In Vitro Regulation of Budding Yeast Bfa1/Bub2 GAP Activity by Cdc5

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Marco Geymonat‡, Ad Spanos, Philip A. Walker‡, Leland H. Johnston, and Steven G. Sedgwick§
From the Divisions of Yeast Genetics and §Protein Structure, National Institute for Medical Research, Mill Hill, London NW7 1AA, Great Britain

The Cdc5 protein of budding yeast is a polo-like kinase that has multiple roles in mitosis including control of the mitotic exit network (MEN). MEN activity brings about loss of mitotic kinase activity so that the mitotic spindle is disassembled and cytokinesis can proceed. Activity of the MEN is regulated by a small GTPase, Tem1, which in turn is controlled by a two-component GTPase-activating protein (GAP) formed by Bfa1 and Bub2. Bfa1 has been identified as a regulatory target of Cdc5 but there are conflicting deductions from indirect in vivo assays as to whether phosphorylation inhibits or stimulates Bfa1 activity. To resolve this question, we have used direct in vitro assays to observe the effects of phosphorylation on Bfa1 activity. We show that when Bfa1 is phosphorylated by Cdc5, its GAP activity with Bub2 is inhibited although its ability to interact with Tem1 is unaffected. Thus, in vivo inactivation of Bfa1-Bub2 by Cdc5 would have a positive regulatory effect by increasing levels of Tem1-GTP so stimulating exit from mitosis.

In budding yeast, the final stages of mitosis are controlled by the mitotic exit network (MEN).1 Once the chromosomes are partitioned equally between mother and daughter cells, the MEN eliminates mitotic kinase activity, a prerequisite for spindle disassembly and cytokinesis (reviewed in Refs. 1 and 2). In the MEN, the key element controlling the pathway is Tem1 (3), a GTPase with biochemical properties similar to those of the Rho family of small GTPases (4). The MEN is negatively regulated by a GTPase with biochemical properties similar to those of the Rho family of small GTPases (4). The MEN involves activities of Cdc15 kinase followed by Dbf2 kinase with its associated binding protein, Mob1 (12). Ultimately, the MEN triggers the long term release of Cdc14 from the nucleolus, which, through a series of dephosphorylation events, reduces mitotic kinase activity (13). Recently, the Cdc14 early anaphase release (FEAR) pathway has been described which controls an initial release of Cdc14 from the nucleolus, which may contribute to optimal activity of the MEN although the precise role of the FEAR remains unclear (14–16).

An important regulator of these activities is the polo-like kinase, Cdc5. Cdc5 has several essential roles in mitosis (12, 17–19) as well as regulation of the MEN and FEAR pathways. Even in the MEN there is evidence for more than one role for Cdc5. Net1, the protein that sequesters Cdc14 to the nucleolus, is phosphorylated in vitro and in vivo by Cdc5 so facilitating release of Cdc14 during anaphase (20, 21). Cdc5 also has a positive but unidentified role at some stage between Tem1 and activation of Dbf2 (12). Furthermore, Cdc5 phosphorylates Bfa1 both in vitro and in vivo during mitosis in normally growing cells (15, 22, 23). Two opposite interpretations have been made on the role of this Bfa1 phosphorylation. Hu and co-workers (23) observed that Bfa1 was unphosphorylated in a nocodazole arrest, a condition where the MEN is kept inactive. For this and several other reasons, they equated phosphorylation of Bfa1 with an active MEN and hence proposed that phosphorylation inhibited Bfa1 GAP activity. However, Lee et al. (12, 22) reached the opposite conclusion. They observed that Bfa1 was highly phosphorylated in a nocodazole arrest and that overexpression of Cdc5 inhibited the Dbf2 activity that normally signifies an active MEN (12, 22).2 By linkin-

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‡ Supported by Association for International Cancer Research Grant 99-008.
§ To whom correspondence should be addressed: Division of Yeast Genetics, National Inst. for Medical Research, Mill Hill, London NW7 1AA, Great Britain. E-mail: ssedgwi@nimr.mrc.ac.uk.

1 The abbreviations used are: MEN, mitotic exit network; GAP, GTPase-activating protein; FEAR, Cdc14 early anaphase release; GST, glutathione S-transferase; MBP, myelin basic protein; PAK, p21-activated kinase; SIN, septation initiation network.

2 L. H. Johnston, unpublished observation.
of GAP activity and so other regulatory mechanisms for the MEN are also discussed.

MATERIALS AND METHODS

Plasmid and Strain Construction—A70N-CDC5 was amplified by PCR using as template a plasmid containing a CDC5 allele harboring two point mutations, S165D and T238D (kindly provided by F. Ulmann and M. Sullivan). The amplified sequence starting at codon 71 of CDC5 was cloned in-frame with GST in a URA3-based plasmid under the control of the GAL1 promoter. The URA3 was then replaced by a TRP1 marker, and the plasmid was introduced into a strain deleted for CDC5 and harboring a CDC5-URA3 plasmid (KKY902-2Bta). The CDC5-URA3 plasmid was eliminated by growth on 5-fluoro-orotic acid to generate strain MGY29. A A70N-CDC5 kinase-dead plasmid expressing GST-Cdc5KD was constructed using a similar strategy but the template was a CDC5 allele harboring an N290A point mutation that inactivates the kinase (23).

Protein Expression and Purification—Tem1, MBP-Bfa1, and GST-Bub2 were prepared as described earlier (4). Yeast harboring plasmids encoding GST-Cdc5 and GST-Cdc5KD were grown to a cell density of 10/10^6/ml in medium supplemented with 2% sucrose as carbon source. After addition of 2% galactose, the cultures were grown for 6 h at 30 °C. Yeast extracts were prepared as described earlier (4). Yeast extracts were prepared as described earlier (4). Yeast harboring plasmids encoding GST-Cdc5 and GST-Cdc5KD were grown to a cell density of 10^9/ml in medium supplemented with 2% sucrose as carbon source. After addition of 2% galactose, the cultures were grown for 6 h at 30 °C and harvested. For GST-Cdc5, growth medium was YEP (1% yeast extract, 2% bacto-peatone), while cells expressing GST-Cdc5KD were in YNB selective minimal medium. Frozen pellets of cells were thawed, resuspended in cold lysis buffer (50 mM Tris, pH 7.5, 250 mM NaCl, 1% Nonidet P-40, 1 mM EDTA, 1 mM dithiothreitol, 10 mM NaF, 50 mM β-glycerophosphate, 1 mM sodium orthovanadate and Complete protease inhibitor mixture (Roche Diagnostics Ltd.), and passed twice through a French pressure cell. Crude protein extract was centrifuged twice at 18,000 × g for 4°C for 20 min. The supernatant was gently mixed with glutathione-agarose beads at 4°C. After 1 h, the beads were packed in a column and washed with washing buffer (50 mM Tris, pH 7.5, 250 mM NaCl, 1% Nonidet P-40, and 1 mM diethiothreitol). GST-Cdc5 protein was eluted in 50 mM Tris, pH 7.5, 100 mM NaCl, 0.1% Nonidet P-40, 1 mM diethiothreitol, and 20 mM reduced glutathione. Elution of protein was monitored by SDS-PAGE. Peak fractions were pooled and dialyzed in 50 mM Tris/HCl, pH 7.5, 100 mM NaCl, 2 mM diethiothreitol, and 25% glycerol. Aliquots were frozen at −80°C.

Phosphorylation of Bfa1—Phosphorylation reactions using radiolabeled ATP were carried out in 30 μl of kinase buffer (50 mM Tris/HCl, pH 7.5, 10 mM MgCl2, 1 mM diethiothreitol) containing 1–10 μg of substrate. 10–50 ng of kinase were added followed by 50 μM ATP and 0.1 μl of [γ-32P]ATP (Amersham Biosciences, 370 MBq/ml, 3000 Ci/mmol). After incubation for 15 min at 30°C, reactions were stopped by adding of 10 μl of × 4 Laemli buffer and heating at 100°C for 2 min. After SDS-PAGE electrophoresis, radioelabeling was visualized by autoradiography. For preparation of phosphorylated Bfa1, the protein was incubated in the same reaction mixture with 10 mM non-radioactive ATP instead of [γ-32P]ATP. Reaction volumes were increased to accommodate up to 100 μg of Bfa1. Incubation was for 4 h. After dialysis against 50 mM Tris/HCl, pH 7.5, 100 mM NaCl, 2 mM diethiothreitol, 25% glycerol, the phosphorylated Bfa1 (1 μg/μl) was stored at −80°C. An identical reaction on Bfa1 was carried out using kinase dead GST-Cdc5KD to provide non-phosphorylated control material.

Nucleotide Exchange and GTPase Assays—Conditions for [γ-32P]GTP exchange and hydrolysis assays were as described elsewhere (4).

RESULTS

Phosphorylation of Bfa1 by Cdc5—The aim of this work was to characterize the effects of Cdc5-dependent phosphorylation on Bfa1. A major technical requirement was to produce Cdc5 in sufficient quantities and with sufficient activity to fully phosphorylate Bfa1 so that any effects of phosphorylation would be reproducible and have their maximal impact. Cdc5 was initially prepared from Escherichia coli and Baculovirus expression systems but was unable to convert Bfa1 completely to an electrophoretically slower, phosphorylated form (data not shown). Eventually, we developed a yeast-based expression system for a GST-Cdc5 derivative, which is activated by S165D and T238D mutations and stabilized by deletion of 70 N-terminal residues (24, 25). In vivo, the activity of this GST-Cdc5 fusion protein was indicated by complementation of a cdc5 deletion mutant under low expression conditions (use of glucose with a GAL promoter) (data not shown). GST-Cdc5KD provided a kinase-dead control and has a N290A mutation (23) and an equivalent 70-residue N-terminal deletion. GST-Cdc5 and GST-Cdc5KD were readily purified (Fig. 1A). The differential activities of affinity-purified GST-Cdc5 and GST-Cdc5KD were confirmed using myelin basic protein as a substrate (Fig. 1B). Conditions for efficient phosphorylation of Bfa1 were established using increasing amounts of GST-Cdc5 (Fig. 1C). With suboptimal levels of Cdc5, multiple isoforms of Bfa1 were visible by protein staining in agreement with the presence of multiple phosphorylation sites in the molecule. However, with 5 ng or more GST-Cdc5 per assay, a single slower migrating form of phosphorylated Bfa1 was detected. No change in the mobility of Bfa1 was produced by treatment with equivalent amounts of GST-Cdc5KD. The efficient and specific phosphorylation of Bfa1 GST-Cdc5 was further confirmed by the differential radioelabeling produced by GST-Cdc5 or GST-Cdc5KD (Fig. 1C). Note that in the following examination of the effects of phosphorylation on Bfa1, optimally phosphorylated Bfa1 was prepared with GST-Cdc5 as described above, while non-phosphorylated Bfa1 was prepared by mock-treatment with catalytically inactive GST-Cdc5KD. Thus, our data are specific to Cdc5 kinase and cannot be attributed to a passive Cdc5-Bfa1 interaction or to unspecified contaminating material in the kinase preparation.

Phosphorylation of Bfa1 by Cdc5 Has No Effect on Inhibition of Tem1 GTP Hydrolysis and Dissociation—The effects of phosphorylation of Bfa1 on Tem1 activity were first examined in the absence of Bub2. Under these conditions unphosphorylated Bfa1 inhibits both the intrinsic GTP hydrolysis and GTP dissociation activities of Tem1 (4). The inhibitory effects of Bfa1 on GTP hydrolysis and GTP dissociation were monitored by loading Tem1 with [γ-32P]GTP in the presence of varying amounts of non-phosphorylated or phosphorylated Bfa1. Amounts of [γ-32P]GTP bound to Tem1 were assayed before and after 5-min
incubation at 30 °C. In the absence of any Bfa1, 50–55% of bound \( \gamma^{-32}P \)GTP was lost from Tem1 in 5 min (Fig. 2). This reflects the loss of \( \gamma^{-32}P \)GTP by the intrinsic GTP hydrolysis and GTP dissociation activities of Tem1 (4). In the presence of non-phosphorylated Bfa1 there was a progressive increase in the amount of \( \gamma^{-32}P \)GTP remaining bound to Tem1 resulting from the inhibition of GTP hydrolysis and GTP dissociation as seen previously (4). When phosphorylated Bfa1 was used, there was a similar inhibition of GTP loss from Tem1 (Fig. 2). Thus, phosphorylation by Cdc5 does not impair the ability of Bfa1 to interact with Tem1 and inhibit GTP dissociation and hydrolysis.

Phosphorylation of Bfa1 by Cdc5 Reduces GAP Activity with Bub2—The effect of phosphorylation of Bfa1 was next examined in assays of GAP activity where Bub2 is also present. These assays were performed by loading Tem1 with \( \gamma^{-32}P \)GTP in the presence of phosphorylated or non-phosphorylated Bfa1. Reactions were then incubated with or without Bub2. In a kinetics experiment, Tem-\( \gamma^{-32}P \)GTP and Bfa1 were incubated with a single amount of Bub2, and the amount of radioactive GTP bound to Tem1 was assayed at frequent intervals during incubation at 30 °C. The addition of Bub2 with non-phosphorylated Bfa1 caused the expected loss of radioactivity (Fig. 3A), which is due to stimulation of Tem1 GTP hydrolysis by Bfa1/Bub2 GAP activity (4). When phosphorylated Bfa1 was used in parallel assays with Bub2, the kinetics of the GAP activity were clearly reduced by ~50%. This level of inhibition was repeatedly detected in multiple assays of GAP activity where levels of filter binding were highly reproducible and varied by no more than 5%. The possibility that the phosphorylation status of Bfa1 was affected by the GAP assay itself was excluded by the observation that there was no change in the level of Bfa1 phosphorylation during the GAP reaction (data not shown). In the absence of any Bub2, ~85–90% of the radioactive \( \gamma^{-32}P \)GTP remained bound to Tem1 throughout (Fig. 3A). This high level of Tem-\( \gamma^{-32}P \)GTP results from the inhibition of the intrinsic GTP dissociation and GTPase activities of Tem1 by Bfa1 as described above (Fig. 2).

To further characterize the influence of Cdc5 phosphorylation on Bfa1, GAP activity was assayed after a fixed time with increasing amounts of Bub2 (Fig. 3B). As seen above, no loss of \( \gamma^{-32}P \)GTP from Tem1 occurred from control reactions incubated with no Bub2, whether or not Bfa1 was phosphorylated.

However, addition of Bub2 resulted in the expected GAP activity as judged by the progressive loss of \( \gamma^{-32}P \)GTP from Tem1 as Bub2 concentrations increased. Significantly, when Bfa1 had been phosphorylated by Cdc5 approximately twice the amount of Bub2 was required to produce the same GAP activity compared with non-phosphorylated Bfa1. Thus, both kinetic and a concentration dependence assays demonstrate that phosphorylation of Bfa1 by Cdc5 reduces the ability of Bfa1 to function as a GAP in association with Bub2.

**DISCUSSION**

We have used an in vitro approach to understand the effects of Cdc5 kinase on the regulation of Tem1 and the MEN. We demonstrate that when Bfa1 is phosphorylated by Cdc5, its GAP activity with Bub2 for Tem1 is reduced. The equivalent result of this activity in vivo would be for Cdc5 to increase Tem1-GTP levels so bringing about positive regulation of the MEN. Our in vitro observation that phosphorylation reduces the GAP activity of Bfa1 is consistent with in vivo results showing that cells with a non-phosphorylatable form of Bfa1 have reduced ability to activate the MEN (23). Nevertheless, it is still somewhat unclear why Hu et al. (23) and Lee et al. (12, 22) reached opposite conclusions on the in vivo effects of phos-
phorylation on Bfa1 activity. One possibility is that the two groups observed different extents of phosphorylation and only complete phosphorylation was inhibitory. This idea stems from more recent observations of Pereira and co-workers (15) who showed that a phosphorylated form of Bfa1 is already present in a metaphase arrest. Upon progression into anaphase, Bfa1 is then further phosphorylated. Although it is not clear whether both changes are caused by Cdc5, there does appear to be two phosphorylation events on Bfa1: one before metaphase that does not inhibit the Bfa1/Bub2 GAP activity and a second during anaphase that could participate in activation of MEN by inhibiting its GAP activity.

In considering how phosphorylation affects Bfa1 and GAP activity, Hu et al. (23) proposed that phosphorylated Bfa1 has a lower affinity for Tem1 as judged by co-immune precipitation. However, our in vitro results show that phosphorylated and non-phosphorylated forms of Bfa1 have equal affinities for Tem1 as judged by their similar abilities to inhibit GTP hydrolysis and dissociation. An alternative mechanism may involve interference in the interaction of phosphorylated Bfa1 with Bub2 rather than with Tem1. It is notable that a C-terminal deletion form of Bfa1 lacking the domain responsible for Bub2 interaction still has partial in vitro GAP activity in combination with Bub2 even though the two proteins can no longer interact (4). This might explain why we observe only a partial inhibition of Bfa1/Bub2 GAP activity after Bfa1 phosphorylation. Moreover, it has been recently shown that Bub2 is also phosphorylated in a cell cycle-dependent manner, and it was proposed that this modification could affect GAP activity (26). Since GAP activity is reduced after phosphorylation of the mammalian GAPs, RGS16 and p120-GAP (27, 28), the concept that this modification could affect GAP activity was supported. Nevertheless, it was not determined whether phosphorylation was inhibitory. This idea stems from our in vitro observations of Pereira and co-workers (15) who noted that complete phosphorylation was inhibitory. This idea stems from our in vitro observations of Pereira and co-workers (15) who noted that complete phosphorylation was inhibitory.

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