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DISTINCT PROPERTIES OF CYCLIN DEPENDENT KINASES COMPLEXES CONTAINING CYCLIN A1 AND CYCLIN A2

Ayesha R. Joshi^{§,‡}, Vaidehi Jobanputra^{§,‡}, Karen M. Lele^{§,‡,¶}, and Debra J. Wolgemuth^{§,‡,¶,⊥,*}

[§]Dept. of Gen. and Dev., Columbia University Medical Center, New York, NY 10032

[‡]Dept. of Ob-Gyn, Columbia University Medical Center, New York, NY 10032

[¶]Institute of Human Nutrition, Columbia University Medical Center, New York, NY 10032

[⊥]Herbert Irving Comprehensive Cancer Center, Columbia University Medical Center, New York, NY 10032

Abstract

The distinct expression patterns of the two A-type cyclins during spermatogenesis and the absolute requirement for cyclin A1 in this biological process *in vivo* suggest that they may confer distinct biochemical properties to their CDK partners. We therefore compared human cyclin A1- and cyclin A2-containing CDK complexes *in vitro* by determining kinetic constants and by examining the complexes for their ability to phosphorylate pRb and p53. Differences in biochemical activity were observed in CDK2 but not CDK1 when complexed with cyclin A1 versus cyclin A2. Further, CDK1/cyclin A1 is a better kinase complex for phosphorylating potentially physiologically relevant substrates pRb and p53 than CDK2/cyclin A2. The activity of CDKs can therefore be regulated depending upon which A-type cyclin they bind and CDK1/cyclin A1 might be preferred *in vivo*.

Keywords

Cyclin A1; cyclin A2; CDK1; CDK2

1. INTRODUCTION

Progression through the cell cycle is driven by cyclin-dependent kinases (CDKs) and their key regulators, the cyclins. Cyclins not only activate the kinase but may also be involved in determining substrate specificity of the cyclin/CDK [1]. Among the mammalian cyclin families, the A-type cyclins present a unique opportunity for investigating the roles cyclins

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*Correspondence to: Debra J. Wolgemuth, CUMC, ICRC, 1130 St. Nicholas Avenue, New York, NY 10032, Tel: 212-851-4754, Fax: 212-923-2090, djw3@columbia.edu.

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play in regulating CDK activity at several levels. There are two sub-types of cyclin A's, cyclin A2 which is virtually ubiquitously expressed, and cyclin A1, whose expression is restricted to the testis [2]. Within the testis, the two A-type cyclins exhibit distinct and non-overlapping patterns of expression [3]. Cyclin A2 is expressed in spermatogonia and pre-leptotene spermatocytes, while cyclin A1 expression begins in late pachytene spermatocytes (Stage IX to XII) and is turned off after the completion of the first meiotic division. In somatic cells, cyclin A2 is believed to function in both G1/S and G2/M phases [4; 5]. This cannot be true for the meiotic cell cycle; rather specific functions for cyclin A2 at G1/S and then cyclin A1 at G2/M would be the prediction.

This prediction was confirmed for cyclin A1 by targeted mutagenesis: spermatocytes lacking cyclin A1 arrest at the first meiotic division (meiotic G2/M) during the transition between diakinesis and metaphase I [6] and undergo apoptosis [7]. Embryonic lethality of cyclin A2-deficient mice [8] has hampered the analysis of its role in the mitotic and meiotic division of the male germ cells. The only other cell cycle protein demonstrated to be essential for meiosis is mouse Cdk2 [9; 10], a cyclin A2 partner. Cdk2-deficient mice male germ cells also arrested in meiosis I, but the arrest was earlier in prophase I, during the mid-pachytene stage.

The specific, non-overlapping expression patterns of cyclin A1 and A2 in both mouse [3] and human [11] testis and the more ubiquitous expression of their likely CDK partners (CDK1 and CDK2) raised the question of whether the two cyclins confer distinct biochemical properties to their Cdk partners at the cellular level. We therefore compared biochemical properties of the kinase complexes formed by human cyclin A1 and cyclin A2 with CDK1 and CDK2. Our analysis revealed differences between cyclin A1- and cyclin A2-containing complexes, especially when associated with CDK1, suggesting that the two A-type cyclins are not functionally redundant with regard to their effects on their Cdk partners.

MATERIALS AND METHODS

Generation of recombinant human cyclin A1 baculovirus

Cyclin A1 was cloned from a human testis cDNA library (BD Biosciences, catalog number 7117-1) by PCR using 5' TGT TCC GGA CAC ATA GAA AGA TAA C3' and 5' CAA CGT GCA GAA GAA TAT GAC G3' as sense and antisense primers respectively. The PCR reaction conditions were 94°C for 10 sec, 60°C for 30 sec. and 68°C for 2 minutes for 25 cycles and contained 1µl of the cDNA, 500uM dNTP, and 2.5 units of Taq DNA polymerase. This PCR product was cloned into pCR II vector and the correct full length human cyclin A1 cDNA was confirmed by sequencing. A FLAG tag and an EcoRI site were generated in this cDNA by PCR and the FLAG-cyclin A1 sub-cloned into pFastBAC1 donor plasmid and baculovirus expressing FLAG-cyclin A1 was generated using the Bac-to-Bac baculovirus expression system (Invitrogen), as per manufacturer's instructions. Recombinant human HA-CDK1, HA-CDK2 and GST-cyclin A2 baculoviruses were kind gifts from Dr. Carol Prives.

Expression and purification of cyclin/CDK complexes

Recombinant complexes were purified from Sf9 cells by co-infection of the cyclin and CDK baculoviruses. The viral preparations were titrated so as to yield 1:1 ratios of the cyclin and the CDK in the complexes formed following infection. After 72 hours, cells were harvested, resuspended in lysis buffer (50 mM Tris-Cl, pH=7.4, 250 mM NaCl, 0.1% NP-40, 5 mM EDTA, 1X mammalian protease inhibitor cocktail (Sigma), 1X phosphatase inhibitor cocktail I (Sigma), 1X phosphatase inhibitor cocktail II (Sigma) and 10% Glycerol) and lysed by passing through a syringe with a 27 gauge needle. Complexes were immunoaffinity purified using anti-HA tagged agarose beads (Sigma) and eluted in 1X TBS containing 100 µg/ml HA-peptide (Sigma) and 0.2% NP40 for two hours at 4°C (2X bed volume). The purified complexes were visualized by resolving the eluate on SDS-PAGE followed by silver staining. The complexes were quantified using BSA as standard. The identity of the protein bands was confirmed by immunoblot analysis.

In vitro kinase assays

The amount of complex was quantified as noted above and the kinase activity of the purified complexes was determined using histone H1 as substrate. Complexes were incubated with 125 µg/ml histone H1 (Roche Diagnostics) in assay buffer (50 mM Tris-HCl, pH=7.4, 4 mM MgCl₂, 1 mM DTT, 100 µM ATP and 10 µCi of γ -³²P-ATP) for 10 min at room temperature (RT), with a total reaction volume of 25 µl, and terminated with 5 µl of 6X Laemmli buffer. Samples were boiled, resolved by SDS-PAGE followed by autoradiography. Sf9 cells infected with cyclin A1 baculovirus alone were used as control.

Determination of kinetic constants

Kinetic constants were determined by *in vitro* kinase assays, using purified complexes and histone H1 as substrate. Briefly, complexes (eluate volumes corresponding to 2–4 ng) were incubated with histone H1 in assay buffer (50 mM Tris-HCl, pH=7.4, 4 mM MgCl₂, 1 mM DTT) at RT in a volume of 20 µl. Five microliters of ATP solution (100 µM ATP and 10 µCi of γ -³²P-ATP, final concentration) in kinase assay buffer was then added. A time of 6 minutes was determined to be in the linear range. The reaction was then spotted onto a p81 phosphocellulose filter (Whatman) and washed in 150 mM phosphoric acid (3x, 15 min each) air dried and counted. First, the amount of complex in the linear range was determined by using saturating concentrations of histone H1 (9.5 µM) and varying the eluate. For determination of V_{max} and K_m , eluate amounts determined to be in the linear range were incubated with varying concentrations of histone H1 (9.2 nM to 9.5 µM) in kinase assay buffer and the pmoles/min of radioactive phosphate incorporated into histone H1 was calculated and analyzed by Michaelis-Menten kinetics using GraphPad Prism4 software. k_{cat} was determined by dividing V_{max} by the moles of complex present in the reaction.

Kinase assays with pRb and p53

Recombinant GST-pRb (amino acids 769–921) and GST-p53 (Santa Cruz) (500 ng) were incubated with amounts of complex normalized to equal histone H1 kinase activity in pRb/p53 kinase assay buffer (50 mM HEPES pH=7.5, 10 mM MgCl₂, 1 mM DTT) in a volume of 20 µl. The reaction was initiated with 5 µl of ATP solution (100 µM ATP and 10

μCi of $\gamma^{32}\text{P}$ -ATP in assay buffer) at 30°C for 20 min. and terminated with 5 μl of 6X Laemmli buffer. Samples were boiled, resolved by SDS-PAGE followed by autoradiography.

Assays with R-roscovitine

To determine IC_{50} values, assays were set up with 2–4 ng of each complex, saturating concentrations (9.5 μM) of histone H1 and various concentrations of R-roscovitine (Calbiochem) for 6 min at RT in 25 μl in histone H1 assay buffer. The final concentrations of R-roscovitine varied from 0–224 μM (based on concentrations previously determined for CDK2 in association with cyclin A2 or cyclin E, [12]). Kinase activity at 0.0 μM R-roscovitine was considered to be 100% and inhibition expressed as percentage of this activity. IC_{50} values were determined by fitting the data to a sigmoidal dose response curve using GraphPad Prism4 software.

RESULTS AND DISCUSSION

Human Cyclin A1 associates with both CDK1 and CDK2 to form active kinase complexes

Cyclin A1 is concurrently expressed with both Cdk's in pachytene-diplotene spermatocytes [3], and we have previously demonstrated co-immunoprecipitation of murine cyclin A1 with both Cdk1 and Cdk2 in testicular lysates [2; 3]. *In vivo*, human cyclin A1 was shown to co-immunoprecipitate with both CDK1 and CDK2 in human embryonal carcinoma cells [11] as well as in prostate cancer cell lines [13]. However, in a yeast two-hybrid assay, human cyclin A1 was reported to interact only with CDK2 and not CDK1 [14]. We therefore determined whether the cyclin A1 protein produced in our baculovirus assay system would interact with both CDKs. Similar to cyclin A2, human cyclin A1 protein co-immunoprecipitated with both CDK1 and CDK2 (Fig. 1A) and the complexes exhibited kinase activity (Fig. 1B).

CDK2 is incompletely phosphorylated when complexed with cyclin A1 as compared to cyclin A2

Association of cyclin A2 with CDK2 leads to substantial conformational changes in CDK2 and an activating phosphorylation on Thr160 of CDK2 by CAK [15]. When phosphorylated on Thr160, CDK2 exhibits an aberrant mobility on SDS-PAGE, migrating faster than the non-phosphorylated form [16]. Although the characteristic faster mobility was observed for CDK2 associated with cyclin A2, the CDK2 associated with cyclin A1 always migrated more slowly (Fig. 1A). This suggested that the CDK2 in the cyclin A1/CDK2 complex was not fully phosphorylated, perhaps due to conformational changes. Cyclin A1 and A2 share 88% identity in the first cyclin box fold; nonetheless, several non-conserved amino acids in the area of interaction with CDK2 were noted that could cause altered binding. For instance, the residues corresponding to His180 and Met190 in the N-terminal helix of cyclin A2, which are involved in contacting CDK2 [17], are replaced by alanine in cyclin A1. Finally, while CDK1 also requires an activating phosphorylation, on Thr161 [18], there has been no evidence for altered or aberrant mobility; correspondingly, there was no difference in the mobility of CDK1 associated with cyclin A1 or cyclin A2.

Differences in CDK2 kinase activity in complexes containing cyclin A1 versus cyclin A2 but not in CDK1 complexes

Kinase activities for cyclin A1/CDK2 and cyclin A2/CDK2 complexes were next analyzed using Michaelis-Menten kinetics (representative experiment is depicted in Figure 1C and values for K_m , k_{cat} and k_{cat}/K_m are summarized in Figure 1(i)). Cyclin A2/CDK2 complexes had a ten-fold higher k_{cat} value ($200.3 \pm 21.2/\text{min}$) as compared to cyclin A1/CDK2 ($31.4 \pm 14.9/\text{min}$) and the k_{cat}/K_m was two-fold higher--most likely reflecting the absence of the activating Thr160 phosphorylation on CDK2 in the cyclin A1/CDK2 complexes.

Kinase assays were also performed for CDK1 complexed with cyclin A1 versus cyclin A2 and K_m , k_{cat} and k_{cat}/K_m were calculated (Fig. 1D and 1(ii)). There was no significant difference between cyclin A1/CDK1 and cyclin A2/CDK1 complexes with regard to activity and K_m , k_{cat} and k_{cat}/K_m values (Fig. 1(ii)).

Interestingly, cyclin A2/CDK2 was catalytically more efficient than cyclin A2/CDK1, as reflected in the values for k_{cat} and k_{cat}/K_m . To the best of our knowledge, such a direct comparison has not been made previously. A less efficient association of cyclin A2 with CDK1 as compared to CDK2 *in vitro* had been documented [19] but the kinetic constants were not determined. We have previously demonstrated that *in vivo* in the mouse testis, cyclin A2 also preferentially associated with Cdk2 as compared to Cdk1 [3].

Determination of IC₅₀ values of R-roscovitine for cyclin A1-containing complexes

The purine analogue R-roscovitine, is a specific inhibitor of CDK1 and CDK2 complexed with cyclin A2 [20] but its effect on cyclin A1/CDK complexes is unknown. We therefore determined the IC₅₀ values of R-roscovitine for cyclin A1/CDK2 and cyclin A1/CDK1 to be 1.6 μM and 3.4 μM respectively, which did not differ significantly from those for cyclin A2/CDK2 and cyclin A2/CDK1, 1.0 μM and 2.1 μM , respectively (Table 1), which were also similar to previously published data [12].

Analysis of kinase activity using pRb as substrate

pRb has been shown to be phosphorylated by cyclin A2/CDK2 *in vitro* [21]. Recombinant cyclin A1/CDK2 complexes were also reported to phosphorylate pRb [22], but the two complexes have not been compared for their phosphorylation kinetics. Phosphorylation of pRb was of particular interest since pRb is expressed in rat spermatocytes in late meiotic prophase [23], similar to mouse and human cyclin A1 [2; 11]. Although the recombinant GST-pRb used in the assay harbors six CDK consensus phosphorylation motifs and four RXL motifs [24], only two of the six potential phosphorylation motifs are phosphorylated by cyclin A2/CDK2 *in vitro* [25]. Both cyclin A1/CDK2 and cyclin A2/CDK2 complexes could apparently phosphorylate more than one site (Fig. 2A; bands 2 and 3 on the Coomassie stained gel and autorad) as suggested from the retarded mobility of the phosphorylated GST-pRb versus the non-phosphorylated form (band 1). However, cyclin A1/CDK2 appeared to be less efficient at phosphorylating GST-pRb, perhaps due to the incompletely phosphorylated CDK2 when it is associated with cyclin A1. Even doubling the amount of cyclin A1/CDK2 (Fig. 2A, lane c) did not lead to complete phosphorylation of GST-pRb

(persistence of bands 2 and 3 in Fig. 2A, lane c). Cyclin A2/CDK2 in contrast, fully phosphorylated pRb even at the lower concentration (Fig. 2A, lanes f and g).

Both cyclin A1/CDK1 and cyclin A2/CDK1 complexes were capable of phosphorylating GST-pRb (Fig 2B), but interestingly, cyclin A1/CDK1 appeared to be more robust (Fig. 2B, lanes b and c). Unlike CDK2-containing complexes, CDK1-containing complexes did not yield a mobility shift of GST-pRb upon phosphorylation (Fig. 2B, lanes b, c, f and g). A similar lack of mobility shift of phosphorylated pRb has also been observed with cyclin D/Cdk4 complexes [21].

Analysis of the kinase activity of the complexes using p53 as substrate

Although p53 has several phosphorylation sites, only Ser315 has been shown to be a substrate for cyclin/CDK complexes *in vitro*, particularly cyclin A2/CDK2 and cyclin B/CDK1 [26; 27]. In the testis, p53 is highly expressed in pachytene spermatocytes [28; 29] and the apoptotic response in cyclin A1-deficient spermatocytes is partially dependent on p53 [30], making it a potentially physiologically relevant *in vivo* substrate for cyclin A1/CDK complexes. Cyclin A1/CDK2 (Fig. 2C, lanes b and c) appeared to be less efficient in phosphorylating GST-p53 as compared to cyclin A2/CDK2 (Fig. 2C, lanes f and g). However, cyclin A1 in association with CDK1 (Fig. 2D, lanes b and c) appeared to phosphorylate GST-p53 more efficiently than cyclin A2/CDK1 (Fig. 2D, lanes f and g).

Both CDK1 and CDK2 are expressed in pachytene spermatocytes in the testis [11], however, the critical catalytic CDK partner for cyclin A1 is not known [7; 31]. The observation that CDK1/cyclin A1 is a better kinase for pRb and p53 suggests that (i) associating with CDK1 may be preferred for phosphorylation of specific substrates and (ii) the cyclin partner is indeed capable of influencing the kinase activity toward specific substrates.

Of the 150 known substrates of CDK1 in yeast (*cdc28*), Clb5/CDK1 was more efficient in phosphorylating 24% of substrates as compared to Clb2/CDK1 [32]. Additionally, even though there were no Clb2/CDK1-specific targets, Clb2/CDK1 was intrinsically a better kinase than Clb5/CDK1. Correspondingly, our analysis of the *in vitro* biochemical properties of the cyclin A1- and cyclin A2-containing complexes also suggest that they can confer distinct biochemical properties to CDK2.

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Figure 1.

(A) Cyclin A1 can associate and form active complexes with CDK1 as well as CDK2 but the CDK2 complexed with cyclin A1 is not completely phosphorylated. Cyclin A2/CDK2 (A2/K2), cyclin A1/CDK2 (A1/K2), cyclin A2/CDK1 (A2/K1), and cyclin A1/CDK1 (A1/K1) were purified from Sf9 cells co-infected with recombinant baculoviruses and visualized by silver staining following SDS-PAGE. The Thr160 phosphorylated form of CDK2 is denoted by [P]-CDK2. (B) Cyclin A1/CDK1 and cyclin A1/CDK2 complexes can both phosphorylate histone H1. *In vitro* kinase assays were performed using purified cyclin A1/CDK1 (A1/K1) and cyclin A1/CDK2 (A1/K2) complexes. Cyclin A1 alone (A1) did not exhibit kinase activity. (C) Cyclin A2/CDK2 is a marginally better kinase than cyclin A1/CDK2. Purified complexes were used in *in vitro* kinase assays as above and the data were analyzed by Michealis-Menten Kinetics. (i) Table summarizing K_m , k_{cat} and

k_{cat}/K_m values, calculated from best-fit values of V_{max} and K_m from three independent experiments. All values are significantly different ($p < 0.05$). **(D) Kinetic parameters of cyclin A1/CDK1 are similar to the kinetic parameters of cyclin A2/CDK1.** *In vitro* kinase assays were set up as above and the data were analyzed by Michaelis-Menten Kinetics. **(ii)** Table summarizing K_m , k_{cat} and k_{cat}/K_m values, calculated as described for (C) from three independent experiments. The difference in the values of K_m , k_{cat} and k_{cat}/K_m was not statistically significant ($p < 0.05$).

Figure 2. Phosphorylation of pRb and p53 by cyclin A1- and cyclin A2-containing complexes (2A and 2B) 500 ng of GST-pRb (amino acids 769–921) were incubated with purified cyclin A1/CDK2 (A, lanes a to d) and cyclin A2/CDK2 (A, lanes e to h) as well as cyclin A1/CDK1 (B, lanes a to d) and cyclin A2/CDK1 (B, lanes e to h). Lanes a and e indicate reactions with no substrate, while lanes d and h are reactions without the complex. The amount of complex in lanes c and g was double the amount used in lanes b and f. The non-phosphorylated form of GST-pRb in panel A is indicated by band 1. Bands 2 and 3 represent phosphorylated and hyper-phosphorylated forms of GST-pRb, respectively. GST-pRb and

[P]-GST-pRb in panel B represent non-phosphorylated and phosphorylated substrates in kinase reactions containing CDK1 complexes. **(2C and 2D)** GST-p53 (500 ng) was incubated with purified cyclin A1/CDK2 (C, lanes a to d) and cyclin A2/CDK2 (C, lanes e to h) as well as cyclin A1/CDK1 (D, lanes a to d) and cyclin A2/CDK1 (D, lanes e to f) as described previously. Similar to assays with pRb, lanes a and e indicate reactions with no substrate, while lanes d and h are reactions without the complex. The amount of complex in lanes c and g was double the amount used in lanes b and f. [P]-GST-p53 designates phosphorylated GST-p53.

Table 1

IC₅₀ values for R-roscovitine.

	Cyclin A2/CDK2	Cyclin A1/CDK2	Cyclin A2/CDK1	Cyclin A1/CDK1
IC ₅₀ (μM)	1.0	1.6	2.1	3.4