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A conserved small GTP-binding protein Alp41 is essential for the cofactor-dependent biogenesis of microtubules in fission yeast

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Abstract The proper folding of tubulins and their incorporation into microtubules consist of a series of reactions, in which evolutionarily conserved proteins, cofactors A to E, play a vital role. We have cloned a fission yeast gene (*alp41*$^+$) which encodes a highly conserved small GTP-binding protein homologous to budding yeast *CIN4* and human ARF-like *Arl2*. *alp41*$^+$ is essential, disruption of which results in microtubule dysfunction and growth polarity defects. Genetic analysis indicates that Alp41 plays a crucial role in the cofactor-dependent pathway, in which it functions upstream of the cofactor D homologue Alp1^D and possibly in concert with Alp21^E.

Key words: ARF-like protein; Cell polarity; Cofactor; GTP-binding protein; Microtubule; Fission yeast

1. Introduction

Microtubules play a pivotal role in a wide variety of cellular processes in all eukaryotes. Microtubules are dynamic structures whose behaviour is influenced by the cell cycle and developmental stage. A number of proteins, including MAPs (microtubule-associated proteins) and molecular motors, are known to regulate this dynamism. A group of further regulators, called cofactors, are involved in microtubule biogenesis. In vertebrates, cofactors were first identified biochemically as activities required for the production of assembly competent heterodimers of α-/β-tubulins in vitro [1–4]. Molecular cloning of these cofactors has revealed that cofactors comprise multiple proteins, consisting of A, B, C, D and E, which are evolutionarily conserved from human to yeast [5–8].

Biochemical analysis performed using purified proteins in vitro led to the proposal that cofactors operate in a pathway comprising two symmetrical branches. After release from the chaperonin complex [9,10], α-tubulins are captured by cofactor B, whilst β-tubulins are captured by cofactor A, which subsequently are replaced by cofactors E and D, respectively. The two pathways (α-tubulin/E and β-tubulin/D) then converge. Finally, cofactor C binds the complex and upon GTP hydrolysis assembly-competent α-/β-tubulin heterodimers are generated [11].

In yeast, both *Saccharomyces cerevisiae* and *Schizosaccharomyces pombe*, genes for cofactor homologues are conserved (except for cofactor C) and have been shown to be important for microtubule biogenesis in vivo ([8,12–21]). Unlike budding yeast in which microtubules can assemble in the absence of cofactors and as a result cofactor homologues are non-essential, in fission yeast, these homologues (Alp11^B, Alp1^D and Alp21^E) are absolutely required for cell viability and microtubule biogenesis [18,20,21]. These proteins constitute a functional cascade in that the pathway functions in a sequential manner (Alp11^B–Alp21^E–Alp1^D) and they are mainly involved in the α-tubulin pathway [21]. However, the cofactor A homologue Alp31^A, proposed to act in the β-tubulin pathway, is dispensable (Radcliffe and Toda, unpublished results).

In budding yeast, the *CIN4* gene, encoding a putative small GTP-binding protein, was identified in genetic screen for mutants defective in microtubule function [12,13]. As described above, despite the differing requirement for cofactor homologues, gene products involved in microtubule biogenesis are conserved between these two yeasts. Here we have cloned the *CIN4* homologue from fission yeast (called *alp41*$^+$, see below). *alp41*$^+$/*CIN4* homologues exist in all eukaryotes. Gene disruption shows that *alp41*$^+$ is essential for cell viability. Genetic analysis indicates that Alp41 plays an essential role in the cofactor-dependent α-tubulin pathway, upstream of Alp1^D.

2. Materials and methods

2.1. Strains, media, genetic methods and nomenclatures

Strains used are HM123 (*h*$^-$*leu1*), DH924 (*h*$^-$*leu1alp11-924*), PR9 (*h*$^-$*leu1ura4his7ade6alp11::ura4*$^+$), DH2-8D (*h*$^-$*leu1alp1-1315* [21]), diploid heterozygous for *alp1*$^+$ (ME1 [18]), *alp11*$^+$, *alp21*$^+$ (PR7 and PR8, respectively [21]) or *alp41*$^+$ (PR29) and *alp41*-deleted haploid suppressed by multicopy plasmids containing *alp1*$^+$ {PR30, *h*$^-$*leu1ura4his7ade6alp41::kanr* containing pDB(*alp1*$^+$)}. YPD (2% dextrose, 2% polypeptone and 1% yeast extract) and YE5S were used as rich media. The standard methods were followed as described [22]. Gene deletions are abbreviated as the gene preceded by Δ such as Δ*alp41*. Resistance to G418 is shown as kan^r.

2.2. Nucleic acid preparation and manipulation

Standard molecular biology techniques were followed as described [23]. Enzymes were used as recommended by the suppliers (New England Biolabs). Nucleotide sequence data reported in this paper are in the DDBJ/EMBL/GenBank databases under accession number AB031326 (*alp41*$^+$).

2.3. Gene disruption

The *alp41*$^+$ gene was deleted using PCR-generated fragments [24]. Dissection of asci from heterozygous diploid cells (PR29) showed that *alp41*$^+$ is an essential gene in which only two spores from one tetrad formed colonies and viable colonies were sensitive to G418.

2.4. Overexpression of the *alp41*$^+$ gene

The entire ORF of the *alp41*$^+$ gene was cloned by PCR into pREP1 under control of the *nmt1* promoter [25], yielding pREP-*alp41*$^+$. Oli-

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gonucleotides used are PO12 (TATGCATATGGGATTATTGAC-TATTTTGAG) and PO13 (TATGGGATCCTTAATAATCAATA-GTTCCCAAC). pREP-*alp41*$^+$ is functional as it suppresses an *alp41* deletion strain.

2.5. Cross-suppression experiments

Diploids heterozygous for *alp1*$^+$ (ME1), *alp11*$^+$ (PR7), *alp21*$^+$ (PR8) or *alp41*$^+$ (PR29) were transformed with multicopy plasmids containing *alp1*$^+$ (pAL100 and pREP-*alp1*$^+$ [18]), *alp11*$^+$ (pREP-*alp11*$^+$), *alp21*$^+$ (pREP-*alp21*$^+$ [21]) or *alp41*$^+$ (pREP-*alp41*$^+$). Leu$^+$ transformants were allowed to sporulate and free spores were directly plated on minimal plates supplemented with adenine and histidine with or without uracil and thiamine (ME1, PR7 and PR8) or on rich YE5S plates containing G418 (100 μg/ml, PR29). If Ura$^+$Leu$^+$ or kan^{r}Leu$^+$ haploid colonies were obtained, in which plasmids (Leu$^+$ prototrophy) were mitotically stable, this plasmid was assigned to be capable of suppressing deletion of the gene of interest. Temperature-sensitive mutants (*alp11-924*, DH924; Δ*alp11*,PR9; and *alp1-1315*, DH2-8D) were also used.

2.6. Indirect immunofluorescence microscopy

Cells were fixed with methanol and primary antibodies (TAT-1, provided by Dr K. Gull) applied, followed by Cy3-conjugated goat anti-rabbit IgG (Sigma).

3. Results and discussion

3.1. alp41$^+$ *encodes a novel GTP-binding protein*

A homology search using budding yeast Cin4 as a query against the *S. pombe* genome database revealed that fission yeast contains one ORF which encodes a protein homologous to this protein (37% identity and 57% similarity, Fig. 1A). The corresponding gene was designated *alp41*$^+$ (altered polarity 41, as the gene is involved in growth polarity control, see below). Alp41 consists of 186 amino acid residues and contain hallmarks for GTP-binding motifs. These include GLDHAGK, WDIGGQ, and NKQD (consensus amino acid residues are underlined and in Fig. 1A these amino acids are marked with asterisks). It is also of note that the position 2 glycine, the site of *N*-myristoylation, is conserved.

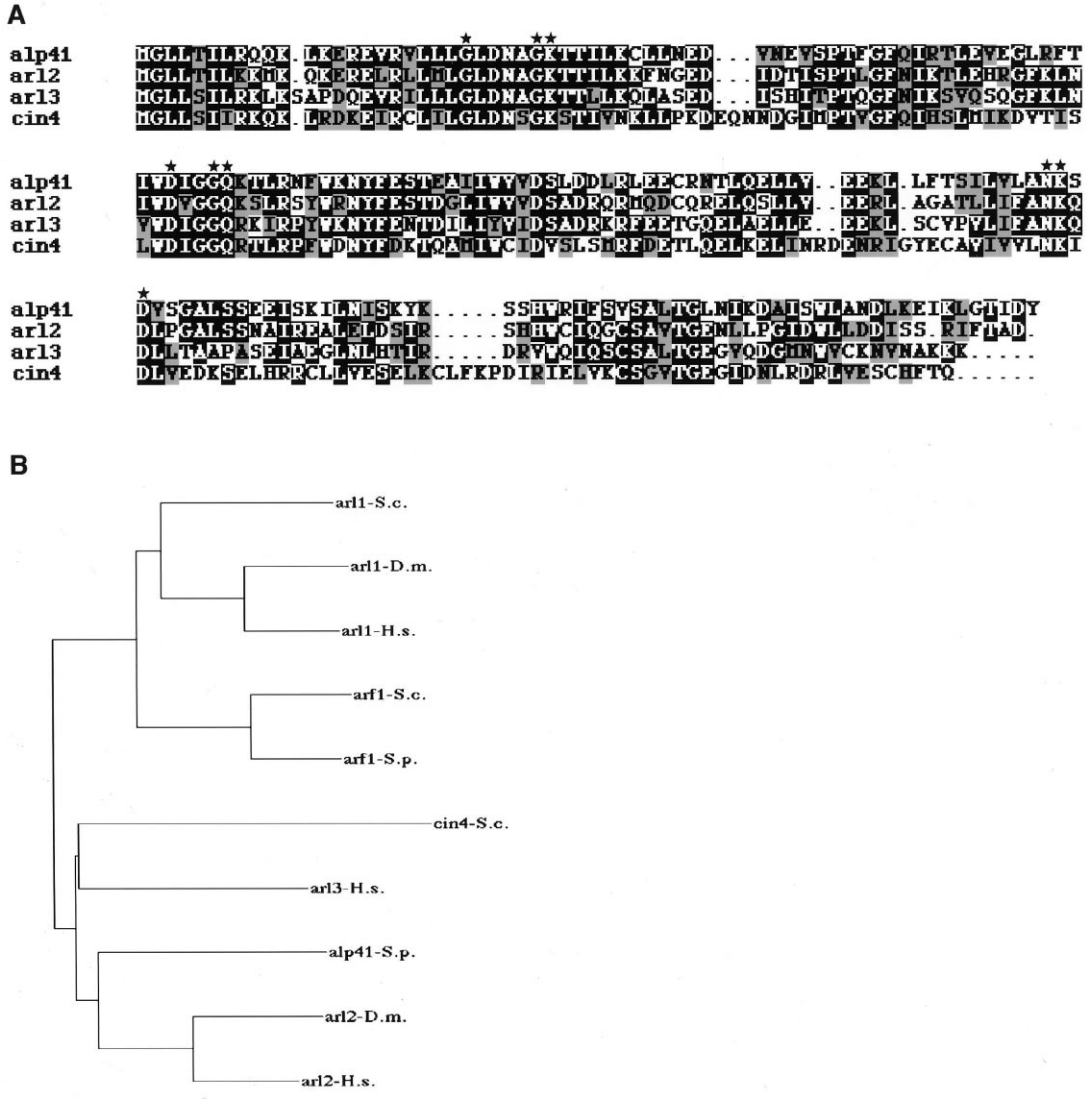

Fig. 1. Alp41 is a homologue of human Arl2 and budding yeast Cin4. A: Comparison of amino acid sequence of fission yeast Alp41, human Arl2 and Arl3 and budding yeast Cin4. Protein sequences were aligned using the CLUSTAL program. Amino acid residues, either identical (black) or conservative (grey), are emphasised. B: Evolutionary phylogeny of various small GTP-binding proteins (ARF and Arl families). Arl1 from human (H. s., accession number L28997), fly (D. m., M61127) and budding yeast (S. c., L28997), Arl2 from human (L13687) and fly (L14923), Cin4 from budding yeast (L36669), Arl3 from human (accession number U071517) and Arf1 from budding yeast (Z74240) and fission yeast (S. p., L09551).

3.2. *Alp41 is evolutionarily conserved and a homologue of metazoan Arl2*

Amino acid comparison between Alp41 and known low molecular weight GTP-binding proteins shows that Alp41 is most homologous to vertebrate Arl2 (52% identity and 73% similarity [26]). Arl represents a group of proteins which belong to the ADP-ribosylation factor (ARF)-like proteins (40% identical to the ARF proteins [27]). The ARF families, originally identified as cofactors for cholera toxin-catalysed ADP-ribosylation of the stimulatory regulatory component of adenylyl cyclase, $G_{S\alpha}$ [28,29], are known to be involved in several distinct steps of intracellular vesicular transport. Unlike the ARF families, the Arl proteins lack ADP-ribosylation activity and are thought to be involved in physiological processes, albeit currently unknown, different from those of ARF [30].

Fig. 1B shows evolutional phylogeny of the ARF and Arl families in various organisms. In addition to Alp41, fission yeast contains homologues for ARF1. It has been shown that both *Drosophila* and human *arl2* genes are expressed ubiquitously in all the tissues examined [26], suggesting that Arl2 is involved in fundamentally important processes in all eukaryotes.

3.3. *alp41$^+$ is essential for cell viability and required for growth polarity control*

As a first step to investigate the cellular function of *alp41*$^+$, gene disruption was performed. The entire ORF was deleted in one of the two chromosomal *alp41*$^+$ genes in a diploid and tetrad analysis performed. It was found that *alp41*$^+$ is essential for cell viability as only two spores are viable (Fig. 2A) and these colonies are sensitive to G418 (the bacterial kanamycin-resistance gene was used as a marker for gene disruption). Microscopic observation of inviable spores indicated that Δ*alp41* spores were capable of germinating, divided several times and arrested with abnormally bent or branched cell morphology (Fig. 2B). In fission yeast it has been shown that microtubules play a crucial role in growth polarity [17,19,31–33]. This terminal morphology of Δ*alp41* cells is very similar, if not identical, to gene disruption of cofactor B and E-encoding *alp11*$^+$ and *alp21*$^+$ [21]. This result strongly suggests that Alp41 is involved in some aspect of the microtubule biogenesis pathways.

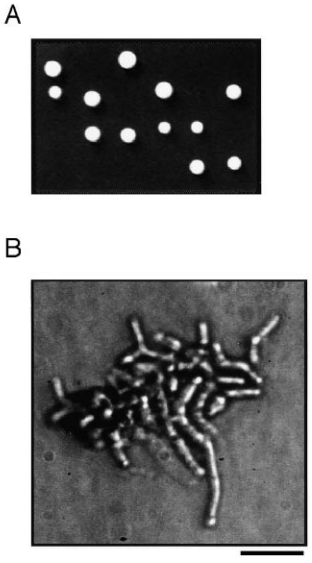

Fig. 2. *alp41*$^+$ is essential for cell viability and its absence leads to polarity defects. A: Gene disruption of *alp41*$^+$. Each tetrad was dissected from a diploid heterozygous for *alp41*$^+$ (PR29) and grown on a rich YE5S plate at 29°C. B: Cell morphology of Δ*alp41* cells which germinated, divided several times and then ceased division is shown. The bar indicates 10 μm.

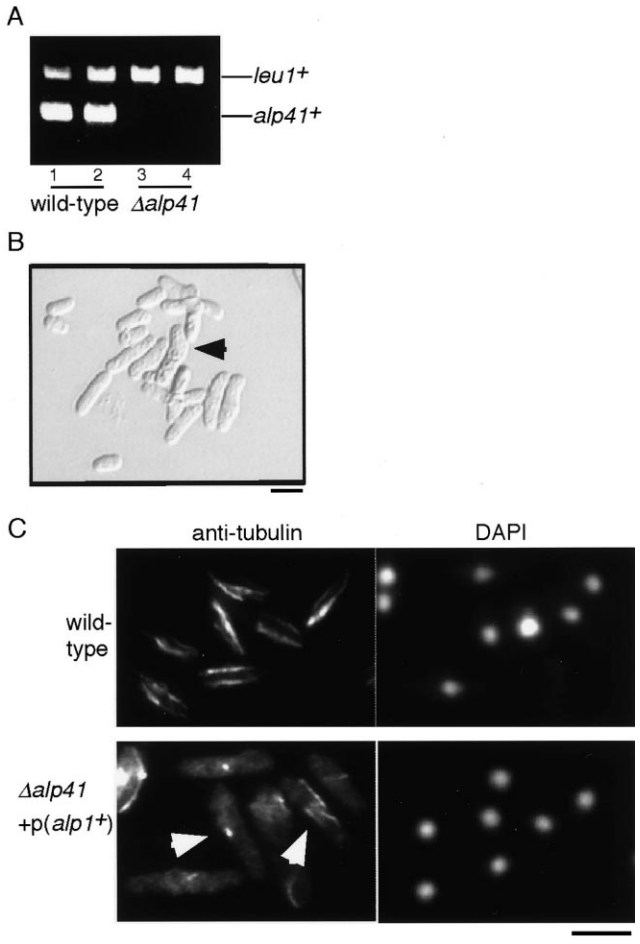

Fig. 3. Bypass of Alp41 requirement by overexpressing the cofactor D-encoding *alp1*$^+$ gene. A: PCR showing deletion of *alp41*$^+$. Genomic DNA was prepared from wild type (lanes 1 and 2) or Δ*alp41* haploid strains containing multicopy plasmids carrying *alp1*$^+$ (PR30, lanes 3 and 4) and PCR performed with four different oligonucleotides as primers. The two corresponded to primers (PO12 and PO13, Section 2) to amplify a whole ORF for *alp41*$^+$ (0.8 kb) and the other two corresponded to those for *leu1*$^+$ amplification (1.2 kb) as a positive control. B: Cell morphology of viable Δ*alp41* cells containing the multicopy *alp1*$^+$ gene. Exponentially growing Δ*alp41* cells containing the multicopy *alp1*$^+$ gene were fixed and photographed. An abnormally bent cell is marked with an arrowhead. C: Immunofluorescence microscopy using an anti-tubulin antibody. Dotted or short microtubules are marked with arrowheads. The bar indicates 10 μm.

3.4. *Alp41 functions upstream of the cofactor D homologue Alp1 in the microtubule biogenesis pathway*

We have previously shown that the cofactor homologues act in a functionally hierarchical manner ($Alp11^B$–$Alp21^E$–$Alp1^D$). This notion is concluded from several experimental data, among which the strongest result is the fact that other-

Table 1
Suppression analysis among cofactor homologues and *alp41*$^+$

Deleted genes	Genes on plasmids				
	vector	*alp11*$^+$	*alp21*$^+$	*alp41*$^+$	*alp1*$^+$
Δ*alp11*	−	+	+	−	+
Δ*alp21*	−	−	+	−	+
Δ*alp41*	−	−	−	+	+
Δ*alp1*	−	−	−	−	+

'+' signifies we obtained Leu$^+$Ura$^+$ or Leu$^+$kan^r haploid cells which contain the deleted gene carrying indicated plasmids, whereas '−' represents failure to obtain Ura$^+$ or kan^r haploid.

wise inviable gene deletion of *alp11*$^+$ is suppressed by augmented levels of either Alp21 or Alp1 and similarly that Δ*alp21* is rescued by higher levels of Alp1, whilst neither *alp11*$^+$ nor *alp21*$^+$ is capable of bypassing Alp1 requirement [21].

To address the possibility that Alp41 plays a role in the cofactor-dependent microtubule pathway, multicopy plasmids containing various genes were introduced in diploid strains heterozygous for each gene and suppression of gene disruption examined by random spore analysis. It transpired that multicopy plasmids containing *alp1*$^+$ are capable of suppressing inviable Δ*alp41*. This was confirmed by PCR (Fig. 3A). Viable Δ*alp41* cells suppressed by multicopy *alp1*$^+$ appear to grow slower than wild type cells and microscopic observation of cell morphology shows that these cells are bent or branched (Fig. 3B), indicative of compromised microtubule function. In fact immunofluorescence microscopy using anti-tubulin antibody shows that these cells do not contain intact microtubules, instead either dotted or short microtubules were observed (Fig. 3C). This is direct evidence that Alp41 regulates microtubule architecture within the cell.

Incomplete suppression in terms of microtubule structures indicates that, despite rescue, suppression by *alp1*$^+$ is not a full bypass of Alp41 function. A similar phenomenon was observed for Δ*alp21* cells rescued by multicopy *alp1*$^+$ (Radcliffe and Toda, data not shown). In contrast, neither *alp11*$^+$ nor *alp21*$^+$ suppressed Δ*alp41*. In addition overexpression of *alp41*$^+$ is unable to bypass the lethal deletion of any of the cofactor homologues (Table 1) and, unlike Alp11^B and Alp1^D [18,21], is not toxic when introduced in wild type cells.

The genetic analysis described above strongly suggests that the GTP-binding Alp41 protein is involved in microtubule biogenesis and most probably functions upstream of Alp1^D. The result in fission yeast is consistent with that in budding yeast as Cin4 appears to function upstream of Cin1^D [13,16]. As multicopy *alp21*$^+$ does not suppress Δ*alp41*, Alp41 might act in concert with Alp21^E. It should be noted that Alp21^E contains leucine-rich repeats (LRRs [21]), which are found in a number of proteins with diverse functions and are believed to be motifs for protein–protein interactions [34]. In budding yeast, the GTP-binding oncoprotein homologues Ras1 and Ras2 bind adenylyl cyclase Cyr1, which contains LRRs, and interestingly in this case LRRs are responsible for this interaction [34]. By analogy to this scenario, it is tempting to speculate that Alp41 binds Alp21^E, which then stimulates some biochemical activity of Alp1^D (Fig. 4). Recently it has been reported that mammalian cofactor D acts as a GTPase-activating protein for β-tubulin [35].

We would like to point out two possible molecular modes of functions of Alp41. One is that Alp41 aids the cofactor-dependent folding of tubulins, which results in formation of functional α-/β-tubulin heterodimers. Perhaps stimulation of the GTPase activity of β-tubulin molecules is a vital function of cofactors as proposed in mammalian cells [11]. The other possibility is that Alp41 may play a part in the intracellular transport of tubulin molecules to the plus ends of microtubules. This role may be analogous to that of the ARF-dependent vesicular transport processes. The differential cellular localisation of Alp11^B and Alp21^E (cytoplasmic [21]) and Alp1^D (microtubules [18]) supports this possibility. No matter how Alp41 functions at the molecular level, these results contribute to the further understanding of microtubule biogenesis and demonstrate that evolutionarily conserved mechanisms regulate microtubule architecture within the cell.

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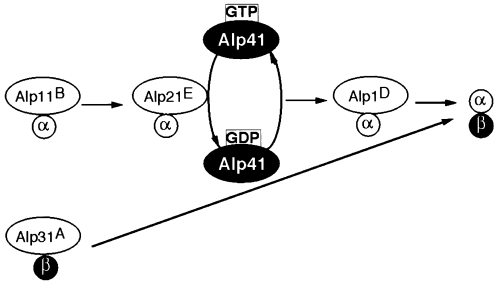

Fig. 4. Model for cofactor-dependent microtubule biogenesis in vivo. The upper pathway (Alp11^B-Alp21^E/Alp41-Alp1^D) is essential for cell division, whilst the lower pathway (Alp31^A) is dispensable. Alp1^D is also proposed to function in the β-tubulin pathway [11].

References

[1] Rommelaere, H., Van Troys, M., Gao, Y., Melki, R., Cowan, N.J., Vandekerckhove, J. and Ampe, C. (1993) Proc. Natl. Acad. Sci. USA 90, 11975–11979.
[2] Kubota, H., Hynes, G., Carne, A., Ashworth, A. and Willison, K. (1994) Curr. Biol. 4, 89–99.
[3] Gao, Y., Vainberg, I.E., Chow, R.L. and Cowan, N.J. (1993) Mol. Cell. Biol. 13, 2478–2485.
[4] Gao, Y., Melki, R., Walden, P.D., Lewis, S.A., Ampe, C., Rommelere, H., Vandekerckhove, J. and Cowan, N.J. (1994) J. Cell Biol. 125, 989–996.
[5] Llosa, M., Aloria, K., Campo, R., Padilla, R., Avila, J., Sánchez-Pulido, L. and Zabala, J.C. (1996) FEBS Lett. 397, 283–289.

[6] Melki, R., Rommelaere, H., Leguy, R., Vandekerckhove, J. and Ampe, C. (1996) Biochemistry 35, 10422–10435.
[7] Tian, G., Huang, Y., Rommelaere, H., Vandekerckhove, J., Ampe, C. and Cowan, N.J. (1996) Cell 86, 287–296.
[8] Tian, G., Lewis, S.A., Feierbach, B., Stearns, T., Rommelaere, H., Ampe, C. and Cowan, N.J. (1997) J. Cell Biol. 138, 821–832.
[9] Lewis, V.A., Hynes, G.M., Zheng, D., Saibil, H. and Willison, K. (1992) Nature 358, 249–252.
[10] Yaffe, M.B., Farr, G.W., Miklos, D., Horwich, A.L., Sternlicht, M.L. and Sternlicht, H. (1992) Nature 358, 245–248.
[11] Lewis, S.A., Tian, G. and Cowan, N.J. (1997) Trends Cell Biol. 7, 479–484.
[12] Hoyt, M.A., Stearns, T. and Botstein, D. (1990) Mol. Cell. Biol. 10, 223–234.
[13] Stearns, T., Hoyt, M.A. and Botstein, D. (1990) Genetics 124, 251–262.
[14] Ursic, D. and Culbertson, M.R. (1991) Mol. Cell. Biol. 11, 2629–2640.
[15] Archer, J.E., Vega, L.R. and Solomon, F. (1995) Cell 82, 425–434.
[16] Hoyt, M.A., Macke, J.P., Roberts, B.T. and Geiser, J.R. (1997) Genetics 146, 849–857.
[17] Mata, J. and Nurse, P. (1997) Cell 89, 939–949.
[18] Hirata, D., Masuda, H., Eddison, M. and Toda, T. (1998) EMBO J. 17, 658–666.
[19] Radcliffe, P., Hirata, D., Childs, D., Vardy, L. and Toda, T. (1998) Mol. Biol. Cell 9, 1757–1771.
[20] Grishchuk, E.L. and McIntosh, J.R. (1999) J. Cell Sci. 112, 1979–1988.
[21] Radcliffe, P.A., Hirata, D., Vardy, L. and Toda, T. (1999) Mol. Biol. Cell 10, 2987–3001.
[22] Moreno, S., Klar, A. and Nurse, P. (1991) Methods Enzymol. 194, 773–782.
[23] Sambrook, J., Fritsch, E.F. and Maniatis, T. (1989) in: Molecular Cloning: A Laboratory Manual, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.
[24] Bähler, J., Wu, J., Longtine, M.S., Shah, N.G., McKenzie III, A., Steever, A.B., Wach, A., Philippsen, P. and Pringle, J.R. (1998) Yeast 14, 943–951.
[25] Maundrell, K. (1990) J. Biol. Chem. 265, 10857–10864.
[26] Cavenagh, M.M., Breiner, M., Schurmann, A., Rosenwald, A.G., Terui, T., Zhang, C., Randazzo, P.A., Adams, M., Joost, H.G. and Kahn, R.A. (1994) J. Biol. Chem. 269, 18937–18942.
[27] Tamkun, J.W., Kahn, R.A., Kissinger, M., Brizuela, B.J., Rulka, C., Scott, M.P. and Kennison, J.A. (1991) Proc. Natl. Acad. Sci. USA 88, 3120–3124.
[28] Kahn, R.A. and Gilman, A.G. (1986) J. Biol. Chem. 261, 7906–7911.
[29] Kahn, R.A. (1994) in: Handbook of Experimental Pharmacology (Dickey, B. and Birnbaumer, L., Eds.), pp. 529–542, Springer-Verlag, Heidelberg.
[30] Clark, J., Moore, L., Krasinska, A., Way, J., Battey, J., Tamkun, J. and Kahn, R. (1993) Proc. Natl. Acad. Sci. USA 90, 8952–8956.
[31] Umesono, K., Toda, T., Hayashi, S. and Yanagida, M. (1983) J. Mol. Biol. 168, 271–284.
[32] Hiraoka, Y., Toda, T. and Yanagida, M. (1984) Cell 39, 349–358.
[33] Sawin, K.E. and Nurse, P. (1998) J. Cell Biol. 142, 457–471.
[34] Kobe, B. and Deisenhofer, J. (1994) Trends Biochem. Sci. 19, 415–421.
[35] Tian, G., Bhamidipati, A., Cowan, N.J. and Lewis, S.A. (1999) J. Biol. Chem. 274, 24054–24058.