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Cdc45p assembles into a complex with Cdc46p/Mcm5p, is required for minichromosome maintenance, and is essential for chromosomal DNA replication

(S-phase/DNA replication/MCM proteins/nuclear localization)

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ABSTRACT **We report the isolation and characterization of *CDC45*, which encodes a polypeptide of 650 amino acids that is essential for the initiation of chromosomal DNA replication in the budding yeast, *Saccharomyces cerevisiae*. *CDC45* genetically interacts with at least two members of the MCM (minichromosome maintenance) family of replication genes, *CDC46* and *CDC47*, which are proposed to perform a role in restricting initiation of DNA replication to once per cell cycle. Like mutants in several MCM genes, alleles of *CDC45* also show a severe minichromosome maintenance defect. Together, these observations imply that Cdc45p performs a role in the control of initiation events at chromosomal replication origins. We investigated this possibility further and present evidence demonstrating that Cdc45p is assembled into complexes with one MCM family member, Cdc46p/Mcm5p. These observations point to a role for Cdc45p in controlling the early steps of chromosomal DNA replication in conjunction with MCM polypeptide complexes. Unlike the MCMs, however, the subcellular localization of Cdc45p does not vary with the cell cycle, making it likely that Cdc45p interacts with MCMs only during the nuclear phase of MCM localization in G_1.**

In order for eukaryotic cells to maintain their genomic integrity it is critical that only a single round of DNA replication occurs in each cell cycle. To explain the underlying mechanism behind this, it has been proposed that cells restrict initiation of DNA replication to once per cell cycle by way of a replication licensing factor [RLF (1)]. In this model, RFL gains entry to the nucleus during mitosis and licenses DNA to be replicated for the following S-phase by associating with replication origins. Once replication origins have initiated a round of replication by generating a single bidirectional replication fork, it is predicted that RLF must be inactivated to prevent re-replication in the same cell cycle and that it can only relicense a nucleus for replication following completion of the next mitosis.

Biochemical analysis of RLF in *Xenopus laevis* egg extracts has resolved it into two main components, the best characterized component consisting of a multiprotein complex composed of polypeptides which are homologues of the *Saccharomyces cerevisiae* proteins Mcm2p, Mcm3p, and Mcm5p/Cdc46p (2–5). The MCM (minichromosome maintenance) family of polypeptides are essential for the initiation of chromosomal DNA replication, minichromosome maintenance, and, in *S. cerevisiae*, are only present in the nucleus from the end of mitosis until the G_1–S transition (4–6). However, MCM homologues in *Schizosaccharomyces pombe* (7, 8), *Drosophila melanogaster* (9), *X. laevis* (10) and mammals (11–14) are located in the nucleus throughout the cell cycle where they associate with chromatin during G_1 and dissociate during S-phase. A common feature of all MCM proteins, however, is that they bind to, and are displaced from, chromatin in a manner consistent with them licensing DNA (15–18).

It is clear from characterization of RLF activity in *Xenopus* that components besides MCM polypeptides are required for licensing activity (19). Cdc45p is a likely component of MCM complexes in yeast based on observations showing that *CDC45* is essential for chromosomal replication and that two MCMs genetically interact with *CDC45* (20). In this report we describe the isolation and characterization of the *CDC45* gene and show that Cdc45p is assembled into a complex with one member of the MCM family, Cdc46p/Mcm5p. This suggests Cdc45p as an additional component of RLF complexes in budding yeast.

MATERIALS AND METHODS

Yeast Strains, Media, Isolation of *CDC45* and DNA Manipulations. Yeast media used in this study are as described in Guthrie and Fink (27). All yeast strains used in this study were isogenic with W303-1a (*MAT***a**, *ura3-52, trp1-1, ade2-1, lys2-801, leu2-3, his3-11, 15, can1-100* [psi$^+$]) and were either generated by direct gene replacement or by backcrossing the original mutant to W303-1a at least three times. The *CDC45* gene was isolated by rescue of the *cdc45-1* cold-sensitive mutation at 12°C in the strain DBY2027 (from D. Botstein, Stanford University) essentially as described (6). Approximately 25,000 transformants were screened. Plasmids were recovered from primary yeast transformants showing plasmid-dependent cold-sensitive rescue by transformation into bacteria, and then tested for complementing activity by retransformation of plasmid DNA into the original cold-sensitive strain. Rescuing plasmids were characterized by restriction mapping. All sequencing was performed on both strands with customized primers using Applied Biosystems *Taq* Dye Deoxy Terminator cycle sequencing kits according to the manufacturers instructions.

A strain expressing Cdc45p(1-650)-GFP was constructed by inserting the gene fusion at the *CDC45* locus by a one-step gene replacement (6). A single HA epitope tag was inserted directly after codon 650 of the *CDC45* open reading frame (ORF) so as to create a tagged Cdc45p derivative (Cdc45p/1-650/YPYDVPDYA) under control of the *CDC45* promoter. c-*myc* epitope tagging of Mcm5p/Cdc46p generated Cdc46p/1-775/EQKLISEEDLNM: the nuclear localization signal (NLS)-tagged version generated Cdc46p/1-775/KRGNSSIG-PNDLSKRKQKKK/EQKLISEEDLNM, so that the NLS was inserted between the *CDC46* ORF and the c-*myc* epitope. The *ubi ts-cdc45* strain was constructed by first constructing a

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Abbreviations: RLF, replication licensing factor; MCM, minichromosome maintenance; *ARS*, autonomously replicating sequence; NLS, nuclear localization signal; GFP, green fluorescent protein.

Data deposition: The sequence reported in this paper has been deposited in the GenBank data base (accession no. U56821).

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ubiDHFRts-CDC45 fusion in the plasmid pPW66R (21) followed by integration at the *CDC45* locus. (Further details are available on request.)

Plasmid Maintenance Assays. Plasmid maintenance assays were performed essentially as described (2, 5). Plasmid-loss rates were determined by $1 - (F/I)^{1/N}$, where I is the initial percentage of plasmid-containing cells and F is the percentage of plasmid-containing cells after N generations.

Immunoprecipitation and Western Blot Analysis. Cells were harvested by centrifugation, washed twice in ice-cold water, and finally resuspended in 0.5 ml lysis buffer (6). Cells were lysed with a half volume of glass beads (425–600 μm; Sigma) in a multibead beater (Biospec Products, Bartlesville, OK) with three 30-sec bursts at maximum intensity at 4°C. Lysates were clarified by centrifugation in a microfuge for 10 min at 4°C and used immediately or snap frozen under liquid nitrogen. Protein extracts (0.5 ml diluted to 0.5 mg/ml in cold lysis buffer) were adsorbed against 50 μl (50% vol/vol slurry) protein A-Sepharose CL-4B beads (Pharmacia) by mixing on a rotating wheel for 30 min at 4°C. Beads were pelleted, the supernatant recovered and mixed for 2 h with primary antibody [1:100 dilution: either affinity purified polyclonal antibody raised to the N terminal 352 amino acids of Mcm5p/Cdc46p (details to be described elsewhere) or affinity purified 12CA5 monoclonal antibody (Babco, Richmond, CA)] after which 50 μl of fresh protein A- Sepharose beads blocked in 1 mg/ml bovine serum albumin (Sigma) was added and mixed for a further 2 h. Immune complexes were recovered after washing beads four times with 1 ml of cold lysis buffer. Immunoprecipitate samples were prepared by boiling 5min in SDS load buffer. Protein samples were resolved by SDS/PAGE and transferred to nylon filters by electroblotting in transfer buffer (50 mM Tris/400 mM glycine/0.1% SDS/20% methanol) for 2 h at 50 V. Filters were blocked in 5% skim milk powder/0.1% Triton X-100 in PBS for 1 h, probed with primary antibodies (1:500 dilution) for 1 h, followed by horseradish peroxidase-conjugated secondary antibodies (1:2500 dilution; Dako) for 1 h in 1% skim milk powder/0.1% Triton X-100 in PBS. Filters were washed three times for 10 min each in 0.1% Triton X-100 in PBS after both the primary and secondary antibody incubations. Samples were detected with the Amersham ECL system.

Other Methods. Flow cytometry was performed by the method described (22). Gap-repair mapping analysis of the *cdc45-1* mutant was as described (3). *In situ* immunolocalization studies were as described previously (6).

RESULTS

***CDC45* Is Essential for Minichromosome Maintenance and for Chromosomal DNA Replication.** Members of the MCM family are required for the activity of autonomously replicating sequences (*ARS*), which control extrachromosomal plasmid replication, and for the function of chromosomal replication origins (5). Because mutant alleles of *CDC45* arrest with a similar *cdc* phenotype to several *mcm* mutants and because *CDC45* genetically interacts with *MCM5/CDC46* and *CDC47* (4), we tested if Cdc45p also displayed some of the replication defects associated with *mcm* mutants. First, we have assessed the requirement for Cdc45p during the cell cycle by constructing a strain where the *CDC45* locus has been modified so that it expresses Cdc45p fused at its N terminus to a signal peptide that confers temperature-sensitive instability (21). Thus, we have effectively created a conditional null mutant *cdc45* strain, where Cdc45p can be rapidly depleted at 37°C, due to chronic instability conferred by the temperature-sensitive degron signal at its N terminus. This strain is inviable at 37°C and uniformly arrests with a 1 M DNA content at the G_1–S boundary as judged by flow cytometry (Fig. 1), with a similar phenotype to the previously characterized cold-sensitive

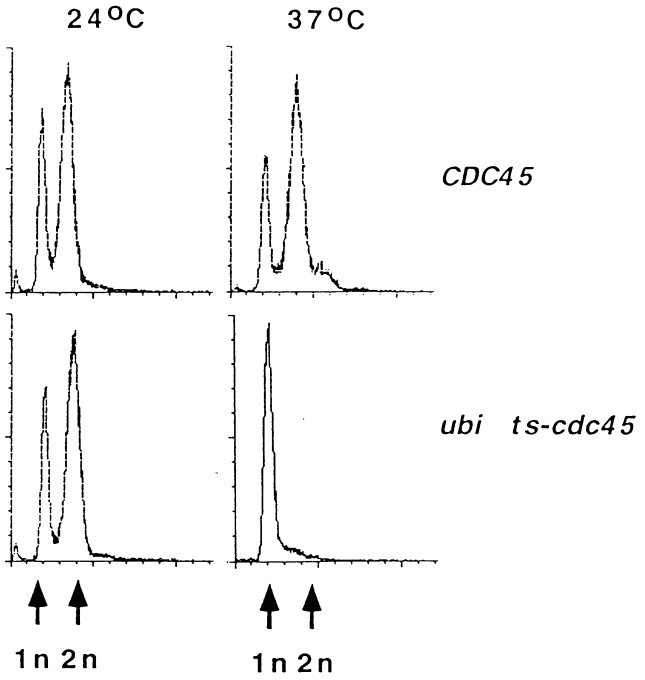

FIG. 1. Depletion of Cdc45p arrests cells late in G_1 with unduplicated DNA. The temperature-sensitive degron approach was used to conditionally deplete cells of Cdc45p by a temperature shift from 24°C to 37°C. The DNA content of *ts ubi-cdc45* (W303-1a background) cells at both their permissive (24°C) and restrictive (37°C) temperatures were measured by flow cytometry and compared with an isogenic wild-type *CDC45* strain under similar conditions. Cells were grown at 24°C to mid-log phase and shifted to 37°C or maintained at 24°C. Three hours after the temperature shift, cultures were fixed, stained, and prepared for flow cytometry.

cdc45-1 mutant (4). This arrest phenotype is indistinguishable from that of many *mcm* mutants which fail to support initiation of chromosomal replication.

Next, we tested if Cdc45p was required for minichromosome maintenance in a strain where Cdc45p was conditionally-depleted (*ts ubi-cdc45*) and in a cold-sensitive mutant (*cdc45-1*), which also arrests at a late stage of G_1 with unreplicated DNA. Several well characterized minichromosomes each carrying a different *ARS* element, were transformed into *cdc45-1*, *ubi ts-cdc45* strains and compared with an isogenic wild-type strain and two *mcm* mutant strains (*cdc46-1* and *cdc47-1*). Plasmid loss under nonselective conditions was measured at 20°C, 25°C, and 30°C (Table 1). This analysis revealed that, like *cdc46* and *cdc47* mutants, cells carrying *cdc45* mutants were severely compromised in their ability to support minichromosome maintenance, a property which is associated with loss or partial loss of plasmid-associated *ARS* activity and function of chromosomal replication origins. Moreover, plasmid instability in the *cdc45-1* strain was partially suppressed (>2.5-fold) by addition of multiple *ARS* elements to a minichromosome, further arguing that *cdc45-1* plays a role in initiation of replication. Like the *mcm5/cdc46-1* mutant, the *cdc47-1/mcm7* mutant also showed a severe minichromosome maintenance defect. These results show that Cdc45p is required for the activity of *ARS* elements and plays an essential role in chromosomal DNA replication, which is likely to involve some role at the level of initiation.

***CDC45* Is Unrelated to the MCM Family.** The *CDC45* gene was isolated by complementation of the *cdc45-1* cold-sensitive mutant allele using a *S. cerevisiae* genomic plasmid library. The *cdc45-1* complementing activity was localized to a 2.5-kb *Spe*I–*Sal*I fragment, with an ORF encoding a predicted polypeptide of 650 amino acids (74.2 kDa; see Fig. 2). To

Table 1. Loss rates of minichromosomes in *cdc45*, *mcm5/cdc46*, and *cdc47* mutant strains

Minichromosome	Temperature	*ARS1*	*ARS501*	*ARSH4*	*HO*
CDC⁺/MCM⁺	20°C	<0.01 (<0.01)	<0.01	<0.01	0.02
	25°C	0.03 (<0.01)	<0.01	0.02	0.01
	30°C	0.04 (0.03)	0.02	0.04	0.03
cdc45-1 (cs)	20°C	0.22 (0.09)	0.18	0.23	0.17
	25°C	0.06 (0.05)	0.03	0.04	0.03
	30°C	0.04 (0.04)	0.03	0.04	0.04
ubi ts-cdc45	20°C	0.04	0.03	0.03	0.05
	25°C	0.05	0.07	0.08	0.08
	30°C	0.25	0.18	0.22	0.20
cdc46-1	20°C	0.04	0.05	0.06	0.03
	25°C	0.06	0.09	0.08	0.06
	30°C	0.27	0.16	0.24	0.19
cdc47-1	20°C	0.03	0.05	0.04	0.03
	25°C	0.04	0.05	0.05	0.02
	30°C	0.20	0.23	0.14	0.17

Numbers in parenthesis indicate the stability in wild-type and *cdc45-1* strains of a plasmid carrying six copies of *ARS1* for comparison with an otherwise identical plasmid carrying a single *ARS1* element.

confirm that the complementing activity defined above was genetically linked to the allele of the original *cdc* mutation, a minimal fragment required for rescue was cloned into a *URA3⁺* integrating plasmid and targeted to the putative *CDC45* locus by homologous recombination in the *cdc45-1* mutant strain. All stable *URA3⁺* transformants were *CDC⁺*, suggesting that an integration rescue had occurred. Tetrad dissection of a cross between the *URA3⁺* integrant and a marker strain (*MATa URA3 CDC45*) confirmed that integrated sequences had targeted to the *CDC45* locus. All 24 *URA⁺* tetrads dissected displayed no conditional lethality at 12°C, indicating the cloned gene to be *CDC45*. To demonstrate that *CDC45* is essential for cell viability, a diploid strain was

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MYYGISQFSE AYNKILRNSS SHSSCQLVIF VSCLNIDALC 40
ATKMLSLLFK KQLVQSQIVP IFGYSELRRH YSQLDDNINS 80
LLLVGFGGVI DLEAFLEIDP QEYVIDTDEK SGEQSFRRDI 120
YVLDAHRPWN LDNIFGSQII QCFDDGTVDD TLGEQKEAYY 160
KLLELDEESG DDELSGDEND NNGGDDEATD ADEVTDEDEE 200
DEDETISNKR GNSSIGPNDL SKRKQRKKQI HEYEGVLEEY 240
YSQGTTVVNS ISAQIYSLLS AIGETNLSNL WLNILGTTSL 280
DIAYAQVYNR LYPLLQDEVK RLTPSSRNSV KTPDTLTLNI 320
QPDYYLFLLR HSSLYDSFYY SNYVNAKLSL WNENGKKRLH 360
KMFARMGIPL STAQETWLYM DHSIKRELGI IFDKNLDRYG 400
LQDIIRDGFV RTLGYRGSIS ASEFVEALTA LLEVGNSTDK 440
DSVKINNDNN DDTDGEEEED NSAQKLTNLR KRWVSNFWLS 480
WDALDDRKVE LLNRGIQLAQ DLQRAIFNTG VAILEKKLIK 520
HLRIYRLCVL QDGPDLDLYR NPLTLLRLGN WLIECCAESE 560
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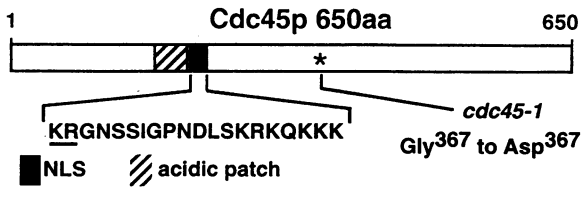

FIG. 2. Predicted amino acid sequence of Cdc45p. Underlined is a region enriched for acidic residues (residues 164–204). A potential bipartite NLS (residues 209–228) is boxed. The glycine residue (underlined at position 367), which is mutated to an asparagine residue in the *cdc45-1* cold-sensitive mutant allele, was determined by the gap-repair method (3); this point mutation is sufficient to confer cold-sensitivity and the *cdc* phenotype associated with the *cdc45-1* mutant. The nucleotide sequence of the *CDC45* ORF together with 5′ and 3′ noncoding regions has been deposited in the GenBank data base (accession no. U56821).

constructed which had one wild-type allele and one allele disrupted with an auxotrophic marker gene: sporulation of the diploid strain generated two viable and two inviable spores for *CDC45* disruptants. Viable spores always lacked the auxotrophic marker used to disrupt the *CDC45* gene, demonstrating the viable spores carried the intact and not the disrupted allele. We conclude that the complementing activity defined here represents the *CDC45* gene.

The nucleotide sequence of the *CDC45* gene together with 3′ and 5′ flanking sequences have been deposited in the GenBank data base (accession no. U56821). Cdc45p does not belong to the MCM family of DNA replication polypeptides, but comparison of known sequences in the data banks reveals it to have 32% amino acid identity and 56% similarity with the Tsd2 polypeptide which is essential for DNA replication in the smut fungus, *Ustilago maydis* (GenBank accession no. U50276).

Cdc45p-HA Assembles into a Complex with Mcm5p/Cdc46p. *CDC45* and *MCM5/CDC46* have been shown to genetically interact in an allele-specific manner (18, 23), suggesting that their encoded polypeptides physically interact and assemble into a complex. By directly immunoblotting cell extracts, we can directly detect epitope-tagged Cdc45p (Cdc45p-HA) with anti-HA monoclonal antibody, or Mcm5p/Cdc46p with rabbit polyclonal antibodies directed against the first 350 amino acids of Cdc46p. To test the possibility that Cdc45p and Mcm5p/Cdc46p assemble into a complex, we immunoprecipitated Mcm5p/Cdc46p or Cdc45p from cell lysates prepared from asynchronous cells, and then reciprocally probed immunoprecipitates after SDS/PAGE and Western blotting to determine if they were assembled into a complex. By this reciprocal immunoprecipitation-immunoblot analysis, we show that Cdc45p is a component of Mcm5p/Cdc46p immunoprecipitates and *vice versa* (Fig. 3). Although this assay shows that both polypeptides can be assembled together in a complex, it is clear that a significant amount of both Cdc45p and Mcm5p/Cdc46p are not assembled together in these complexes. This could be due to complex instability or perhaps because only a fraction of these polypeptides associate in a complex (see *Discussion*). Our results do, however, confirm inferences from genetic studies that Cdc45p associates with MCMs in multiprotein complexes.

Cdc45p Is Located in the Nucleus Throughout the Cell Cycle. Genetic and biochemical evidence firmly establishes that Cdc45p can assemble into functional complexes with MCM polypeptides such as Cdc46p/Mcm5p. As the localization of MCMs are strictly cell cycle-regulated, we tested if this was also true for Cdc45p. This is an important issue, given that

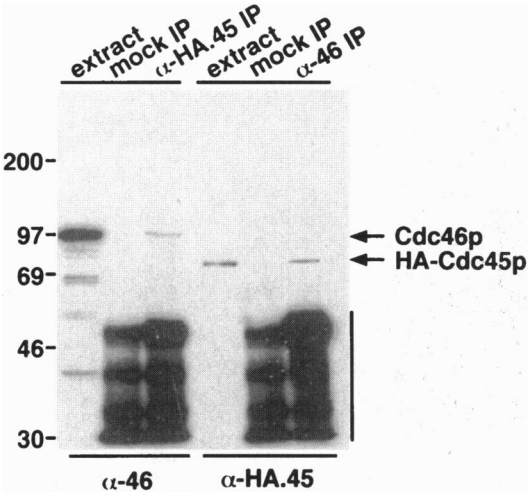

FIG. 3. Cdc45p assembles in a complex with Mcm5p/Cdc46p in crude whole cell extracts. Cdc45p-HA or Mcm5p/Cdc46p was immunoprecipitated from crude cell lysates (200 μg total protein) with either anti-Mcm5p/Cdc46p rabbit polyclonal antibody (lane 6), HA monoclonal antibody (lane 3), or protein A-Sepharose beads alone (lanes 2 and 5). Mcm5p/Cdc46p and Cdc45p-HA immunoprecipitates were resolved on 8% SDS polyacrylamide gels, Western blotted, and probed with HA monoclonal and anti-Mcm5p/cdc46p antibodies, respectively. The control reactions in lanes 2 and 5 demonstrate that protein A-Sepharose alone does not immunoprecipitate either Cdc45p or Mcm5p/Cdc46p, and the 12CA5 and Mcm5p/Cdc46p antibodies alone do not crossreact with Mcm5p/Cdc46p and Cdc45p, respectively. Lanes 1 and 4 show detection of polypeptides directly from crude cell lysates (200 μg) when probed with anti-HA and anti-Mcm5p/Cdc46p antibodies, respectively.

Cdc45p interacts with a polypeptide whose localization changes with the cell cycle. Localization of Cdc45p was initially assessed in a strain expressing a Cdc45p-green fluorescent protein (GFP) fusion, using tubulin staining and differential interference contrast microscopy to determine cell cycle position. GFP, when expressed by itself in yeast, does not show any specific localization pattern and generally accumulates evenly between the cytosol and nucleus (ref. 23; S.D., unpublished), making it suitable for nuclear localization experiments. In an asynchronous cell population, the Cdc45p–GFP fusion was always nuclear and displayed no cell cycle-regulated changes (Fig. 4), in contrast to the localization of MCM polypeptides (4–6). A similar pattern of localization was also seen in viable, unfixed cells using a GFP fusion (result not shown). We have confirmed these observations using a second approach where Cdc45p was tagged with a single HA-epitope (Fig. 5*A*). Both the epitope-tagged and GFP fusion were shown to be functional by complementing the *cdc45-1* mutation at 12°C (data not shown). To unequivocally demonstrate that Cdc45p was nuclear throughout the cell cycle, its localization was assessed using cell cycle mutants and cell cycle inhibitors which arrest cells at well-defined points of the cell cycle. This approach confirmed results from asynchronous cells, placing Cdc45p in the nucleus during early (*cdc28*, α-factor) and late (*cdc34*) G_1, S-phase (hydroxyurea), G_2 (*cdc9*, *cdc13*), early (nocodazole), and late (*cdc15*) mitosis (Fig. 5*B* and Table 2). This is quite surprising as Cdc46p/Mcm5p has been reported to shuttle in and out of the nucleus at specific stages of the cell cycle.

A sequence closely matching the canonical bipartite NLS (24) has been located at residues 209–228 of Cdc45p (see Fig. 2). Mutation of two basic residues normally crucial for NLS function, residues K209 and R210 to N209 and I210, abolished nuclear import (Fig. 5*A*). By itself, the wild-type Cdc45p NLS

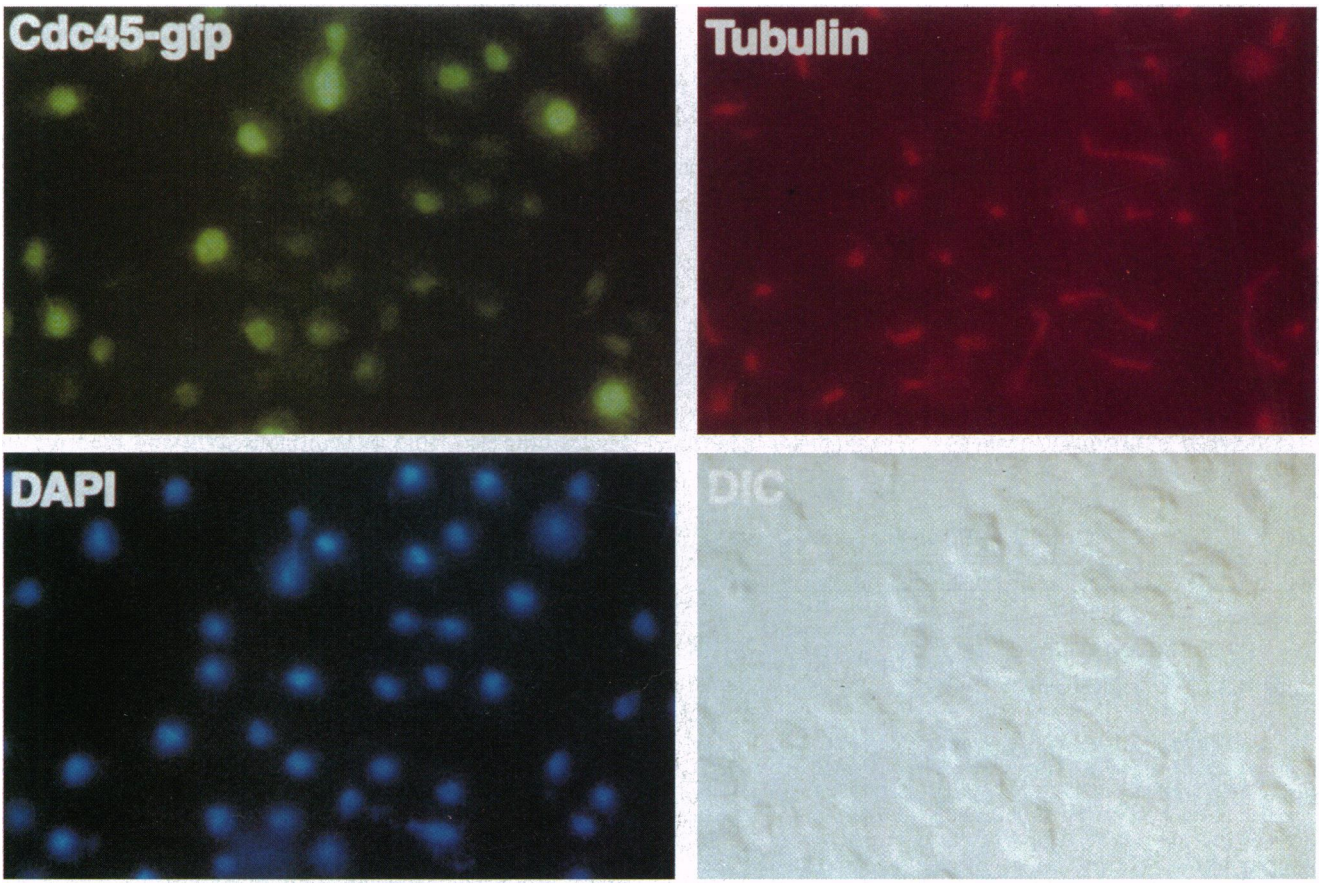

FIG. 4. Cdc45p is nuclear throughout the cell division cycle. An asynchronous culture of cells expressing Cdc45p–GFP were fixed, visualized by differential interference contrast (DIC) microscopy, and stained for Cdc45p–GFP, tubulin (spindles), and 4′,6-diamidino-2-phenylindole (DAPI) (DNA).

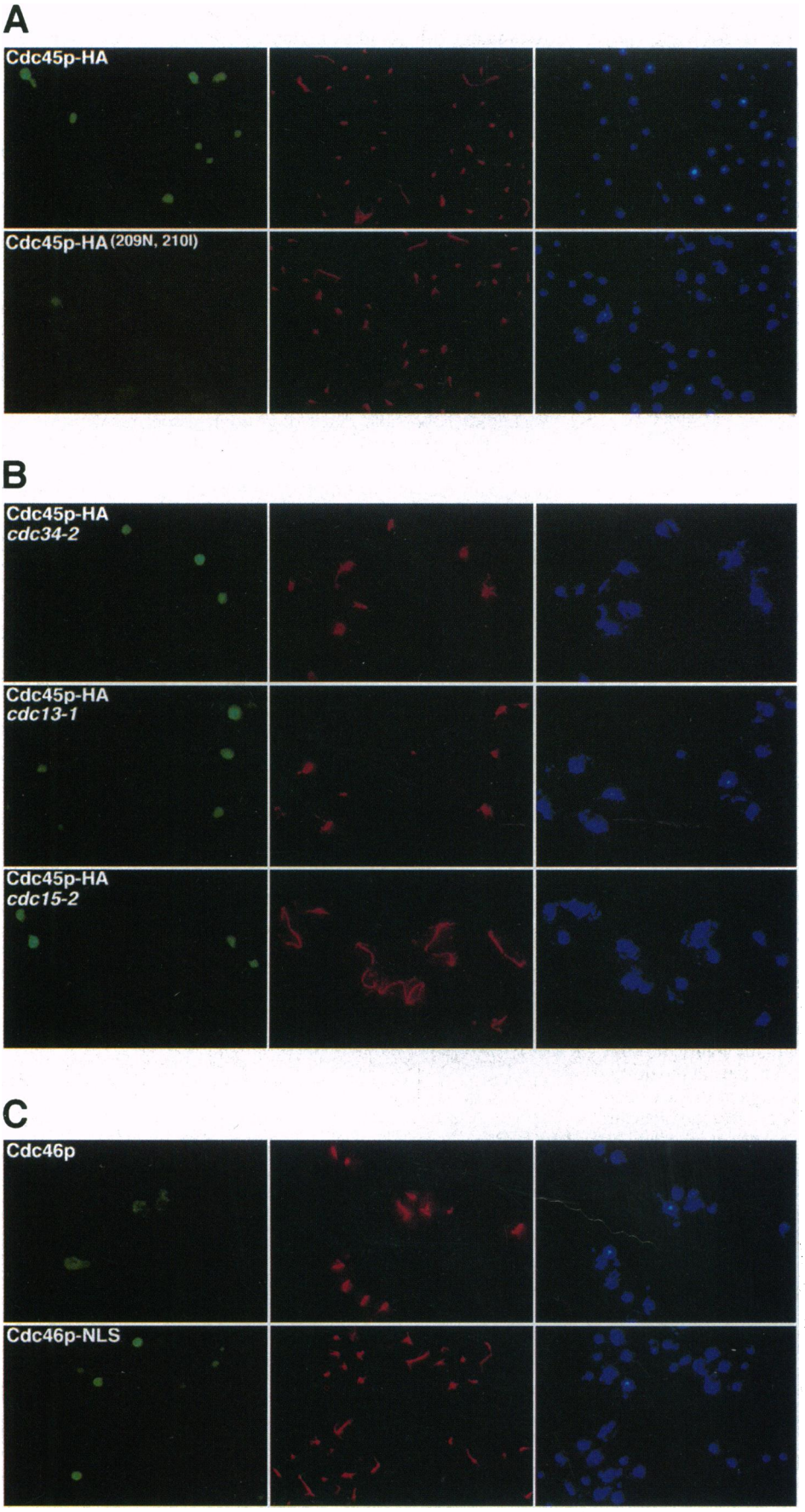

FIG. 5. Localization of Cdc45p to the nucleus requires a bipartite NLS. (*A*) Two basic residues (209K, 210R) predicted to be important for function of the putative Cdc45p bipartite NLSs were mutated to N and I, respectively. Localization of HA-epitope tagged Cdc45p (wild type, Cdc45p-HA; NLS mutated, Cdc45p-HA^{209N,210I}) was then assessed in unsynchronized cells by indirect immunofluorescence. (*Left* to *Right*) Cdc45p-HA staining, tubulin, 4′,6-diamidino-2-phenylindole (DAPI). (*B*) Localization of Cdc45p in well-defined cell cycle blocks. Localization of Cdc45p was determined in *cdc* mutants arrested under nonpermissive conditions (37°C). (*Top* to *Bottom*) *cdc34-2* (late G_1 block), *cdc13-1* (G_2 block), and *cdc15-2* (late M-phase block). (*Left* to *Right*) Cdc45p-HA, tubulin, and DAPI staining. (*C*) The 209–228 region of Cdc45p functions as an autonomous NLS. The putative bipartite NLS of Cdc45p (amino acids 209–228) was fused to the C terminus of a c-*myc*-tagged derivative of Cdc46p/Mcm5p, which carries an uncharacterized nuclear localization defect. (*Upper*) Cdc46p (Cdc46p-derivative, no NLS). (*Lower*) Cdc46p-NLS (Cdc46p$^{1-775}$/Cdc45p$^{209-228}$/MYC). Note that only a fraction of the cells were stained with HA-epitope in comparison to GFP staining. This has been observed previously in the detection of other tagged polypeptides and is likely to be caused by the fixation conditions used.

was sufficient to confer constitutive nuclear localization to a derivative of Cdc46p which does not normally localize to the nucleus (Fig. 5*C* and Table 2). Hence, residues 209–228 of Cdc45p functions as a constitutive (noncell cycle regulated) NLS, consistent with its nuclear localization throughout the cell cycle.

Table 2. Subcellular localization of Cdc45p in different cdc and inhibitor blocks

	Mutant/ inhibitor	Cell cycle position	Localization
Cdc45p		Asynchronous	$nuc^{GFP,HA}$
	cdc28-4	E.G_1	nuc^{GFP}
	α-factor	E.G_1	nuc^{GFP}
	cdc34-2	L.G_1	nuc^{HA}
	cdc4	L.G_1	nuc^{HA}
	HU	S	nuc^{GFP}
	cdc13	G_2	nuc^{HA}
	cdc9	G_2	nuc^{HA}
	Nocodazole	Metaphase	nuc^{GFP}
	cdc15	Anaphase	$nuc^{GFP,HA}$
$Cdc45p^{209N,210I}$		Asynchronous	$cyto^{HA}$
Cdc46p*		G_1	$cyto^{MYC}$
		G_2	$cyto^{MYC}$
Cdc46p*–Cdc45p NLS		G_1	nuc^{MYC}
		G_2	nuc^{MYC}

Cell cycle arrest was achieved by growth of cells for approximately one generation time in the presence of α-factor, nocodazole, or hydroxyurea, or by shifting *ts cdc* mutants to the restrictive temperature for a similar length of time. Epitope-tagged/GFP-Cdc45p was expressed from an integrated copy of the gene at the *URA3* locus (*MATα, CDC45, CDC45-Tag::ura3*). Localization of GFP, HA-tagged Cdc45p or MYC-tagged Cdc46p was assessed where indicated. A summary of the data obtained for the Cdc45p NLS mutant ($Cdc45p^{209N,210I}$), Cdc46p* (Cdc46p derivative that does not localize to the nucleus), and Cdc46p*–Cdc45p NLS fusion are shown (see Fig. 5).

DISCUSSION

We have shown that in budding yeast, *CDC45* performs an essential role in minichromosome maintenance and in chromosomal DNA replication (Fig. 1 and Table 1). To characterize the role *CDC45* plays in the initiation process, we have investigated its role in relation to the well-characterized MCM polypeptides, which have previously been shown to be components of RLF in *Xenopus*. To this end, we isolated the *CDC45* gene by rescue of the *cdc45-1* cold-sensitive mutation. *CDC45* is predicted to encode a polypeptide of 650 amino acids (74.2 kDa), which does not share any obvious homology with the MCM family of proteins (Fig. 2). However, Cdc45p shares significant amino acid identity (32%) and similarity (56%) with the *U. maydis* polypeptide, Tsd2, which apparently also has a role in DNA replication (GenBank accession no. U50276). Extensive similarity between both polypeptides extends along their entire length, though significant gaps need to be incorporated in an alignment to permit the match between Cdc45p and Tsd2. Several features appear to have been conserved between both polypeptides, most notably the bipartite NLS and a region rich in acidic residues. The extent of sequence identity and conservation of certain structural features between Cdc45p and Tsd2 suggests to us that they may be functional homologues.

Although Cdc45p assembles into a complex with Mcm5p/Cdc46p, our analysis does not distinguish between the possibility of this being due to direct or indirect interactions. The Cdc45p–Mcm5p assemblies detected here could be part of large MCM complexes characterized previously, where three and possibly more MCMs assemble together to form part of RLF activity (25). Genetic evidence does, however, suggest that Cdc45p interacts with at least one other member of the MCM family, Cdc47p (20). Experiments are in progress to determine if Cdc45p associates with other MCMs and if it is a component of the large MCM complexes described previously. Now that Cdc45p has been shown to interact with a component of the yeast RLF machinery, the possibility exists that Cdc45p could also be part of the RLF activity described in metazoans (19) where MCMs plus additional components are necessary for licensing replication to once only in each cell cycle. Although Cdc45p family members have not yet been reported outside of *Saccharomyces* and *Ustilago,* the conserved nature of the replication machinery, in particular the MCM family of polypeptides, makes it likely that Cdc45p family members will play a conserved role in control of replication in higher eukaryotes. This role is likely to involve control of replication mechanisms in conjunction with the MCM family of polypeptides. We are currently investigating this possibility.

Our results clearly show that (*i*) Cdc45p forms complexes with Cdc46p/Mcm5p and (*ii*) the subcellular localization of Cdc45p does not completely overlap with MCM polypeptides throughout the cell cycle. We propose two scenarios to reconcile these observations. First, a very small proportion of Mcm5p/Cdc46p may remain associated with Cdc45p in the nucleus while most of the cells MCM pool is exported into the cytoplasm during S-phase. There is, however, no evidence to indicate the presence of residual MCM in the nucleus during G_2 phase (5, 6, 20). Second, it is possible that Cdc45p only associates with MCMs during the period when they are found in the nucleus (in G_1). This would imply that interactions between these polypeptides occurs principally only during G_1 phase of the cell cycle. If this were the case, the assembly of Cdc45p into complexes with MCMs could be a rate-limiting level of control in determining the activity of MCM–RLF complexes in *Saccharomyces* and would provide another tier of control in the formation of replication preinitiation complexes (26). Further experiments are now required to define the exact role of Cdc45p in control of DNA replication, when it is assembled into complexes with MCMs, and how this polypeptide influences the activity of replication origins in conjunction with MCMs.

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