Published in final edited form as:

Curr Genet. 2018 June ; 64(3): 677–680. doi:10.1007/s00294-017-0782-8.

O Cdc7 kinase where art thou?

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Abstract

Although Cdc7 protein kinase is important for regulating DNA replication in all eukaryotes and is a target for cancer therapy, it has never been localized in cells. Recently, a novel molecular genomic method was used by our laboratory to localize Cdc7 to regions of chromosomes. Originally, mutations in the *CDC7* gene were found in the classic *cdc* mutant collection of Lee Hartwell (Hartwell, et al. 1973). The *CDC7* gene was found to encode a protein kinase called DDK that has been studied for many years, establishing its precise role in the initiation of DNA replication at origins. Recently, clinical studies are underway with DDK inhibitors against DDK in cancer patients. However, the conundrum is that Cdc7 has never been detected at origins of replication even though many studies have suggested it should be there. We used “Calling Card” system in which DNA binding proteins are localized to the genome via retrotransposon insertion and deep-sequencing methods. We have shown that Cdc7 localizes at many regions of the genome and was enriched at functional origins of replication. These results are consistent with DDK’s role in many additional genomic processes including mutagenesis, chromatid cohesion, and meiotic recombination. Thus, the main conclusion from our studies is that Cdc7 kinase is found at many locations in the genome, but is enriched at functional origins of replication. Furthermore, we propose that application of the Calling Card system to other eukaryotes should be useful in identification of functional origins in other eukaryotic cells.

Keywords

DDK; Cdc7; Yeast; DNA replication; origins; chromatin; calling cards

Introduction: DDK and DNA replication

DDK (Dbf4-Dependent Kinase or Cdc7-Dbf4 kinase) is a conserved Ser/Thr protein kinase that regulates the initiation of DNA replication, meiotic recombination, mutagenesis, and chromatid cohesion (for recent reviews, see (Araki 2016, Matsumoto and Masai 2013, Rossbach and Sclafani 2016). The role of DDK is conserved in evolution as expression of human DDK can substitute for yeast DDK in yeast cells (Davey, et al. 2011). Yet despite the extensive knowledge of DDK’s important functions in the cell, there was scant evidence for its localization to replication origins and to other chromosomal regions. DDK is a distant cousin of CDKs (cyclin-dependent kinases), which also regulate DNA replication and the

cell cycle (Benanti 2016, Machin, et al. 2016). DDK is present at constant levels through the cell cycle but is regulated by the binding of an unstable, regulatory subunit called Dbf4 to form an active heterodimer. Hence the useful term “DDK” for Dbf4-dependent kinase was coined (Nasmyth 1996). DDK phosphorylates many chromatin-bound proteins to regulate a number of important chromosomal processes. From both *in vivo* and *in vitro* studies, the role of DDK in the initiation of DNA replication is known to be phosphorylation of one or more MCM (Mini-Chromosome Maintenance) proteins of the Mcm2–7 helicase bound to origins to activate the helicase by forming the CMG (Cdc45-GINS-MCM) holoenzyme and allow binding of the replisome (Fig. 1).

DDK has multiple chromatin-bound substrates

Like most protein kinases, DDK has many substrates, but only one is essential for DNA replication and cell division during mitotic growth (Rossbach and Sclafani 2016). This explains why my laboratory was able to isolate a the *mcm5-bob1* (“bypass of block”) mutation that can bypass the essential role and suppress the lethality of a *cdc7* and *dbf4* deletions (Hardy, et al. 1997, Jackson, et al. 1993). This bypass allowed for the discovery of other substrates such as Rev7 protein involved in translesion DNA synthesis (Brandao, et al. 2014), the Mer2 protein during meiosis (Wan, et al. 2008), and potentially proteins present at the kinetochore (Natsume, et al. 2013). In the latter study, Dbf4 was localized to the yeast kinetochore by ChIP, however Dbf4 binding to replication origins was weak. Surprisingly, Dbf4 binding was only measured in G1-arrested cells, in which Dbf4 protein levels are very low.

The conundrum: Dbf4 binds to origins, but where is Cdc7?

Using the yeast one-hybrid system and ARS-LacZ reporters, Dbf4 protein was shown to interact with origins of replication at the A region (ARS consensus site), which was necessary and sufficient for binding (Dowell, et al. 1994). However, Cdc7 protein in the same assay was unable to bind the ARS1-lacZ reporter (Dowell, et al. 1994, which was also later reported [Hardy, 1996 #1386]). These studies suggested that Dbf4 may direct Cdc7 to origins (Dowell, et al. 1994). Later experiments measured binding of Cdc7 and Dbf4 to bulk chromatin, showing that Cdc7 was constitutively bound to chromatin throughout the cell cycle, even when Dbf4 was low in G1-phase, but increased levels of chromatin-bound Cdc7 were observed during S phase (Weinreich and Stillman 1999). As mentioned above, Dbf4 protein was found weakly bound to origins in G1 phase, but origin binding by Dbf4 was not measured in S phase or asynchronously bound cells and Cdc7 at origins was not studied (Natsume, et al. 2013). Again, the main emphasis of this study was to show DDK has an important role at kinetochores, which was observed at many levels. Therefore, we were faced with the fact that after more than 20 years since the original one-hybrid study (Dowell, et al. 1994), localization of Cdc7 to origins was still not been seen even though every model of initiation shows DDK bound to origins.

The Calling Card System adapted for DNA replication proteins

We reasoned one possibility for the omission of Cdc7 in ChIP experiments could be that binding of Cdc7 to origins was weak, as one might expect for an enzyme-substrate complex (Rossbach, et al. 2017). We also decided to use the Calling Cards method, which was developed to map the binding site of transcription factors in yeast (Wang, et al. 2008, Wang, et al. 2007) and in mammalian cells (Wang, et al. 2012). Because this method measures the history of chromatin or DNA binding events of proteins, we thought it might be more sensitive and also yield more interesting data than just looking at a single S phase in a synchronous culture. The method is based on the fact that the yeast Sir4 protein directs integration of a Ty5 retrotransposon. The Ty5, whose expression is induced, is marked with a selectable marker and has a molecular bar code enabling localization by deep-sequencing methods in a strain with a query protein fused to Sir4. Thus, each time Cdc7-Sir4 protein binds DNA we get an insertion or permanent “Calling Card” mark nearby in the genome.

Our reasoning about weak binding due to an enzyme-substrate relationship was correct in that integrations were low when wild-type Cdc7-Sir4 was used, but increased seven-fold when a kinase-dead cdc7KD-Sir4 mutant was used that can bind substrates but not catalyze phospho-transfer. This increase allowed us to obtain enough sequencing data to accurately map the insertion events, which were rare and only occurred once per cell. Consistent with its role in many chromatin-processes, the kinase-dead Cdc7 protein was found at many genomic sites and not specifically at origins of replication. If we classified origins based on their timing, early replicating regions had the most insertions. This consistent with data showing the majority of origins fire in the first 20–40 min of S phase (Yang, et al. 2010).

We also analyzed the role of Dbf4 protein by using a cdc7KDDC-sir4 construct in which the C-terminal 50 amino acids of Cdc7, which is necessary and sufficient for Dbf4 binding (Jackson, et al. 1993), is deleted. The cdc7KDDC-sir4 protein produced a higher number of unique insertions indicating it bound to more sites. When we analyzed functional ARS sites using the OriDB (Origin Database) (Siow, et al. 2012) rather than ORC or MCM sites we saw a peak of insertions within 100bp of the ARS consensus site that was reduced when Dbf4 was not bound. From these data, we believe Cdc7 is bound to specific sequences that will become origins of replication, consistent with active Cdc7-Dbf4 complexes activating origins of replication. Thus, the Cdc7 protein has a narrower distribution of bound sites when Dbf4 is present indicating that Dbf4 may direct Cdc7 to specific sites such as origins (Fig. 1) as suggested originally (Dowell, et al. 1994). We saw no enrichment of the kinasedead Cdc7 protein at TSS (transcription-initiation sites), but an increase in specific insertions at pericentric regions consistent with DDK’s role at kinetochores (Natsume, et al. 2013).

We also performed ChIP analysis with a highly overexpressed tagged Cdc7 construct in synchronized cultures. Although the signal at several ARSs was weak, it was significantly above that seen in the untagged control and was absent at distal regions from the ARS. The tagged kinase-dead Cdc7 protein stayed longer on the chromatin in S phase as expected as we are blocking catalysis.

Conclusions and Future Uses

From our genome-wide studies, we resolve the conundrum and show that DDK localizes to many regions of the genome, but is enriched at functional origins of replication (Rossbach, et al. 2017). The results are consistent with DDK's role in the initiation of DNA replication but also in DDK's roles in additional genomic processes including mutagenesis, chromatid cohesion, and meiotic recombination. Our use of the Calling Card method to map the binding sites of a DNA replication protein instead of a transcription factor shows the versatility of the method. Indeed, as proposed (Wang, et al. 2008, Wang, et al. 2007), the method can be used for any protein that binds DNA or chromatin in the cell. One possibility is to use the mammalian system (Wang, et al. 2012) to map functional origins of replication in different developmental contexts. Because it has been difficult to map mammalian origins of replication and there is no consensus binding site for known origins (Leonard and Mechali 2013), application of the Calling Card method to human DDK and to other proteins should identify functional origins that are used over many cell cycles. Furthermore, our studies are highly significant because inhibitors of DDK are in clinical trials against cancer and the Calling Card system could be used to help determine their effects on cells (Sasi, et al. 2014, Swords, et al. 2010).

Acknowledgments

We thank Rob Mitra (Wash U.) and Mark Johnston (U. Colorado) for providing both ideas and technical assistance for the Calling Card system. This work was supported by a PHS award to R.A.S. (R01 GM35078) and a Research Scholar grant (RSG-13-216-01-DMC) from the American Cancer Society to J.R.H. Shared Resource core facilities used were supported by the University of Colorado Cancer Center grant (P30CA046934).

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Figure 1.

Model of DDK Function and Localization during the cell cycle. In G1 phase, Cdc7 protein is bound to the chromatin, but not enriched at origins, which have a MCM2-7 double hexamer bound to the ORC. As cell proceed into S phase, Dbf4 increase the concentration on Cdc7 near origins that will be activated. Origins are activated by the active DDK (Cdc7-Dbf4) phosphorylation of the MCM2-7 double hexamer causing the binding of Cdc45 and GINS proteins to form the CMG active helicase and a functional replisome.