Report

14-3-3 Family Members Act Cooperatively to Regulate Mitotic Progression

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ABSTRACT

The mitosis promoting phosphatase, cdc25C, is a target of both the DNA replication and DNA damage checkpoint pathways. These pathways regulate cdc25C function, in part, by promoting the association of cdc25C with 14-3-3 proteins, which results in the retention of cdc25C in the cytoplasm. To determine which 14-3-3 proteins were required to regulate cdc25C function, we tested the ability of various 14-3-3 family members to form a complex with and negatively regulate cdc25C in human cells. Two 14-3-3 family members, 14-3-3ε and 14-3-3γ specifically formed a complex with cdc25C but not with the 14-3-3 binding defective cdc25C mutant, S216A. In addition, 14-3-3ε and 14-3-3γ inhibited the ability of cdc25C, but not the S216A mutant, to induce premature chromatin condensation (PCC) in U-2OS cells. These results suggested that the reduction in PCC by 14-3-3ε and 14-3-3γ was due to inhibition of cdc25C function. In contrast, 14-3-3α was unable to form a complex with cdc25C, but was able to inhibit the ability of both wild type cdc25C and S216A to induce PCC. This suggests that 14-3-3α regulates entry into mitosis independently of cdc25C and 14-3-3ε and 14-3-3γ. Thus, specific members of the 14-3-3 family of proteins may act cooperatively to maintain the DNA replication checkpoint by regulating the activity of different cell cycle proteins.

INTRODUCTION

Cdc25C is a dual specificity protein phosphatase that dephosphorylates and activates the cyclin B/cdc2 complex thus initiating the process of mitosis in mammalian cells.1-5 In human cells, cdc25C has been shown to be a target of both the DNA replication and the DNA damage checkpoints.6,7 The checkpoint pathways regulate cdc25C function through phosphorylation of specific residues in cdc25C that prevent its premature activation in S and G2 phases. It has been demonstrated that during interphase, a major site of phosphorylation in human cdc25C is a serine residue at position 216 (S216).8 Conversely, S216 is not phosphorylated during mitosis suggesting that phosphorylation of this residue may contribute to the negative regulation of cdc25C activity during interphase.6,9 Consistent with the above hypothesis, expression of a cdc25C mutant that substituted alanine for serine 216 (S216A) induced premature entry into mitosis by override of a DNA replication checkpoint and a γ-radiation induced DNA damage checkpoint.6,7

Several kinases that promote the phosphorylation of the S216 residue in cdc25C have been identified. Piwnica-Worms and colleagues purified a kinase from HeLa cells, C-TAK1, that specifically phosphorylates residue S216 in vitro.8,22 Cdc25C can also be phosphorylated by chk1, a DNA damage checkpoint kinase first identified in fission yeast.5,9,12 Experiments performed in the fission yeast Schizosaccharomyces pombe and in human cells has shown that cdc25C can also be phosphorylated by a third kinase cds1/chk2.13-16 Both chk1 and chk2 specifically phosphorylate cdc25C on S216 in vitro.6,9,10,13-15 Therefore, the S216 residue in cdc25C may be a substrate for multiple kinases that specifically inhibit its activity in response to the S-phase or DNA damage checkpoints.17

It has been previously demonstrated that the induction of the mitotic program in cells undergoing S-phase leads to premature chromatin condensation (PCC).18 The mitotic program can be induced by the transient over expression of cdc25C resulting in the induction of PCC in the transfected cells.6,7,18 The induction of PCC by the over expression of cdc25C is not blocked when cells are arrested in S-phase with the drug hydroxyurea (HU).6,7 suggesting that the induction of PCC reflects an override of the DNA replication checkpoint. The S216A mutant of cdc25C induced higher levels of PCC than the wild type cdc25C suggesting that phosphorylation of S216 by the checkpoint kinases Chk1 or Chk2 could be required for the inactivation of cdc25C at the DNA replication checkpoint.6,7

KEY WORDS

14-3-3, cdc25C, mitosis, checkpoints
Evidence from papers describing mice lacking either chk1 or ATR indicate that the DNA replication checkpoint may be under the control of ATR and chk1 as loss of either component leads to premature mitosis and early embryonic lethality in the mouse. Further, it has been demonstrated that chk2 can be phosphorylated in response to an S-phase checkpoint in mammalian cells lacking ATM in a manner that is presumably ATR dependent. In the same cells chk2 cannot be activated by the presence of DNA damage induced by either γ-radiation or the drug topotecan. These results suggest that ATR is required to mediate genome stability during S-phase.

Phosphorylation of cdc25C at S216 by chk1, chk2 or C-TAK1 in vitro results in the generation of a binding site for the 14-3-3 family of proteins in residues 213–218 of cdc25C. A consensus binding site for 14-3-3 was first identified by Muslin et al. as RSXpS[L/E/A/M]P as well as identification of a second consensus sequence, RX[Ar/S][+]/pS[L/E/A/M]P (where Ar is an amino acid with an aromatic side chain and + an amino acid with a basic side chain). A peptide corresponding to the first sequence is present in residues 213–218 of cdc25C. Wild type cdc25C but not the S216A mutant can bind to 14-3-3 proteins in human cells. Similarly, X. laevis cdc25c has been shown to form a complex with the 14-3-3ε and 14-3-3ζ proteins in egg extracts and 14-3-3 ε and 14-3-3ζ cdc25C protein in vitro when cdc25C is phosphorylated by either chk1 or chk2.6

Although cdc25C function may be negatively regulated by complex formation with 14-3-3 proteins in vivo, association of 14-3-3 proteins with cdc25C does not appreciably decrease cdc25C phosphatase activity in vitro. This leads to the question of how 14-3-3 proteins affect cdc25C function in response to DNA damage. In both S. pombe and X. laevis, the association of cdc25C with 14-3-3 proteins could block the nuclear accumulation of cdc25 prior to mitosis. Previous work from this laboratory has demonstrated that the endogenous human cdc25C protein was localized in the cytoplasm during interphase in multiple cell types. We also observed that cdc25C was transported to the nucleus during late G2 just before the initiation of mitosis. A similar result was reported earlier for an exogenously expressed cdc25C protein. The region in cdc25C required for the cytoplasmic localization during interphase was mapped to the 14-3-3 binding site at residue S216. The cdc25C mutant, S216A, did not bind to 14-3-3 proteins, showed a pan cellular localization and induced higher levels of PCC than the wild type cdc25C protein. Thus, in human cells the cytoplasmic sequestration of cdc25C by 14-3-3 proteins may negatively regulate mitotic progression when checkpoint pathways are activated. Conversely, phosphorylation of S214 or S285 in human and X. laevis cdc25C respectively inhibits complex formation with 14-3-3 proteins and results in override of checkpoint function. Although seven different 14-3-3 genes have been identified in mammalian cells, it is not known whether a specific 14-3-3 protein modulates cdc25C function in response to the DNA replication or DNA damage checkpoints. To address this issue we tested the ability of five different 14-3-3 proteins to form a complex with and inhibit cdc25C function in vivo. Two 14-3-3 proteins, 14-3-3ε and 14-3-3γ, formed a complex with cdc25C in vivo and inhibited the ability of cdc25C to induce PCC. In contrast, another family member, 14-3-3ζ, was unable to bind to either wild type cdc25C or the S216A mutant but was able to inhibit the ability of both proteins to induce PCC. These results suggest that specific 14-3-3 proteins act independently to inhibit mitotic progression.

**Materials and Methods**

**Cell Strains and Transfections.** The human osteosarcoma cell line, U2-OS was obtained from the ATCC and cultured in Dulbecco’s modified Eagle’s medium (DMEM) [Celgro] supplemented with 10% Fetal Clone-I serum [Hyclone], 100 U penicillin per ml, and 100 μg streptomycin per ml. Cells were transfected by calcium phosphate precipitation as described previously. Cells were transfected on a cover-slip in a 35 mm dish with 5 μg of each cdc25C, 1.5 μg of pBSK and 0.5 μg of the HA 14-3-3 plasmids per dish.

**Plasmids.** The MYC tagged cdc25C and S216A constructs have been described previously. The GST14-3-3 constructs have been previously described. Consistent with this model, two 14-3-3 homologues were cloned as BamHI–XhoI fragments downstream of the HA epitope tag in pCDNA3 (Invitrogen). 14-3-3β and 14-3-3δ were amplified using the following primer pairs: 5′ ggg gga tcc ATG ACC ATG GAC AAA AGT GAGG 3′ and 5′ aaa ctc gag TTA GGT GTT CTC TCC TCC AGC 3′, 5′ gag tcc ATG AGA GCC AGT CTG ATCC 3′ and 5′ aaa ctc gag TCA GCT GTG GGG CTC CTGGG 3′ respectively (non-coding residues in lowercase and coding residues in upper case) and were then cloned into pCDNA 3 as described above.

**Antibodies.** Tissue culture supernatants of the mouse monoclonal hybridomas anti-HA (12CA5) and anti-MYC (9E-10) were used at a dilution of 1:50 for immunofluorescence analysis and Western blots. The anti-cdc25 rabbit polyclonal antibody (C-20, Santa Cruz) was used at a dilution of 1:1000 for immunofluorescence. The secondary antibodies goat anti-mouse immunoglobulin G (IgG) Rhodamine and goat anti-rabbit IgG FITC (Boehringer Mannheim) were diluted 1:1000 for immunofluorescence assays. The secondary antibodies goat anti-mouse HRP and goat anti-rabbit HRP (Pierce) were diluted 1:1000 for Western blot analysis. Where noted antibodies were cross-linked to Protein A Sepharose with Dimethyl pimelidate as described.

**Immunoprecipitation and Western Blots.** Whole cell extracts of U-2OS cells were prepared in EBC buffer (50mM Tris-HCl [pH 8.0], 120mM NaCl, 0.5% Nonidet P-40 [NP40], 10 μg/ml of aprotinin, 0.1μg/ml of leupeptin, 0.1mM phenylmethylsulfonylfluoride, 50mM NaF, 1 mM sodium orthovanadate, 1mM EDTA). Extracts were cleared by centrifugation at 12,000 x 10-15 minutes at 4°C. To perform the in vitro 14-3-3 binding assays, E. coli strains expressing GST fusions to the different 14-3-3 constructs were induced to synthesize the fusion protein as described. The bacterial pellets were lysed by sonication in PBS containing 1% Triton X-100 and incubated with glutathione sepharose beads (Pharmacia) for 1 h at 4°C with rocking. The beads were washed 3 times with NET-N (20 mM Tris-HCl[pH 8.0], 100 mM NaCl, 1 mM EDTA, 0.5% NP40). The beads were suspended in an equal volume of NET-N and stored at 4°C. Approximately equal amounts of GST fusion protein or anti-cdc25C (TC113) antibodies’ and protein A Sepharose were added to EBC lysates of U-2OS cells and the reactions rocked overnight at 4°C. The complexes were washed three times with NET-N and resolved in a 10% SDS-PAGE gel.
RESULTS

Binding of cdc25C to 14-3-3 Proteins In Vitro. To identify the specific 14-3-3 protein that bound to cdc25C, GST fusions of the various 14-3-3 proteins or GST alone were synthesized in E. coli and purified on glutathione sepharose beads. These fusion proteins were incubated with U-2OS cell lysates and the bound cdc25C was detected by Western blotting. As shown in Figure 1A, all the 14-3-3 proteins used in these studies (14-3-3ζ, 14-3-3ɛ, 14-3-3σ, 14-3-3β and 14-3-3γ) bound to cdc25C with varying affinities (Fig. 1A, lanes 3–7). The relative amounts of the GST fusion proteins used in this assay is shown in Figure 1B. In this experiment, the amount of GST 14-3-3ζ used in the binding assay was much lower than the other fusion proteins resulting in a low signal for the co-precipitated cdc25C (Fig. 1A, lane 3).

In contrast, neither GST alone nor a 14-3-3ζ mutant that is defective for substrate binding, GST14-3-3ζK49E (Fig. 1A, lanes 2 and 7), were able to bind to cdc25C. Similarly, the GST 14-3-3ζ fusion proteins did not recognize the S216A mutant of cdc25C (data not shown). These results suggest that binding of the 14-3-3 proteins to cdc25C in vitro required both the ligand recognition domain of cdc25C and phosphorylation of the S216 residue in cdc25C.

Binding of cdc25C to 14-3-3 Proteins In Vivo. To further confirm these results we tested the ability of the exogenously expressed 14-3-3 proteins to bind too exogenously expressed cdc25C or a cdc25C mutant defective for 14-3-3 binding, S216A.6,7 It has been shown previously that exogenously expressed human cdc25C could form a complex with 14-3-3ζ.7 MYC epitope tagged wild type cdc25C (WT) or S216A were transfected into U-2OS cells with either the vector alone or expression constructs for the different HA-epitope tagged 14-3-3 cDNAs. Extracts from the transfected cells were prepared and immunoprecipitations performed with antibodies to either the HA or MYC epitopes. As shown in Figure 2, all the 14-3-3 proteins were expressed at relatively equal levels (Fig. 2, lanes 2–6 and 8–12, fourth panel) while no signal was detected in cells transfected with the vector alone (Fig. 2, lanes 1 and 7). Similarly the MYC tagged cdc25C constructs were expressed in all the transfectants (Fig. 2, lanes 1–12, first panel). In contrast to the results observed in the in vitro assay not all the 14-3-3 proteins formed a complex with cdc25C in vivo. Both 14-3-3ζ and 14-3-3γ were able to co-precipitate the wild type cdc25C protein (Fig. 2, lanes 2 and 3, second panel) but were unable to bind to the S216A mutant (Fig. 2, lanes 8 and 9, second panel) as expected, a result consistent with previously reported data.6 The other 14-3-3 isoforms were unable to co-precipitate either the wild type cdc25C (Fig. 2, lanes 4–6, second panel) or the S216A mutant protein (Fig. 2, lanes 10–12, second panel). In similar fashion wild type cdc25C formed a complex with 14-3-3ζ and 14-3-3γ in vivo as demonstrated by its ability to co-precipitate these 14-3-3 isoforms (Fig. 2, lanes 2 and 3, second panel) but did not form a complex with 14-3-3ζ, 14-3-3β or 14-3-3σ (Fig. 2, lanes 4–6, third panel). S216A was unable to form a complex with any HA-14-3-3 protein above background levels (Fig. 2, lanes 7–12, third panel). These results suggest that the 14-3-3ζ and 14-3-3γ proteins are specifically able to form a complex with cdc25C in vivo while the other 14-3-3 proteins do not bind to cdc25C in vivo.

Effect of 14-3-3 Proteins on cdc25C Function. To test whether binding by 14-3-3 proteins had an effect on cdc25C function we tested the ability of the various 14-3-3 proteins to inhibit the ability of cdc25C to induce premature chromatin condensation (PCC). PCC assays were performed in U-2OS cells as previously described7 to compare the effects of 14-3-3 proteins on cdc25C or on the S216A mutant. As shown in Figure 3, wild type cdc25C induces PCC in approximately 30% of the transfected cells while the S216A mutant induced PCC in greater than 40% of the transfected cells. Co-transfection of 14-3-3ζ and 14-3-3γ with cdc25C resulted in an inhibition of the ability of cdc25C to induce PCC (decreasing the percentage of cells undergoing PCC to approximately 18% and 15% respectively) while neither protein was able to inhibit PCC by the S216A mutant protein, which is consistent with the binding data shown above. 14-3-3ζ and 14-3-3β were unable to inhibit PCC by either wild type cdc25C or the S216A mutant. However, to our surprise, 14-3-3γ inhibited the ability of both wild type cdc25C and the S216A mutant of cdc25C to induce PCC (decreasing the levels of PCC to approximately 13% and 20% respectively) (Fig. 3). These results suggest that while 14-3-3ζ and 14-3-3γ inhibit PCC by binding to and inactivating cdc25C function, 14-3-3γ inhibits PCC by a cdc25C independent mechanism, as it is able to inhibit both the wild type cdc25C protein as well as the 14-3-3 binding defective mutant S216A. Further the mechanism of action of 14-3-3γ is distinct from the 14-3-3ζ and 14-3-3γ proteins.
DISCUSSION

The results presented herein suggest that the 14-3-3 proteins regulate progression through the cell cycle by targeting different cell cycle proteins. 14-3-3ε and 14-3-3γ both formed a complex with cdc25C in vivo and were able to inhibit the ability of exogenously expressed cdc25C to induce PCC and over-ride an S-phase checkpoint. Neither 14-3-3ε nor 14-3-3γ could bind to or inhibit the activity of the S216A mutant of cdc25C. In contrast, 14-3-3σ, whose expression is induced by DNA damage in a p53 dependent manner, did not bind to either cdc25C or the S216A mutant but did inhibit the ability of both cdc25C and S216A to induce PCC. These results suggest that 14-3-3σ targets cell cycle proteins other than cdc25C and regulates mitotic progression differently than 14-3-3ε and 14-3-3γ.

Effect of 14-3-3 Family Members on cdc25C Function. Our data demonstrate that the physical interaction between cdc25C and 14-3-3ε and 14-3-3γ has functional consequences as both proteins are able to inhibit the ability of cdc25C to induce PCC thereby suggesting that these Figure 3 (Left). Inhibition of PCC induced by cdc25C by 14-3-3 proteins. U2 OS cells transfected with the indicated MYC tagged cdc25C constructs and the HA-tagged 14-3-3 constructs were immunostained with the anti-MYC polyclonal antibody (A-14 Santa Cruz), the anti-HA antibody (12CA5) and DAPI. More than a 100 MYC positive, cdc25C expressing cells were counted and the percentage of cells containing condensed fragmented chromatin was determined in three independent experiments.

Binding of cdc25C to Different 14-3-3 Family Members. The 14-3-3 family of proteins is a highly conserved family of nine proteins that bind to at least two different consensus peptides that contain conserved phospho-serine residues. Due to the inherent conservation of these proteins it could be assumed that all of them would be capable of forming a complex with any 14-3-3 binding protein. Indeed our in vitro binding results demonstrate that all of the 14-3-3 proteins tested in this assay were able to bind to cdc25C in vitro. This is in marked contrast to the results observed in vivo. Only 14-3-3ε and 14-3-3γ were able to form a complex with cdc25C in cells while 14-3-3ζ, 14-3-3β and 14-3-3σ were unable to bind to cdc25C in vivo. This complex was specific and required phosphorylation of cdc25C at the S216 residue as a point mutant of cdc25C, S216A, was unable to bind to any 14-3-3 protein consistent with previously reported results. A report from Mils et al. shows that cdc25B interacts with the 14-3-3β, 14-3-3η and 14-3-3ζ in a yeast two hybrid assay and interacts strongly with the 14-3-3ζ and 14-3-3η proteins in vivo, but bound to all the 14-3-3 isoforms tested weakly in vitro. These results suggest that in addition to sequences required for binding to the 14-3-3 consensus binding site, other factors determine 14-3-3 ligand interactions in vivo, especially in determining which 14-3-3 family member binds to a specific ligand. These factors could include, but are not limited to, proteins that maybe required for mediating a stable interaction between 14-3-3 proteins and their ligands in vivo or post-translational modifications of the different 14-3-3 family members and their ligands. 14-3-3 proteins inhibit the dephosphorylation of S287 in X. laevis cdc25C by PP2A resulting in maintenance of checkpoint function. It is possible that 14-3-3 proteins that do not form a complex with cdc25C in vivo are unable to maintain a stable complex with and maintain phosphorylation of S216 in vivo.
proteins maybe required for regulation of cdc25C by the DNA replication and DNA damage checkpoints. Neither protein was able to inhibit PCC induced by the 14-3-3 binding mutant of cdc25C, S216A, suggesting that the inhibition of cdc25C function required a physical complex between cdc25C and 14-3-3ε and 14-3-3γ and that they mediate their effects on cell cycle progression through direct inhibition of cdc25C function, presumably by maintaining the cytoplasmic localization of cdc25C in interphase as suggested by us and others.\(^7,32\) Inhibition of chk1 kinase activity in vivo results in the disruption of the cdc25C 14-3-3 complex, the entry of cdc25C into the cytoplasm and entry into mitosis even in the presence of damaged DNA.\(^41\) Regulation of cdc25 function and localization by 14-3-3 proteins is an evolutionarily conserved pathway in eukaryotes and has been demonstrated in the fission yeast S. pombe, X. laevis and human cells.\(^7,26,30-32,42\) A phylogenetic analysis of the 14-3-3 isoforms used in this report indicates that 14-3-3ε and 14-3-3γ are the oldest and most similar to the fission yeast 14-3-3 homologue rad24 (Fig. 4A).\(^43\) Therefore our observations that 14-3-3ε and 14-3-3γ regulate cdc25C function are consistent with the evolutionary conservation of this pathway.

In contrast to 14-3-3ε and 14-3-3γ, 14-3-3σ was able to inhibit the ability of cdc25C to induce PCC despite being unable to form a stable complex with cdc25C. A trivial explanation for this result would be that the interaction is unstable and therefore undetectable in our assay system. This is belied by our result that 14-3-3σ could inhibit the ability of the S216A mutant of cdc25C to induce PCC. The S216A mutant of cdc25C does not bind to 14-3-3σ either in vivo (Fig. 2) or in vitro (data not shown) suggesting that 14-3-3σ inhibits cell cycle progression by targeting proteins other than cdc25C. A likely candidate for the cell cycle protein inhibited by 14-3-3σ is the cdk1/cyclin B1 complex. 14-3-3σ has been shown to regulate both the sub cellular localization and the activity of cdk1 by promoting its association with the mitosis inhibiting kinase wee1.\(^44,45\) Therefore, 14-3-3σ could inhibit PCC by cdc25C by inactivating cdk1 function. This is consistent with our reported result that induction of PCC by cdc25C in U-2OS cells requires the expression of cyclin B1.\(^7\) Further, over expression of wee1 can inhibit PCC induced by cdc25C.\(^18\) These results are consistent with with the notion that PCC by cdc25C can be blocked by inhibiting a downstream event, in this case either the accumulation of active cdk1/ cyclin B1 in the nucleus or inhibiting the activity of the cdk1/cyclin B1 complex. In addition to these effects on cdk1 14-3-3σ may target other proteins that play a role in mitotic progression.

All these results lead us to propose the following model by which 14-3-3 proteins inhibit progression through mitosis under conditions of incomplete S-phase (Fig. 4B). 14-3-3ε and 14-3-3γ may bind to and induce the cytoplasmic localization of cdc25C in interphase or in response to checkpoint pathways. 14-3-3σ may inhibit PCC by exogenously expressed cdc25C probably by inhibiting the nuclear transport of cdk1/cyclin B or possibly by promoting the association of cdk1 with wee1 in the nucleus. Alternatively, 14-3-3σ may target other cellular proteins that lie either upstream or downstream of cdc25C and are required for mitotic progression.
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