Microbiology (2007), **153**, 1519–1529 DOI 10.1099/mic.0.2006/004283-0

Transduction of centrifugation-induced gravity forces through mitogen-activated protein kinase pathways in the fission yeast *Schizosaccharomyces pombe*

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Received 13 November 2006
Revised 9 January 2007
Accepted 16 January 2007

Centrifugation of cells of *Schizosaccharomyces pombe* in liquid medium prompted a marked activation of Sty1 and Pmk1, which are the effector mitogen-activated protein kinases (MAPKs) of the stress-activated protein kinase pathway and the cell-integrity pathway, respectively. Transduction of the centrifugation signals showed a sensitivity threshold above which the response was dependent on time and temperature. Centrifugation-induced phosphorylation of Sty1 and Pmk1 required the presence of the main functional components of the respective signalling cascades, i.e. Wak1 or Win1 plus Wis1, and Mkh1 plus Pek1. The transcription factor Atf1 also became phosphorylated in a Sty1-dependent way upon centrifugation. Hypergravity was an important factor in the activation of Sty1 induced by centrifugation, whilst activation of Pmk1 was mostly due to gravity-associated shear forces. Centrifugation did not increase cell survival against other stresses. Rather, the increased gravitational forces produced a delay in the cell cycle, probably related to alterations in the actin-polarization pattern. Phosphorylation of the MAPK Sty1 was needed for the depolarization of actin patches induced by the centrifugation stress.

INTRODUCTION

Survival under stress depends upon the modulation of inducible genes that enable cells to cope with the altered surroundings. Transduction of environmental signals into changes in gene expression underlies the adaptive response to external challenges, including accommodation of physical forces (Treisman, 1996; Kyriakis & Avruch, 2001). Mitogen-activated protein kinase (MAPK) pathways are key mechanisms for the conversion of signals into intracellular phosphorylation events and have been evolutionarily well conserved in all eukaryotes. The basic functional module of each MAPK pathway is composed of three sequentially acting protein kinases that become activated in response to triggering stimuli (Waskiewicz & Cooper, 1995; Marshall, 1995). Mitogen-activated protein kinase kinase kinases (MAPKKKs) phosphorylate and activate mitogen-activated protein kinase kinases (MAPKKs), which in turn phosphorylate and activate MAPKs. The effector MAPKs control, among others, the activity of downstream transcription factors so that the outcome of the cascade activation is a change in the expression programme of responsive target genes.

Yeast cells are a suitable model system to study signalling mechanisms and stress responses. In the fission yeast *Schizosaccharomyces pombe*, sensing and transduction of oxidative stresses, heat shock, temperature downshifts and osmotic changes have been studied in some detail (Degols *et al.*, 1996; Shiozaki *et al.*, 1998; Wilkinson & Millar, 1998; Quinn *et al.*, 2002; Soto *et al.*, 2002; Madrid *et al.*, 2004, 2006). Biochemical and genetic evidence supports the existence in this yeast of three distinct MAPK phosphorylation pathways, including the stress-activated protein kinase (SAPK) pathway (composed of MAPKKKs Wak1/Wis4/Wik1 and Win1, MAPKK Wis1 and MAPK Sty1/Spc1/Phh1, which is highly similar to mammalian p38 kinase) (Shiozaki & Russell, 1995), the cell-integrity pathway (which consists of MAPKKK Mkh1, MAPKK Pek1/Skh1 and MAPK Pmk1/Spm1, which is homologous to mammalian ERK1/2) (Zaitsevskaya-Carter & Cooper, 1997) and the mating pheromone-responsive MAPK Spk1 pathway (Toda *et al.*, 1991).

Because life develops on Earth under rather constant gravity forces, changes in this physical factor may induce important

Abbreviations: MAPK, mitogen-activated protein kinase; MAPKK, mitogen-activated protein kinase kinase; MAPKKK, mitogen-activated protein kinase kinase kinase; Ha6H, epitope comprising haemagglutinin antigen plus six histidine residues; SAPK, stress-activated protein kinase; YES, yeast extract plus supplements.

physiological effects. Several studies have demonstrated the effects of microgravity on the regulation of microbial gene expression (Johanson *et al.*, 2002; Nickerson *et al.*, 2004). The results gathered may lead to a better understanding of cell behaviour under reduced-gravity space-flight conditions and disclose features of life that cannot be normally observed in ground-based experiments. However, comparatively less attention has been paid to cell responses under hypergravity conditions, even though many experimental protocols in current molecular and cell biology include centrifugation steps in which high gravitational forces are reached. In this context, a technical observation initially revealed that MAPK Sty1 became activated by phosphorylation when cells from *S. pombe* cultures were recovered by centrifugation instead of filtration (Shiozaki *et al.*, 1998). However, no further studies on this stimulation of MAPK Sty1 have been reported, except for the indication that centrifugation-induced Sty1 activation can be precluded in the cold (Soto *et al.*, 2002) and that such stimulation mimics some effects of heat shock-induced Sty1 activation (Petersen & Hagan, 2005). In this work, we have characterized the molecular mechanisms by which centrifugation signals transduce into cells of the fission yeast to potentially modulate gene expression. Our results indicate that the SAPK signalling pathway is stimulated in response to hypergravitational conditions and that centrifugation-induced forces also signal through the Mkh1–Pek1–Pmk1 cell-integrity pathway in liquid media. Transduction of these centrifugation signals does not appear to trigger any obvious stress response of adaptive value. However, centrifuged cells slow down growth temporarily by arresting the cell-division cycle at G_2/M, probably due to actin cytoskeletal alterations induced by the centrifugation stress.

METHODS

Strains and culture media. The *S. pombe* strains employed in this study are listed in Table 1. They were grown routinely with shaking at 28 °C in yeast extract plus supplements (YES) medium (Moreno *et al.*, 1991). The culture medium was supplemented with adenine, leucine, histidine or uracil (100 mg l^{-1}; Sigma). Solid medium was made by the addition of 2 % (w/v) bacto-agar (Difco). In experiments performed with *cdc25-22* thermosensitive mutant strains, the cells were grown in YES medium to an OD_{600} of 0.2 at 25 °C (permissive temperature), shifted to 37 °C for 3.5 h and released from growth arrest by transfer back to 25 °C.

Centrifugation-stress treatment. Yeast cultures were grown to mid-exponential phase (OD_{600} = 0.8–1.0) at 28 °C. Samples (50 ml) of the cultures were placed in 50 ml Falcon tubes and subjected to centrifugation at various ***g*** values in a refrigerated centrifuge with a swinging-bucket rotor (model Z360K; Berthold Hermle AG). At different times, the cell pellets were collected, washed with cold PBS and analysed directly as indicated below. In parallel, one sample was harvested by filtration from the cultures as a control (1 ***g***). When the centrifugal forces applied were too low to sediment the cells within a given centrifugation period, the cells were harvested by filtration immediately after the treatment and washed as above.

Purification and detection of activated Sty1–Ha6H, Atf1–Ha6H and Pmk1–Ha6H proteins. To analyse Sty1 phosphorylation, total cell homogenates were prepared under native conditions, employing chilled acid-washed glass beads and lysis buffer [10 % glycerol, 50 mM Tris/HCl (pH 7.5), 150 mM NaCl, 0.1 % Nonidet NP-40, plus specific protease and phosphatase inhibitor cocktails for fungal and yeast extracts; Sigma]. The lysates were removed and cleared by centrifugation at 20 000 ***g*** for 15 min. Sty1 tagged with an

Table 1. Strains used in this study

All strains are *leu1-32 ura4-D18 ade6-M210* (or *ade6-M216*), except for PPG148, which is *ura4-D18*.

Strain	Genotype	Source
JM1521	h^+ *his7-366 sty1:Ha6H(ura4*$^+$*)*	Buck *et al.* (2001)
JM1821	h^- *atf1:Ha6H(ura4*$^+$*)*	Buck *et al.* (2001)
NT146	h^- *atf1::ura4*$^+$	T. Toda, Cancer Research UK London Research Institute, UK
VB1828	h^+ *mak1::LEU2 sty1:Ha6H(ura4*$^+$*)*	Buck *et al.* (2001)
VB1829	h^- *mak2::LEU2 sty1:Ha6H(ura4*$^+$*)*	Buck *et al.* (2001)
VB1934	h^- *mak3::kanR sty1:Ha6H(ura4*$^+$*)*	Buck *et al.* (2001)
TS-1	h^- *his7-366 mcs4::ura4*$^+$ *sty1:Ha6H(ura4*$^+$*)*	Soto *et al.* (2002)
TS-2	h^- *his7-366 wak1::ura4*$^+$ *sty1:Ha6H(ura4*$^+$*)*	Soto *et al.* (2002)
TS-3	h^- *his7-366 wis1::his7*$^+$ *sty1:Ha6H(ura4*$^+$*)*	Soto *et al.* (2002)
TS-4	h^- *his7-366 sty1::ura4*$^+$ *atf1:Ha6H(ura4*$^+$*)*	Soto *et al.* (2002)
MI900	h^+ *pyp2:13myc(KanMX6)*	A. Franco, National Institute for Medical Research, Mill Hill, London, UK
MI901	h^- *sty1::ura4pyp2:13myc(KanMX6)*	This work
TP319-13c	h^- *pmk1::ura4*$^+$	T. Toda, Cancer Research UK London Research Institute, UK
MI200	h^+ *pmk1:Ha6H(ura4*$^+$*)*	Madrid *et al.* (2006)
MI202	h^+ *mkh1::ura4*$^+$ *pmk1:Ha6H(ura4*$^+$*)*	Madrid *et al.* (2006)
MI203	h^+ *pek1::kanMX6 pmk1:Ha6H(ura4*$^+$*)*	Madrid *et al.* (2006)
MI204	h^+ *sty1::ura4*$^+$ *pmk1:Ha6H(ura4*$^+$*)*	Madrid *et al.* (2006)
PPG148	h^- *cdc25-22*	S. Moreno, Consejo Superior de Investigaciones Científicas, Salamanca, Spain

epitope comprising haemagglutinin antigen plus six histidine residues (Ha6H) was purified by using Ni^{2+}-NTA–agarose beads (Qiagen), as reported previously (Soto *et al.*, 2002). The purified proteins were resolved in 10 % SDS-PAGE gels, transferred to nitrocellulose filters (Amersham Biosciences) and incubated with either mouse anti-haemagglutinin (Ha) (clone 12CA5; Roche Molecular Biochemicals) or mouse anti-(phospho-p38) (Cell Signaling Technology) antibodies. The immunoreactive bands were revealed with an anti-mouse horseradish peroxidase-conjugated secondary antibody (Sigma) and the ECL system (Amersham Biosciences). For Atf1–Ha6H purification, the harvested cells were lysed into denaturing lysis buffer [6 M guanidine hydrochloride, 0.1 M sodium phosphate, 50 mM Tris/HCl (pH 8.0)] and the Atf1 protein was isolated by affinity precipitation on Ni^{2+}-NTA–agarose beads as described previously (Shiozaki & Russell, 1997). The purified proteins were resolved in 6 % SDS-PAGE gels, transferred to nitrocellulose filters (Amersham Biosciences) and incubated with a mouse anti-Ha antibody (12CA5). The immunoreactive bands were detected as described above. Ha6H-tagged Pmk1 was purified and resolved from cell extracts as described for Sty1. As reported earlier (Madrid *et al.*, 2006), the purified Pmk1–Ha6H fusion protein was assayed by Western blotting with mouse anti-Ha antibody (12CA5) for total Pmk1 protein or with a polyclonal anti-(phospho-p42/44) antibody (Cell Signaling Technology) to detect activated Pmk1 protein. Western blot analysis of the Pyp2–13myc fusion protein was performed with whole-cell extracts employing a mouse monoclonal anti-*c*-myc antibody (clone 9E10; Roche Molecular Biochemicals) and an anti-Cdc2 antibody (PSTAIRE; Sigma Chemical) as loading control.

RNA isolation and hybridization. Yeast cultures grown to an OD_{600} of 0.5–0.7 were either subjected to centrifugation or recovered by filtration and incubated at 28 °C. At different times, cells from 30 ml culture were collected and total RNA preparations were obtained essentially as described by Moreno *et al.* (1991) and resolved through 1.5 % agarose–formaldehyde gels. Northern (RNA) hybridization analyses were performed as described by Soto *et al.* (2002). Probes for *pyp2* and *leu1* were prepared as reported previously (Madrid *et al.*, 2004). *leu1* mRNA was employed as an internal standard for the RNA amount loaded in each lane.

Viability assays. Centrifuged and control cell samples were plated on YES agar plates and incubated at 28 °C for 3 days or assayed by the methylene blue test according to Arnold (1972). To study the effect of centrifugation on the development of thermotolerance, yeast cultures were centrifuged at 800 ***g*** for 5 min, incubated at 28 °C for 30 min and then subjected to a thermal stress by incubating at 48 °C. Cell samples collected at different times were diluted appropriately and spread in triplicate onto YES plates. The viability of the cells was measured by their ability to form colonies after incubation at 28 °C for 4 days. The survival fraction was calculated as a percentage relative to control samples that received no heat treatment.

Fluorescence microscopy. Cell fixation, calcofluor white staining of cell walls and septa, DAPI (4′,6-diamidino-2-phenylindole) staining of nuclei and rhodamine–phalloidin staining of F-actin were performed as described by Alfa *et al.* (1993). Images were taken on a Leica DM 4000B fluorescence microscope with a ×100 objective, captured with a cooled Leica DC 300F camera and IM50 software and then imported and processed with Adobe PhotoShop 6.0. Quantitative determinations of nuclei, septa and polarized actin involved the analysis of at least 300 cells per strain.

Reproducibility of results. All experiments were repeated at least three times with similar results. Representative results are shown.

RESULTS

Sty1 activation following centrifugation

Strain JM1521 of *S. pombe*, which harbours a genomic copy of *sty1* tagged with Ha6H (Table 1), was used to examine whether simulated gravity forces above the normal value elicited MAPK Sty1 activation. The Sty1–Ha6H protein was purified by affinity chromatography and its activation was assessed by Western immunoblotting with anti-(phospho-p38) antibodies. Duplicated samples were probed with an antibody against the Ha6H tag to normalize for protein content. The results shown in Fig. 1 not only confirm that hypergravity can be transduced to Sty1 phosphorylation (Shiozaki *et al.*, 1998), but also disclose the existence of a certain minimal threshold value of about 200 ***g***, above which the phosphorylation of Sty1 was induced up to a saturating value (Fig. 1a). For a given gravitational force, Sty1 activation was dependent on the time of centrifugation (Fig. 1b). The rate of Sty1 activation also increased when the temperature was raised in the centrifugation chamber, and shorter delays were then observed for the activation of Sty1 during centrifugation [compare Fig. 1(b) and (c)]. Moreover, the centrifugation-induced signal was transient and Sty1 activity decayed significantly after disappearance of the stressing stimulus (Fig. 1d).

Transduction of centrifugation stress through the SAPK pathway

We dissected the function of the various components of the SAPK signalling pathway in *S. pombe* to establish their involvement in the transduction of the centrifugation signal. Previous studies have shown that an early step in the response of *S. pombe* to mild oxidative stresses includes a two-component system regulated by histidine kinases Mak2 and Mak3 (Buck *et al.*, 2001). We explored the possibility that the same sensors were involved in the hypergravity-induced transduction pathway leading to activation of Sty1. However, phosphorylation of Sty1 occurred following centrifugation of mutant strains lacking Mak1, Mak2 or Mak3 and in double or triple mutants (not shown), indicating that the transduction mechanism is independent of this initial two-component system (Fig. 2a). In contrast, induction of the response was abolished completely in strain TS-1, which is disrupted in the response regulator Mcs4 (Fig. 2a). This upstream component of the SAPK pathway acts in the conserved phospho-relay system initiated by the histidine kinases and triggers the activation of the alternative MAPKKKs Wak1 and Win1 (Buck *et al.*, 2001). We also studied the role of MAPKKK Wak1 in the activation process after exposure of strain TS-2 (*wak1*Δ) to altered gravitational forces by centrifugation. In this case, Sty1 activation increased only slightly compared with the control strain (Fig. 2a), similar to previous results obtained under osmostress (Soto *et al.*, 2002), indicating that Wak1 is a key element in the regulation of Sty1 activation under hypergravity. However, the existence of a decreased but still detectable activation in the absence of Wak1 reveals that, to

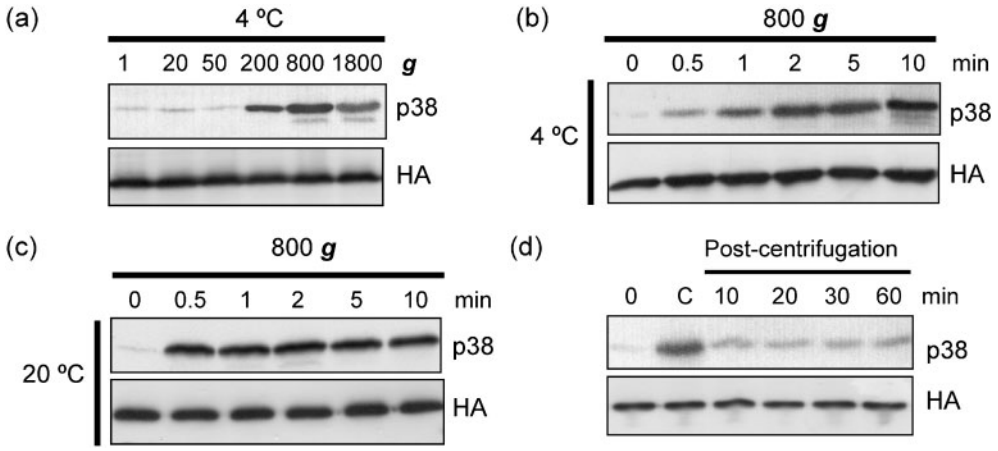

Fig. 1. Activation of MAPK Sty1 following centrifugation. Cells from control strain JM1521, carrying an Ha6H-tagged chromosomal version of the *sty1* gene, were analysed for Sty1 phosphorylation by immunoblotting with anti-(phospho-p38) antibodies as indicated in Methods after being centrifuged under different conditions: (a) cells subjected for 5 min to the indicated ***g*** values at 4 °C; (b) cells centrifuged for the indicated time intervals at 800 ***g*** and 4 °C; (c) cells centrifuged as above, but at 20 °C; (d) cells centrifuged at 800 ***g*** (time C) for 10 min at 4 °C, maintained thereafter at 28 °C for the time indicated before being processed for Sty1 analysis. Lanes labelled as 1 ***g*** in (a) and 0 min in (b), (c) and (d) correspond to control, non-centrifuged cells recovered by filtration.

some extent, the alternative MAPKKK Win1 probably transmits the signal to the Wis1–Sty1 cascade in a branched pathway (Fig. 2a). Moreover, as for other stresses (Shiozaki & Russell, 1995), the function of MAPKK Wis1 is critical to ensure Sty1 activation, as *wis1*-disrupted strain TS-3 revealed no phosphorylation of Sty1 by centrifugation-induced gravity forces, as opposed to control strain JM1521 (Fig. 2a).

Sty1-dependent phosphorylation of Atf1 occurs during centrifugation stress

The transcription factor Atf1 functions downstream of the SAPK phosphorylation cascade and its phosphorylation by MAPK Sty1 under different stress conditions induces the expression of several stress-responsive genes (Soto *et al.*, 2002). The sensitivity of Sty1 to hypergravity prompted us to explore whether the phosphorylation status of Atf1 changed under increased gravitational forces. In this regard, it is relevant to mention that Atf1 of unstressed cells migrates in polyacrylamide gels as a single protein band of approximately 85 kDa that undergoes a specific Sty1-dependent band shift due to phosphorylation under different stresses (Soto *et al.*, 2002). As shown in Fig. 2(b), centrifugation of cells from strain JM1821, with Ha6H-tagged Atf1 (Table 1), resulted in Atf1 phosphorylation. However, Atf1 purified from the *sty1*-disrupted strain TS-4 subjected to similar conditions migrated unchanged, corresponding to the unphosphorylated form (Fig. 2b). These results indicate clearly that Sty1 is the only MAPK that phosphorylates Atf1 *in vivo* during hypergravity conditions.

The expression of genes regulated by Atf1 in cells exposed to centrifugation was also examined. We performed Northern and Western blot analyses for *pyp2*, which is a representative Atf1 target gene. As indicated in Fig. 2(c), transcription of *pyp2* increased in wild-type cells on centrifugation, whereas it was blocked in *sty1*Δ cells. Similarly, the level of a Pyp2–12myc fusion protein was enhanced upon centrifugation and this increase was dependent on Sty1 function (Fig. 2d)

Transduction of centrifugation stress through the Pmk1 cell-integrity pathway

MAPK Pmk1 has been involved in cell-wall construction, morphogenesis, cytokinesis and ion homeostasis (Zaitsevskaya-Carter & Cooper, 1997). More recently, it has been shown that Pmk1 is activated in multiple stress situations, including hypo- or hypertonic stress, glucose deprivation, presence of cell wall-damaging compounds and oxidative stress, leading to the suggestion that this pathway might reinforce the SAPK signalling pathway in the control of survival and adaptation to sublethal stressing conditions (Madrid *et al.*, 2006). We therefore examined the possibility of activation of the MAPK Pmk1 pathway by increased centrifugation forces. In these experiments, we used strain MI200, which harbours a genomic copy of *pmk1* tagged with Ha6H (Table 1). The purified Pmk1–Ha6H fusion protein was assayed by Western blotting with a polyclonal anti-(phospho-p42/44) antibody to detect activation of the Pmk1 protein (Madrid *et al.*, 2006). Fig. 3(a) illustrates that, above a certain value, which was roughly coincident with the threshold for activation of the SAPK pathway, the phosphorylation of Pmk1 was also dependent on the effective centrifugation force. Moreover, under a constant centrifuge-induced artificial gravity, Pmk1 activation was

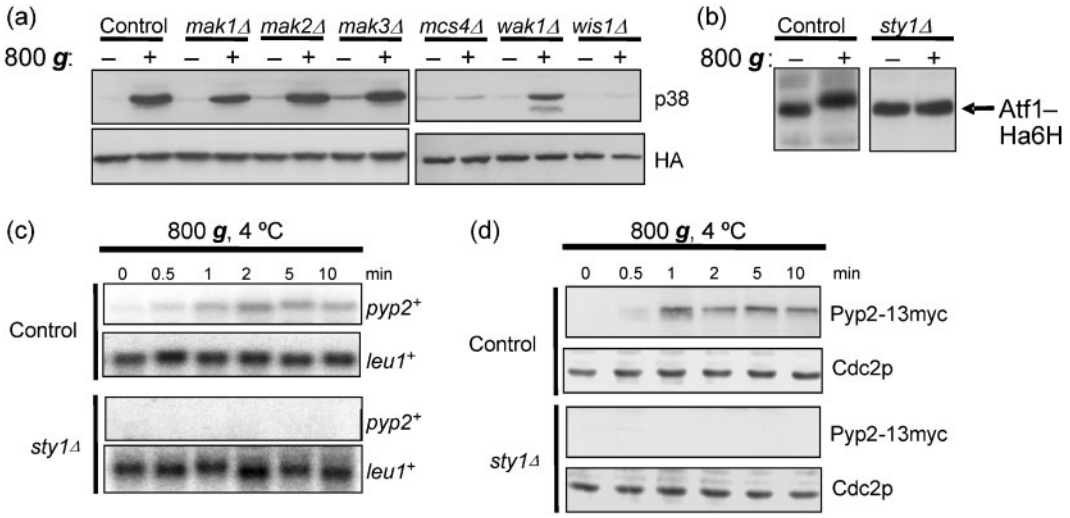

Fig. 2. Elements of the SAPK pathway involved in transduction of the centrifugation signal. (a) Cells from control strain JM1521 and from strains VB1828 (*mak1*Δ), VB1829 (*mak2*Δ), VB1934 (*mak3*Δ), TS-1 (*mcs4*Δ), TS-2 (*wak1*Δ) and TS-3 (*wis1*Δ), carrying an Ha6H-tagged chromosomal version of the *sty1* gene, were centrifuged at 800 ***g*** for 5 min at 4 °C. Sty1 was purified by affinity chromatography and activated Sty1 was detected by immunoblotting with anti-(phospho-p38) antibodies. To determine loaded Sty1, immunoblotting with anti-Ha antibodies was used. (b) Sty1-dependent phosphorylation of the transcription factor Atf1 following centrifugation. Cells from control strain JM1821 and strain TS-4 (*sty1*Δ) carrying a chromosomal copy of the Ha6H-tagged *atf1* gene were recovered either by filtration (–) or after centrifugation at 800 ***g*** for 10 min (+). The purified Atf1–Ha6H protein was analysed by SDS-PAGE followed by immunoblotting with anti-Ha antibodies. Centrifuged control cells showed a typical Atf1 shift that was absent in filtered control cells and in *sty1*Δ cells. (c) *pyp2* expression under centrifugation. Strains JM1521 (control) and TS-4 (Δ*sty1*) were grown at 28 °C in YES medium to mid-exponential phase and subjected to centrifugation at 800 ***g*** for the times indicated. Total RNA was extracted, denatured, transferred to nylon membranes and hybridized with ^{32}P-labelled probes for the *pyp2* and *leu1* genes. (d) Pyp2 protein levels. Cells from strains MI900 (control) and MI901 (*sty1*Δ), carrying a 13myc-tagged chromosomal version of the *pyp2* gene, were centrifuged at 800 ***g*** at 4 °C. At different times, cells were collected and Pyp2 was detected by immunoblotting with anti-*c*-myc antibodies. Anti-Cdc2 antibody was used as a loading control.

related to the temporal length of the stressing stimuli (Fig. 3b). On the other hand, the transmission of the gravity-stress signal through the Pmk1 cell-integrity pathway required the function of both MAPKKK Mkh1 and MAPKK Pek1, suggesting a linear transduction course (Fig. 3c). Activation of Pmk1 induced by centrifugation was also observed in the absence of Sty1 (Fig. 3c). As MAPK Sty1 negatively controls the basal level of MAPK Pmk1 activation (Madrid *et al.*, 2006), *sty1*-deleted cells were characterized by a comparatively high phosphorylation level in the absence of gravitational stimulus (zero time).

Contribution of gravitational and shearing forces to activation of MAPK Sty1 and Pmk1

Shear and frictional forces may contribute significantly to the total effect acting on cells during centrifugation (van Loon *et al.*, 2004). To interpret the significance of the above results better, experiments were performed by centrifuging cell samples in the absence of liquid medium to minimize induced shear. For this purpose, cells were recovered by filtration and the resulting filters were introduced into test tubes that were then centrifuged. Under these conditions, Sty1 became phosphorylated, although to a lower extent than in cells centrifuged in liquid medium (Fig. 4). These results suggest that hypergravity is in fact a main component of the centrifugation stress that activates Sty1, although it is not exclusive, as shear forces appear to contribute to such activation. Notably, however, we were unable to show clear activation of Pmk1 in filter-supported cells under the same experimental design (Fig. 4, centrifuged pellet). Hence, in contrast to Sty1, activation of Pmk1 during centrifugation in liquid media appears to be mostly due to induced inertial forces other than hypergravity.

Viability in response to hypergravity and sensitivity of centrifuged cells to a heat shock

We analysed the viability of *S. pombe* cells in response to hypergravity and whether Sty1 plays a role in such survival. As indicated in Fig. 5(a), wild-type cells showed a similar ability to grow before and after centrifugation, and this result was not altered by the absence of Sty1 or Pmk1.

In many instances, yeast cells subjected to one type of sublethal stress acquire enhanced tolerance to other stresses (Attfield, 1997). This overlapping cross-effect (the so-called

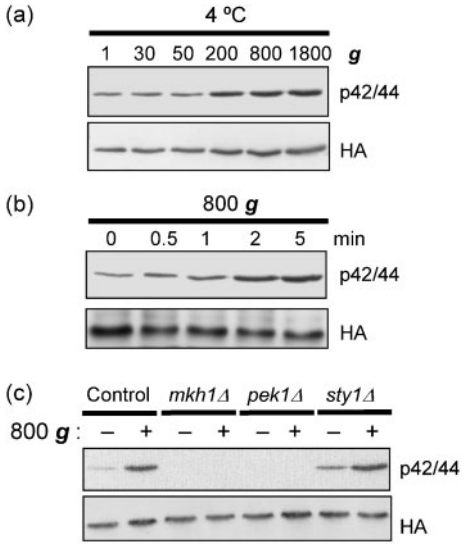

Fig. 3. Transduction of the centrifugation signal through the Mkh1–Pek1–Pmk1 cell-integrity pathway. (a) Control strain MI200, carrying an Ha6H-tagged chromosomal version of *pmk1*, was subjected to centrifugation in YES medium at the indicated ***g*** values for 5 min at 4 °C. Pmk1–Ha6H was purified by affinity chromatography. Activated Pmk1 was detected by immunoblotting with anti-(phosho-p42/44) antibody and total Pmk1 was detected with anti-Ha antibody as a loading control. (b) Cells were centrifuged at 800 ***g*** for the indicated time at 4 °C and processed as indicated above. (c) Centrifugation-induced activation of Pmk1 is channelled through MAPKKK Mkh1 and MAPKK Pek1. Cells from strains MI200 (control), MI202 (*mkh1*Δ), MI203 (*pek1*Δ) and MI204 (*sty1*Δ) growing in YES medium were recovered by either filtration (–) or centrifugation at 800 ***g*** for 5 min (+). Pmk1 was purified by affinity chromatography and activated and total Pmk1 was detected as indicated above.

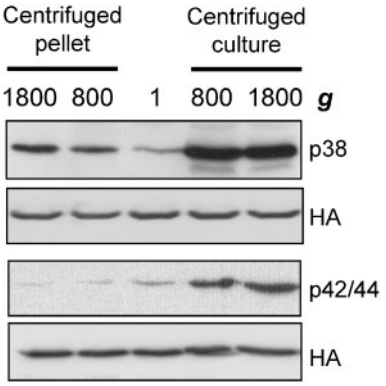

Fig. 4. Differential activation of Sty1 and Pmk1 by centrifugation of cells in filter or liquid medium. Cells from strains JM1521 (Ha6H-tagged Sty1) and MI200 (Ha6H-tagged Pmk1) were grown at 28 °C in YES medium to mid-exponential phase. Aliquots of cells were recovered in both cases by filtration on Millipore 0.45 μm HA nitrocellulose membrane filters and the filters were then centrifuged at either 800 or 1800 ***g*** for 10 min at 4 °C or kept as a non-centrifuged control (1 ***g***). In parallel, equivalent volumes of liquid cell cultures were centrifuged as above. Activated Sty1 or Pmk1 was detected by immunoblotting using anti-(phospho-p38) or anti-(phospho-p42/44) antibodies, respectively, and total Sty1 and Pmk1 was detected by immunoblotting with anti-Ha antibodies as indicated in Methods.

general stress response) is caused by the concerted expression of multiple Atf1-dependent stress-related genes whose induction may be triggered by different stressors. We compared the survival rate of both control and centrifugation-treated cells after a thermal upshift to 48 °C for different times by measuring their ability to grow in solid medium or to reduce the methylene blue dye. The assays were performed at different post-centrifugation intervals to allow for the expression of possible centrifugation-induced protective genes. In either case, no significant differences in heat tolerance were found (Fig. 5b). These results support the hypothesis that centrifugation treatment does not confer any gross adaptive advantage to *S. pombe* cells confronted with unrelated stimuli.

Centrifugation induces a delay at the G_2/M transition

Eukaryotic cells arrest the cell cycle in response to different environmental stresses. For example, *S. pombe* cells undergo a G_2/M delay after a hyperosmotic shock (Rupes *et al.*, 1999). To test whether centrifugation induces a similar effect on the cell cycle, *cdc25-22* cells (strain PPG148) were grown at 25 °C to exponential phase and shifted to 37 °C for 3.5 h to synchronize the cells in G_2. Once arrested, the cells were either centrifuged at 200 ***g*** for 5 min or incubated without centrifugation for the same period, before being placed at the permissive temperature of 25 °C. Finally, we estimated the emergence of binucleated and septated cells in aliquots taken at different times. As shown in Fig. 6(a), compared with untreated cells, centrifugation induced a modest but reproducible delay of approximately 15 min in the commitment to mitosis (and consequently cell separation). The arrest was clearly smaller than that observed in cultures treated with 0.6 M KCl, which is included in Fig. 6(b) for comparative purposes. The extent of the delay in the commitment of mitosis after centrifugation did not increase when higher centrifugation forces (800 ***g***) were used, suggesting a low saturation level for this effect. These results confirm that cell perturbation induced by centrifugation promotes a transient cell-cycle arrest in *S. pombe*, even at low ***g*** forces.

Actin depolarization induced by centrifugation is a consequence of Sty1 activation

It has recently been reported that centrifugation of *S. pombe* cells at moderate ***g*** forces (2900 ***g***) promotes actin depolarization from cell tips and a block in cell growth. However, this effect appears to be transient, and resumption

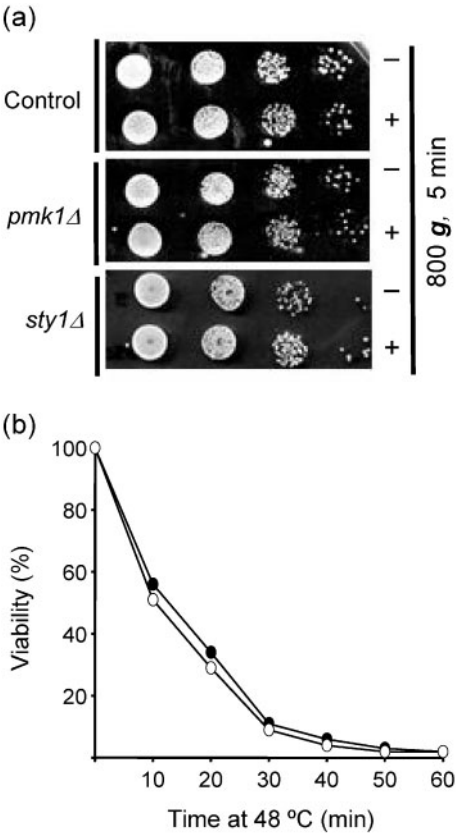

Fig. 5. Centrifugation does not affect cell viability or cross-protection against thermal stress. (a) Strains JM1521 (control), TS-4 (*sty1*Δ) and TP319-13c (*pmk1*Δ) were grown in YES medium to an A_{600} of 0.5 and either centrifuged at 800 ***g*** for 5 min or left untreated. Finally, samples containing 10^5, 10^4, 10^3 or 10^2 cells were spotted onto YES plates and incubated for 3 days at 28 °C before being photographed. (b) Yeast cultures from strain JM1521 were either centrifuged at 800 ***g*** for 5 min (○) or left untreated (●). Both cultures were incubated at 28 °C for 30 min and subjected to a thermal stress by incubating at 48 °C. Cell samples collected at different times were diluted appropriately and spread in triplicate onto YES plates. The survival fraction was calculated as a percentage relative to control samples that received no heat treatment.

of tip growth after stress is promoted by phosporylation at Ser402 of polo kinase Plo1 (Petersen & Hagan, 2005). Such work suggests that actin depolarization after stress might be due to Sty1 activation. To test the validity of this suggestion, we first analysed the kinetics of actin depolarization/repolarization in cells from strain JM1521 centrifuged for 5 min at 200 ***g***, which is the lowest centrifugal force known to promote significant Sty1 activation (Fig. 1). Under these conditions, the percentage of cells with polarized actin dropped quickly 10 min after centrifugation, and the return of actin patches to the cell poles was almost complete after 30–40 min (Fig. 7a, b). The actin depolarization induced by centrifugation was more evident in cells subjected to higher ***g*** forces (2900 ***g***; Fig. 7a, b). If the remodelling of the actin cytoskeleton elicited by centrifugation was a consequence of Sty1 activation, cells from a *sty1*-deleted strain should be defective in actin depolarization after centrifugation. This prediction appears to be correct, as the percentage of cells with polarized actin from *sty1*Δ strain TS4 subjected to either 200 ***g*** (not shown) or 2900 ***g*** did not change significantly after centrifugation (Fig. 7a, b). Hence, Sty1 activation mediated by centrifugation promotes actin depolarization in *S. pombe*. When actin localization was studied in *atf1*Δ mutant cells upon centrifugation, the results indicated that, unlike *sty1*Δ cells, those lacking Atf1 function still showed actin depolarization (Fig. 7a, b). This suggests that activated Sty1 regulates actin organization independently of the Atf1 transcription factor. Similarly, the contribution of Pmk1 to actin depolarization was studied in *pmk1*Δ cells and, in contrast to *sty1*Δ cells, they showed a wild-type pattern of actin depolarization after centrifugation (not shown).

DISCUSSION

Gravity has shaped life on Earth. In the course of evolution, specialized gravireceptory cells have appeared in many multicellular organisms (Monshausen & Sievers, 2002; Lai *et al.*, 2006). However, single cells, devoid of specialized organs, are also able to sense altered gravity forces. The experimental amenability of yeasts favours investigation of the basics of how gravity-induced signals are converted into molecular and biochemical inputs. In particular, we have focused on the fission yeast *S. pombe* to clarify whether this unicellular system could represent a 'minimal system' able to sense alterations in this physical force. We have shown the existence of centrifugation-induced signal-transduction pathways and explored the effects of their activation under gravitational stress. Transduction of centrifugation-induced signals appears to involve at least two different pathways, the SAPK and cell-integrity pathways.

Several studies during space flights and simulated microgravity conditions have suggested that low gravity affects proliferation and differentiation of both eukaryotic and prokaryotic cells (Zayzafoon *et al.*, 2005). In contrast, the effects of the reverse situation, i.e. hypergravity, are scarcely known, despite the fact that centrifugation procedures are often used in biological studies. Centrifugation forces developed in swinging-bucket rotors whose axes are parallel to the normal gravity vector simulate high-gravity fields. In *S. pombe*, such conditions promote a markedly elevated phosphorylation of the MAPKs Sty1 and Pmk1, provided that a minimal response threshold is exceeded. This threshold explains the absence of gravity-induced effects on cells during the incubation of flask cultures in conventional, non-reciprocal gyrorotatory shakers. As judged from the basal phosphorylation values of the MAPKs Sty1 and Pmk1 obtained in our experiments, standard gyrorotatory shakers only produce a gravity-like mechanical signal whose intensity is well below that required for the cellular signal-response chain of gravity perception.

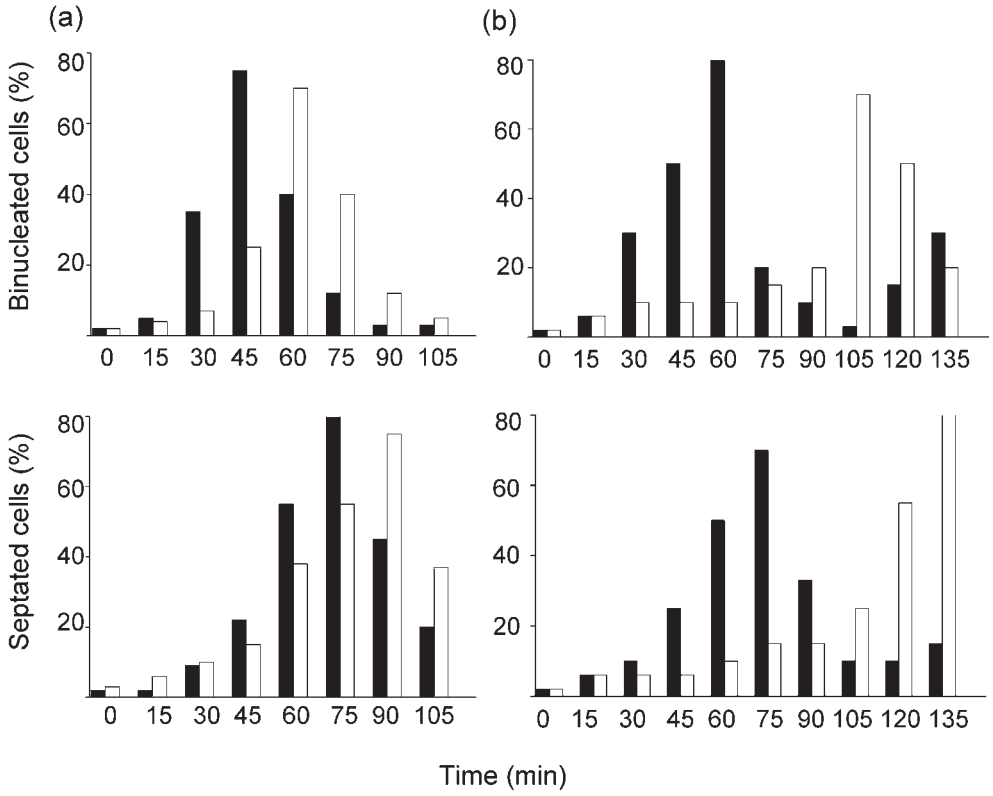

Fig. 6. Centrifugation induces a delay in the cell-division cycle. (a) Cells of strain PPG148 (*cdc25-22*) were arrested at 37 °C and, before being placed at the permissive temperature for growth (25 °C), they were either centrifuged at 200 ***g*** for 5 min (empty bars) or membrane-filtered and incubated without centrifugation for the same time period (filled bars). Binucleated and septated cells were determined by staining in aliquots taken at different times. (b) For comparison, the arrested cells of strain PPG148 were placed at 25 °C and the cultures were left untreated (filled bars) or treated with 0.6 M KCl (empty bars). In all cases, a minimum of 300 cells per sample was screened.

Although the intensity of the phosphorylation signal prompted by centrifugation appears stronger in MAPK Sty1 than in MAPK Pmk1, this does not imply that the stress signal is transmitted preferentially via the SAPK pathway relative to the cell-integrity pathway. Western immunoblot assays for phosphorylated Sty1 and Pmk1 were performed, using two different antibodies. Consequently, the sensitivity of our experimental analyses reflects different affinities towards phosphorylated Thr and Tyr residues within the respective proteins, invalidating direct quantitative comparisons based on the intensity of the blot reactions.

There are conflicting results about the effect of centrifugation on the survival of yeast cells. Some reports indicate that yeasts do not lose viability and are scarcely damaged during centrifugation at high speed (Nakamura & Schlenk, 1973), whereas others show a decreased ability for colony formation after artificial hypergravitational stress by long-term centrifugation (Yoshida *et al.*, 1999). Although our experiments were performed at comparatively lower ***g*** values, we did not find any significant difference in viability between centrifuged and control samples, regardless of the method for cell-survival determination.

Mechanical forces of fluid-shear stress and gravitational forces are different physical entities, and the use of centrifugation as a tool to generate gravity-like accelerations may subject the samples to unwanted inertial forces (Nickerson *et al.*, 2004). Hence, it could be argued that the activation of Sty1 during centrifugation was due only to associated stresses produced in liquid media, such as local heating or shearing forces. However, several observations suggest that this is unlikely. First, the basal level of phosphorylation of Sty1 and Pmk1 is almost undetectable in cells cultured at 28 °C, a temperature value that is never exceeded under temperature-controlled centrifugations. Thus, although the gravitational effect on cells may show dependence on thermal conditions, like other metabolic responses, it does not seem to be caused by increases in temperature. Second, the stimulation of MAPK Sty1, but not that of Pmk1, is still observed by centrifugation of cells supported on filters, i.e. when the linear displacement of cells along fluid columns is virtually nil. This suggests that an increased gravity force appears to be, by itself, a main component of the signal responsible for the activation of the SAPK transduction pathway, irrespective of the contribution of additional stress forces. An intriguing conclusion of

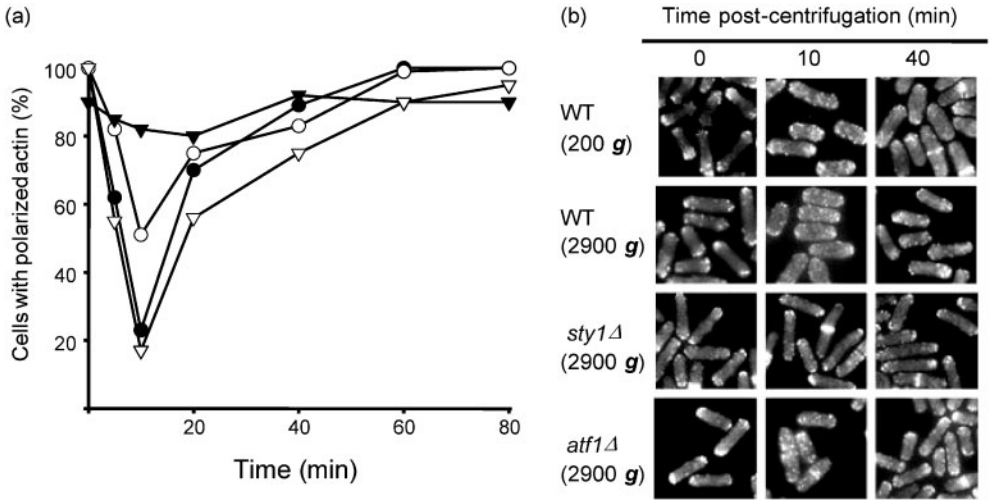

Fig. 7. Effect of centrifugation on actin depolarization is mediated by Sty1 function. (a) Mid exponential-phase cells from strains JM1521 (control), TS-4 (*sty1*Δ) and NT146 (*atf1*Δ) were centrifuged at 200 or 2900 ***g*** for 5 min in YES medium and cultured again in the same medium. After centrifugation, the cells were fixed and stained with rhodamine–phalloidin at timed intervals for localization of polarized actin patches. At least 300 non-mitotic cells were screened per sample. Symbols: ○, JM1521 cells after 200 ***g***; ●, JM1521 cells after 2900 ***g***; ▼, TS-4 cells after 2900 ***g***; ▽, NT146 cells after 2900 ***g***. (b) Localization of actin by fluorescence microscopy in control (wt), *sty1*Δ and *atf1*Δ cells at several times after centrifugation at 200 or 2900 ***g*** for 5 min at 4 °C.

this study is that mechanisms different from those that account for the activation of MAPK Sty1 (a combination of increased gravitational force plus shearing) operate for the activation of MAPK Pmk1 during centrifugation. Activation of the cell-integrity pathway is triggered by centrifugation only in liquid medium, suggesting that Pmk1 phosphorylation results exclusively from the effect of shearing forces associated with the centrifugation process. Whether the fission yeast funnels the centrifugation signal into the cell-integrity pathway as a friction-induced input or as a mechanical stimulus resulting from stretching deformations of the cell wall or plasma membrane is at present unknown.

For simplicity, the centrifugation signalling pathways can be separated into three sequential steps: perception of gravity or associated forces, signal transduction and cell response. As outlined above, the sensing mechanisms initiating transduction of the signals in fission yeast are unknown. In the case of the SAPK pathway, our results rule out the participation of histidine kinases Mak1–3 in early molecular steps. Activation of this pathway also entails phosphorylation of the transcription factor Atf1, and we have determined that there is hypergravity-inducible gene expression in *S. pombe*. Enhanced transcription of Atf1-dependent genes occurs under various physical and chemical stresses and underlies the general stress response, so that it is not uncommon for yeasts to exhibit some degree of crosstalk in response to different environmental stimuli (Attfield, 1997). Although centrifugation activates signal-transduction systems involved in the transcriptional control of cross-protection, we have been unable to detect any gross adaptive phenotypic alteration after centrifugation, suggesting that activation of these systems may be necessary, but not sufficient, for induction of protection. This situation is strikingly reminiscent of what has been described following activation of the p53 signalling pathway induced by hypergravity in human cell lines (Okaichi *et al.*, 2004).

There have been suggestions that mechanical and physical forces may be sensed initially as local deformations or perturbations of the cell surface and that mechanosensitive systems might promote changes in cell-signalling pathways, which are converted subsequently into molecular responses (Nickerson *et al.*, 2004; Ingber, 2006). Another different view is that the triggering factor results from the intracellular polarized displacement of some cell components under high-gravity fields, by analogy with what happens in statenchyma cells of higher plants and the otolith-related cells of animals (Monshausen & Sievers, 2002; Lai *et al.*, 2006). Indeed, subcellular organelles move inside *S. pombe* along the hypergravity-force vector because of their differential density, thus affecting cell behaviour (Daga & Chang, 2005). Irrespective of the nature of the sensors, the impact of altered gravity is probably related closely to the functional state of membranes and contractible elements of the cytoskeleton (Lewis *et al.*, 1998; Tabony *et al.*, 2001). The involvement of these cell components may be critical in the processes of reception and realization of gravitational stimuli, as demonstrated by the noticeable alterations in the molecular organization of membranes and cytoskeleton under altered gravity conditions on a variety of cells (Goldermann & Hanke, 2001; Tairbekov, 2004). In this

respect, our results are consistent with the observation that application of centrifugation to *S. pombe* cells perturbs the pattern of actin patches in the cell tips and blocks cell growth temporarily (Petersen & Hagan, 2005). Notably, we show that the displacement of actin from the tip of these cells during centrifugation is Sty1-dependent, thus providing an additional function for this kinase and the SAPK pathway. As a whole, the above results support the hypothesis that *S. pombe* may be a powerful genetic model that is useful in the dissection of the molecular mechanisms of gravity perception and response.

ACKNOWLEDGEMENTS

A. N. and M. M. are predoctoral fellows from Fundación Séneca (Región de Murcia) and Ministerio de Educación, Cultura y Deporte (FPU), respectively. This work was supported by grants BFU2005-01401/BMC from MEC and 00475/PI/04 from Fundación Séneca, Spain.

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Edited by: M. Molina