Bipartite Binding of a Kinase Activator Activates Cdc7-related Kinase Essential for S Phase*

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Dfp1/Him1 protein of fission yeast, Schizosaccharomyces pombe, encodes the regulatory subunit for Hsk1 kinase, a homologue of budding yeast Cdc7 kinase essential for initiation and progression of the S phase of the cell cycle. This protein binds and activates Hsk1 kinase, which phosphorylates the MCM2 protein. Comparison of the amino acid sequences of the Cdc7 regulatory subunits from various eukaryotes revealed the presence of three small stretches of conserved amino acid sequences, namely Dbf4 motifs N, M, and C. We report here that the Dbf4 motif M, a unique proline-rich motif, and the Dbf4 motif C, a C_H2-type zinc finger motif, are essential for mitotic functions of Dfp1/Him1 protein as well as for full-level activation of Hsk1 kinase. In vitro, a small segment containing the Dbf4 motif M or C alone binds to and partially activates Hsk1. Co-expression of these two segments augments the extent of activation. Furthermore, a fused polypeptide containing only Dbf4 motifs M and C without any spacer can activate Hsk1 and is capable of rescuing the growth defect of him1 null cells. Insertion of a long stretch of amino acids between the motif M and motif C can be tolerated for mitotic functions. On the other hand, internal deletion of Dbf4 motif N, which has some similarity with the BRCA C-terminal domain motif, results in a defect in hydroxyurea-induced checkpoint responses and sensitivity to methyl methane sulfonate, yet mitotic functions and kinase activation are intact. In one-hybrid assays with budding yeast Dbf4, motif N mutants exhibit reduced interaction with a replication origin. Our observations suggest the molecular architecture of Cdc7/Dbf4-related kinase complexes at the origins, in which they are tethered to replication machinery through Dbf4 motif N and the catalytic subunits are activated through bipartite binding of Dbf4 motifs M and C of the regulatory subunits.

Saccharomyces cerevisiae CDC7 is a temperature-sensitive mutant defective in initiation of DNA replication (1). Initial experiments indicated that ongoing protein synthesis was not required for completion of the S phase once the function of Cdc7 was executed (2, 3). dbf4 (dumbbell former), isolated from independent screening (4), showed terminal phenotypes similar to those of cdc7(ts). Later, DBF4 was re-isolated as a multi-copy suppressor of cdc7(ts) (5). Subsequent genetic and biochemical evidence showed that DBF4 encodes a regulatory or activation subunit for Cdc7 protein (6).

Structural and functional homologues of Cdc7 have been noted in fission yeast, human, mouse, and Xenopus (7–10). The kinase domains are particularly well conserved and identity between yeast and human is 45%. Activation subunits for these Cdc7-related kinases have been isolated through interaction screening or in data base searches (11–17). Expression of the activation subunit is cell cycle-regulated and is accumulated during the S phase (13, 17–21). Accordingly, Cdc7-dependent kinase activity is high during the S phase. Despite functional similarity to cyclins in apparent periodic appearance and kinase activation during the cell cycle, Dbf4 and cyclins share no apparent sequence similarity. Compared with the Cdc7 catalytic subunits, Dbf4 and activation subunits for Cdc7-related kinases from other eukaryotes are more diverged. There is less than 25% identity between Dbf4 and Dfp1/Him1, the fission yeast homologue of Dbf4, and no overall homology was evident between yeast and mammalian Dbf4 homologues. However, alignment of known Dbf4 homologues revealed two stretches of amino acids (Dbf4 motif N and Dbf4 motif C) conserved in all the known Dbf4/Him1-related molecules (13). We discovered another stretch of amino acids (Dbf4 motif M), which is also conserved in all the Dbf4-related molecules (22).

With fission yeast Dfp1/Him1 protein as a model, we have now generated a series of deletion and point mutants and examined the functions of these conserved motifs of Dbf4-related molecules regarding in vivo functions, binding to the catalytic subunit, kinase activation, and interaction with replication origins. We describe here essential functions of Dbf4 motif M and Dbf4 motif C for mitotic cell cycle and kinase activation and the potential of each motif to serve as an independent Hsk1 binding module. The combination of these two motifs can activate the kinase activity of Hsk1. We also found that Dbf4 motif N, related to BRCA C-terminal domain motif although not essential for mitotic function and kinase activation, plays crucial roles in the DNA replication checkpoint as well as in recovery from DNA damage-induced cell cycle arrest and may be involved in interaction with replication machinery or chromatin.

EXPERIMENTAL PROCEDURES

Yeast Strains, Media, and Genetics—Schizosaccharomyces pombe strains were grown in rich (YES) or minimal (EMM) 1 medium containing the required supplements. General genetic manipulation (23) and

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transformation (24) were done as described. To induce expression from the nmt1 or modified nmt1 promoter (25), cells were grown to mid-exponential phase in EMM containing 10 μg/ml thiamine, spun down, and washed three times with EMM lacking thiamine before being resuspended in fresh medium lacking thiamine. Cell survival analysis for DNA replication block or DNA damage was done as described (26).

Construction of Deletion Derivatives of Dfp1/Him1—Deletion derivatives were PCR-constructed using sets of primers listed on Table I. For PCR, we used Taq DNA polymerase Hi-Fi (Roche Molecular Biochemicals) as outlined by the supplier. The amplified fragment, digested with Sall and BamHI, was then inserted into the pREP41-HA vector (25). For expression in insect cells, the resulting expression vector on pREP41-HA was first digested with NdeI, filled in with the Klenow fragment, and digested with BamHI. The insert DNA containing the HA tag and him1 coding frame was re-cloned into the Smal-BglII site of pVL1933, an insect cell expression vector.

Construction of Internal Deletion and Point Mutants—Internal deletions and point mutations were introduced during two consecutive runs of PCR. The first PCR was done using sets of N1/antisense primers and BamR/sense primers (Table I). The resulting two fragments were isolated and used for the second PCR in the presence of N1 and BamR primers. The resulting fragment was subcloned into pREP41-HA and pVL1933 vectors as described above, and the presence of the expected mutations was confirmed by sequencing. The construction of motif N mutants of Dbf4 motif C was done on pGAD424. Dbf4 fusion plasmids (27) were used as a scaffold to engineer Dbf4 motif C and M. The resulting plasmids simultaneously expressed HA-tagged 113-amino acid motif M and 65-amino acid linker sequence. To construct a plasmid expressing Dbf4 motif M and Dbf4 motif C, the pStl-Smal fragment of pREP41-motif M containing nmt1-driven Dbf4 motif C was cloned at the Smal site on pREP41-motif M using a pStl-Smal adapter. The resulting plasmid simultaneously expressed HA-tagged 113-amino acid motif M and 65-amino acid motif C polypeptides.

Preparation of Extracts from Insect Cells, Immunoprecipitation, and in Vitro Kinase Assays—Expression of proteins in insect cells and preparation of extracts were done as previously described (17). Immunoprecipitation and kinase assays were also done as described (10, 17).

Antibodies—Anti-HA monoclonal antibody (12CA5) was purchased from Berkeley Antibody Co. Rabbit anti-Hsk1 antibodies pep1 (9) and Hsk1C were developed against an oligopeptide (corresponding to residues 378–506) of Hsk1 protein and bacterially produced GST-Hsk1C were developed against an oligopeptide (corresponding to residues 423–578 of Hsk1 protein) and bacterially produced GST-Hsk1 antibodies pep1 (9).

Kinase Activation through Bipartite Binding

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Three Stretches of Amino Acids Conserved in Cdc7 Regulatory Subunits—Despite the striking functional conservation of regulatory subunits for Cdc7-related kinases, the level of con-
### Table I

**Oligonucleotides used for construction of plasmids**

| Name of mutant | Primer at N terminus or anti-sense primer | Primer at C terminus or sense primer |
|----------------|-------------------------------------------|--------------------------------------|
| Full-length     | 5'-TAC GCG TCG ACA TGC ACC TAG GAA GAT GTC CT-3' (N1) | 5'-ACT TGG GAT CCT GGT AAA TC-3' (HmR) |
| Dell (1–176)    | 5'-TAC GCG TCG ACA TGC ACC TAG GAA GAT GTC CT-3' | 5'-GCG GAT CCT CGA GTC CAC CAA ATT GCT GAA TTT G-3' |
| Dell (1–223)    | 5'-TAC GCG TCG ACA TGC ACC TAG GAA GAT GTC CT-3' | 5'-GCG GAT CCT CGA GTC CAC CAA ATT GCT GAA TTT G-3' |
| Dell (1–335)    | 5'-TAC GCG TCG ACA TGC ACC TAG GAA GAT GTC CT-3' | 5'-GCG GAT CCT CGA GTC CAC CAA ATT GCT GAA TTT G-3' |
| Del4 (1–511)    | 5'-TAC GCG TCG ACA TGC ACC TAG GAA GAT GTC CT-3' | 5'-GCG GAT CCT CGA GTC CAC CAA ATT GCT GAA TTT G-3' |
| Del5 (104–545)  | 5'-TAC GCG TCG ACA TGC ACC TAG GAA GAT GTC CT-3' | 5'-ACT TGG GAT CCT GGT AAA TC-3' |
| Del6 (177–545)  | 5'-TAC GCG TCG ACA TGC ACC TAG GAA GAT GTC CT-3' | 5'-ACT TGG GAT CCT GGT AAA TC-3' |
| Dell (223–545)  | 5'-TAC GCG TCG ACA TGC ACC TAG GAA GAT GTC CT-3' | 5'-ACT TGG GAT CCT GGT AAA TC-3' |
| Del7 (336–545)  | 5'-TAC GCG TCG ACT CCT TGG TGG CTA AAA ATG AC-3' | 5'-ACT TGG GAT CCT GGT AAA TC-3' |
| Del10 (223–480) | 5'-TAC GCG TCG ACA AAG TTT GCA ACC CGG TGT TA-3' | 5'-GCG GAT CCT CGA GGC TTT TAT AAG AGT AGT CCT T-3' |
| Motif M (223–335) | 5'-TAC GCG TCG ACA AAG TTT GCA ACC CGG TGT TA-3' | 5'-GCG GAT CCT CGA GGC TTT TAT AAG AGT AGT CCT T-3' |
| Motif C (480–545) | 5'-TAC GCG TCG ACA AAG TTT GCA ACC CGG TGT TA-3' | 5'-GCG GAT CCT CGA GGC TTT TAT AAG AGT AGT CCT T-3' |
| Him1Δ154–194    | 5'-TAC GCG TCG ACA AAG TTT GCA ACC CGG TGT TA-3' | 5'-GCG GAT CCT CGA GGC TTT TAT AAG AGT AGT CCT T-3' |
| Him1Δ177–222    | 5'-TAC GCG TCG ACA AAG TTT GCA ACC CGG TGT TA-3' | 5'-GCG GAT CCT CGA GGC TTT TAT AAG AGT AGT CCT T-3' |
| Motif M mutant   | 5'-TAC GCG TCG ACA AAG TTT GCA ACC CGG TGT TA-3' | 5'-GCG GAT CCT CGA GGC TTT TAT AAG AGT AGT CCT T-3' |
| 3E              | 5'-TAC GCG TCG ACA AAG TTT GCA ACC CGG TGT TA-3' | 5'-GCG GAT CCT CGA GGC TTT TAT AAG AGT AGT CCT T-3' |
| Motif C (for M+C; 485–545) | 5'-TAC GCG TCG ACA AAG TTT GCA ACC CGG TGT TA-3' | 5'-GCG GAT CCT CGA GGC TTT TAT AAG AGT AGT CCT T-3' |
| Linker D64      | 5'-TAC GCG TCG ACA AAG TTT GCA ACC CGG TGT TA-3' | 5'-GCG GAT CCT CGA GGC TTT TAT AAG AGT AGT CCT T-3' |
| Gal4AD          | 5'-TAC GCG TCG ACA AAG TTT GCA ACC CGG TGT TA-3' | 5'-GCG GAT CCT CGA GGC TTT TAT AAG AGT AGT CCT T-3' |
| Dfb4-N-2E       | 5'-TAC GCG TCG ACA AAG TTT GCA ACC CGG TGT TA-3' | 5'-GCG GAT CCT CGA GGC TTT TAT AAG AGT AGT CCT T-3' |
| Dfb4-N-GL       | 5'-TAC GCG TCG ACA AAG TTT GCA ACC CGG TGT TA-3' | 5'-GCG GAT CCT CGA GGC TTT TAT AAG AGT AGT CCT T-3' |
molecule in fission yeast, the function of which is specific for sporulation (29).

Complementation Activity of Deletion Derivatives of Dfp1/Him1—To better understand functions of each conserved domain in Dfp1/Him1 protein, we generated a series of N-terminal and C-terminal deletion derivatives. In generating deletions, we made use of the information on the exon-intron organization of spo6+ (29). We selected end points of deletions at positions of introns, since exon junctions often define the structural boundaries (30). The truncated proteins were expressed on plasmid pREP41 under the control of the attenuated nmt1 promoter. The mitotic function of each deletion, defined as the potential to rescue the growth of him1 null cells when expressed on a plasmid, was examined by introducing them into the him1+/− heterozygous diploid cells (Fig. 2B). The N-terminal deletion up to the amino acid 222 (Del 7) did not significantly affect the potential to complement the growth defect of the him1 disruptant. Further deletion up to the position 335, which removed Dbf4 motif M (Del 8), resulted in loss of complementation activity. On the other hand, deletion of the C-terminal 35 amino acids, removing a part of Dbf4 motif C (Del 4), led to reduced complementing activity; the mutant protein restored the growth only in medium lacking thiamine, in which nmt1 promoter was activated. Further deletion up to position 480, which removed the entire Dbf4 motif C (Del11), led to a complete loss of complementation activity. Thus, C-terminal 323 amino acids are sufficient for mitotic function of Dfp1/Him1 protein, and both Dbf4 motif M and C may be essential for this function.

Regions of Dfp1/Him1 Required for Binding and Activation of Hsk1—We then expressed each deletion derivative in insect cells to determine interactions with and activation of Hsk1 protein (Fig. 3). Each mutant Dfp1/Him1 was co-expressed either with wild-type Hsk1 or the kinase-attenuated mutant of Hsk1 (K129R-K130S) to calibrate the extent of activation of the kinase by the mutant proteins. The Dfp1/Him1 deletions were tagged with HA at the N terminus, and immunoprecipitation was done either with anti-HA or anti-Hsk1 antibody. Binding to Hsk1 was evaluated by co-immunoprecipitation of the two proteins with either antibody. Kinase assays were done on the immunoprecipitates with GST-SpMCM2N used as a substrate, as described in the previous section. We first confirmed that the expression of Hsk1 and each deletion derivative of Dfp1/Him1 was at a similar level in insect cell extracts (data not shown). Consistent with the result of mitotic functions, Del5 (104–545) bound to Hsk1 and activated its kinase activity as efficiently as did the wild type. Del6 (177–545) and Del7 (223–545) also similarly activated the kinase by the mutant proteins. The Dfp1/Him1 deletions were tagged with HA at the N terminus, and immunoprecipitation was done either with anti-HA or anti-Hsk1 antibody. Binding to Hsk1 was evaluated by co-immunoprecipitation of the two proteins with either antibody. Kinase assays were done on the immunoprecipitates with GST-SpMCM2N used as a substrate, as described in the previous section. We first confirmed that the expression of Hsk1 and each deletion derivative of Dfp1/Him1 was at a similar level in insect cell extracts (data not shown). Consistent with the result of mitotic functions, Del5 (104–545) bound to Hsk1 and activated its kinase activity as efficiently as did the wild type. Del6 (177–545) and Del7 (223–545) also bound to and activated both Hsk1 wild type and K129R-K130S, as indicated by their potential to cause a mobility shift of GST-SpMCM2N protein, although Del7 was only partially active. Therefore, Dbf4 4-motif N (151–195) may also contribute to kinase activation and/or recognition of the substrate. Alternatively, conformational change induced by the deletion may be inhibitory for functions.

On the other hand, the C-terminal deletion, which profoundly affected the mitotic function of Him1, also affected its kinase activation. Del4 (1–511) and Del11 (1–480), lacking the C-terminal 34 and 65 amino acids, respectively, could activate the wild-type Hsk1 but not the attenuated Hsk1K129R-K130S. Hsk1K129R-K130S-Del4 or Hsk1K129R-K130S-Del11 combination generated very little phosphorylated and mobility-shifted forms of GST-SpMCM2N, whereas full-level phosphorylation of MCM2 was observed in combination with the wild-type Hsk1. This was the case even with Del3 (1–335). Del8 (336–545) and Del10 (223–480) also similarly activated the wild-type Hsk1 but not K129R-K130S, although the level of
MCM2 phosphorylation was reduced compared with former deletions containing the N-terminal regions. This is consistent with findings that deletion of the N-terminal segment of Dfp1/Him1, although not essential for mitotic functions, decreased its potential to activate Hsk1 for phosphorylation of MCM2. The level of kinase activation was particularly affected in immunoprecipitates with anti-HA antibody, which suggests that the presence of the HA antibody may be inhibitory for kinase activation and/or recognition of the substrate in the N-terminal deletions.

The results indicate that both N-terminal and C-terminal regions can independently interact with Hsk1 and activate kinase activity in vitro. Del1 (1–176), Del2 (1–222), and Del12 (336–480), which lack both Dbf4 motif M and C, showed very little or no binding and activation of Hsk1. However, Del10 (223–480), carrying the Dbf4 motif M, was capable of activating the wild-type Hsk1 and underwent extensive phosphorylation. In conclusion, activation of Hsk1 kinase, as revealed by the potential to activate the K129R-K130S mutant, required at minimum the C-terminal 323 amino acids, containing both Dbf4 motif M and Dbf4 motif C, which are also sufficient to maintain its mitotic function. However, either the N-terminal 335 amino acids or the C-terminal 210 amino acids containing Dbf4 motif M or Dbf4 motif C, respectively, were sufficient for binding and activation of the wild-type Hsk1 for MCM2 phosphorylation. Binding and activation was completely lost in the absence of both motifs.

A Mutation in Dbf4 Motif M Results in Impaired Mitotic Functions and Kinase Activation—Our findings suggest that both Dbf4 motif M and Dbf4 motif C play crucial roles in kinase activation and mitotic function of Dfp1/Him1 protein. To determine whether Dbf4 motif M plays essential roles on full-length Dfp1/Him1 protein, we generated a mutant Dfp1/Him1 in which conserved residues in this motif (two aromatic residues, Tyr-311 and Tyr-291, and one aspartic acid, Asp-286) were replaced with alanine (Fig. 2A). The mutant protein, expressed on the pREP81 vector under the highly attenuated nmt1 promoter, was able to rescue growth of the him1 null cells on plates without thiamine, although the growth of the transfor- 
mants was slower than seen in the wild-type cells (Fig. 4A). The levels of the Dfp1/Him1 protein in transformants in the absence of thiamine were similar between the wild-type and the mutant (Fig. 4B). After the addition of thiamine to the culture grown in medium lacking thiamine, growth of the motif M mutant strain was almost completely suppressed, whereas the strain carrying pREP81-Him1 (wild type) grew after the addition of thiamine (Fig. 4A). Fluorescence-activated cell sorter analyses of DNA content in asynchronous culture of the motif

**Fig. 2.** Alignment of the three conserved motifs of Dbf4-related molecules and Dfp1/Him1 mutants characterized in this study. A, locations of the characterized three conserved motifs in Dfp1/Him1 protein are indicated as striped regions. The dark gray portion indicates the region that shares 25% identity with S. cerevisiae Dbf4, whereas the light gray portions represent less conserved regions. The sequence alignments of the three motifs between Dbf4, Dfp1/Him1, and huASK are shown in addition to the positions of point mutations in Dbf4 motif N and Dbf4 motif M mutants generated. Amino acid substitutions at the conserved proline residues of Dbf4 motif M in dbf4-1, 2, and 3 temperature-sensitive mutants (38) as well as those at conserved valine residues in nimO18 mutant (12) are also indicated. The amino acid residues conserved in more than two members are indicated by white letters in black backgrounds. aa, amino acids. B, schematic drawing of Dfp1/Him1 mutants constructed and analyzed in this study. The gray and striped regions represent the N-terminal HA tag and the three motifs, respectively. The numbers in parentheses indicate the amino acid residues present in each deletion derivative. For small motif M and motif C polypeptides, the lengths of amino acids are also indicated. The potential of each derivative, when expressed on the pREP41 vector in the presence of thiamine, to rescue the growth of the him1 null mutant is indicated for each mutant. + + + and ++ indicate full growth recovery and slower cell growth. + indicates that the mutant can complement only in the absence of thiamine, where the protein is overexpressed. Him1 FL, full-length Him1.
M mutant after the addition of thiamine showed an increase in 1C cells (Fig. 4C, right; 3 h) followed by an increase of less than 1C DNA content (Fig. 4C, right; 6–24 h). These results indicate that the motif M mutant cells first arrest at the G1/S boundary, then proceed to premature mitosis in the absence of DNA synthesis. A significant portion of the cells exhibited the cut phenotype, indicative of problems in coupling of DNA synthesis and mitosis (data not shown).

The kinase activation by the motif M mutant was then examined in vitro. The motif M mutant bound to Hsk1 with an efficiency similar to that seen in the wild-type (data not shown) but failed to fully activate its kinase activity. The mobility-shifted form of MCM2 was not detected in combination with the K129R-K130S mutant, and the extent of the Him1 hyperphosphorylation was also significantly reduced (Fig. 4D, lanes 5, 6, 11, and 12). These results indicate that the motif M mutant is defective in full-level activation of Hsk1 kinase.

**Bipartite Hsk1 Binding Modules on Dfp1/Him1 Protein**—

The results of deletion and mutation analyses indicate that the presence of either Dbf4 motif M or Dbf4 motif C may be sufficient for binding to Hsk1 and for partial kinase activation in vitro. We then asked if small segments containing Dbf4 motif M or Dbf4 motif C would be sufficient for binding to Hsk1. The 113-amino acid segment from amino acids 223 to 335 (Fig. 2B, Motif M) or the 65-amino acid segment from amino acids 481 to 545 (Fig. 2B; Motif C) was co-expressed with Hsk1 in insect cells. The mutant Dbf4 motif M containing three amino acid substitutions (Motif M mutant) was also similarly expressed. Either polypeptide containing the Dbf4 motif M or the Dbf4 motif C was co-immunoprecipitated with Hsk1 protein (Fig. 5A, lanes 1, 2, 7, and 8; data not shown), thus indicating that both motifs are capable of independently binding to Hsk1. The mutant Dbf4 motif M polypeptide could not bind to Hsk1 (Fig. 5A, lanes 3, 4, 9, and 10), suggesting that the mitotic defect of the motif M mutant was due to its reduced efficiency of interaction with Hsk1, although this effect could not be detected on the full-length Dfp1/Him1 protein in our immunoprecipitation assay. Kinase assays with the immunoprecipitate containing motif M or motif C complexed with Hsk1 indicated that both the wild-type Hsk1 and associated motif M or motif C polypeptide could be autophosphorylated (Fig. 5B, lanes 1 and 5). However, the level of autophosphorylation of Hsk1K129R-K130S was lower either with motif M or motif C compared with the full-length Dfp1/Him1 protein (Fig. 5B, lanes 2, 6, and 8). Similarly, very little MCM2 phosphorylation (appearance of a mobility-shifted form) was detected with either polypeptide in a complex with wild-type Hsk1 (Fig. 5B, lanes 1 and 5), indicating that additional segments of Dfp1/Him1 are required for kinase activation. We further narrowed down the two binding regions by constructing plasmids expressing 56-amino acid (250–305) and 60-amino acid (276–335) segments containing motif M and 58-amino acid (488–545) and 37 amino acid (488–524) segments containing motif C (Fig. 5C). Immunoprecipitation with anti-HA antibody followed by detection with anti-Hsk1 antibody plus kinase assays indicated that the 86- and 60-amino acid motif M and 58-amino acid motif C were able to associate with Hsk1 (Fig. 5D, lanes 3–8), but the 37-amino acid motif C was not (Fig. 5D, lanes 9 and 10). Autophosphorylation of the
wild-type Hsk1 was detected with the 86-amino-acid motif M and 58-amino-acid motif C and with the 60-amino-acid motif M to a lesser extent (Fig. 5D, lanes 3, 5, and 7). Phosphorylation of the 86-amino-acid motif M and 58-amino-acid motif C supported autophosphorylation of Hsk1K129R-K130S to a significant extent (Fig. 5D, lanes 4 and 8), the 60-amino-acid motif M, which bound to Hsk1, showed the level of Hsk1K129R-K130S autophosphorylation at about 20% that shown by the 86-amino-acid motif M polypeptide (Fig. 5D, lane 6). These results define the minimal segment required for Hsk1 binding within the 60- and 58-amino-acid segments of motif M and motif C, respectively, and suggest that the 60-amino-acid motif M segment, capable of binding to Hsk1, lacks amino acids essential for full-level activation of Hsk1 kinase.

We recently found that the co-expression of the N-terminal and C-terminal halves of ASK, the activation subunit for huCdc7, together with huCdc7 in mammalian cells could reconstitute an active kinase. Therefore, we expressed both motif M and motif C polypeptides together with Hsk1 protein in insect cells. When the immunoprecipitates were assayed for kinase reactions, more efficient phosphorylation of both polypeptides (as revealed by more extensive mobility shift) was observed with wild-type Hsk1 protein (Fig. 5B, lanes 11 and 15). The level of Hsk1K129R-K130S phosphorylation also increased (Fig. 5B, lanes 12 and 16). This stimulation was not observed when the motif M mutant was coexpressed with motif C (Fig. 5B, lanes 13, 14, 17, and 18). These results suggest a possibility that the motif M and motif C polypeptides form a ternary complex with Hsk1, which then may lead to stimulation of Hsk1 kinase activity. It should be noted that MCM2 phosphorylation was not stimulated even in the presence of both motif M and motif C (Fig. 5B, compare lanes 1, 2, 5, 6, 15, and 16).

Only Motif M and Motif C Are Sufficient for Mitotic Activity and Kinase Activation—The above results suggest the possibility that only motif M and motif C segments are sufficient for mitotic activity of Dfp1/Him1 protein and that the segment connecting the two motifs may be dispensable. The amino acid sequences between Dbf4 motif M and Dbf4 motif C are not conserved between species, and the length also varies, with 170, 346, and 37 amino acids in Dfp1/Him1, Dbf4, and ASK, respectively. To determine if these sequences contribute to mitotic activity and kinase activation of Dfp1/Him1 protein, we constructed a plasmid expressing a fused polypeptide composed of only small segments of motif M and motif C. The resulting polypeptide contains 113 amino acids (223–335) of motif M, 5

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* N. Sato, M. Sato, K. Arai, and H. Masai, unpublished data.
Amino acids derived from an unrelated linker sequence and 61 amino acids (485–545) of motif C. Surprisingly, this small polypeptide could support growth of him1 null cells (Fig. 6A, construct b). This result clearly indicates that the intervening sequences between motif C and motif M are not required for mitotic activity and kinase activation of Dfp1/Him1 protein and that the presence of the two conserved motifs is sufficient. We then determined if insertion of an exogenous sequence between the two motifs could be tolerated. We found that the presence of the two conserved motifs is sufficient. We additional mutants of this motif and examined their properties (data not shown). However, all the mutants exhibited mild sensitivity to HU, and cut cells were generated after exposure to HU to the level about 50 to 60% that of the corresponding wild-type him1 null (Fig. 6A). The C-terminal half of the motif N is needed for mitotic function. We reported that mutations to HU-mediated replication (Fig. 7A). Him1Δ177–222 lacks the C-terminal half of the motif N, including conserved serine/threonine residues. Him1Δ154–222 lacks the entire motif N as well as additional amino acids, whereas Him1Δ3E contains serine/threonine (Ser-183, Thr-187, and Thr-191) to glutamic acid substitutions. All the above mutants except for Him1Δ3E could support a near normal growth in him1 null cells when expressed from the attenuated nmt1 promoter on pREP41 (Fig. 2B), and the levels of kinase activation were comparable with those of the wild-type (data not shown). However, all the mutants exhibited mild sensitivity to HU, and cut cells were generated after exposure to HU to the level about 50–60% that of the cds1 null mutant (data not shown). The Cds1 protein is known to be required for S-phase checkpoint control downstream of Rad3/ATM kinase. Him1 3A, Him1 3E, Del6, and Del 7 were also sensitive to MMS. These results further confirm our previous observation and strongly support our conclusion that Dfh4 motif N is specifically involved in response of cells to HU-mediated replica-
Kinase Activation through Bipartite Binding

**FIG. 6.** Only the Dbf4 motif M and Dbf4 motif C regions are sufficient for mitotic functions of Dfp1/Him1, and the intervening region can be deleted or replaced with other sequences of different lengths. A, schematic drawing of the plasmids expressing fusion polypeptides of motif M and motif C stretches containing various intervening sequences and their properties. The level of growth complementation is expressed as in Fig. 2B. + + or + in the Hsk1 activation in vitro column indicates phosphorylation of exogenous MCM2 substrate or activation of only autophosphorylation but not that of substrate phosphorylation, respectively. ND, not determined. aa, amino acids. B, in vitro kinase assays with motif M and motif C fusion polypeptide (containing a 5-amino acid spacer; construct b shown in A) expressed in insect cells. Combinations of Hsk1, wild-type (WT), or mutant as indicated and Dfp1/Him1 (full-length (FL) or M-C fusion (M-C)) were expressed in insect cells, and in vitro kinase assays were done on immunoprecipitates (IP) prepared by anti-HA (lanes 1–5) or by anti-Hsk1 (Hsk1C) (lanes 6–10) antibody. The products were run on 8% (upper and lower) or 15% (middle) SDS-PAGE. Upper panel, autoradiogram of kinase assay; middle panel, blot with anti-Hsk1 antibody; lower panel, blot with anti-Hsk1 (pep1) antibody. IgG indicates immunoglobulin heavy and light chains.

**Dbf4 Motif N Is Required for Interaction with Replication Origins in Vivo—**Dowell et al. (31) report that a one-hybrid assay could detect the interaction of *S. cerevisiae* Dbf4 protein with replication origins in a sequence-specific manner. The segment on Dbf4 involved in this interaction was mapped to positions 81–278, which contained the motif N. Therefore, we asked if the BECA C-terminal domain-like motif N is involved in the interaction of Dbf4 protein with replication origins. We constructed Dbf4 mutants ΔN, GL, and 2E, in which the N-terminal half of Dbf4 motif N (residues 126–160) is deleted or conserved LG at position 158–159 was changed to GL or the conserved threonine residues at positions 171 and 175 were substituted for by glutamic acid, respectively. These mutants were expressed as fusions with the activation domain of the Gal4 transcription factor on plasmid pGAD424. The resulting plasmids rescued the temperature-sensitive growth of *dbf4Δ-1(ts) mutant* (data not shown). These results are consistent with our conclusion that Dbf4 motif N is not required for mitotic functions and interaction with the catalytic subunit in Dfp1/Him1. To determine if these motif N mutants could interact with replication origins in one-hybrid assays, wild-type Dbf4 for the control could interact with wild-type ARS sequences but not with mutant ARS-containing base substitutions within the ACS (ARS consensus sequence), indicating that the assay indeed measures the sequence-specific interaction of Dbf4 protein with replication origins. In contrast to the wild type, all three motif N mutant Dbf4 plasmids showed a background level of LacZ activity (Fig. 7C), thus indicating loss of specific interaction with replication origins. Therefore, Dbf4 motif N is specifically required for interaction with replication machinery or chromatin components at the origins.

**DISCUSSION**

It is now well established that Cdc7-related kinases are widely conserved and play pivotal roles in initiation of eukaryotic DNA replication (14, 32–35). Although the catalytic subunits encoded by Cdc7-related genes share significant homology in kinase-conserved domains, structures of the regulatory subunits are unexpectedly diverged (13). Through comparison of Dbf4, Dfp1/Him1, and ASK, the regulatory subunits from budding yeast, fission yeast, and human, respectively, we identified and reported three stretches of amino acids that are conserved in all the three molecules (13, 17, 22). In the present work, using fission yeast Dfp1/Him1 protein as a model, we carried out detailed analyses of structures involved in various functions of this essential regulator of the Cdc7 kinase, the ultimate switch for DNA replication.

**Dissection of Dfp1/Him1 Protein; Hsk1 Binding, Kinase Activation, and Mitotic Function—**Brown and Kelly (11) report that Dfp1/Him1 changes the substrate specificity rather than activating Hsk1 kinase activity per se. Hsk1 expressed in insect cells possesses significant autophosphorylation activity on its own and to some extent can phosphorylate exogenous substrates. The Hsk1K129R-K130S mutant we generated shows very little autophosphorylation activity by itself, which means that its intrinsic kinase activity is significantly impaired. However, its autophosphorylation as well as MCM2 phosphorylation activities can be activated almost to the wild-type level in the presence of Dfp1/Him1 protein. Use of the Hsk1K129R-K130S mutant facilitated the measurement of the potential of various mutant Dfp1/Him1 proteins to activate the kinase reactions mediated by Hsk1.

The Dfp1/Him1 mutants we characterized can be classified into eight types depending on the conserved motifs they carry (Fig. 8). The type C or D, lacking or mutated in either Dbf4 motif M or C, respectively, lost mitotic activity completely or partially, and this correlates with their attenuated kinase activation, as revealed by their lack of potential to activate attenuated Hsk1 mutant Hsk1K129R-K130S. Truncation mutants of nimO protein, the Aspergillus homologue of Dbf4, lacking the motif C segment was reported to be incapable of complementing the *nimO* deletion (12). Similarly, C-terminal truncation of budding yeast Dbf4 was shown to lose complementation activity of a *dbf4* null mutant (5). Both type C and D can bind to Hsk1 and activate the wild-type Hsk1 *in vitro*, and type F or G, containing only Dbf4 motif M or C, respectively, can bind to Hsk1. However, Hsk1 binding and, thus, kinase activation is completely lost in type E lacking both Dbf4 motif M and C. In contrast, Dbf4 motif N is dispensable for mitotic functions and full-level kinase activation; the C-terminal 323 amino acids are sufficient for complementing growth defect of *him1* null cells. However, type B mutants lacking or mutated in Dbf4 motif N are partially defective in DNA replication checkpoint control and are sensitive to DNA damages.

Our results indicate that Dfp1/Him1 protein interacts with Hsk1 through two separate binding domains, Dbf4 motif M and C. Dbf4 motif C is related to C2H2-type zinc finger motif
(12), which is often involved in protein-protein interactions (36), whereas Dbf4 motif M, characterized by the presence of conserved multiple proline residues, is not related to any known motifs in the data base and, hence, may be a novel protein-interacting motif. Our earlier two-hybrid analyses showed that the C-terminal 50 amino acids containing Dbf4
motif C could interact with Hsk1, findings consistent with data obtained in the immunoprecipitation studies. In each complex containing the 113-amino acid motif M or 65-amino acid motif C, the Dfp1/Him1-derived polypeptide was phosphorylated. However, MCM2 phosphorylation was not stimulated by Hsk1K129R-K130S as well as that of motif M and motif C polyptides but not phosphorylation of MCM2 (Fig. 5B). Although we cannot conclude from these experiments that the three molecules form a ternary complex, it is possible that simultaneous binding of motif M and motif C to distinct regions on Hsk1 may synergistically stimulate its kinase activity, whereas interaction with exogenous substrates may require an additional segment of Dfp1/Him1 protein.

The presence of two Hsk1 binding regions is consistent with data by Hardy and Pautz (37) that two separate regions on Dbf4 protein can bind to Cdc7 in two-hybrid assays. Furthermore, the two Dbf4 regions mapped in the above studies, 241–416 and 573–695 of Dbf4, contain Dbf4 motif M and Dbf4 motif C, respectively. Furthermore, the mutation in the temperature-sensitive dbf4K129R–K130S, or dbf4K129R–K130S, as a proline to leucine substitution at position 277 and a proline to leucine or serine substitution at position 308 within Dbf4 motif M (Ref. 38; Fig. 2A). These two proline residues are conserved in Dbf4 motif M. Furthermore, nimO18 mutation was identified to be a substitution of a rather conserved valine residue within Dbf4 motif M with glutamic acid (12). These findings support our conclusion that Dbf4 motif M is crucial for functions of Cdc7 regulatory subunits.

Dbf4 Motif N Is Required for Replication Checkpoint Functions and Is Involved in Interaction with Chromatin—Our results also indicate that domains on Dfp1/Him1 protein essential for mitotic functions and those involved in replication checkpoint functions could be separated. Sensitivity to HU observed with some of our mutants may not be simply due to additive effects of HU on S phase defects. The motif N mutants are sensitive to HU and develop cut cells in the presence of HU despite their proficiency for kinase activation and mitotic functions. The sequence similarity of motif N to the BRCA C-terminal domain-like domain on Cut5 protein (17) suggests the presence of a common factor that may interact with this domain. The amino acids 81–278 of Dbf4 protein were previously noted to be sufficient for interaction with ARS in one-hybrid assays (31). This region contains the entire Dbf4 motif N. Our results with one-hybrid assays showed that Dbf4 motif N is required for interaction with replication origins in vivo. It is an intriguing possibility that motif N interacts with replication machinery assembled at the origin, and this interaction, although dispensable for mitotic functions, may be important for signal transmission of DNA replication checkpoint control. Identification of interacting molecules with the motif N will aid in identifying molecular architecture involving Dbf4-related molecules at the origins.

Mode of Cdc7 Kinase Activation by Cdc7 Regulatory Subunits—It was concluded that kinase activation to a full extent requires the presence of both Dbf4 motif M and C. Since these two motifs are not related in amino acid sequences, it is likely that the two domains bind to distinct domains on Hsk1 protein. The kinase activities of budding yeast Cdc7 and human Cdc7 strictly depend on association with corresponding regulatory subunits. Given the high degree of amino acid conservation of these motifs, we predict that activation of Cdc7-related kinase by Dbf4-related regulatory subunits may commonly involve these two conserved domains. A segment of
ASK, the activator of huCdc7, containing either motif M or motif C, can independently interact with huCdc7, and a segment containing both Dbf4 motif M and Dbf4 motif C of ASK is sufficient for activation of huCdc7 in vivo and in vitro. The coexpression of two independent N-terminal and C-terminal huCdc7 binding segments of ASK with huCdc7 resulted in activation of autophosphorylation. Although both 60-amino acid (276–335) and 86-amino acid (249–335) Dbf4 motif M segments bound to Hsk1 with similar efficiency, a higher level of kinase activation was observed with the latter polypeptide. This suggests that the 27 amino acids (249–275) are required for kinase activation. LKX\_KX\_RD (254–266), present immediately N-terminal to Dbf4 motif M, is conserved in budding yeast Dbf4 and ASK and may play important roles in activation of Hsk1 kinase.

The sequences and lengths between motif M and motif C diverged even between budding and fission yeasts. Polypeptides containing only Dbf4 motif M and C but lacking the spacer sequence rescued the growth defect of \_kin1\_ null cells. Furthermore, insertion of 37-amino acid or 346-amino acid spacer sequences derived from ASK or Dbf4, respectively, between the motif M and motif C on the same plasmid did not affect complementing activity. Thus, our results demonstrate that simultaneous binding of motif M and motif C segments to the catalytic subunit is sufficient for kinase activation as well as for mitotic functions and that the intervening region serves merely as a flexible spacer peptide that connects the two conserved motifs for coordinated binding to the catalytic subunit (Fig. 8). This is in contrast to activation of Cdk by cyclins, where all the Cdk binding activity is contained in a single domain of \(-100\) amino acids in length called the cyclin box (39). A unique feature of the Cdc7 kinase molecule essential for DNA replication, provides an important clue to the progression of human cells can be manipulated through modulation of activities of the huCdc7-ASK kinase complex.

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REFERENCES
1. Selanfani, R. A. (2000) *J. Cell Sci.* 113, 2111–2117
2. Hartwell, L. H. (1971) *J. Mol. Biol.* 59, 183–194
3. Hartwell, L. H. (1975) *J. Bacteriol.* 115, 966–974
4. Johnston, L. H., and Thomas, A. P. (1982) *Mol. Gen. Genet.* 186, 439–444
5. Kitada, K., Johnston, L. H., Sugino, T., and Sugino, A. (1992) *Genetics* 131, 971–979
6. Jackson, A. L., Pahl, P. M. B., Harrison, K., Rosamond, J., and Selanfani, R. A. (1993) *Mol. Cell Biol.* 13, 2899–2908
7. Sato, N., Arai, K., and Masai, H. (1997) *EMBO J.* 16, 4340–4351
8. Kim, J. M., Sato, N., Yamada, M., Arai, K., and Masai, H. (1998) *J. Biol. Chem.* 273, 23248–23257
9. Masai, H., Miyake, T., and Arai, K. (1995) *EMBO J.* 14, 3094–3104
10. Jong, W., and Hunter, T. (1997) *Proc. Natl. Acad. Sci. U. S. A.* 94, 14320–14325
11. Brown, G., and Kelly, T. (1998) *J. Biol. Chem.* 273, 22083–22090
12. James, S. W., Bullock, K. A., Gygax, S. E., Kraynack, B. A., Matura, R. A., MacLeod, J. A., McNeal, K. K., Prasarkauskas, K. A., Scacheri, P. C., Shenefiel, H. L., Tohin, H. M., and Wade, S. D. (1999) *J. Cell Sci.* 112, 1313–1324
13. Kumagai, H., Sato, N., Yamada, M., Mahony, D., Segherzzi, W., Lees, E., Arai, K., and Masai, H. (1999) *Mol. Cell. Biol.* 19, 5083–5093
14. Johnston, L. H., Masai, H., and Sugino, A. (1999) *Trends Cell Biol.* 9, 249–252
15. Jiang, W., McDonald, D., Hope, T. J., and Hunter, T. (1999) *EMBO J.* 18, 5793–5799
16. Lepke, M., Putter, V., Staib, C., Knießl, M., Berger, C., Hoehn, K., Nanda, I., Schmid, M., and Grummt, F. (1999) *Mol. Gen. Genet.* 262, 220–229
17. Takeda, T., Ogino, K., Matsui, E., Cho, M., Kumagai, H., Miyake, T., Arai, K., and Masai, H. (1999) *Mol. Cell. Biol.* 19, 5535–5547
18. Brown, G., and Kelly, T. (1999) *Proc. Natl. Acad. Sci. U. S. A.* 96, 8443–8448
19. Cheng, L., Collyer, T., and Hardy, C. (1999) *Mol. Cell. Biol.* 19, 4270–4278
20. Rizki, M. F., Santocanale, C., Drury, L. S., and Difflay, J. F. (2000) *Mol. Cell. Biol.* 20, 242–248
21. Weinreich, M., and Stillman, B. (1999) *EMBO J.* 18, 5334–5346
22. Masai, H., and Arai, K. (2000) *Biochem. Biophys. Res. Commun.* 18, 228–232
23. Gutz, H., Heslot, H., Leupolz, U., and Leprieur, N. (1974) *Handbook of Genetics* (King, R. C., ed) pp. 395–446, Plenum Press, New York
24. Ohtoshi, A., Miyake, T., Arai, K., and Masai, H. (1997) *Mol. Cell. Biol.* 17, 4360–4365
25. Kihara, M., Nakai, W., Asano, S., Suzuki, A., Kitada, K., Kawasaki, Y., Ikeda, H., Arai, K., and Masai, H. (2001) *Mol. Biol. Cell* 12, 1275–1279
26. Al-Khodairy, F., Fotou, E., Sheldrick, K. S., Griffiths, D. J. F., Lehmann, A. R., and Carr, A. R. (1994) *Mol. Cell. Biol.* 14, 8443–8453
27. Ohtoshi, A., Miyake, T., Arai, K., and Masai, H. (1999) *Mol. Biol. Cell* 7, 2208–2219
28. Masai, H., Arai, K., and Masai, H. (1997) *Mol. Gen. Genet.* 254, 562–570
29. Li, J. J., and Hershkowitz, I. (1998) *Science* 263, 1870–1874
30. Nakamura, T., Kishida, M., and Shima, S. (2000) *Genes Cells* 5, 463–479
31. Doi, M. (1985) *Adv. Biophys.* 19, 91–131
32. Ohtoshi, A., Miyake, T., Arai, K., and Masai, H. (1997) *Mol. Cell. Biol.* 17, 4360–4365
33. Masai, H., Arai, K., and Masai, H. (2000) *J. Biol. Chem.* 275, 29042–29052
34. Masai, H., Sato, N., Arai, K., and Masai, H. (1999) *Front. Biosci.* 4, 135–140
35. Sato, N., Arai, K., and Masai, H. (1997) *Mol. Microbiol.* 11, 805–810
36. Mackay, J. P., and Crossley, M. (1998) *Trends Biochem. Sci.* 23, 1–4
37. Hardin, P. J. F., and Pautz, A. (1996) *Mol. Cell. Biol.* 16, 6775–6782
38. Hardin, P. J. F., and Pautz, A. (1996) *Mol. Cell. Biol.* 16, 6775–6782
39. Kihara, M., Nakai, W., Asano, S., Suzuki, A., Kitada, K., Kuchiy, Y., Husson, J. H., and Sugino, A. (1997) *J. Biol. Chem.* 272, 11413–11418
40. Bahman, M., Buck, V., White, A., and Rosamond, J. (1988) *Biochim. Biophys. Acta* 951, 355–363
41. Shellman, Y. G., Schauer, I. E., Oshore, G., Dohrmann, P., and Selanfani, R. A. (1998) *Mol. Gen. Genet.* 259, 429–436
42. Hanks, S. K., Quinn, A. M., and Hunter, T. (1998) *Science* 241, 42–52
43. Takeda, T., Ogino, K., Tatebayashi, K., Ikeda, H., Arai, K., and Narumi, H. (2000) *Mol. Cell Biol.* 12, 1257–1274

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3. H. Masai, E. Matsui, K. Ogino, and K. Arai, unpublished result.
Bipartite Binding of a Kinase Activator Activates Cdc7-related Kinase Essential for S Phase
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