ATP-DEPENDENT PRE-REPLICATIVE COMPLEX ASSEMBLY IS FACILITATED BY Adk1p IN BUDDING YEAST

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Pre-replicative complex (pre-RC) assembly is a critical part of the mechanism that controls the initiation of DNA replication, and ATP binding and hydrolysis by multiple pre-RC proteins are essential for pre-RC assembly and activation. Here we demonstrate that Adk1p (Adenylate kinase) plays an important role in pre-RC assembly in Saccharomyces cerevisiae. Isolated from a genetic screen, adk1G20S cells with a mutation within the nucleotide binding site are defective in replication initiation. adk1Δ cells are viable at 25°C but not at 37°C. Flow cytometry indicates that both the adk1-td (temperature-inducible degron) and adk1G20S mutants are defective in S phase entry. Furthermore, Adk1p binds to chromatin throughout the cell cycle and physically interacts with Orc3p, while the Adk1G20S protein has a reduced ability to bind chromatin and Orc3p without affecting the cellular ATP level. In addition, Adk1p associates with replication origins by ChIP assay. Finally, Adk1-td protein depletion prevents pre-RC assembly during the M-to-G1 transition. We suggest that Adk1p regulates ATP metabolism on pre-RC proteins to promote pre-RC assembly and activation.

The initiation of DNA replication governs the genome duplication in eukaryotes which occurs once and only once per cell cycle. In S. cerevisiae, replication initiation is critically controlled by pre-RC assembly which includes sequential loading of ORC (Origin Recognition Complex) (1-3), Noc3p (4), Cdc6p (5-8), Cdt1p (9), and MCM proteins (Mcm2-7p; minichromosome maintenance) (10, 11) onto ARS (autonomously replicating sequence) elements. In addition, ATP binding on and hydrolysis by Cdc6p and several subunits of ORC are essential for pre-RC assembly (12-15). At least ten components (Orc1p, Orc4p, Orc5p, Cdc6p, and Mcm2-7p) of pre-RCs are ATPases (16) containing the Walker A and B motifs required for ATP binding and hydrolysis. Mutation analysis showed that all conserved ATP binding motifs of these proteins are essential for cell viability (12, 17, 18). There have been numerous reports on the roles of binding and hydrolysis of ATP in pre-RC assembly. In S. cerevisiae, ATP binding of Orc1p activates ORC assembly and ORC binding to replication origins (1). Mutation within the Orc1p ATP-binding site prevents ORC binding to DNA in vitro and is lethal in vivo (19). Other studies indicated that mutations in the Walker A motif eliminate the ATP binding and hydrolysis activities of Orc1p (20, 21). When ATP binds to Orc1p, an initial round of chromatin loading of MCM proteins is permitted, while ATP hydrolysis is required for other rounds of MCM loading in vitro (13, 22). Human ORC assembly in vitro is dependent on ATP binding and impaired by mutations in Orc4p or Orc5p ATP binding sites (23, 24). In S. cerevisiae, Cdc6p binding to ORC, which is dependent on the Cdc6p ATPase activity, changes the ORC structure and contributes to pre-RC assembly (25). Cdc6p mutated in the Walker B motif cannot interact with Orc1p in vitro (26) and shows decreased chromatin loading and lethality in vivo (18). Similar mutation in human Cdc6p also eliminates its ATP binding and hydrolysis activities (27). However, the enzyme(s) that may
regulate ATP metabolism during pre-RC assembly has not been reported.

Adenylate kinases are phosphotransferases that catalyze the inter-conversion reaction of ATP + AMP ↔ 2ADP and control nucleotide metabolic processes thus the cell growth rate in eukaryotes (28). Adk1p is important for cell proliferation but not essential for cell viability by gene disruption analysis in S. cerevisiae (29), and two isozymes of Adk1p, termed Adk2p and Ura6p, have been found (30-32). In S. pombe, the Adk1p homolog is required for cell viability (33). In mammalian tissues, 6 isozymes, designated AK1 to AK6, have been identified and characterized (34-36). These adenylate kinases show functional similarities in ATP metabolism.

From a random EMS (ethane methyl sulfonate) mutagenesis followed by a phenotypic screen, which we called the IDR (initiation of DNA replication) screen, to identify genes involved in or regulating replication initiation in S. cerevisiae (37, 38; L. Ma, YZ, T.C. Chan, D. Feng, J. Wang, X. Fu, Y. Lu and CL, submitted), we isolated an adk1G20S mutant that loses a single-ARS plasmid at a high rate and a multiple-ARS plasmid at a reduced rate. We show that both the adk1-td and adk1G20S mutants have replication initiation defects, suggesting that Adk1p plays an important role in DNA replication initiation. Furthermore, we demonstrate that Adk1p binds to pre-RC components and replication origins and becomes essential for pre-RC assembly and cell viability at 37°C.

**EXPERIMENTAL PROCEDURES**

**Plasmids, Strains, and Antibodies**- The original adk1G20S P138L mutant was isolated from the IDR screen after EMS mutagenesis of the parental strain YL36 (ade2-1 ade3-1 ura3-1 his3-11,15 trpl-1 leu2-3,112 can-100), a derivative of W303-1A with the addition of the ade3-1 mutation. The integration plasmids pJJ244-ADK1, pJJ244-adk1G20S, and pJJ244-adk1P138L bearing the full length ADK1, adk1G20S and adk1P138L, respectively, generated by site-directed mutagenesis (except for ADK1), were integrated into the ura3 locus of the adk1A strain to construct the ADK1 wild-type strain and adk1 mutants (39). The adk1-td-HA strain was constructed in the GAL-UBR1 background as described (40) using the PCR product generated with the forward primer 5'-CATTAACGTTCCTCGTGTAGGATCCACTTCTTGAGCTCCAC-3' and the reverse primer 5'-GCACCAGGTGGCCAAATTTAAGGACATTGCAGGATCCGATCCG-3'.

The ADK1-HA strain (3xHA-tagged ADK1 at the endogenous ADK1 locus) was constructed in the W303-1A background, and the pJJ244-ADK1-HA and pJJ244-adk1G20S-HA strains were constructed in the integrated pJJ244-ADK1 and pJJ244-adk1G20S background, respectively, using the one-step C-terminal tagging method (41) to transform the respective yeast cells with a PCR fragment amplified by the forward primer 5'-AACCTCCTGCTACTGTTTGGGCTGACATCTTGAACAAGCTAGGTAAAGGATCGGATCCCCGGGT-3' and the reverse primer 5'-AATTTAAAAAAAGAAAAGATATTTAGAAACATTGCAGCAAGTGCGCTAGTATTTAAGAATTCGAGC-3'.

The anti-HA (12CA5) antibody was from Roche Applied Science. Anti-ORC and anti-MCM antibodies were gifts from Bruce Stillman (Cold Spring Harbor Laboratory).

**Protein Extraction, Co-immunoprecipitation, Immunoblotting, ChIP assay and ATP assay**- Protein extraction, co-IP, ChIP assays were performed as described (4, 42). ATP assay were performed as described (43). After yeast cell wall was digested (4), the ATPLite™ assay system (PerkinElmer – USA) and 10⁷ cells per sample were used for each measurement.

**Cell Synchronization, Fluorescence Activated Cell Sorting (FACS) Analysis and Chromatin Binding**- Cell cycle block and release with α-factor, hydroxyurea (HU) or nocodazole (Noc.) were carried out as described (4). adk1-td cells were cultured to early log phase and then arrested with
the cell cycle inhibitors in YPD or SCM (synthetic complete medium)-based selective medium containing 0.1 mM CuSO$_4$ at 25°C. YPRG medium (2% raffinose and 0.5% galactose) without CuSO$_4$ was then used to induce GAL-UBR1 expression at 25°C for 1 hr and to degrade the Adk1-td protein at 37°C for 1 hr. FACS analysis and chromatin binding assays were performed as described (4, 42, 44).

RESULTS

**adk1**$^{G20S}$ mutant cells have defects in DNA replication initiation- We carried out a sensitive yeast phenotypic screen with randomly mutagenized yeast cells to identify proteins related to replication initiation using a pair of tester plasmids, p1ARS and p8ARSs (4). It is known that mutants in genes that function in or regulate DNA replication initiation exhibit high plasmid loss rates in p1ARS transformants and lower plasmid loss rates in p8ARSs transformants (4, 6, 45-47). Therefore we employed these plasmids to identify mutants defective in DNA replication initiation. Among many mutants in known and unknown replication-initiation proteins (37, 38; L. Ma, YZ, T. Chan, D. Feng, J. Wang, X. Fu, Y. Lu and CL, submitted), an adk1$^{G20S}$ mutant was identified to be a replication initiation mutant.

The original adk1 mutant isolated from our screen contains two point mutations: G20S within the putative nucleotide binding site (1$^{\text{G}}$GPPGAGKST$^{21}$; ref. 48) and P138L. We separated the two mutations, integrated them separately into an adk1$^\Delta$ strain, and then examined the plasmid loss rates of the integrants using a colony color sectoring assay (49) and a colony size assay. Cells in the ade2-1 ade3-1 background turn red on the non-selective YPD plates when they contain p1ARS or p8ARSs bearing the reporter gene ADE3-2. In the colony color sectoring assay, the white/red sectoring morphology of the colony indicates plasmid loss rate of the cells in the colony (red cells contain the plasmid, and white cells have lost the plasmid). The adk1$^{G20S}$ mutant lost p1ARS at a high rate and p8ARSs at a lower rate, while the adk1$^{P138L}$ mutant lost both plasmids at low rates (Fig. 1A). Therefore, the G20S, but not the P138L mutation, was responsible for the replication initiation phenotypes of the original adk1 mutant isolated from the screen.

In the colony size assay, the background of the mutants is leu2-1, thus the adk1$^\Delta$/pRS416-adk1$^{G20S}$ cells (the pRS416 vector contains the URA3 marker) need to carry p1ARS or p8ARSs bearing the LEU2 marker in order to grow on SCM-Ura-Leu (synthetic complete medium lacking uracil and leucine) plates. When mutant cells have replication initiation defects, p1ARS transformants form small colonies because of the high rate of p1ARS loss while p8ARSs transformants form bigger ones. Consistent with results from the colony color assays, the adk1$^{G20S}$ mutant exhibited smaller colony size with p1ARS than p8ARSs on SCM-Ura-Leu plates, while the difference in colony size disappeared on SCM-Ura plates which were non-selective for LEU2 (Fig. 1B). Furthermore, quantitative plasmid assays (Fig. 1C) confirmed the plasmid loss phenotypes of the adk1$^{G20S}$ mutant. These results suggest that the adk1$^{G20S}$ mutant is defective in DNA replication initiation.

**ADK1 becomes essential for viability at 37°C**- Consistent with a previous report that ADK1 is important for growth but not essential for viability at normal growth temperatures (29), the adk1$^\Delta$ cells that we generated could grow at 25°C, but they grew more slowly than wild-type cells (Fig. 1D). However, adk1$^\Delta$ cells were not viable at 37°C (Fig. 1D), indicating that ADK1 is essential for cell viability at 37°C. To further confirm this conclusion, we generated a temperature-inducible degron adk1-td strain, transformed the adk1-td cells with pRS416-ADK1 or with the pRS416 vector alone, and then tested the transformants at 25°C and 37°C. The adk1-td cells transformed with pRS416 were viable at 25°C but not at 37°C, while the adk1-td cells transformed with pRS416-ADK1 grew normally at both temperatures (Fig. 1E). These results confirm that ADK1 is essential for cell viability at 37°C.

**The adk1$^{G20S}$ mutant and ADK1 wild-type cells have similar in vivo ATP levels and growth rates**- To rule out the possibility that the plasmid loss phenotypes of the adk1$^{G20S}$ mutant resulted from an altered cellular ATP level, we measured the in vivo ATP levels in log phase wild-type, adk1$^{G20S}$ and adk1$^\Delta$ cells. Wild-type and adk1$^{G20S}$ mutant
cells have similar ATP levels in vivo, while \( \text{adkl} \Delta \) cells have a much lower ATP level (Fig. 2A). These results indicate that the replication initiation defects of the \( \text{adkl}^{G20S} \) cells did not result from ATP homeostasis imbalance.

We also measured cell growth rates of the wild-type and \( \text{adkl} \) mutants at 25°C. Compared to wild-type cells, the growth rates of the \( \text{adkl}^{G20S} \) and \( \text{adkl}^{P138L} \) mutants were only slightly reduced (Fig. 2B). Together with the data on the cellular ATP levels, these results support the notion that the \( \text{adkl}^{G20S} \) mutant cells exhibit allele-specific defects in replication initiation not being associated with ATP homeostasis or growth rate.

*Adk1p is required for the G1-to-S transition at 37°C* - To examine the role of Adk1p in the cell cycle, we examined the DNA contents of wild-type, \( \text{adkl} \text{-td} \) and \( \text{adkl}^{G20S} \) cells by flow cytometry. The cells were first arrested in G1 phase by \( \alpha \)-factor. After Adk1-td protein degradation in galactose-containing medium (galactose induces the over-expression of Ubr1p to facilitate td protein degradation; ref. 40) at 37°C, the \( \text{adkl} \text{-td} \) cells were released from the G1 block into fresh medium at 37°C. Control experiments were performed with glucose-containing medium at 25°C. \( \text{adkl} \text{-td} \) cells at 25°C (Fig. 2C) and wild-type cells at both temperatures (Fig. 2C, D) entered and completed S phase after release from the \( \alpha \)-factor block. In contrast, \( \text{adkl} \text{-td} \) cells at 37°C were mostly defective in S phase entry, with only a small fraction of the cells started to enter S phase at 150 min after \( \alpha \)-factor removal (Fig. 2D). These results suggest that Adk1p is essential for the G1-to-S transition at 37°C. The \( \text{adkl}^{G20S} \) mutant cells showed a notable delay in the G1-to-S transition compared to wild type cells at both 25°C and 37°C (Fig. 2C, D). On the other hand, Adk1p is not required for S phase progression after the cells were released from the early S phase block by hydroxyurea (HU; Fig. S1A, B), or for mitosis after the cells were released from the G2/M block by nocodazole (Noc.; Fig. S1C, D), at 25°C or 37°C.

*Adk1p binds to chromatin throughout the cell cycle, and the \( \text{adkl}^{G20S} \) mutant protein has reduced chromatin binding* - To determine if Adk1p binds chromatin for its role in replication initiation and to understand the basis for the \( \text{adkl}^{G20S} \) mutant phenotypes, we carried out chromatin binding assays with \( \text{ADK1-HA} \) and \( \text{adkl}^{G20S-HA} \) cells. In asynchronous cells, the \( \text{ADK1-HA} \) protein has much reduced chromatin binding compared to wild-type Adk1p using Orc3p as the loading control (Fig. 3A). To examine the cell cycle pattern of chromatin association of the Adk1p and \( \text{adkl}^{G20S} \) proteins, \( \text{ADK1-HA} \) and \( \text{adkl}^{G20S-HA} \) cells were arrested in G1 phase by \( \alpha \)-factor and then released into fresh medium at 25°C. Samples were collected for chromatin binding assays at different time points after release. Adk1p-HA was more or less constant in both the supernatant and chromatin fractions throughout the cell cycle, while the constant chromatin binding of Orc3p and the cell cycle-regulated chromatin association of Mcm2p were as expected (Fig. 3B; see the accompanying FACS data in Fig. 3C). In \( \text{adkl}^{G20S-HA} \) cells, Orc3p was constant while the release of Mcm2p from chromatin was delayed (Fig. 3D) in accordance with the slower cell cycle progression of the \( \text{adkl}^{G20S-HA} \) cells (Fig. 3E) compared to wild-type cells (Fig. 3C). Consistent with the results from the asynchronous cells, the Adk1p-HA protein was reduced in the chromatin fractions and increased in the supernatant fractions (Fig. 3D) compared to the wild-type Adk1p (Fig. 3B).

*Adk1p physically interacts with Orc3p, and the \( \text{adkl}^{G20S} \) mutant protein has mostly lost the interaction with Orc3p* - To test if Adk1p physically interacts with ORC, co-IP assay was carried out with asynchronous \( \text{ADK1-HA} \) and \( \text{adkl}^{G20S-HA} \) cells. Orc3p could be co-immunoprecipitated with Adk1p-HA by the anti-HA antibody (Fig. 3F, lane 6, lower panel), but not by the control mouse IgG (Fig. 3F, lane 5) from the \( \text{ADK1-HA} \) cell extracts. Untagged strain gave a negative co-IP signal as expected (Fig. 3F, lane 3). For \( \text{adkl}^{G20S-HA} \) cells, no obvious co-IP of Orc3p could be detected (Fig. 3F, lane 6, lower panel). These results suggest that Adk1p physically interacts with ORC in vivo and the \( \text{adkl}^{G20S} \) mutant protein has mostly lost this interaction.

*Adk1p binds to replication origins in vivo* - After knowing that Adk1p binds to chromatin and interacts with Orc3p, we examined if the Adk1-HA protein associated with replication origins in vivo by ChIP assay with anti-HA antibody, with anti-Orc3 antibody and mIgG as the positive and negative controls, respectively. Specific PCR
primer pairs were used to amplify two replication origins, ARS1 and ARS501, and their corresponding non-ARS control sequences, R2.5 and (501+11 kb), respectively. When anti-Orc3 (Fig. 3G, lane 4) or anti-HA (Fig. 3G, lane 5) antibody was used, ARS1 and ARS501, but not the non-ARS control regions, were detected in the immunoprecipitated chromatin DNA. Negative control for PCR without DNA template (Fig. 3G, lane 2), untagged strain control (Fig. 3G, lane 3) and IgG control produced no ChIP signal (Fig. 3G, lane 6). Together with the results of interaction of Adk1p with chromatin and ORC, these ChIP data suggest that Adk1p binds to replication origins probably through its interaction with ORC and perhaps other pre-RC proteins.

Adk1p is required for pre-RC assembly at 37°C- During the M-to-G1 transition, MCM proteins, known as the last markers for pre-RC assembly, are loaded onto chromatin at replication origins. We examined the in vivo pre-RC assembly efficiency by detecting Mcm2p and Cdc6p on chromatin when the Adk1p-td protein was depleted. adk1-td cells were arrested at the G2/M boundary by nocodazole. After Adk1p-td protein degradation, the cells were released from the G2/M block into G1 phase in α-factor-containing medium at 37°C and then harvested at various time points for chromatin binding assays (Fig. 4A) and for FACS analysis to monitor cell cycle progression (Fig. 4B). In the control experiment in which the Adk1p-td protein was not degraded at 25°C, Mcm2p and Cdc6p were successfully loaded onto chromatin as expected (Fig. 4A, lanes 1-5). In contrast, when the Adk1p-td protein was degraded at 37°C, Mcm2p and Cdc6p were mostly absent on chromatin (Fig. 4A, lanes 6-10), suggesting that the chromatin association of MCM proteins and Cdc6p was significantly impaired. Orc3p was constant in the chromatin fractions at different time points at both temperatures. In the supernatants, Orc3p and Mcm2p were constant at both temperatures, while Cdc6p is cell cycle-regulated as expected (Fig. 4A, lanes 1-10). These results reveal that Adk1p is critical for pre-RC assembly at 37°C without affecting the levels of MCM proteins and Cdc6p in vivo.

We also examined the efficiency of pre-RC assembly during the M-to-G1 transition in adk1G20S-HA cells. Compared to wild-type cells at 25°C and 37°C, Mcm2p chromatin loading were delayed and reduced in adk1G20S-HA cells (Fig. 4C; FACS data in Fig. 4D, E), suggesting that the Adk1G20S mutant protein is partially defective in promoting pre-RC formation.

Adk1p is dispensable for pre-RC maintenance in G1 phase at 37°C- In G1 phase, MCM proteins are maintained on chromatin. To determine if Adk1p is required for pre-RC maintenance in G1 phase, we examined Mcm2p in the chromatin fractions when Adk1p-td was depleted in α-factor-containing medium at 37°C. Mcm2p was stable in both the chromatin and supernatant fractions when the Adk1p-td protein was stable at 25°C or degraded at 37°C (Fig. 4F; FACS data in Fig. 4G), suggesting that the chromatin association of MCM proteins in G1 phase does not require Adk1p. Since Adk1p is required for the G1-to-S transition at 37°C (Fig. 2D), we suggest that ATP metabolism is important for the pre-RC activation and/or the transition from pre-RC to pre-IC (pre-initiation complex) in addition to its function in pre-RC assembly.

DISCUSSION

Our study reveals that Adk1p facilitates the ATP-dependent pre-RC assembly and that Adk1p becomes essential for pre-RC assembly and cell viability at 37°C. The data from the plasmid loss assays show that the adk1G20S mutant is specifically defective in DNA replication initiation. On the other hand, the adk1G20S mutant and wild-type cells have similar in vivo ATP levels and growth rates, indicating that the replication initiation defects of the adk1G20S mutant do not result from an ATP homeostasis imbalance. We also show that Adk1p physically interacts with ORC, chromatin and replication origins, and that Adk1p is essential for pre-RC assembly and activation at 37°C. These physical interaction and functional data support a direct role of Adk1p in promoting pre-RC assembly and activation.

Our data also indicate that the Adk1G20S mutant protein has weakened association with chromatin and ORC compared to wild-type Adk1p. These results provide the probable molecular basis for the defects of the adk1G20S mutant cells in pre-RC assembly and replication initiation. In addition,
in a study unrelated to DNA replication, the Adk1<sup>G20S</sup> mutant protein has been reported to have a lower adenylate kinase activity than wild-type Adk1p (48). Therefore, we suggest that the Adk1<sup>G20S</sup> mutant protein has a reduced ability to regulate ATP metabolism at pre-RCs due to the impairment of both its enzymatic activity and of its association with ORC and perhaps other pre-RC proteins, resulting in decreased efficiencies of pre-RC assembly and activation.

Adk1p has two isozymes, Ura6p and Adk2p, and Ura6p can partly compensate for the loss of function of Adk1p in <i>S. cerevisiae</i> (31, 32). Since adk1Δ cells are viable with growth defects under normal growth conditions, it is possible that the physiological role of Adk1p in ATP-dependent pre-RC assembly can be partially compensated by Ura6p and/or Adk2p at normal growth temperatures when the Adk1p activity is absent. This is consistent with the report that Adk1p contributes about 90% to the total adenylate kinase activity in yeast cells (29). We show that Adk1p becomes essential for pre-RC assembly and for cell viability at 37°C, while it is not required for S phase progression or mitosis, consistent with Adk1p playing a critical role in pre-RC assembly. It is possible that a higher cellular adenylate kinase activity is needed for faster ATP metabolism at 37°C, thus the 10% remaining adenylate kinase activity provided by Ura6p and Adk2p is not sufficient for cell viability.

In conclusion, we propose that Adk1p may catalyze the inter-conversion reaction of ATP + AMP ↔ 2ADP and regulate ATP metabolism directly on pre-RC proteins, so as to facilitate the ATP binding- and hydrolysis-dependent pre-RC assembly and activation. Adk1p has also been reported to interact with Cdc14p (50), which dephosphorylates multiple pre-RC components and is required for pre-RC assembly (38), hence it is also possible that Adk1p may regulate phosphorylation and dephosphorylation processes of pre-RC components during pre-RC assembly. This study reveals a novel function of Adk1p in the ATP-dependent pre-RC assembly and opens a new avenue to investigate the mechanism and cell cycle control of DNA replication initiation.

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FOOTNOTES

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FIGURE LEGEENDS

FIG. 1. The adklG20S mutant cells have replication initiation defects, and Adk1p is essential for cell viability at 37°C.

(A) Colony color sectoring assays of different adkl mutants. ADK1 wild-type strain (YL36) and different adkl mutants transformed with p1ARS and p8ARSs separately were streaked onto YPD plates and incubated at 25°C for 3-5 days to form colonies. (B) Colony size assays of the adklG20S mutants. adklΔ cells containing pRS416-adklG20S were transformed with p1ARS and p8ARSs
separately, spread onto the plasmid-loss testing plate (SCM-Leu-Ura) and the control SCM-Ura plate (pRS416 contains the URA3 marker) and incubated at 25°C for 5 days to allow colony formation. (C) Quantitative plasmid loss assay for wild-type and adk1G20S mutant. (D) Ten-fold serial dilutions of log phase adk1Δ cells were spotted onto YPD plates and incubated at 25°C and 37°C to test cell viabilities. (E) adk1-td cells transformed with pRS416 or pRS416-ADK1 were streaked onto SCM-Ura/RG (R, raffinose; G, galactose) plates at 25°C and 37°C to test cell viabilities.

FIG. 2. The ADK1 wild-type and adk1G20S mutant cells have similar ATP levels and cell growth rates in vivo, and the adk1-td and adk1G20S mutant cells are defective at the G1-to-S transition.

(A) ATP levels in log phase wild-type, adk1G20S, and adk1Δ cells were measured with total cell extracts. (B) The cell growth rates of the adk1Δ cells transformed with pRS416-ADK1, pRS416-adk1G20S, or pRS416-adk1P138L in SCM-Ura medium at 25°C were measured by optical density at 600 nm (O.D.600). (C, D) Wild-type, adk1-td and adk1G20S mutant cells were arrested in G1 phase by â-factor at 25°C. After Adk1-td protein degradation in YPRG medium at 37°C, cells were released from the â-factor block into fresh YPRG at 37°C (D). Cells in the control experiments were cultured in YPD at 25°C (C). The adk1G20S mutant cells were cultured in YPD at 25°C (C) or 37°C (D). Aliquots of cells were analyzed by FACS and bud counting.

FIG. 3. Adk1p binds to ORC and chromatin at replication origins, and the Adk1G20S mutant protein has reduced binding to chromatin and ORC.

(A) Asynchronous ADK1-HA and adk1G20S-HA cells were examined by chromatin binding assay. (B-D) ADK1-HA (B, C) and adk1G20S-HA (D, E) cells were released from â-factor block, and aliquots of cells were collected at various time points at 25°C for chromatin binding assay using anti-Orc3, anti-Mcm2 and anti-HA antibodies (B, D) and for FACS analysis (C, E). (F) Adk1-HA or Adk1G20S-HA protein was immunoprecipitated from extracts of ADK1-HA or adk1G20S-HA cells, respectively, with anti-HA antibody. ADK1 untagged strain and mouse IgG were used as the negative controls. Immunoprecipitates were blotted with anti-HA and anti-Orc3 antibodies, followed by the HRP-conjugated secondary anti-Mouse IgG-light chain antibody. WCE, whole cell extracts; IgG L.C., immunoglobulin light chains. (G) Anti-Orc3 and anti-HA ChIP assays were performed with asynchronous ADK1 untagged and ADK1-HA cells. ARS1, ARS501 and the control sequences, R2.5 and (501+11 kb), in the chromatin immunoprecipitates by anti-Orc3, anti-HA or the control mouse IgG and in the input chromatin were examined by PCR. No DNA, PCR without DNA template.

FIG. 4. Adk1p is essential for pre-RC assembly in vivo at 37°C, and the adk1G20S mutant cells are partially defective in pre-RC assembly

(A-E) adk1-td (A, B), ADK1 wild-type (C, D) or adk1G20S-HA (C, E) cells were arrested at the G2/M boundary in YPD medium containing nocodazole (Noc.) at 25°C. After the Adk1-td protein was depleted in YPRG medium at 37°C for 1 hr, adk1-td cells were released into â-factor-containing YPRG medium at 37°C. ADK1 wild-type and adk1G20S-HA cells were shifted to 37°C for 1 hr before being released from the nocodazole block into â-factor-containing YPD medium at 37°C. Control cells were kept in YPD medium at 25°C. Cells at various time points after release from the nocodazole block were harvested for chromatin binding assays (A, C) and FACS analysis (B, D, E). (F, G) adk1-td cells were arrested in G1 phase with â-factor-containing YPD medium at 25°C. After Adk1-td protein degradation in YPRG at 37°C, the cells were kept in G1 phase at 37°C for chromatin binding assays (F) and FACS analysis (G). Control cells were kept in â-factor-containing YPD medium at 25°C.
Figure 1

A) Comparison of ADK1 (WT) and adk1G20S, adk1P138L with p1ARS and p8ARSs.

B) adk1 Δ/pRS416-ADK1 ΔG20S comparison with p1ARS and p8ARSs.

C) Graph showing % Loss per generation for adk1G20S and ADK1 (WT).

D) Growth assay comparison between YPD/25°C and YPD/37°C for ADK1 (WT) and adk1Δ.

E) adk1-td growth comparison between SCM-Ura/25°C and SCM-Ura/RG/37°C.
Figure 2
Figure 3
Figure 4
ATP-dependent pre-replicative complex assembly is facilitated by Adk1p in budding yeast

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