A Charged Amino Acid Cluster in the C-Terminal Domain of Human Cyclin A is Required for Activation of Cyclin-Dependent Kinase

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Summary: Cyclins are regulatory subunits of cyclin-dependent kinases (CDKs) and required for the activation of CDKs. Human cyclin A interacts with CDK via its central cyclin box domain, while the function of the C-terminal domain is largely unknown. We isolated a novel mutation lacking any associated CDK activity at a cluster of charged amino acids in the C-terminal domain. This mutant showed marked protein instability. It did not activate CDK even when stabilized with the proteasome inhibitor, suggesting that the charged amino acid cluster in the C-terminal domain is required for the activation of CDK and is also involved in the protein stability of human cyclin A.

Key Words: cyclin A, cyclin-dependent kinase (CDK), CDK activation, protein stability

Introduction

Key transitions within the cell cycle of eukaryotes are controlled by cyclin-dependent kinases (CDKs). The kinase activity of CDKs depends on their association with the activating subunits, the cyclins. Cyclins are differentially expressed during the cell cycle and specific cyclin-CDK complexes are assembled and activated at different cell cycle stages. Each of these cyclin-CDK complexes executes a unique cell cycle function, which is essential for cell cycle progression.

One of the two major functions of cyclin is to promote activation of CDK. This is ascribed to "cyclin box", a conserved domain within all cyclins that is necessary for binding to CDK. The activation of CDK can be detected by assaying for the kinase activity using a non-specific CDK substrate such as histone H1. The other major function of cyclin is to target CDK to its specific substrates. Presumably, cyclin-specific domains other than the cyclin box are responsible for that function. However, there is only limited information on the exact cyclin domains determining the substrate specificity. Systematic analyses of the domains specific for each cyclin would be very important for clarifying the cyclin-specific substrates, which represent a major gap in our current knowledge of cell cycle control.

Cyclin A plays some important roles in vertebrate cell cycle control since it is essential for the onset of both DNA replication and mitosis. It has been implicated by previous deletion analyses of Xenopus cyclin A that it can be separated into three domains: the N-terminal domain which is essential for a periodic degradation of the cyclin A protein, the central cyclin box domain, and the C-terminal domain whose function is largely unknown. More recently, X-ray crystallography studies on human cyclin A-CDK complex have revealed that cyclin A interacts with CDK via the cyclin box domain and the C-terminal domain is basically free of the CDK interaction. The C-terminal domain contains several clusters of charged amino acid residues which are situated on the protein surface. This fact has prompted us to analyze the function of the C-terminal domain by targeting these charged clusters for mutagenesis. Here we show that one of such clusters is essential for the CDK activation and is also involved in the protein stability.
Materials and Methods

1. Isolation of a novel C-terminal mutation, cyclin A 410, causing the loss of CDK activation.

We have introduced alanine-scanning mutations at seven sites within the C-terminal domain of human cyclin A. cDNAs containing these mutations were transiently transfected to NIH3T3 cells and expressed as myc-tagged proteins. The exogenous proteins were then immunoprecipitated with anti-myc antibodies and assayed for their associated histone H1 kinase activity. We found that one of those mutants, cyclin A410, in which three consecutive charged amino acid residues are substituted by alanines (Fig. 1A), lacked any associated H1 kinase activity (Fig. 1B). All the other C-terminal mutants exhibited normal H1 kinase activity, which is comparable to the wild type (data not shown). A control mutant cyclin A211, which carries an alanine substitution at a highly conserved arginine residue within the cyclin box (Fig. 1A), also showed no associated H1 kinase activity (Fig. 1B). This is quite reasonable since the Arg211 residue is conserved among all the cyclins and its alteration has been shown to abolish CDK activation. On the other hand, the cyclin A410 is a novel C-terminal domain mutation which causes the loss of CDK activation.

2. The cyclin A410 mutation, as well as the cyclin box mutation cyclin A211, causes protein instability.

We next checked the expression level of the cyclin A 410 and cyclin A211 proteins. Fig. 2A shows anticyclin A immunoblotting of the extracts from NIH3T3 cells transiently transfected with the wild-type and the mutant cyclin A cDNAs. The steady-state amount of both mutant cyclin A proteins were remarkably decreased as compared to the wild-type protein. Since cyclin A has been known as one of the targets for proteasome-mediated proteolysis, we asked if the low expression level of the mutant cyclin A proteins was due to an acceleration of their proteolysis. NIH3T3 cells were transiently transfected with the wild-type and mutant cDNAs in the presence of a proteasome inhibitor LLnL or a calpain inhibitor LLM, which is structurally related to LLnL but does not inhibit the proteasome. Because LLnL blocks the degradation of ubiquitinated proteins but does not inhibit the conjugation of ubiquitin to the target proteins, LLnL treatment results in the accumulation of polyubiquitinated proteins. As shown in Fig. 2B, the exogenous cyclin A proteins, either wild-type or mutant, were greatly stabilized by treatment with LLnL but not with LLM, indicating that the low expression of the mutant pro-
proteins is indeed due to an acceleration of proteasome-mediated proteolysis. When treated with LLnL, ladders of slower and faster migrating species were detected in the mutant protein blots, while only one slow migrating band was apparent in the wild-type protein blot. These results also suggest that the mutant cyclin A proteins are polyubiquitinated and targeted to proteolysis at a much higher efficiency than the wild-type protein.

Instability of the cyclin A211 mutant protein was unexpected, since previous studies on Xenopus oocyte cyclin A have shown that an equivalent mutation at the conserved arginine residue exhibits the opposite phenotype\(^{10}\). The *Xenopus* mutant protein is resistant to proteolysis, suggesting that binding to CDK is necessary for cyclin A degradation. However, the human cyclin A used in this study belongs to a somatic-type subfamily (cyclin A\(_2\)), which is structurally distinct from a germ-line type (cyclin A\(_1\)) including *Xenopus* oocyte cyclin A. Several lines of evidence have suggested that these two types are differentially regulated in terms of protein stability\(^{10}\). We therefore propose that the human somatic-type cyclin A is more easily targeted to proteolysis when it is dissociated from CDK. This also seems to account for the instability of the cyclin A410 protein since it possesses no intrinsic CDK-activating function like the cyclin A211 protein (see below).

3. The cyclin A410 mutation site is essential for activating CDK.

To discern the intrinsic effects of the cyclin A211 and cyclin A410 mutations on CDK activation, we assayed the H1 kinase activity associated with the mutant proteins when stabilized. NIH3T3 cells were transfected with increasing amounts of the myc-tagged cyclin A cDNAs in the presence of LlNl, followed by immunoprecipitating their extracts with anti-myc anti-
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Fig. 3 Cyclin A410 mutant exhibits loss of the CDK activation even when stabilized by LLnL treatment
(A) Anti-myc immunoprecipitation of myc-tagged cyclin A proteins stabilized by LLnL treatment. NIH3T3 cells were transfected with vector alone (vec, 20 μg) or 5, 10, and 20 μg each of the myc-tagged cyclin A cDNAs in the presence of LLnL. The cell extracts were immunoprecipitated and immunoblotted with anti-myc antibodies. H and L represent heavy and light chains of IgG, respectively.
(B) Histone H1 kinase assay of the anti-myc immunoprecipitates prepared as in (A).

bodies and assaying for the associated H1 kinase activity. Fig. 3A shows that the amounts of the mutant cyclin A proteins in the immunoprecipitates were almost comparable to that of the wild-type protein. Nevertheless, as shown in Fig. 3B, the H1 kinase activities associated with the mutant cyclin A proteins were as low as background level. These results indicate that those mutations abolish the CDK-activating function of cyclin A independently of their effect on the protein stability.

As mentioned above, the cyclin A211 mutant is expected to exhibit the CDK activation-negative phenotype because the mutation resides within the cyclin box. In contrast, the cyclin A410 mutation resides in the C-terminal domain of cyclin A. The only mutational analysis reported to date of the C-terminal domain is the deletion analysis of Xenopus oocyte cyclin A, which has shown that its C-terminal 14-amino acid region is essential for the CDK activation. This region is also conserved at the C-terminus of the human cyclin A, but the cyclin A410 mutation site is not located within the corresponding region. It is also difficult to compare our results directly with the Xenopus cyclin A data, since a human cyclin A mutation within the C-terminal region, which is essential for the CDK activation in Xenopus, did not affect the CDK activation (data not shown). The cluster of three charged amino acid residues, where the cyclin A410 mutation resides, does not directly interact with the CDK polypeptide chain, according to the X-ray crystallography. Instead, the cluster might be required for the integrity of the structure of cyclin box domain since it is located in the proximity of the α-helix containing the Arg211 residue. In any case, our identification of this charged amino acid cluster as an essential site for the protein stability and the CDK activation should provide insight into the C-terminal domain-specific functions of human cyclin A.

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References
ヒトサイクリン A の C 末端領域に存在する荷電アミノ酸クラスターの 1 つはサイクリン依存性キナーゼの活性化に必要である

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要約：サイクリンはサイクリン依存性キナーゼ（CDK）の制御サブユニットであり、CDK の活性化に必要である。ヒトサイクリン A はその中央にあるサイクリンボックス領域を介して CDK と相互作用するが、その C 末端側領域の機能は殆ど未知である。我々は、C 末端領域の荷電アミノ酸クラスターの 1 つにおける変異として CDK 活性を伴わない変異を新規に分離した。この変異は顕著なタンパク不安定性を示したが、プロテアソーム阻害剤によってタンパクを安定化させても CDK 活性を伴わなかったことより、その C 末端領域の荷電アミノ酸クラスターが CDK 活性化に必要であり、またタンパク安定性にも関与することが示唆された。

キーワード：サイクリン A、サイクリン依存性キナーゼ（CDK）、CDK 活性化、タンパク安定性

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