Binding of HTm4 to KAP/CDK2/Cyclin A complex enhances the phosphatase activity of KAP, dissociates Cyclin A, and facilitates KAP dephosphorylation of CDK2

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Cdk2 activation requires phosphorylation of Thr160 and dissociation from cyclin A. The T loop of cdk2 contains a regulatory phosphorylation site at Thr160. An interaction between KAP and cdk2 compromises the interaction between cdk2 and cyclin A, which permits access of KAP, a Thr160-directed phosphatase, to its substrate, cdk2. We have reported that KAP is bound and activated by a nuclear membrane protein, HTm4. Here, we present in vitro data showing the direct interaction between the HTm4 C-terminus and KAP tyrosine residue 141. We show that this interaction not only facilitates access of KAP to Thr160 and accelerates KAP kinetics, but also forces exclusion of cyclin A from the KAP/cdk2 complex.

HTm4 (MS4A3) is the third member of subfamily A in an extensive membrane-spanning 4-domain gene family. These genes are only loosely related at the sequence level, but their encoded proteins share a common four-transmembrane topology, including CD20 (MS4A1) and FcεRI (MS4A2). To date, few functions for the MS4 family of proteins have been ascribed. However, a diverse functionality is beginning to emerge. These functions include roles such as cell surface signaling...
receptors and intracellular adapter proteins (1-5).

Cell cycle progression is regulated by the sequential activation, and inactivation, of the cyclin-dependent kinases (cdks). Cdk, themselves, are substrates for regulatory phosphorylation and dephosphorylation. The phosphorylation status of cdks is controlled by a discrete class of regulatory kinases and phosphatases (6). Cdk activity is controlled by activatory and inhibitory proteins, the latter exemplified by p21 and p27. Binding of inhibitors such as p21 and p27 to different cyclin/cdk complexes is sufficient to arrest the cell cycle (7-12). Conversely, inactive, monomeric cdk can be activated via association with a specific cyclin, and the concurrent phosphorylation of a conserved and essential threonine residue, such as Threonine-160 (Thr160) in cdk2, which is located within the activation segment (T-loop) of the kinase.

In addition to cyclin association, full activation of cdk2 requires phosphorylation of Thr160 and dephosphorylation of Thr14 and Tyr15. Thr160 of cdk2 is phosphorylated by CAK (cdk-associated kinase), while its dephosphorylation is critical for inactivation. This dephosphorylation is executed by a serine/threonine directed phosphatase, KAP. It has been shown that exogenous expression of KAP slows the G1 phase cell cycle progression in HeLa cells, and that aberrant KAP transcripts are detected in some hepatocellular carcinomas(13,14). These observations suggest that KAP has the same biological effects as cdk inhibitors, though their modes of actions are different. KAP can bind to cdk2 either in the presence or absence of cyclins (15-17). However, KAP can only dephosphorylate cdk2 when cyclin A is degraded or disassociated, in a mechanism which may control access of KAP to its substrate Thr160 (16). Cyclin binding may therefore control access of KAP to its substrate p-Thr160. We previously reported that HTm4 interacts directly with the KAP via its carboxy-terminal (18,19), and that exogenous expression of HTm4 leads to dephosphorylation of cdk2 and cell cycle arrest at the G0/G1 phase. Here, we show that the presence of HTm4 in the KAP/cdk2/cyclin A complex controls cdk2 activity in a dual fashion. First, HTm4 binding causes exclusion of cyclin A from its interaction with cdk2. Second, HTm4 binding potentiates KAP enzymatic activity and causes conformational changes that regulate access to Thr160.

MATERIALS AND METHODS

Subcloning, expression, purification and characterization of HTm4 and KAP

Human HTm4 was RT-PCR cloned from total RNA extracted from human peripheral blood mononuclear cells from healthy volunteers with informed consent, using the following 5' and 3' primers: (forward primer: hHTm4Sfo1:5'gta-tag-gcg-cct-ccc-acg-aag-ttg-ata 3'; reverse primer: hHTm4XaRev: 5'ata-gtt-tag-cgg-ccc-ttc-cct-cta-cag-aat-tgg-gag-gtg-agg 3'). The PCR product was subcloned into the pQE-Tri System-HisStrep-1 vector (Qiagen). An amino-terminal His-tag was conferred during subcloning.

Recombinant HTm4 was purified from transformed JM109 E. coli as follows. Protein production was induced by the addition of IPTG to bacterial liquid culture in logarithmic phase. Bacteria were harvested by centrifugation at 6000 rpm for 30 min. The bacterial pellet was lysed by sonication on ice (20KW, 15 min) in 50 ml of N buffer (20mM Tris-HCl (pH 8.0) containing 0.1M NaCl, 1 mM PMSF, 1 mM β-mercaptoethanol). The bacterial lyse was centrifuged at 20x10⁴ g at 4 °C for 30 min. The remaining pellet was lysed in 40 ml of N buffer containing 6M Guanidine-HCl. Proteins were bound to a 20 ml nickel-chelated sepharose column (Amersham) and were refolded by exposure to a 6 to 0 M
urea gradient at 0.2 ml/min. The refolded protein sample was eluted with a gradient of N buffer containing 0.5 M imidazole and 0.3% deoxycholate. The peak fraction containing HTm4 was collected and purified by XArrest agarose after His-tag cleavage with 4U of Factor Xa (Novagen). Wild-type KAP (KAPwt), inactive KAP (C140S), and mutant KAP (KAPt) were expressed and purified as described previously (20).

**Western Blotting of HTm4**

For the Western blot, 10 µl of purified protein was boiled in SDS sample buffer and resolved by 15% PAGE. After electrotransfer to PVDF, the membrane (BioRad) was blocked followed by either incubation with either anti-His-tag antibody diluted at 1:5,000 (MBL), or anti-HTm4 antibody (Zymed), and HRP conjugated anti-rabbit IgG. Signals were detected using ECL (Amersham).

**Protein Interaction Analyses (Surface Plasmon Resonance Assay)**

The immobilization of proteins to sensor chips (CM5 chip) was performed using a carbodiimide covalent linkage protocol (Amersham). KAP was coupled to the sensor chip at 3200 Resonance Units (RU). Non-specific human immunoglobin was coupled to the surface of the sensor chip (anti-β2m antibody BBM.1) at 2900 RU. Protein interaction assays were carried out using the BioSensor Biacore 2000 (Biacore). An HTm4 C-terminal peptide composed of 22 amino acids (CNANCCNSREEISSPPNSV) was synthesized. Either full length HTm4 protein (20 µM), or HTm4 C-terminal peptide (1mM) in 50 mM HEPES containing 1 mM EDTA, 1 mM DTT, 5% glycerol and 0.1% Tween 20 was examined at a flow rate of 10 µl/min.

**Protein Activity Assays**

KAP phosphatase activity was examined by the detection of hydrolysis of para-nitrophenyl-phosphate (pNPP) (WAKO) as described (20). The time course of phosphatase activity for KAP was examined. 20 µl of reaction mixture containing 50 mM Tris-HCl (pH 7.5), 0.15 M NaCl, 2 mM DTT, 0.5 mM EDTA, 3 mM pNPP, 40 µM KAP, 4 µM cdk/cyclin A (Upstate) and 20 µM HTm4 was incubated at 30°C for the indicated times. The reaction was halted by the addition of 0.1 M NaOH. The phosphatase activity in 5 µl of each sample was measured as A410. To investigate the effect of HTm4 on KAP, KAP phosphatase activity was assessed with different HTm4 concentrations in the presence of pNPP. Reaction velocity was measured in the presence of 0.0025 to 20 µM HTm4. All reaction mixtures contained 40 µM KAP and 4 µM active cdk2/cyclin A. Activities of either wild-type or C140S (inactive) KAP were examined in the different reaction mixtures: With or without 20 µM HTm4 (C-terminal peptides/non-related peptides (NRP) at 0.1 mM), or 4 µM active cdk2/cyclin A (cdk2 monomer).

**Kinetic Measurements**

In order to investigate the effect of HTm4 on KAP, the initial reaction velocity of KAP was assessed using different concentrations of active cdk2/cyclin A as the substrate in the presence or absence of HTm4. The results were further analyzed using a Lineweaver-Burk plot.

**KAP dephosphorylation of peptide or protein substrates**

KAP dephosphorylation of phosphorylated proteins or peptide substrates was examined using the Ser/Thr Phosphatase Assay Kit 1 (Upstate) according to the manufacturer’s instructions. Phosphorylated Thr160 of cdk2 and K-R-pT-I-R-R peptide were used as substrates for the assay. Concentrations of KAP and HTm4 were both 20 µM; concentrations of p-cdk2 and peptide substrates (K-R-pT-I-R-R) were 10 and 25 µM, respectively. Reactions were performed
in pNPP Ser/Thr Assay Buffer, containing 50 mM Tris-HCl (pH 7.0), and 100 µM CaCl₂. In order to examine the importance of the HTm4 C-terminal region, and to confirm the KAP phosphatase activity enhancement effect of HTm4, an HTm4 C-terminal peptide composed of 22 amino acids (CNANCCNSREEIISPPNSV) was synthesized. The phosphatase enhancement activity of the HTm4 C-terminal region was examined using the assay protocol described above. All assays were performed in triplicate, with the average values and standard deviations displayed on the plot (Fig. 2-4).

**Circular Dichroism Spectroscopy**

Circular Dichroism (CD) spectroscopic measurements were performed on a Jasco spectropolarimeter (Jasco, Easton, MD). Spectra measurements were recorded from 250nm to 320nm. The spectra change of KAP was measured by comparing two different KAP samples in the same solution (20 mM Tris-HCl, pH7.2, 0.15 M NaCl). Sample A contained 5 µM of KAP and an excess amount of HTm4 C-peptide (50 µM). The contents of sample B were identical to those in sample A, except for HTm4 C-peptide. The difference in spectra was further converted to parameter [Mean Residue Ellipticity] and plotted on the graph in Fig. 4.

**Cyclin A Dissociation Assay**

The status of the cdk2/cyclin A complex was examined in the presence and absence of HTm4. Recombinant cdk2/cyclin A (Upstate) and His-tagged KAP were combined in vitro, and incubated in the absence or presence of either 1 or 10 µM of HTm4. The samples were added to a Ni-magnet bead matrix (Toyobo) in order to capture His-tagged KAP protein and any associated binding partners. The bound fractions were resolved by SDS-PAGE, and Western blotted for the presence of cyclin A (Santa Cruz Biotech). An alkaline phosphatase conjugated to anti-rabbit IgG (EY Lab) was used to visualize the amount of bound cyclin A present in the KAP/cdk2 complex. Ten µl of 10 µM cdk2/cyclin A (Upstate), and 10 µl of 10 µM His-tagged KAP were mixed in 20 mM Tris-HCl (pH 7.5) buffer containing 0.15 M NaCl, 1mM β-mercaptoethanol and 0.1% Tween 20. After incubating at room temperature for 5 min, either 10 µl of 10 µM HTm4 (without His-tag) or its buffer were added to the reaction mixture. After incubating at room temperature for 5 min, 5 µl of Ni-Magnet beads (Toyobo) were added to each mixture. The fractions bound to His-tagged KAP were washed with the reaction buffer, and boiled for SDS-PAGE. The gel was stained with a silver staining kit (ATTO).

**RESULTS**

**Direct binding of KAP and HTm4 in vitro**

We have previously shown that HTm4 and KAP phosphatase may be co-immunoprecipitated from hematopoietic cells (18). Since these data form the basis for a novel model where HTm4 is a component of the cell cycle machinery, it is important that we determine whether the interaction is direct, or if it involves an intermediary protein. We have since established a purified protein-protein interaction system that allows us to examine whether HTm4 and KAP interact directly. His-tagged HTm4 was produced in *E. coli*, and purified by fast protein liquid chromatography (FPLC) (Fig. 1a). The eluted peak corresponding to HTm4 was visualized by Coomassie Blue staining (Fig. 1b) and then analyzed by Western blot. Both anti-HTm4 and anti-His antibodies detected a 27kDa band corresponding to HTm4 (Fig. 1c-d). Surface plasmon resonance (SPR) was used to detect direct binding of purified HTm4 to KAP. Full length KAP protein was coupled to a sensor chip CM-5 shell (Amersham). Compared to
either un-coupled or non-specifically-coupled chips (coupled to anti-β2m antibody BM.1), an immediate increase in mass was observed when either full length HTm4 (Fig. 2a) or a C-terminal peptide from HTm4 (Fig. 2b) was passed over KAP-coupled sensor chips. Moreover, pre-mixing HTm4 with KAP abolished the KAP/HTm4 selective binding (data not shown). These in vitro data confirm that HTm4 and KAP interact directly, and that the HTm4 C-terminus is necessary and sufficient to mediate binding to KAP.

**KAP phosphatase activity is enhanced in the presence of HTm4**

HTm4 is a direct binding partner for KAP, as shown in our previous yeast two-hybrid data. We hypothesize that HTm4 may regulate the enzymic activity of the KAP phosphatase. In the current experiment, KAP phosphatase activity was measured using pNPP as a generic substrate for KAP. In the presence of HTm4 and the cdk2/cyclin A binding complex, a biphasic kinetic for KAP activity was observed. As shown in Fig. 3a, after addition of pNPP substrate, an exponential phase (in the first 20 min) was followed by a plateau phase (after 30 minutes). The presence of HTm4 linearly increased the phosphatase activity of KAP (y = 1.45x + 0.01; r² = 0.98) (Fig. 3b). Without HTm4, the V_max and K_m for KAP phosphatase activity were 1.30 nM/min and 2.68 µM, respectively (Fig. 3c-d). However, when HTm4 was present, the V_max of KAP phosphatase activity dramatically increased to 17.5 nM/min. A parallel increase in K_m (to 8.33 µM) was also observed. Taken together, these data suggest that the presence of HTm4 directly affects the activity of the KAP phosphatase.

**KAP dephosphorylation of peptide or protein substrates**

KAP dephosphorylation of phosphorylated proteins or peptide substrates was examined using the Ser/Thr Phosphatase Assay Kit 1 (Upstate) according to the manufacturer’s instructions. Phosphorylated Thr160 of cdk2 (p-cdk2) and K-R-pT-I-R-R peptides were used as substrates for the assay. Concentrations of KAP and HTm4 were both 20 µM; concentrations of p-cdk2 and peptide substrates (K-R-pT-I-R-R) were 10 and 25 µM, respectively. Reactions were performed in pNPP Ser/Thr Assay Buffer, containing 50 mM Tris-HCl (pH 7.0), and 100 µM CaCl₂. To examine the necessity of the HTm4 C-terminal region, and to confirm the KAP phosphatase activity enhancement effect of HTm4, a peptide containing the last 22 amino acids of the HTm4 C-terminus (CNANCNSREEISSPPNSV) was synthesized. The phosphatase enhancement activity of the HTm4 C-terminal region was examined using the assay protocol described above. All assays were performed in triplicate, with the average values and standard deviations displayed on the plot.

We further evaluated the major factors affecting KAP phosphatase activity. As we have shown, KAP phosphatase activity is significantly enhanced in the presence of HTm4. When an inactive KAP mutant (C140S) is substituted for the wild-type KAP, the phosphatase activity is almost completely abolished. The inactive KAP mutant thus serves as a negative control (15,20). We found that when HTm4 was absent, or was substituted with a non-related peptide (NRP), KAP phosphatase activity was almost completely inhibited compared to control reactions containing wild-type KAP, HTm4 and cdk2/cyclin A complex (Fig. 3e). It is interesting to note that a cdk2/cyclin A complex, rather than either a monomeric cdk2 or cyclin A, was required for a stronger KAP phosphatase activity induced by HTm4. Additionally, our data suggest that KAP activity is optimal in the context of a multi-component protein complex that contains its substrate, cdk2, and the HTm4 protein. Our data demonstrate that wild-type KAP and HTm4 are essential for KAP phosphatase activity.
Based on our previous yeast-two-hybrid and immunoprecipitation data, we aimed to confirm whether the C-terminal region of HTm4, alone, was sufficient to mediate the HTm4-KAP interaction, and to induce the KAP phosphatase activity. To this end, we utilized the same synthesized peptide containing the hypothesized KAP binding domain (CNANCCNSREEISSPPNSV), as described above. When introduced into our in vitro KAP activity assay, the HTm4 C-peptide (C-Pep) showed a comparable enhancement effect on KAP phosphatase activity compared to HTm4 wild-type protein (Fig. 3e). These data both confirm our previous observation that the C-terminal of HTm4 mediates direct binding of HTm4 to KAP, and show that the C-terminus is the functional domain of HTm4 that regulates KAP activity.

HTm4 controls the phosphatase activity of KAP towards its physiological substrates

The data presented above demonstrate that HTm4 enhances the activity of the KAP phosphatase towards a generic substrate compound, pNPP. We asked whether HTm4 could also modify the activity of KAP towards its physiological substrates. We evaluated the ability of KAP to dephosphorylate two of its substrates (Fig. 3f). First, we examined cdk2 with a phosphorylated Thr160 (p-cdk2), which acts as a natural substrate for KAP. We then used a synthesized threonine phosphopeptide (p-Pep), K-R-pT-I-R-R (Upstate). We found that HTm4 increases the dephosphorylating activity of KAP towards phosphorylated cdk2 by over 6-fold, when cdk2 is presented in the context of the cdk2/cyclin A complex. We also demonstrated that HTm4 potentiates dephosphorylation of the synthesized phosphopeptide K-R-pT-I-R-R. However, no KAP dephosphorylation activity could be detected if HTm4 was not presented. Interestingly, no dephosphorylation effect was observed when phosphorylated cdk2 was presented in the cdk2/cyclin A binding complex. These data imply that the presence of HTm4 confers a substrate-specific enhancement of KAP phosphatase activity.

Structural change of KAP by adding C-peptide of HTm4

Based on our observation that C-peptide of HTm4 protein enhanced KAP activity, we hypothesized that the binding of HTm4 C-peptide to KAP molecule induced KAP conformational change. In order to confirm this hypothesis, we examined conformational changes of KAP in the presence or absence of the C-peptide by using Circular Dichroism (CD) Spectroscopy. The CD spectrum of a protein in the “near-uv” spectral region (250-350nm) can be sensitive to certain aspects of tertiary structure. At these wavelengths the chromophores are the aromatic amino acids and disulfide bonds, and the CD signals they produce are sensitive to the overall tertiary structure of the protein. Signals in the region from 250-270 nm are attributable to phenylalanine residues, signals from 270-290 nm are attributable to tyrosine, and those from 280-300 nm are attributable to tryptophan. Disulfide bonds give rise to broad weak signals throughout the near-uv spectrum. As shown in Fig. 4, we found a spectral change at 270-280 nm in the difference spectrum of KAP between sample A (with HTm4 C-peptide) and sample B (without C-peptide). The 270-290 nm difference spectrum demonstrated a conformational change in tyrosine when HTm4 C-peptide was added into KAP solution. Because HTm4 C-peptide has no tyrosines residues, this major change in the spectrum must be caused by conformational changes in KAP’s tyrosine residues. Hence, this result demonstrated that HTm4 could cause direct conformational/tertiary structural change in KAP after its binding to KAP by C terminal.
HTm4 dissociates inhibitory cyclin A from the cdk2/cyclin A binding complex.

The activity of cdk2 is controlled by its protein-protein interactions with KAP and cyclin A. The binding of KAP phosphatase to the cdk2/cyclin A complex dephosphorylates, and hence activates, cdk2. However, the binding of cyclin A inhibits the ability of KAP to dephosphorylate cdk2. Dissociation of cyclin A by HTm4 facilitates cdk2 dephosphorylation by KAP. Interestingly, HTm4 enhances the KAP dephosphorylation effect even on phosphorylated cdk2 that is presented in a complex with cyclin A. This result suggests that HTm4 may facilitate cdk2 activation by alleviating the inhibitory effect of cyclin A. Our model suggests that HTm4 may dually regulate cdk2 activity, via KAP, and by physically excluding cyclin A from its inhibitory interaction with cdk2.

To verify our hypothesis, we used an affinity purification method to evaluate the cdk2/cyclin A interaction both with and without HTm4. We isolated His-tagged KAP protein using a Ni²⁺ binding column. Without HTm4, cyclin A remained in the His-purified KAP/cdk2/cyclin A binding complex (Fig. 5a). However, when purified HTm4 (after His-tag cleavage) was added to the KAP/cdk2/cyclin A reaction mixture, cyclin A dissociated from the complex in a dose-dependent manner, and the remaining protein complex was found to contain only KAP/cdk2/HTm4 (Fig. 5b). These observations demonstrate that HTm4 inactivates cdk2 by activating KAP, and by causing concomitant dissociation of cyclin A from cdk2. Together with the observation that HTm4 enhances KAP phosphatase activity, we suggest that HTm4 is positioned as a key regulator in the activation of KAP and for cdk2 activity.

DISCUSSION

Mechanism for HTm4-induced dissociation of cyclin A from cdk2/cyclin A

In silico modeling was used to probe the potential nature of the HTm4 regulatory interaction that promotes KAP phosphatase activity, and potentially leads to the exclusion of cyclin A. Based on our findings, we show that KAP undergoes conformational changes following allosteric binding to the HTm4 C-terminal domain, and that these conformational changes may subsequently facilitate the interaction between the KAP substrate and its active site. To examine this hypothetical model, we produced a steric structure model using the coordinates of cdk2/cyclin A (1JST) (21) and cdk/KAP (1FQ1) (20) from the Protein Data Bank (PDB). These structures were overlapped utilizing Homology in Insight II software (MSI). The resulting model complex is shown in Fig. 6a. Both cdk structures significantly overlap, and the overall RMS deviation is 0.92 Å, with the exception of the T-loop region. In this region, marked differences in cdk structure can be noted, depending on whether coordinates are derived from the cdk2/cyclin A (blue loop), or the cdk2/KAP complex (red loop). There is a deep concave face, as shown in Fig. 6b. We hypothesize that the concave face forms a potential docking site for the HTm4 C-terminus. On the reverse side of the interface, p-Thr160 of cdk2 can be inserted into the entrance of the active site of KAP in the absence of cyclin A, while a small molecule of substrate such as pNPP may still be able to enter the entrance even in the presence of cyclin A. This finding may explain why KAP activity towards pNPP is enhanced at moderate levels in the presence of cyclin A.

Mode of structural change of the cdk2 T-loop in the absence and presence of HTm4

The T-loop is a conserved segment in cdks, which plays an important role in cdk
activation. In the cdk2/cyclin A complex, phosphorylation is associated with the mobility of T-loop (12). However, viral cyclins (k-, h-cyclins) can still activate cdk6 without T-177 phosphorylation, which corresponding to T-160 phosphorylation in cdk2. The degree of interaction between T-loop of cdk6 and V-cyclin is showed to be critical, because the interaction area between cdk6 and v-cyclin was 20% larger than that of cdk2 and cyclin A (15). This increased interaction is contributed mainly by the T-loop of cdk6. Hence, the movement of T-loop is critical for cdk activation.

In the cdk2/cyclin A activation model, van der Waals (VW) interactions exists between Ala151, Phe152 and Tyr159 of cdk2 T-loop and Phe267, Ile182 and Ile270 of cyclin A (Fig 7a) (6). However, in our KAP/cdk2/cyclin A complex model, the second cdk2/cyclin A interaction (Phe152-Ile182) is completely abolished if the T-loop is moved due to the interaction of KAP (Fig. 7b). This result demonstrates that binding of KAP to cdk2 weakens the interactions between cdk2 and cyclin A. Moreover, it is reported that interactions between the T-loop of cdk2 and KAP comprise VW interactions among residues Tyr141 and Phe53 of KAP with residue His161 of cdk2 (20).

Besides the VW interaction between the T-loop of cdk2 and cyclin A, a specific ionic interaction also exists between the -NH at Arg157 of cdk2 and the –OH of Glu268 of cyclin A in cdk2/cyclin A complex. This interaction disappears when the cyclin A molecule is dissociated, as seen in the cdk2/KAP complex (Fig. 8a). Thus, the extension of the T-loop in cyclin A/cdk2 (blue) is inhibited by this ionic interaction.

In PDB models, more than twenty sets of coordinates are deposited for cdk2, both with and without cyclin A or its inhibitors. We investigated each structure of the T-loop and measured dihedral angles between Val154 and Pro155, which are conserved in several cdks. Fig. 8b shows amino acid multi-alignments for several cdks. When cdk2 is associated with cyclin A, the T loop is shortened with cis-Pro at position 155. Conversely, without cyclin A, the loop is extended with trans-Pro (Table 1). When bound to KAP (without cyclin A), the T-loop of cdk2 shows extension in the trans form. According to the predicted crystal structure of cdk2/CyclinA, this cis-trans conversion at Pro 155 is thought to occur automatically because the residue Pro155 would no longer be accessible to an isomerase (Fig 8c).

When cyclin A dissociates from cdk2, the T loop will extend through cis-trans conversion at the Pro155 residue, and p-Thr160 can then be inserted into the active site of KAP. Thus, the susceptibility of p-Thr160 to KAP phosphatase occurs in response to HTm4 binding to KAP. Pro155 commonly exists in cdk3, cdk5 but not cdk4, cdk5 as shown in Fig. 8b, while its counter part residue Glu258 is conserved in cyclins A, B, E, and C but not in cyclin D. This disparity suggests that mechanisms for cdk inactivation by KAP may differ in between cdk species and hence between different phases of the cell cycle.

Binding site of HTm4

In Fig. 8d, we show the close allosteric position between Thr160 of cdk2 and Tyr84, Tyr141 of KAP, which suggests that the concave face of KAP accepts the HTm4 C-terminus via the Tyr141 residue of KAP. Immediately following HTm4 binding, the conformational change of the Tyr141 residue of (Fig. 4) will weaken the VW interaction between cdk2 and cyclin A, and then affect continuously alongside T-loop (from residues 142 to 165) involving Ala151, Phe152 and Tyr159. This HTm4 binding eventually dissociates cyclin A from cdk2, thereby inducing a structural change of cdk2. At the reverse side of the interface, p-Thr160 of cdk2 is inserted into the entrance of the active site of KAP, after cyclin A is released.
Model of action mechanism for HTm4

HTm4 has four transmembrane domains (22). Topologically, this structure provides two tails that are available for protein-protein interactions (23). HTm4 localizes to the nuclear membrane. We hypothesize that the tails of HTm4 extend into the nuclear lumen, and tether the KAP/cdk2/cyclin A complex (Fig. 9). The interaction between HTm4 and the KAP/cdk2/cyclin A complex is likely reversible, and may occur only at certain stages in the cell cycle. HTm4 significantly affects the activity of KAP, cdk2 and cyclin A. HTm4 also regulates cdk2 activity in a dual fashion, by concurrently activating KAP activity and facilitating the accessibility of Thr160 to KAP via causing dissociation of inhibitory cyclin A (Fig. 9). In conclusion, we show that HTm4 regulates the cell cycle in hematopoietic cells through its ability to control cdk2 status.

REFERENCES


FOOTNOTES

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The abbreviations used in this paper are: cdk, cyclin-dependent kinase; Thr160, Threonine-160; CAK, cdk-associated kinase; KAP, cdk-associated phosphatase; KAPwt, wild-type KAP; KAPt, mutant KAP; RU, Resonance Units; pNPP, para-nitro phenyl-phosphate; NRP, non-related peptides; CD, Circular Dichroism; FPLC, fast protein liquid chromatography; SPR, Surface plasmon resonance; p-cdk2, Phosphorylated cdk2; p-Pep, threonine phosphopeptide; PDB, Protein Data Bank; VW, van der Waals.
FIGURE LEGENDS

Figure 1. Purification and Characterization of HTm4. (a) Elution of His-HTm4 from a nickel column. His-tagged HTm4 was expressed in E.coli, refolded with the column methods described above, and eluted with a gradient concentration of 0.5 M imidazole. (b) The eluted peak corresponding to HTm4 was visualized by Coomassie Blue staining. (c) Western blot analysis of HTm4 fraction of E.coli lysate. The HTm4-containing fraction was confirmed by Western blot using an anti-HTm4 antibody. (d) Western analysis of HTm4 fraction of E.coli lysate. HTm4-containing fraction was confirmed by western blot with anti-His tag antibody.

Figure 2. KAP and HTm4 Interaction by Biosensor analysis. (a) Binding of full length HTm4 protein to KAP. The interactions of HTm4 with immobilized proteins were analyzed on a sensor chip (CM-5) cell. KAP was coupled to the surface of the sensor chip at 3200 Resonance Units (RU); non-specific human immunoglobulin (NS) was coupled to the surface of the sensor chip (anti-β2m antibody BBM.1) at 2900 RU; uncoupled sensor chip (UC). Binding of full-length HTm4 protein (20 µM) in 50 mM HEPES containing 1 mM EDTA, 1 mM DTT, 5 % glycerol and 0.1 % Tween 20 was examined at a flow rate of 10 µl/min. (b) Binding of HTm4 C-terminal to KAP. The interactions of HTm4 with immobilized proteins were analyzed on a sensor chip (CM-5) cell. KAP was coupled to the surface of the sensor chip at 3200 Resonance Units (RU); non-specific human immunoglobulin (NS) was coupled to the surface of the sensor chip (anti-β2m antibody BBM.1) at 2900 RU; uncoupled sensor chip (UC). Binding of HTm4 C-terminal peptide (1mM) in 50 mM HEPES containing 1 mM EDTA, 1 mM DTT, 5 % glycerol and 0.1 % Tween 20 was examined at a flow rate of 10 µl/min.

Figure 3. HTm4 enhances KAP phosphatase activity. (a) Kinetics of wild-type KAP phosphatase activity. 20 µl of reaction mixture containing 50 mM Tris-HCl (pH 7.5), 0.15 M NaCl, 2 mM DTT, 0.5 mM EDTA, 3 mM pNPP (WAKO), 40 µM KAP, 4 µM cdk2/cyclin A (Upstate) and 20 µM HTm4 was incubated at 30ºC for the indicated times. The reaction was halted by the addition of 0.1 M NaOH. The phosphatase activity in 5 µl of each sample was measured at 410 nm. (b) Presence of HTm4 linearly increases KAP phosphatase activity. KAP phosphatase activity was assessed in the presence of increasing amounts of HTm4 using p-Nitrophenyl Phosphate (pNPP) as the KAP substrate. The reaction velocity was measured in the presence of 0.0025 to 20 µM HTm4. All reaction mixtures contained 40 µM KAP and 4 µM active cdk2/cyclin A. Reactions were repeated in triplicate. Their average values and standard deviations are shown. (c) Lineweaver-Burk Plot. KAP phosphatase activity was assessed using the Ser/Thr Assay Kit 1 (Upstate) with different concentrations of active cdk2/cyclin A (from 4 to 20 µM) as the substrate in the presence or absence of 20 µM and 40 µM HTm4. V_{max} and K_{m} for the samples in the presence and absence of HTm4 are 17.5, 1.30 nM/min, and 8.33, 2.68 µM, respectively. (d) Dixon Plot. KAP phosphatase activity was assessed using the Ser/Thr Assay Kit 1 (Upstate) with different concentrations of active cdk2/cyclin A (from 4 to 20 µM) as the substrate in the presence or absence of 20 µM and 40 µM HTm4. Kinetic data was further analyzed by Dixon Plot analysis. From the equation shown, we conclude that HTm4 binds more readily to KAP/cdk2 than to KAP alone, since the crossing point between the two lines (in the presence and absence of HTm4) is located in the third quadrant (-x, -y). (e) Effects of HTm4 on KAP phosphatase activity towards pNPP substrate. Activities of either wild-type or C140S inactive mutant KAP were examined in the different reaction mixtures, with or without 20 µM HTm4 (its C-terminal peptides/non-related peptides (NRP) at 0.1 mM), or with 4 µM active
cdk2/cyclin A (cdk2 monomer). (f) Effects of HTm4 on KAP phosphatase activity with phosphorylated cdk2 or cdk2-derived peptide as its substrate. Phosphorylated threonine 160 (pT160) from cdk2/cyclin A, active, and phosphopeptide (K-R-pT-I-R-R) (Upstate) were used as substrates to assess KAP activity. Assays were performed using the Ser/Thr Phosphatase Assay Kit 1 (Upstate). Concentrations of KAP and HTm4 were both 20 µM, and concentrations of p- cdk2 and peptide (K-R-pT-I-R-R) were 10 and 25 µM, respectively. Reactions were made in the pNPP Ser/Thr Assay Buffer, which contains 50 mM Tris-HCl (pH 7.0), and 100 µM CaCl2. The reaction was allowed to proceed for 30 min at 30 °C, and absorbance was measured at 650 nm.

Figure 4. Plot of KAP CD spectra Change. Spectra Measurements were recorded from 250 nm to 320 nm. The spectra change of KAP was measured by comparing two different KAP samples in the same solution (20 mM Tris-HCl, pH 7.2, 0.15 M NaCl). Sample A contains 5 µM of KAP and an excess amount of HTm4 C-peptide (50 µM). Sample B was formulated as Sample A, with the exception of the HTm4 C-peptide. The difference in spectra was further converted to parameter [Mean Residue Ellipticity] and plotted.

Figure 5. Effects of formation of the cdk2/cyclin A complex in the presence or absence of HTm4. (a) In the absence of HTm4. Ten µl of 10 µM cdk2/cyclin A (Upstate) and 10 µM of His-tagged KAP were mixed, and the same amount of 10 µM of HTm4 (left), 1 µM of HTm4 (middle), or its buffer (right) were added to the mixture. After that 5 µl of Ni-Magnet beads (Toyobo) were added. Bound fractions were electrophoresed by SDS-PAGE and then detected by Western blotting with anti-cyclin A antibody (Santa Cruz Biotechnology) as the primary antibody, and alkaline phosphatase-conjugated anti-rabbit IgG (EY Lab) was used as the secondary antibody. The band of cyclin A is indicated with an arrow. (b) HTm4 released cyclin A. Ten µl of 10 µM cdk2/cyclin A (Upstate) and 10 µl of 10 µM of His-tagged KAP was mixed in 20 mM Tris-HCl (pH 7.5) buffer containing 0.15 M NaCl, 1 mM 2-mercaptoethanol and 0.1 % Tween 20. After incubation at room temperature for 5 min, 10 µl of 10 µM of HTm4 (without His-tag), or its buffer were added to the mixture, and allowed to incubate at room temperature for 5 min. 5 µl of Ni-Magnet beads (Toyobo) were added to each mixture. The bound fractions through His-tagged KAP were washed with the Tris buffer and boiled for SDS-PAGE. The gel was then silver stained (ATTO). Bands of cyclin A, cdk2 and KAP are indicated with arrows.

Figure 6. KAP, cdk2 and cyclin A Complex Model. (a) KAP, cdk2 and cyclin A interaction. Coordinates from cdk2/cyclin A and cdk2/KAP from the PDB have been structurally aligned. A CPK trace has been labeled. Both phosphorylated Thr160 residues of the T-loops are shown on the CPK model. The red T-loop is from cdk2/KAP, and the blue T-loop is from cdk2/cyclin A. Extension of the blue T-loop is inhibited by an ionic interaction between Arg-157 of the T-loop of cdk2 and cyclin A Glu-268, as shown in Fig. 7a. (b) Stereo view of model complex cdk2 (Yellow)/KAP (Blue) and cyclin A (Pink). This view shows the reverse side of Fig. 7c, in a CPK model. The green molecule is Thr160, and phosphorus is labeled with red. The cyclin A molecule is viewed from the back of the cdk2/KAP complex.

Figure 7. Van der Waals intereaction in KAP, cdk2 and cyclin A. (a) Van der Waals interaction between T-loop of cdk2 (red) and Cyclin A (green) in cdk2/Cyclin A complex. (b) The second interaction (between Phe152-Ile182) is completely abolished by moving the T-loop.

Figure 8. KAP, cdk2 and cyclin A complex models. (a) Existence of ionic interaction. Existence of ionic interaction of between Arg157 of the T-loop of cdk2 and cyclin A Glu268 in cdk2/cyclin A
complex, but not in the cdk2/KAP complex. The calculated distances are based on coordinates of cdk2/cyclin A in PDB and our model in cdk2/KAP. The numbers on the top of our alignment are from the amino acid sequence of cdk2. Pro and phosphorylation site of Thr are colored with pink and yellow. Conserved Arg157 is indicated with an arrow (c) Stereo view of the T-loop side in cdk2/CyclinA (1JST). Pro155 of cdk2 (carbon: green, oxygen: red, nitrogen: dark blue) is seen in the deep concave. The pro residue seems not to be accessible by an isomerase. Right cyan is cdk2 and left white molecule is cyclin A. (d) This view is a reverse side of Fig. 6a (KAP, cdk2 active site) and is also shown by CPK model. Thr160 of cdk2 (Yellow) and cyclin A (pink) are seen on the back side of the cdk2/KAP complex (KAP: blue; cdk2: white). We can stereographically see a broad concave that HTm4 C-terminal domain would bind. Tyrosines residues 87 (dark blue) and 141 (green) of KAP represents the candidate conformational change sites after the HTm4-C terminal binding shown by CD difference spectrum (Fig. 4).

Figure 9. Proposed mechanism of action for HTm4. Cdk2 is activated by cyclin A binding and Thr160 phosphorylation. We propose that the active form of cdk2 binds KAP and HTm4, and is hence tethered to the nuclear membrane. The active cdk2/cyclin A will not be inactivated by KAP dephosphorylation at Thr160 until cyclin A is dissociated by HTm4. Thus HTm4 plays a key role in cell cycle regulation via the HTm4-KAP-cdk2 cascade.

Table 1. Angles between Val154 and Pro155 in the T-loop of cdk2 from the PDB. Coordinates from PDB of cdk2 with or without cyclin A or KAP were downloaded from PDB and angles were measured.

<table>
<thead>
<tr>
<th>PDB ID</th>
<th>(degree)</th>
<th>TL</th>
<th>Cyclin A/KAP</th>
</tr>
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<tbody>
<tr>
<td>1JSU</td>
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<td>cis</td>
<td>S (+)/(-)</td>
</tr>
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<td>S (+)/(-)</td>
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</tr>
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<td>E (-)/(+).</td>
</tr>
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<tr>
<td>1PW2</td>
<td>177.23</td>
<td>trans</td>
<td>E (-)/(-)</td>
</tr>
</tbody>
</table>

(TL: T-loop, E: extended, S: shortened)
Figure 1.
Figure 2.
Figure 3.

(a) Phosphatase Activity for pNPP ($\Delta A_{410nm}$) over time (Min).

(b) Phosphatase Activity (A410nm) vs. HTm4 (mM).

(c) Phosphatase Activity ($\Delta A_{650nm}$) with and without HTm4.

(d) $1/V$ vs. $1/S$ graph showing crossing point.

(e) Phosphatase Activity for pNPP ($\Delta A_{410nm}$) for various conditions.

(f) Phosphatase Activity for p-CDK2/p-T peptide ($\Delta A_{650nm}$) for different combinations of CDK2, Cyclin A, and HTm4.
Figure 4.

![Graph showing mean residue ellipticity vs wavelength (nm). The x-axis represents wavelength from 250 to 320 nm, and the y-axis represents mean residue ellipticity in degrees cm² dmol⁻¹. The data points are scattered, indicating a trend in ellipticity with respect to wavelength.]
Figure 5.

(a) HTm4 (µM) 10 1 0

100 kD
60 kD
20 kD

Anti-Cyclin A

(b) HTm4 (µM) M 10 0

75 kD
37 kD
25 kD

Cyclin A
CDK2
KAP
HTm4
Figure 6.
Figure 7.
Figure 8.

a

b

Cdk2  AIKLADFGGLARAFGVPTYTHEVVTLwyapeill
Cdk3  AIKLADFGGLARAFGVPLRTYTHEVVTLwyapeill
Cdk5  ELKLADFGGLARAFGVRCYSAEVTLwyRPPDVLF
Cdk4  TVKLADFGGLARISYQMalTPVVTLwyAPEVLL
Cdk6  QIKLADFGGLARISFQMaTSVVTLwyAPEVLL

Cdk2  AIKLADFGGLARAFGVPTYTHEVVTLwyapeill
Cdk3  AIKLADFGGLARAFGVPLRTYTHEVVTLwyapeill
Cdk5  ELKLADFGGLARAFGVRCYSAEVTLwyRPPDVLF
Cdk4  TVKLADFGGLARISYQMalTPVVTLwyAPEVLL
Cdk6  QIKLADFGGLARISFQMaTSVVTLwyAPEVLL

Cdk2  AIKLADFGGLARAFGVPTYTHEVVTLwyapeill
Cdk3  AIKLADFGGLARAFGVPLRTYTHEVVTLwyapeill
Cdk5  ELKLADFGGLARAFGVRCYSAEVTLwyRPPDVLF
Cdk4  TVKLADFGGLARISYQMalTPVVTLwyAPEVLL
Cdk6  QIKLADFGGLARISFQMaTSVVTLwyAPEVLL

Cdk2  AIKLADFGGLARAFGVPTYTHEVVTLwyapeill
Cdk3  AIKLADFGGLARAFGVPLRTYTHEVVTLwyapeill
Cdk5  ELKLADFGGLARAFGVRCYSAEVTLwyRPPDVLF
Cdk4  TVKLADFGGLARISYQMalTPVVTLwyAPEVLL
Cdk6  QIKLADFGGLARISFQMaTSVVTLwyAPEVLL

Cdk2  AIKLADFGGLARAFGVPTYTHEVVTLwyapeill
Cdk3  AIKLADFGGLARAFGVPLRTYTHEVVTLwyapeill
Cdk5  ELKLADFGGLARAFGVRCYSAEVTLwyRPPDVLF
Cdk4  TVKLADFGGLARISYQMalTPVVTLwyAPEVLL
Cdk6  QIKLADFGGLARISFQMaTSVVTLwyAPEVLL
Figure 9.
Binding of HTm4 to KAP/CDK2/Cyclin A complex enhances the phosphatase activity of KAP, dissociates cyclin A, and facilitates KAP dephosphorylation of CDK2
Masanobu Chinami, Yoshihiko Yano, Xing Yang, Saira Salahuddin, Helen Turner, Taro Shirakawa, David Barford and Chaker N. Adra

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