 8741–8748
- Honda, A., Nogami, M., Yokozeki, T., Yamazaki, M., Nakamura, H., Watanabe, H., Kawamoto, K., Nakayama, K., Morris, A. J., Frohman, M. A. and Kanaho, Y. (1999) Phosphatidylinositol 4-phosphate 5-kinase α is a downstream effector of the small G-protein ARF6 in membrane ruffle formation. *Cell* **99**, 521–532
- Godi, A., Pertile, P., Meyers, R., Marra, P., Di Tullio, G., Iurisci, C., Luini, A., Corda, D. and De Matteis, M. A. (1999) ARF mediates recruitment of PtdIns-4-OH kinase- β and stimulates synthesis of PtdIns(4,5) P_2 on the Golgi complex. *Nat. Cell. Biol.* **1**, 280–287
- Desrivières, S., Cooke, F. T., Parker, P. J. and Hall, M. N. (1998) MSS4, a phosphatidylinositol-4-phosphate 5-kinase required for organization of the actin cytoskeleton in *Saccharomyces cerevisiae*. *J. Biol. Chem.* **273**, 15787–15793
- Homma, K., Terui, S., Minemura, M., Qadota, H., Anraku, Y., Kanaho, Y. and Ohya, Y. (1998) Phosphatidylinositol-4-phosphate 5-kinase localized on the plasma membrane is essential for yeast cell morphogenesis. *J. Biol. Chem.* **273**, 15779–15786
- Jurado, L. A., Chockalingam, P. S. and Jarrett, H. W. (1999) Apocalmodulin. *Physiol. Rev.* **79**, 661–682
- Levitani, I. B. (1999) It is calmodulin after all! Mediator of the calcium modulation of multiple ion channels. *Neuron* **22**, 645–648
- Ohya, Y. and Botstein, D. (1994) Diverse essential functions revealed by complementing yeast calmodulin mutants. *Science* **263**, 963–966
- Kubler, E., Schimmoller, F. and Riezman, H. (1994) Calcium-independent calmodulin requirement for endocytosis in yeast. *EMBO J.* **13**, 5539–5546
- Geli, M. I., Wesp, A. and Riezman, H. (1998) Distinct functions of calmodulin are required for the uptake step of receptor-mediated endocytosis in yeast: the type I myosin Myo5p is one of the calmodulin targets. *EMBO J.* **17**, 635–647
- Okano, H., Cyert, M. S. and Ohya, Y. (1998) Importance of phenylalanine residues of yeast calmodulin for target binding and activation. *J. Biol. Chem.* **273**, 26375–26382
- Guthrie, C. and Fink, G. R. (1991) *Guide to Yeast Genetics and Molecular Biology, Methods in Enzymology*, vol. 194, Academic Press, New York
- Stephens, L. R., Hughes, K. T. and Irvine, R. F. (1991) Pathway of phosphatidylinositol(3,4,5)-trisphosphate synthesis in activated neutrophils. *Nature (London)* **351**, 33–39
- Dulic, V., Egerton, M., Elguindi, I., Raths, S., Singer, B. and Riezman, H. (1991) Yeast endocytosis assays. *Methods Enzymol.* **194**, 697–710
- Benedetti, H., Raths, S., Crausaz, F. and Riezman, H. (1994) The END3 gene encodes a protein that is required for the internalization step of endocytosis and for actin cytoskeleton organization in yeast. *Mol. Biol. Cell.* **5**, 1023–1037
- D'Hondt, K., Heese-Peck, A. and Riezman, H. (2000) Protein and lipid requirements for endocytosis. *Annu. Rev. Genet.* **34**, 255–295
- Davis, J. N., Rock, C. O., Cheng, M., Watson, J. B., Ashmun, R. A., Kirk, H., Kay, R. J. and Roussel, M. F. (1997) Complementation of growth factor receptor-dependent mitogenic signaling by a truncated type I phosphatidylinositol 4-phosphate 5-kinase. *Mol. Cell. Biol.* **17**, 7398–7406
- Jost, M., Simpson, F., Kavan, J. M., Lemmon, M. A. and Schmid, S. L. (1998) Phosphatidylinositol-4,5-bisphosphate is required for endocytic coated vesicle formation. *Curr. Biol.* **8**, 1399–1402
- Ohya, Y. and Botstein, D. (1994) Structure-based systematic isolation of conditional-lethal mutations in the single yeast calmodulin gene. *Genetics* **138**, 1041–1054
- Schmidt, A. and Hall, M. N. (1998) Signaling to the actin cytoskeleton. *Annu. Rev. Cell. Dev. Biol.* **14**, 305–338
- Levin, D. E., Bowers, B., Chen, C. Y., Kamada, Y. and Watanabe, M. (1994) Dissecting the protein kinase C/MAP kinase signalling pathway of *Saccharomyces cerevisiae*. *Cell. Mol. Biol. Res.* **40**, 229–239
- Martin, H., Rodriguez-Pachon, J. M., Ruiz, C., Nombela, C. and Molina, M. (2000) Regulatory mechanisms for modulation of signaling through the cell integrity Sit2-mediated pathway in *Saccharomyces cerevisiae*. *J. Biol. Chem.* **275**, 1511–1519
- Ketela, T., Green, R. and Bussey, H. (1999) *Saccharomyces cerevisiae* mid2p is a potential cell wall stress sensor and upstream activator of the PKC1-MPK1 cell integrity pathway. *J. Bacteriol.* **181**, 3330–3340
- Verna, J., Lodder, A., Lee, K., Vagts, A. and Ballester, R. (1997) A family of genes required for maintenance of cell wall integrity and for the stress response in *Saccharomyces cerevisiae*. *Proc. Natl. Acad. Sci. U.S.A.* **94**, 13804–13809
- Hendricks, K. B., Wang, B. Q., Schnieders, E. A. and Thorner, J. (1999) Yeast homologue of neuronal frequenin is a regulator of phosphatidylinositol-4-OH kinase. *Nat. Cell. Biol.* **1**, 234–241
- Janmey, P. A. (1994) Phosphoinositides and calcium as regulators of cellular actin assembly and disassembly. *Annu. Rev. Physiol.* **56**, 169–191
- Stossel, T. P. (1989) From signal to pseudopod. How cells control cytoplasmic actin assembly. *J. Biol. Chem.* **264**, 18261–18264
- Delley, P. A. and Hall, M. N. (1999) Cell wall stress depolarizes cell growth via hyperactivation of RHO1. *J. Cell. Biol.* **147**, 163–174

- 44 Nonaka, H., Tanaka, K., Hirano, H., Fujiwara, T., Kohno, H., Umikawa, M., Mino, A. and Takai, Y. (1995) A downstream target of RHO1 small GTP-binding protein is PKC1, a homolog of protein kinase C, which leads to activation of the MAP kinase cascade in *Saccharomyces cerevisiae*. *EMBO J.* **14**, 5931–5938
- 45 Kamada, Y., Qadota, H., Python, C. P., Anraku, Y., Ohya, Y. and Levin, D. E. (1996) Activation of yeast protein kinase C by Rho1 GTPase. *J. Biol. Chem.* **271**, 9193–9196
- 46 Nakamura, T., Ohmoto, T., Hirata, D., Tsuchiya, E. and Miyakawa, T. (1996) Genetic evidence for the functional redundancy of the calcineurin- and Mpk1-mediated pathways in the regulation of cellular events important for growth in *Saccharomyces cerevisiae*. *Mol. Gen. Genet.* **251**, 211–219
- 47 Garrett-Engle, P., Moilanen, B. and Cyert, M. S. (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
- 48 Zhao, C., Jung, U. S., Garrett-Engle, P., Roe, T., Cyert, M. S. and Levin, D. E. (1998) Temperature-induced expression of yeast FKS2 is under the dual control of protein kinase C and calcineurin. *Mol. Cell. Biol.* **18**, 1013–1022
- 49 Mizunuma, M., Hirata, D., Miyahara, K., Tsuchiya, E. and Miyakawa, T. (1998) Role of calcineurin and Mpk1 in regulating the onset of mitosis in budding yeast. *Nature (London)* **392**, 303–306
- 50 Andrews, P. D. and Stark, M. J. (2000) Type 1 protein phosphatase is required for maintenance of cell wall integrity, morphogenesis and cell cycle progression in *Saccharomyces cerevisiae*. *J. Cell. Sci.* **113**, 507–520
- 51 Singer-Kruger, B., Nemoto, Y., Daniell, L., Ferro-Novick, S. and De Camilli, P. (1998) Synaptojanin family members are implicated in endocytic membrane traffic in yeast. *J. Cell Sci.* **111**, 3347–3356
- 52 Cremona, O., Di Paolo, G., Wenk, M. R., Luthi, A., Kim, W. T., Takei, K., Daniell, L., Nemoto, Y., Shears, S. B., Flavell, R. A. et al. (1999) Essential role of phospho-inositide metabolism in synaptic vesicle recycling. *Cell* **99**, 179–188
- 53 Schmidt, A., Bickle, M., Beck, T. and Hall, M. N. (1997) The yeast phosphatidylinositol kinase homolog TOR2 activates RHO1 and RHO2 via the exchange factor ROM2. *Cell* **88**, 531–542
- 54 Madaule, P., Axel, R. and Myers, A. M. (1987) Characterization of two members of the rho gene family from the yeast *Saccharomyces cerevisiae*. *Proc. Natl. Acad. Sci. U.S.A.* **84**, 779–783
- 55 Helliwell, S. B., Schmidt, A., Ohya, Y. and Hall, M. N. (1998) The Rho1 effector Pkc1, but not Bni1, mediates signalling from Tor2 to the actin cytoskeleton. *Curr. Biol.* **8**, 1211–1214
- 56 Helliwell, S. B., Howald, I., Barbet, N. and Hall, M. N. (1998) TOR2 is part of two related signaling pathways coordinating cell growth in *Saccharomyces cerevisiae*. *Genetics* **148**, 99–112

Received 14 March 2002/17 June 2002; accepted 21 June 2002

Published as BJ Immediate Publication 21 June 2002, DOI 10.1042/BJ20020429