Purification of Hsk1, a Minichromosome Maintenance Protein Kinase from Fission Yeast*

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Members of the Cdc7 family of protein kinases are essential for the initiation of DNA replication in all eukaryotes, but their precise biochemical function is unclear. We have purified the fission yeast Cdc7 homologue Hsk1 approximately 30,000-fold, to near homogeneity. Purified Hsk1 has protein kinase activity on several substrates and is capable of autophosphorylation. Point mutations in highly conserved regions of Hsk1 inactivate the kinase in vitro and in vivo. Overproduction of two of the mutant hsk1 alleles blocks initiation of DNA replication and deranges the mitotic checkpoint, a phenotype consistent with a role for Hsk1 in the early stages of initiation. The purified Hsk1 kinase can be separated into two active forms, a Hsk1 monomer and a heterodimer consisting of Hsk1 complexed with a copurifying polypeptide, Dfp1. Association with Dfp1 stimulates phosphorylation of exogenous substrates but has little effect on autokinase activity. We have identified Dfp1 as the fission yeast homologue of budding yeast Dbf4. Purified Hsk1 phosphorylates the Cdc19 (Mcm2) subunit of the six-member minichromosome maintenance protein complex purified from fission yeast. Since minichromosome maintenance proteins have been implicated in the initiation of DNA replication, the essential function of Hsk1 at the G1/S transition may be mediated by phosphorylation of Cdc19. Furthermore, the phosphorylation of critical substrates by Hsk1 kinase is likely regulated by association with a Dbf4-like co-factor.

The initiation of DNA replication at eukaryotic chromosomal origins is generally considered to be a two-stage process (reviewed in Refs. 1 and 2). The first stage involves the ordered assembly of initiation factors onto the replication origin to form a complex that is competent to initiate DNA synthesis. This assembly is likely nucleated by the origin recognition complex, which is bound to replication origins in a sequence-specific manner throughout the cell cycle (3, 4). From late M phase through G1 phase the Cdc18/Cdc6 protein associates with origins in an origin recognition complex-dependent manner (5, 6). Finally, a family of six proteins termed minichromosome maintenance proteins (MCMs)1 associates with the preinitiation complex, a reaction that depends on Cdc18/Cdc6 (6, 7). In the second stage of initiation, these competent preinitiation complexes are triggered to initiate DNA synthesis by the action of additional protein factors, believed to be one or more protein kinases.

The Schizosaccharomyces pombe gene hsk1+ was cloned by homology to the Saccharomyces cerevisiae CDC7 gene (8). CDC7 and hsk1+ are absolutely required for the initiation of DNA replication (8, 9). CDC7 encodes a cell cycle-regulated protein kinase whose activity peaks at the G1/S transition, as demonstrated by immunoprecipitation analysis (10–13). Cdc7 interacts genetically and in two-hybrid assays with Dbf4 (12, 14, 15), a potential regulatory subunit of the kinase. Expression of DBF4 is required at the G1/S transition for initiation of DNA replication (16) and for Cdc7 kinase activity (12). It has been suggested that Dbf4 may also play a role in targeting Cdc7 kinase to replication origins (15).

The recent cloning of Cdc7 homologues from S. pombe, Xenopus laevis, and H. sapiens (8, 17–19) indicates that regulation of initiation of Cdc7-family proteins may be a conserved feature of eukaryotic DNA replication. Strong candidates for targets of Cdc7 kinases at the G1/S transition are the members of the MCM family of proteins. The MCM family has six members, all of which are essential proteins that function in DNA replication (reviewed in Ref. 20). These proteins form heteromeric complexes that interact with chromatin and replication origins in vivo (5–7, 21, 22). The MCM proteins appear to dissociate from chromatin and replication origins as S phase proceeds (6, 7, 23–25). Several lines of experimental evidence link Cdc7 function with that of the MCM proteins. First, an allele of mcmm5 in budding yeast is a bypass suppressor of cdc7 and dbf4 mutants (26). Second, immunoprecipitates of human Cdc7 protein phosphorylate Mcm2 and Mcm3 fusion proteins purified from bacteria (17). Finally, budding yeast Cdc7/Dbf4 expressed in insect cells phosphorylates GST-Mcm2, -Mcm3, -Mcm4, and -Mcm6 fusion proteins in vitro (27). Although these data are consistent with the possibility that Cdc7 kinase may phosphorylate one or more MCM subunits at G1/S, heteromeric MCM complexes have not yet been directly tested as substrates.

Although the importance of Cdc7 kinase for progression into S phase is clear, the nature of the requirement for Cdc7 is unclear, and the relevant substrates of the Cdc7 kinase at G1/S are unknown. In the present study we describe the purification of Hsk1 from fission yeast. Purified Hsk1 contains kinase and autokinase activity, and this activity depends on the presence of highly conserved protein kinase sequence motifs. A subset of mutant hsk1 alleles displays a dominant-negative phenotype, including accumulation of G1 cells, indicating a role for hsk1+ in regulating the G1/S transition. The purified Hsk1 kinase

polymerase chain reaction; DTT, dithiothreitol; PAGE, polyacrylamide gel electrophoresis.

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1 The abbreviations used are: MCM, minichromosome maintenance proteins; GST, glutathione S-transferase; HA, hemagglutinin; PCR,

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contained a second polypeptide, Dfp1. The Dfp1 polypeptide is homologous to budding yeast Dbf4 and forms a heterodimeric complex with Hsk1. The presence of Dfp1 stimulates Hsk1 kinase phosphorylation of exogenous substrates but has no effect on autokinase activity. We tested the ability of purified Hsk1 kinase to phosphorylate the six-member MCM complex purified from fission yeast and found that Cdc19 (Mcm2) was specifically phosphorylated. The essential function of Hsk1 in entry into S phase may therefore be mediated by phosphorylation of Cdc19 (Mcm2). The phosphorylation of Cdc19 and other critical substrates of Hsk1 kinase is likely regulated by association with a Dbf4-like co-factor.

MATERIALS AND METHODS

Strains and Plasmids—Basic fission yeast genetic and molecular biology techniques were used (28). Cells were grown in Edinburgh minimal medium plus required supplements.

The fission yeast strain GBY358 expresses Hsk1 fused to the COOH terminus to six histidines and three hemagglutinin (HA) epitopes (29) and was constructed as follows. The hsk1 open reading frame was amplified using PCR of S. pombe cDNA and cloned into pSLF172 (30) to give pSLF172-hsk1. The first 575 base pairs of the hsk1 open reading frame, the nmt1 promoter, and the ars1 sequences were removed from pSLF172-hsk1 by restriction enzyme cleavage and religation. A six histidine-encoding NotI linker was inserted between the hsk1 ORF and the 3HA tag. The resulting plasmid was linearized near the middle of the remaining hsk1 sequences with XhoI and introduced into an h- leu1-32 ura4-D18 ade6-M210 strain. ura+ transformants were selected, and a strain with a single integration at the hsk1 locus was identified by southern hybridization analysis. The only copy of hsk1 in GBY358 is the tagged copy, indicating that Hsk1–6His-3HA is functional.

Expression of wild-type and mutant hsk1–3HA for immunoprecipitations, kinase assays, and phenotypic analysis was performed in an h- leu1-32 ura4-D18 ade6-M210 strain carrying pSLF172-hsk1 (either wild-type hsk1–3HA (strain GBY276) or the indicated point mutants). Point mutations in pSLF172-hsk1 were generated using QuickChange PCR (Stratagene). Transformants were isolated in the presence of 5 μg/ml thiamine, and expression was induced by the removal of thiamine as described (31).

GBY357, the diploid strain carrying a disruption of hsk1-1, has the genotype h- leu1-32 ura4-D18 ade6-M210 strain carrying pSLF172-hsk1 (either wild-type hsk1–3HA (strain GBY276) or the indicated point mutants). Point mutations in pSLF172-hsk1 were generated using QuickChange PCR (Stratagene). Transformants were isolated in the presence of 5 μg/ml thiamine, and expression was induced by the removal of thiamine as described (31).

For flow cytometry, cells were fixed in 70% ethanol, harvested, and stained in 2 μg/ml propidium iodide in 50 mM sodium citrate. Flow cytometry was performed using a FACScan and CellQuest software (Becton-Dickinson). Fixation and staining with 4',6-diamidino-2-phenylindole dihydrochloride and calcofluor, which stain DNA and septa, respectively, was as described (28).

Immunoprecipitation—To prepare cell extracts for immunoprecipitation, 4 g of cells were resuspended in 4 ml of 2 °C lysis buffer (2 × LB 100 mM HEPES, 2 mM EDTA, 20% (v/v) glycerol, 20 mM sodium pyrophosphate, 100 mM NaF, 2 mM Na3VO4, 2 mM DTT, 2 mM phenylmethylsulfonyl fluoride, 40 μg/ml leupeptin, 40 μg/ml 1-cholesterol-4-tosylamide-7-amino-2-heptanone-HCl, 20 μg/ml aprotinin, 20 μg/ml bestatin, 2 μg/ml pepstatin A, pH 7.5). The suspension was transferred to two 15-ml Corex centrifuge tubes, and 0.5-mm glass beads were added to the meniscus. The cells were broken by vortexing 15 times for 30 s each, alternating with incubation on ice for 30 s. After removal of the beads by centrifugation, NaCl and Tween 20 were added to 250 mM and 0.05%, respectively. The lysate was stirred for 15 min and clarified by centrifugation at 20,000 rpm in a Sorvall SS-34 rotor for 30 min at 2 °C. Protein concentrations were determined by the method of Bradford (Bio-Rad). Extract (1 mg) was incubated with 3 μg of 12CA5 anti-HA monoclonal antibody (29) or 9E10 anti-myc monoclonal antibody (BAbCo) for 2 h at 4 °C followed by the addition of 10 μl of protein A-Sepharose or protein G-Sepharose (Amersham Pharmacia Biotech). After further incubation at 4 °C for 1 h, beads were collected by centrifugation, washed 4 times with 1 ml of 1 × LB plus 250 mM NaCl, 0.05% Tween 20 and twice with 1 ml of 1 × LB plus 25 mM NaCl, 0.05% Tween 20.

Expression of GST-Hsk1 Fusion Proteins in Escherichia coli—DNA fragments encoding amino acids 1–122 of Hsk1 and 1–530 of Cdc19 were generated by PCR and cloned into pGEX4T-1 (Amersham). The fusion proteins were purified using glutathione-Sepharose as directed by the manufacturer.

Purification of Hsk1—50 g of GBY358 cells (which carry a single copy of hsk1-6his-3HA integrated at the hsk1 locus) were resuspended in 50 ml of 2 × LB-EDTA-DTT (2 × LB lacking EDTA and DTT) and adjusted to a final volume of 100 ml with H2O. Cells were lysed in a DynoMill (Glen Mills) with 0.5-mm glass beads by 10 pulses of 30 s each with cooling at −10 °C. After cell lysis, NaCl and Tween 20 were added to 250 mM and 0.05%, respectively. The lysate was stirred for 15 min at 0 °C and then clarified by centrifugation in a Sorvall SS-34 rotor at 20,000 rpm for 30 min at 2 °C. The extract was adsorbed in batch to 5 ml of Tulon-Sepharose (CLONTECH) for 2 h at 4 °C. The resin and washes were applied to a mono Q HR 5/5 column (Amersham) and washed with 5 volumes of 1 × LB-EDTA-DTT plus 250 mM NaCl, 0.05% Tween 20, and then with 5 volumes of 1 × LB-EDTA-DTT plus 250 mM NaCl, 0.05% Tween 20, 5 mM imidazole. The column was eluted with 1 × LB-EDTA-DTT plus 175 mM NaCl, 0.05% Tween 20, 75 mM imidazole. Fractions containing Hsk1 were identified by immunoblot analysis and were pooled. 120 μg of anti-HA monoclonal antibody 12CA5 was added, followed by gentle mixing at 4 °C for 2 h. 100 μl of Protein A-Sepharose was added, and the incubation continued for 1 h. Immune complexes were centrifuged by precipitation and washed 4 times with 1 × WASH (20 mM Tris-HCl, 250 mM NaCl, 1 mM EDTA, 10% glycerol, 0.05% Tween 20, 1 mM DTT, 10 μg/ml leupeptin, 10 μg/ml aprotinin, 0.5 mM phenylmethylsulfonyl fluoride, pH 8.0). Hsk1 was eluted by the addition of 200 μl of 2 mg/ml HA.11 peptide (BABCo) in 1 × WASH followed by incubation for 16 h at 4 °C. The eluate was recovered, and the resin was washed twice with 200 μl of 1 × WASH. The eluate and the washes were pooled and dialyzed against 50 mM Tris, 1 mM EDTA, 10% (v/v) glycerol, 0.05% Tween 20, 1 mM DTT, 1 μg/ml leupeptin, 1 μg/ml aprotinin, 0.5 mM phenylmethylsulfonyl fluoride, pH 8.0 (mM buffer) plus 50 mM NaCl. The dialysate was applied to a mono Q HR 5/5 column (Amersham) and eluted with a 10-ml linear gradient of NaCl (50 to 500 mM in mM buffer). Fractions (0.5 ml) were collected and assayed for Hsk1 protein kinase activity. Protein concentrations were determined by the method of Bradford (Bio-Rad). Protein in the pooled fractions from the 12CA5 immunoaffinity and mono Q chromatography steps was quantitated by Bradford (Bio-Rad). Bovine serum albumin was used as the standard. Hsk1 was purified to apparent homogeneity by gel filtration on a Superdex 200 HR 10/30 column. Native gel analysis was performed using Laemml discontinuous gels with 0.02% Tween 20 but lacking

2 http://www.sanger.ac.uk/Projects/S_pombe/blast_server.shtml
**RESULTS**

**Hsk1 Immunoprecipitates Contain Protein Kinase Activity—**

The sequence of hsk1 contains a number of motifs typical of protein kinases. Additionally, immunoprecipitates of the homologous protein in budding yeast have been shown to have kinase activity (10, 11). To test Hsk1 for protein kinase activity, we fused Hsk1 to a triple HA epitope tag and overexpressed the protein in fission yeast (strain GBY276). Immunoprecipitates of extracts from this strain were incubated in the presence of [γ-32P]ATP, and the products were analyzed by SDS-PAGE and autoradiography (Fig. 1). A radiolabelled polypeptide was evident at the molecular weight expected for Hsk1 in the anti-HA immunoprecipitate but not when the immunoprecipitation was performed with a control antibody (419). This phosphorylated polypeptide comigrated exactly with the Hsk1 polypeptide as detected by immunoblot analysis (not shown), indicating that Hsk1 is associated with a protein kinase that can phosphorylate Hsk1. To determine whether the kinase activity is intrinsic to Hsk1, we expressed a series of mutant forms of Hsk1 and tested them for kinase activity in immunoprecipitates (Fig. 2). All of the point mutations affected sites within conserved domains that have previously been shown to be important for the essential in vivo function of the budding yeast homologue of Hsk1, Cdc7 (38–40). These included mutations in the ATP binding site (K129A), a conserved kinase domain (D216N), and a potential phosphorylation site (K129A). This phosphorylated polypeptide comigrated exactly with the Hsk1 polypeptide as detected by immunoblot analysis (not shown), indicating that Hsk1 is associated with a protein kinase that can phosphorylate Hsk1. To determine whether the kinase activity is intrinsic to Hsk1, we expressed a series of mutant forms of Hsk1 and tested them for kinase activity in immunoprecipitates (Fig. 2). 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FIG. 3. Overexpression of two Hsk1 point mutants causes dominant defects in initiation of DNA replication and in a mitotic checkpoint. Cells carrying hsk1” or one of two point mutant alleles, hsk1D216N or hsk1T291E, under the control of a thiamine-repressible promoter were grown to mid-logarithmic phase in the presence (Promoter off) or absence (Promoter on) of thiamine. Cells were harvested, fixed, and stained with 4',6-diamidino-2-phenylindole dihydrochloride and calcofluor to stain the nuclei and septa. Representative cut or anucleate cells are indicated by the arrows, and the percentage of cut and anucleate cells is indicated on each micrograph. Samples of cells grown with the promoter on were also analyzed by flow cytometry to measure DNA content. The positions of 1C and 2C DNA contents, and the percent of cells with <2C DNA content are indicated on the histograms.

Overexpression of Mutant Hsk1 Proteins Causes a Dominant-negative Phenotype—We examined the phenotypic consequences of overexpressing wild-type and mutant alleles of hsk1 under the control of the strong nmt1 promoter. This promoter is activated by the removal of thiamine from the growth medium, and maximal expression occurs approximately 16 h after the removal of thiamine. Cells were examined 20 h after the removal of thiamine (Fig. 3) by microscopy and flow cytometry. Cells overexpressing hsk1” (Fig. 3), K129A, or T291A (data not shown) were normal in appearance and had the typical 2C DNA content by flow cytometry. In contrast, the mutants D216N and T291E both contained a large percentage of cells with abnormal morphology, including anucleate cells and “cut” cells in which septation has occurred in the absence of nuclear division (Fig. 3). When these cells were examined by flow cytometry, an accumulation of cells with a 1C DNA content was apparent, indicating a delay in entry into S phase. Cells with a less than 1C DNA content were also observed by flow cytometry, consistent with the presence of cut and anucleate cells. This phenotype indicates that a fraction of the cells underwent mitosis and cytokinesis in the absence of DNA replication. A similar phenotype has previously been observed in cells deficient in genes that are important for the initiation of DNA replication, such as cdc18”, orp1, or the gene encoding DNA polymerase a (41–43). That these dominant negative alleles of hsk1 have a similar phenotype indicates that hsk1” has an important role in the initiation of DNA replication.

We also examined whether any of these mutant hsk1 alleles retained activity in vivo. Plasmids containing the wild-type hsk1” gene or one of the mutant hsk1 alleles were introduced into a diploid strain harboring a disruption of one chromosomal hsk1 gene. Diploid transformants were induced to sporulate. Haploid cells carrying the plasmid were selected and screened for the presence of the hsk1 disruption. When the plasmid carried the wild-type hsk1” gene, approximately half of the haploid transformants contained the hsk1 chromosomal deletion (15/25 haploid cells were ura’), indicating that hsk1” rescues this strain (note that in tetrad analysis the untransformed diploid strain produced no viable spores carrying the hsk1 disruption, confirming that hsk1” is an essential gene). In contrast, when the plasmid carried a mutant hsk1 allele, all of the haploid transformants contained the intact chromosomal copy of hsk1” (0/37 haploids were ura’ for K129A, 0/69 for T291A, 0/64 for T291E, 0/77 for D216N). These data indicate that all of the tested mutations inactivate hsk1 function in vivo.

Purification of Hsk1—Although several Cdc7 kinase family members have associated kinase activity in immunoprecipitation analysis, the enzyme has not been purified from any organism. To facilitate purification we fused hsk1 to a DNA segment encoding six histidine residues and three HA epitope tags and integrated the resulting chimeric gene in a single copy at the hsk1 locus. The resulting strain, GBY358, expresses Hsk1–6his-3HA under the control of the wild-type hsk1” promoter. We used a combination of affinity and conventional column chromatography to purify Hsk1 approximately 30,000-fold, to near homogeneity (Table I). Our purification procedure makes no assumptions about the subunit structure of the kinase and utilizes only mild elution conditions in an effort to preserve the structure of the enzyme as it exists in vivo. In the final step of the purification, chromatography on a mono Q anion exchange column, the Hsk1 polypeptide, autokinase activity, and protein kinase activity on an exogenous substrate (recombinant Hsk1 NH2-terminal fusion protein produced in bacteria) all precisely co-eluted (Fig. 4). SDS-polyacrylamide gels stained with silver show that the purified Hsk1 migrates as a single band and is nearly homogeneous. We conclude that purified Hsk1 has protein kinase and autokinase activities. We tested the ability of purified Hsk1 to phosphorylate a variety of other substrates (data not shown). Hsk1 phosphorylates histone H1 poorly relative to myelin basic protein and casein. The specific activity of the purified Hsk1 was 37 units/mg with myelin basic protein as the substrate and 19 units/mg with casein.

Hsk1 Is an MCM Kinase—Members of the MCM family of proteins have been implicated in Cdc7 kinase function in both budding yeast and metazoans. To test directly whether any MCM family members are Hsk1 substrates, we purified the entire MCM complex of six polypeptides from fission yeast as described by Adachi et al. (36). A silver stain of the purified MCM complex separated by SDS-PAGE is shown in Fig. 5A. The purified MCM proteins eluted from the final column of the purification, a Superdex 200 gel filtration column, with an apparent molecular mass of approximately 600 kDa, consistent with previous reports (36). The co-elution of all six MCM proteins at a high molecular mass suggests they are present as a multimeric complex or complexes. We incubated the MCM complex with purified Hsk1 in a kinase assay and separated the products by SDS-PAGE. A phosphorylated polypeptide of approximately 120 kDa was detected (Fig. 5B). Phosphorylation of the 120-kDa peptide was dependent upon the presence of

| Table I |
|---|
| **Fraction** | **Protein** | **Hsk1** | **Purification** |
| Extract | 2621 | 80 | 1 |
| Talon-Sepharose pool | 10.72 | 46 | 141 |
| 12CA5 pool | ~0.012 | 5.2 | ~14,344 |
| Mono Q pool | ~0.003 | 2.7 | ~29,508 |

*a Arbitrary units from quantitative immunoblot analysis of each fraction. 
*b Estimated by densitometry of SYPRO orange-stained SDS-polyacrylamide gels.

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Kinase assays also include a negative control reaction without added mono Q load, and fractions through the peak of Hsk1 (Cdc19 (Mcm2) subunit).

Kinase activities were assayed for autokinase activity and for kinase activity (using GST-MCM complex as a substrate. A phosphorylated protein co-migrating with Cdc19 is indicated. The purified MCM complex does not contain MCM kinase activity.

Purification of Hsk1

FIG. 5. Hsk1 phosphorylates the purified MCM complex on the Cdc19 (Mcm2) subunit. A, silver-stained SDS gel of the purified MCM complex from fission yeast, with the position of each of the six subunits indicated. B, purified Hsk1 was assayed for kinase activity using the MCM complex as a substrate. A phosphorylated protein co-migrating with Cdc19 is indicated. The purified MCM complex does not contain MCM kinase activity. C, kinase assays with purified Hsk1 and the MCM complex were silver-stained (silver) to detect the six MCM proteins and then autoradiographed (kinase). D, protein kinase assays as in C were immunoblotted (western) to detect Cdc19 and Hsk1 and then autoradiographed (kinase).

A, 12CA5 peptide eluate pool (12CA5 pool), mono Q load, and fractions through the peak of Hsk1 (31–37) are shown. Kinase assays also include a negative control reaction without added kinase (0).

FIG. 6. Purified Hsk1 kinase contains two polypeptides. A, the mono Q pool of purified Hsk1 was assayed for kinase activity (kinase) and subjected to immunoblot analysis with 12CA5 (western) to identify the Hsk1 pool. The open triangle indicates the position of p90, the polypeptide that co-purifies with Hsk1. The faster mobility phosphoprotein is, likely, a breakdown product as it accumulates during the purification and during storage. B, the mono Q pool of purified Hsk1 kinase was incubated under protein kinase assay conditions, denatured by boiling, and then immunoprecipitated with an irrelevant monoclonal antibody (control) or with anti-HA tag monoclonal 12CA5 (α-HA). Antibody-bound and -unbound fractions were fractionated on SDS-PAGE and autoradiographed. A sample of auto phosphorylated Hsk1 kinase before immunoprecipitation (UT) is included. C, the mono Q pool of purified Hsk1 kinase was fractionated on SDS-PAGE and silver-stained. Two polypeptides of similar mobility are evident.

Two Forms of Hsk1 Kinase—It has been proposed that Cdc7 kinase activity requires association of Cdc7 with Dbf4 (12). Although interactions between Cdc7 and Dbf4 have been detected by two hybrid assays, it is not known whether these proteins form a direct complex or whether Dbf4 is a subunit of the active protein kinase. Our most purified fraction appeared to migrate as a single band on silver-stained SDS gels. However, we noted the presence of a phosphorylated polypeptide of slightly slower mobility than Hsk1 in kinase assays of the purified protein (open triangle Fig. 6A). To determine whether the polypeptide of slower mobility (initially termed p90) was a hyperphosphorylated form of Hsk1, we subjected the kinase reaction products to immunoblot analysis, using 12CA5 antibody (open triangle Fig. 6A). To pursue this possibility further, we incubated purified Hsk1 in a kinase assay and denatured the products by boiling. The epitope-tagged Hsk1 polypeptide was immunoprecipitated with 12CA5 antibody (Fig. 6B, anti-HA bound). Under conditions where approximately 65% of the Hsk1 was precipitated, all of the p90 remained in the supernatant (Fig. 6B, anti-HA unbound). Again, we concluded that p90 is not a modified form of Hsk1 but rather is a co-purifying protein that is also a substrate of the Hsk1 kinase.

We have been able to resolve the Hsk1 and p90 polypeptides on SDS-polyacrylamide gels (Fig. 6C), but clearly these two proteins have very similar mobilities.

Since p90 co-purifies with Hsk1 through a purification procedure that enriches the latter by 30,000-fold, it seemed highly likely that the two polypeptides are tightly complexed. To verify this assumption, the most highly purified Hsk1 preparation (mono Q fraction) was fractionated further on a Superdex 200 gel filtration column, and the fractions were assayed individually for Hsk1 and p90 (Fig. 7A). We were able to separate Hsk1 into two fractions, one containing both Hsk1 and p90 (fractions 19 through 25) and one containing Hsk1 alone (fractions 27 through 33). We used native gel analysis to determine the molecular masses of the complexes present in each fraction (Fig. 7B). Fraction 23, which contained both Hsk1 and p90, yielded a single complex that had a molecular mass of 139 kDa.
by native gel electrophoresis. This result is consistent with a heterodimeric complex of Hsk1 polypeptide and p90. Fraction 31, which contained only Hsk1, yielded a complex with a molecular mass of 72 kDa, consistent with a monomer of Hsk1. An additional species was observed in fraction 31, with a molecular weight consistent with homodimeric Hsk1, but was not analyzed further.

*p90 Activates Hsk1 Kinase Phosphorylation of Exogenous Substrates*—We tested the ability of the Hsk1 and Hsk1/p90 fractions to phosphorylate two exogenous substrates, a GST fusion containing the amino-terminal 122 amino acids of Hsk1 (for Hsk1) and One Cdc19 (for p90) and Superdex fraction 31 (containing Hsk1) were electrophoresed under non-denaturing conditions, transferred to nitrocellulose, and probed for Hsk1 using 12CA5 antibody. The 72-kDa Hsk1 and the 139-kDa Hsk1/p90 complex are indicated. C. kinase assays were performed using GST-Hsk1-1-122 or GST-Cdc19 as substrates. Assays contained either no added kinase (0), the Hsk1/p90 Superdex fraction (50), or the Hsk1 Superdex fraction (31).

**FIG. 7. Two forms of Hsk1 kinase.** A, the mono Q pool of Hsk1 was fractionated on a Superdex 200 gel filtration column. Column fractions were assayed for Hsk1 by immunoblot analysis, probed with 12CA5 antibody (top), and assayed for kinase activity (bottom). The positions of Hsk1 and p90 are indicated, as are the elution positions of the gel filtration standards. *Asterisks* indicate fraction 23, which contains both Hsk1 and p90, and fraction 31, which contains only Hsk1. B, the Hsk1 mono Q pool, Superdex fraction 23 (containing Hsk1 and p90), and Superdex fraction 31 (containing Hsk1) were electrophoresed under non-denaturing conditions, transferred to nitrocellulose, and probed for Hsk1 using 12CA5 antibody. The 72-kDa Hsk1 and the 139-kDa Hsk1/p90 complex are indicated. C. kinase assays were performed using GST-Hsk1-1-122 or GST-Cdc19 as substrates. Assays contained either no added kinase (0), the Hsk1/p90 Superdex fraction (50), or the Hsk1 Superdex fraction (31).

**FIG. 8. p90 is the fission yeast Dbf4 homologue Dfp1.** A. Hsk1 was immunoprecipitated from a strain expressing untagged Dfp1 (right two lanes) or 6his-3myc-tagged Dfp1 (left two lanes). The immunoprecipitates (IP) were incubated in the absence (−) or presence (+) of ATP in kinase assay buffer, and the products were separated by SDS-PAGE and transferred to nitrocellulose. The immunoblots were probed with 9E10 (α-myc) to detect Dfp1 or with 12CA5 (α-HA) to detect Hsk1. B. extracts from the strain expressing Hsk1–6his-3HA and Dfp1–6his-3myc (GBY396) were immunoprecipitated with a control monoclonal antibody, 12CA5 to precipitate Hsk1, or 9E10 to precipitate Dfp1. Immunoprecipitates were incubated with [γ−32P]ATP under kinase assay conditions, and the products were separated by SDS-PAGE. The autoradiograph is shown, and the positions of p90 and Hsk1 are indicated.

To further confirm the identity of p90, we again prepared extracts from GBY396, which expresses myc-tagged Dfp1 and HA-tagged Hsk1. We immunoprecipitated either Hsk1 or Dfp1 and incubated the immunoprecipitates with [γ−32P]ATP under kinase assay conditions. The products of the reactions were analyzed by SDS-PAGE (Fig. 8B). When the Hsk1 polypeptide was immunoprecipitated with anti-HA antibody (Fig. 8B, lane 2), the phosphorylated products were identical to those observed previously with the purified Hsk1 kinase (see Fig. 6A), i.e. one comigrated precisely with Hsk1 and the other with p90. The same result was obtained when Dfp1 was immunoprecipitated with anti-myc antibody (Fig. 8B, lane 3). We conclude that p90 is Dfp1, the fission yeast homologue of DBF4.

**DISCUSSION**

Members of the Cdc7 family of protein kinases are absolutely required for the initiation of DNA replication and are highly conserved throughout Eukaryota. We have purified the *S. pombe* Cdc7 homologue, Hsk1, to apparent homogeneity. This is the first description of the purification of a Cdc7 kinase from any source. Analysis of the purified protein has allowed us to characterize the catalytic activity of the Hsk1 protein kinase, investigate its substrate specificity, and define its subunit structure.

To explore the functional role of Hsk1, we examined the activity of several Hsk1 point mutants in *vitro* and *in vivo*. Mutations were introduced into the ATP binding motif (K129A), a conserved kinase domain (D216N), and a residue
that is believed to correspond to the cyclin-dependent kinase-activating kinase phosphorylation site in cyclin-dependent kinases (T291A and T291E (38)). All of the mutations examined inactivated the kinase activity of Hsk1 in vitro and inactivated the essential function of Hsk1 in vivo. Surprisingly, however, these mutant Hsk1 proteins produced very different phenotypes upon overexpression. Although overexpression of wild-type Hsk1 and the K129A and T291A mutants had no effect on an otherwise wild-type strain, overexpression of the D216N and T291E mutants delayed progression from G1 into S phase and deranged the checkpoint that normally prevents mitosis when DNA replication is defective. A similar dominant phenotype has been described following overexpression of the analogous Cdc7 mutants, D163N and T281E (39), although it is not known whether these mutant proteins retain kinase activity. This phenotype is reminiscent of that observed when proteins with important roles in the initiation of DNA replication (e.g. Cdc18, Orp1, polo) are depleted. It is not clear why some mutants lacking kinase activity should exhibit a dominant negative phenotype, whereas others do not. All of the mutant proteins were expressed at similar levels to wild-type Hsk1, so the phenotype does not appear to correlate with an unusual stability or instability of the mutant proteins. It is particularly interesting that two kinase-negative mutants carrying mutations at the same amino acid residue (T291A and T291E) cause dramatically different phenotypes when overexpressed. If these mutant proteins block initiation of DNA replication by competing for limiting Dfb4 protein, as has been suggested for the analogous mutants in budding yeast (39, 40), then the K129A and T291A mutants may be unable to interact with fission yeast Dfp1. The K129A mutant affects the conserved lysine residue that is probably part of the ATP binding domain, and the T291 mutant affects a potential phosphoacceptor site, (40) raising the possibility that ATP binding or threonine 291 phosphorylation might affect the interaction of Hsk1 with Dfp1.

Significantly, we have found that purified Hsk1 kinase phosphorylates the MCM protein complex purified from fission yeast. Although the phosphorylation of MCM proteins expressed as GST fusions has been described (17, 27), this is the first indication that purified Cdc7-family kinases can phosphorylate MCM proteins in a multimeric complex as they are believed to exist in vivo. Both MCMs and Hsk1 are essential for initiation of DNA replication, and both are likely present at replication origins at the G1/S transition. Phosphorylation of MCMs by Cdc7-family kinases may regulate the essential function of the MCM complex in initiation. Furthermore, we found a clear preference for the phosphorylation of Cdc19 (Mcm2) by Hsk1. This is of particular interest, as a recently described heteromeric MCM complex with helicase activity appears to lack Mcm2 (45), and the helicase activity of this complex is inhibited by the addition of purified Mcm2 (46). One model for the role of Hsk1 in regulating the initiation of DNA replication is that Hsk1 activates MCM helicase activity by phosphorylating Mcm2, thereby relieving the inhibitory effect of Mcm2. Mutagenesis of the Hsk1 phosphorylation site(s) in Cdc19 will allow us to determine the consequences of this phosphorylation in vivo.

In budding yeast, Cdc7 is believed to associate either directly or indirectly with Dfb4, and Cdc7 kinase activity depends on Dfb4 in vitro (12). We identified a second polypeptide in the purified Hsk1 mono Q fraction with significant homology to Dfb4. This polypeptide, which we called Dfp1, co-purifies with Hsk1 through two affinity steps and a conventional ion exchange step (an ∼30,000-fold purification), indicating that Dfp1 is specifically and tightly associated with Hsk1. The native molecular mass of the Hsk1-Dfp1 complex was approximately 139 kDa. Thus, our experiments provide biochemical evidence for the formation of a stable heterodimer of Hsk1 and its regulatory subunit Dfp1.

Experiments in budding yeast have indicated that Dfb4 is required for Cdc7 kinase activity (12), but the nature of this requirement is poorly defined. We found that monomeric Hsk1 retains autokinase activity, indicating that association of Hsk1 with the Dfb4 homologue Dfp1 is not required for protein kinase activity per se. However, when we measured phosphorylation of two exogenous substrates, GST-Hsk11–122 and GST-Cdc19, we found that Dfp1 caused a dramatic stimulation of Hsk1 kinase activity, indicating that the biochemical role of Dfp1 is to modify the substrate specificity of the catalytic subunit, Hsk1. These findings are in agreement with models of Cdc7 kinase activation based upon genetic studies which proposed that kinase activation requires direct binding of Cdc7 to Dfb4 in a manner analogous to the activation of cyclin-dependent kinases by cyclins (40). Activation of Hsk1 by Dfp1 appears to be mechanistically distinct from cyclin/cyclin-dependent kinase activation, however, because Hsk1 retains kinase activity in the apparent absence of Dfp1. Association with Dfp1 could cause changes in the substrate binding site of Hsk1 rather than altering the conformation of the ATP binding site and catalytic residues as seen upon cyclin binding to cyclin-dependent kinase (47). It is also possible that Dfp1 contacts substrates directly to extend the specificity of the kinase. In vivo studies in budding yeast have shown that the transcription of DBF4 is cell cycle-regulated and peaks at the G1/S transition (48). If expression of Dfp1 protein is similarly regulated, then association of Dfp1 with Hsk1 could play an important role in ensuring that Hsk1 kinase can efficiently phosphorylate substrates critical for progression into S phase.

We have also noted phosphorylations of the Hsk1 kinase that may play important roles in regulating kinase activity. Hsk1 monomer and Hsk1-Dfp1 heterodimer both have similar Hsk1 autokinase activity, suggesting that this activity could potentially play a role that is distinct from Dfp1-regulated phosphorylation of substrates such as Cdc19. Additionally, Dfp1 itself is an excellent substrate of Hsk1 kinase. Although it is not yet clear whether Dfp1 phosphorylation has a regulatory role, there are a number of possibilities. For example, phosphorylation of Dfp1 could alter the substrate specificity of the enzyme, regulate Dfp1-Hsk1 interactions, and/or control the stability of Dfp1. Another possibility is that phosphorylation of Dfp1 might change the subcellular or subnuclear localization of the Hsk1 kinase, thereby regulating its access to critical substrates. It has been proposed that in budding yeast Dfb4 targets Cdc7 to replication origins (15), and Dfp1 may play a similar role in fission yeast. Although speculative, regulation of this interaction by phosphorylation of Dfp1 would represent an interesting (and testable) mechanism for controlling the initiation of DNA replication.

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