Protein Kinase CK2 Is Inhibited by Human Nucleolar Phosphoprotein p140 in an Inositol Hexakisphosphate-dependent Manner*

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Protein kinase CK2 is a ubiquitous protein kinase that can phosphorylate various proteins involved in central cellular processes, such as signal transduction, cell division, and proliferation. We have shown that the human nucleolar phosphoprotein p140 (hNopp140) is able to regulate the catalytic activity of CK2. Unphosphorylated hNopp140 and phospho-hNopp140 bind to the regulatory and catalytic subunits of CK2, respectively, and the interaction between hNopp140 and CK2 was prevented by inositol hexakisphosphate (InsP6). Phosphorylation of α-casein, genimin, or human phosphatidylcholine transfer protein-like protein by CK2 was inhibited by hNopp140, and InsP6 recovered the suppressed activity of CK2 by hNopp140. These observations indicated that hNopp140 serves as a negative regulator of CK2 and that InsP6 stimulates the activity of CK2 by blocking the interaction between hNopp140 and CK2.

The protein kinase CK2, formerly known as casein kinase II, is a serine/threonine protein kinase that has been found in virtually all tissues and cell lines and is responsible for the phosphorylation of more than 300 cellular proteins (1). The cellular function of CK2 is related to essential processes such as cell cycle, proliferation, or signal transduction since the proteins phosphorylated by CK2 are involved in DNA replication and transcription, translation, and signal transduction (2–5). The catalytic subunit of CK2 is a serine/threonine protein kinase that has been found in virtually all tissues and cell lines and is responsible for the phosphorylation of more than 300 cellular proteins (1). The cellular function of CK2 is related to essential processes such as cell cycle, proliferation, or signal transduction since the proteins phosphorylated by CK2 are involved in DNA replication and transcription, translation, and signal transduction (2–5). The catalytic subunit of CK2 and suppresses CK2 activity (20). Small molecules that can regulate CK2 activity have also been characterized. Polyanions, such as heparin, inhibit CK2 (21), where as polycations, such as spermine or polylysine, moderately activate the enzyme (22, 23). Recently, highly phosphorylated forms of inositols were shown to regulate the activity of CK2. Inositol hexakisphosphate (InsP6) stimulated the catalytic activity of CK2 in cell extracts or in partially purified fraction of CK2 by 2–3-fold but failed to activate purified recombinant CK2 (24), suggesting that the cell extracts contain an unidentified negative regulator whose inhibitory activity was blocked by InsP6.

Several proteins that could interact with CK2 and affect the activity of the enzyme have been identified. Among these proteins, the FACT (facilitates chromatin transcription) complex (3, 18) and Pin1 (a peptidylprolyl isomerase) (19) were shown to modulate CK2 substrate specificity. In addition, the tumor suppressor adenomatous polyposis coli (APC) (25) binds to the catalytic subunit of CK2 and suppresses CK2 activity (20). Small molecules that can regulate CK2 activity have also been characterized. Polyanions, such as heparin, inhibit CK2 (21), whereas polycations, such as spermine or polylysine, moderately activate the enzyme (22, 23). Recently, highly phosphorylated forms of inositols were shown to regulate the activity of CK2. Inositol hexakisphosphate (InsP6) stimulated the catalytic activity of CK2 in cell extracts or in partially purified fraction of CK2 by 2–3-fold but failed to activate purified recombinant CK2 (24), suggesting that the cell extracts contain an unidentified negative regulator whose inhibitory activity was blocked by InsP6.

One of the proteins that can interact with CK2 is hNopp140, a nucleolar phosphoprotein (25). It can be highly phosphorylated by CK2, and the cellular function of hNopp140 is related to both the biogenesis of the nucleolus and the cell cycle (26). In this study we investigated the interaction between hNopp140 and CK2 and demonstrated that hNopp140 could negatively regulate the catalytic activity of CK2. Furthermore, we showed that InsP6 abolished the negative regulation of CK2 by...
hNopp140. These findings provide insight into the roles of hNopp140 and phosphoinositides in CK2 regulation.

**EXPERIMENTAL PROCEDURES**

**Materials**—Protein kinase CK2 was purchased from New England Biolabs (Ipswich, MA). d-myo-inositol 1,2,3,4,5,6-hexakisphosphate (InsP₆), d-myo-inositol 1,2,3,4,5,6-hexakisphosphate (InsP₆), d-myo-inositol 1,2,3,4,5,6-hexakisphosphate (InsP₆), and d-myo-inositol 1,2,3,4,5,6-hexakisphosphate (InsP₆) were kindly provided by Prof. Young-Seuk Bae (Kyungpook National University). The amplified DNA fragments—encoding human CK2α and CK2β subunits—were amplified by PCR from the pT7-CK2α and pT7-CK2β plasmids, respectively, which were kindly provided by Prof. Young-Seuk Bae (Kyungpook National University). The amplified DNA fragments were inserted into the cloning sites of the pGEX-4T1 vector, producing GST-CK2α and GST-CK2β, in which CK2α or CK2β was fused at the C terminus of GST. The plasmids were then transformed into E. coli DH5α, and the expression of GST-CK2α or GST-CK2β was induced using 0.5 mM isopropyl-

**Plasmid Construction and Protein Purification**—Recombinant hNopp140 was expressed in Escherichia coli BL21(DE3) and purified as previously described (25). The DNA fragments encoding human CK2α and CK2β subunits were amplified by PCR from the pET21a or the HindIII and XhoI cloning sites of the pET28a (Novagen) vector, producing GST-CK2α and GST-CK2β. The expressions of recombinant GST-CK2α and anti-CK2α antibodies were obtained from Santa Cruz Biotechnology. All other consumables were of reagent grade.

**Protein Kinase CK2 Is Inhibited by hNopp140**

**RESULTS**

hNopp140 Inhibits the Catalytic Activity of CK2—the effect of hNopp140 on the catalytic activity of CK2 was examined in vitro using α-casein as a substrate. Phosphorylation of α-casein by the CK2 holoenzyme was significantly reduced as the concentration of unphosphorylated hNopp140 increased (Fig. 1A). The activity of CK2 decreased to 40% of the control level in the presence of an equimolar ratio of hNopp140 and further decreased to 20% of control in the presence of 0.7 μM hNopp140 (Fig. 1C). Because hNopp140 is a substrate of CK2 (26), the inhibitory activity of phospho-hNopp140 was examined to eliminate the effects of competitive inhibition that might be caused by the presence of unphosphorylated

GST Pulldown Assay—Unphosphorylated hNopp140 or phospho-hNopp140 (2 μg) were mixed with 1 μg GST-CK2α and/or GST-CK2β in PBST (PBS containing 0.1% Tween 20) at 4 °C for 2 h, then further incubated with 20 μl of GST-agarose beads (50% suspension) (Peptron, Korea). After washing the beads 5 times with PBST, the proteins bound to the resin were eluted by adding 2 × SDS-PAGE sample buffer and separated by SDS-PAGE. The proteins on the gel were transferred onto a polyvinylidene difluoride membrane. The membrane was blocked with 5% nonfat dried milk for 1 h, reacted with 0.2 μg/ml mouse anti-hNopp140 antibodies in PBST for 1 h, and then incubated with 0.1 μg/ml sheep anti-mouse IgG conjugated to horseradish peroxidase for 1 h. After successive washes with PBST, immunoblots were visualized using an enhanced chemiluminescence system (Pierce) as described in the manufacturer’s instruction manual.

**Cell Culture and Transfection**—The cDNA encoding hNopp140, flanked by BamHI and SalI restriction sites, was cloned into the mammalian expression vector pCMV-Tag3B (Strategene, La Jolla, CA) to generate pCMV-hNopp140, which expressed a full-length hNopp140 with a Myc tag at the N terminus. For transformation, 293T cells were grown in 10-cm culture dishes and maintained in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum. Sub-confluent cells were transiently transfected with pCMV-hNopp140 DNA (4 μg/dish) mixed with the Lipofectamine PLUS reagent (Invitrogen), according to the manufacturer’s protocol. Cells were harvested ~48 h after transfection.

**Immunoprecipitation**—Harvested cells were lysed in lysis buffer (50 mM Tris-HCl, 150 mM NaCl, 1% Nonidet P-40, 1 mM phenylmethylsulfonyl fluoride, and 1% protease inhibitor mixture, pH 7.4) for 2 h at 4 °C, and cell debris was removed by centrifugation at 10,000 × g for 30 min. The cell lysate was incubated with 1 μg of anti-hNopp140 antibodies in lysis buffer at 4 °C for 2 h. After incubation with protein G-Plus agarose (Santa Cruz Biotechnology) for 2 h, the mixture was centrifuged for 10 s, and the supernatant was removed. After washing the agarose beads five times with lysis buffer, the proteins bound to the beads were eluted by adding SDS-PAGE sample buffer and separated by SDS-PAGE. Proteins in the gel were transferred onto a polyvinylidene difluoride membrane and visualized with specific antibodies using an ECL-chemiluminescence method (Pierce) as described in the manufacturer’s protocol.

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As shown in Fig. 1, B and C, phospho-hNopp140 is as effective in inhibiting the activity of CK2 as unphosphorylated hNopp140. The inhibitory activity of hNopp140 on CK2 was also tested using geminin, a regulator of eukaryotic DNA replication (27) and a substrate of CK2 (28). As shown in Fig. 1 D, recombinant geminin was effectively phosphorylated by CK2, and the CK2-dependent phosphorylation was significantly reduced in the presence of hNopp140 (Fig. 1 D), although the inhibitory effect of hNopp140 was weaker than those with α-casein as substrate.

Specific Interaction between hNopp140 and CK2 Is Modulated by Phosphorylation—To dissect the interaction between hNopp140 and CK2, binding of the catalytic and regulatory subunits of CK2, GST-CK2α or GST-CK2β, respectively, to phospho-hNopp140 or unphosphorylated hNopp140 was examined. When GST-CK2α was incubated with hNopp140 and precipitated with glutathione beads, only phospho-hNopp140 co-precipitated with CK2α. In contrast, GST-CK2β was not able to bind phospho-hNopp140 but did bind to unphosphorylated hNopp140 (Fig. 2). These results indicate that hNopp140 binding to the different subunits of CK2 depended on its phosphorylation state; phospho-hNopp140 binds to CK2α, and unphosphorylated hNopp140 binds to CK2β.

Because hNopp140 is highly phosphorylated in cell extracts (26), hNopp140 would normally bind to the catalytic subunit of CK2 and repress its catalytic activity. To investigate whether hNopp140 could affect the assembly of CK2 subunits, the interaction between hNopp140 and CK2binding of the catalytic and regulatory subunits of CK2, GST-CK2α or GST-CK2β, respectively, to phospho-hNopp140 or unphosphorylated hNopp140 was examined. When GST-CK2α was incubated with hNopp140 and precipitated with glutathione beads, only phospho-hNopp140 co-precipitated with CK2α. In contrast, GST-CK2β was not able to bind phospho-hNopp140 but did bind to unphosphorylated hNopp140 (Fig. 2). These results indicate that hNopp140 binding to the different subunits of CK2 depended on its phosphorylation state; phospho-hNopp140 binds to CK2α, and unphosphorylated hNopp140 binds to CK2β.

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Thus, the inhibition of CK2 by hNopp140 might be due to reduced accessibility of substrate proteins to the active site of CK2 or induction a conformational change to a less active state.

The Repressed Activity of CK2 by hNopp140 Is Stimulated by InsP₆—Previously, CK2-dependent phosphorylation of PCTP-like protein was shown to be repressed by an unidentified factor(s), and the repressed CK2 activity was stimulated in the presence of inositol polyphosphates such as InsP₆ (24). To test whether inositol polyphosphates could stimulate the repressed activity of CK2 by hNopp140, the effect of various inositol phosphates on the CK2-dependent phosphorylation of geminin by hNopp140 was examined by measuring the amount of hNopp140 bound to the CK2 subunits, and the extracted proteins were separated by SDS-PAGE and analyzed by immunoblotting (WB) with an anti-hNopp140 antibody. However, the stimulatory effect of various inositol phosphates were not clearly observed due to the marginal difference of the phosphorylation level in the presence or absence of 25 μM InsP₆. In contrast, InsP₆ significantly increased the reduced CK2-dependent phosphorylation of geminin by hNopp140 (Fig. 4C). Likewise, the reduced CK2-dependent phosphorylation of geminin by hNopp140 was also stimulated by InsP₆ (Fig. 4D). However, the relative stimulatory effects of various inositol phosphates were not clearly observed due to the marginal difference of the phosphorylation level in the presence or absence of hNopp140 (data not shown). These results indicated that InsP₆ regulates CK2 by stimulating the repressed activity of CK2 by hNopp140, and the InsP₆-dependent regulatory property of hNopp140 was well matched to the unidentified cellular factor that regulated CK2 in InsP₆-dependent manner (24).

InsP₆ Inhibits the Interaction between hNopp140 and CK2—The binding affinity of hNopp140 to CK2 and the InsP₆-dependent stimulation of hNopp140-repressed CK2 activity suggested that InsP₆ might interfere the interaction between hNopp140 and CK2. The effect of InsP₆ on this interaction was examined by measuring the amount of hNopp140 bound to GST-CK2α or GST-CK2β in the presence or absence of InsP₆ using a GST pulldown assay. Phospho-hNopp140 did not coprecipitate with GST-CK2α when the proteins were incubated in the presence of 25 μM InsP₆ (Fig. 5A). In contrast, the binding...
of unphosphorylated hNopp140 to GST-CK2β was only slightly reduced by InsP₆. We further tested the effect of InsP₆ on the interaction between hNopp140 and CK2 in cell extracts by immunoprecipitation using anti-hNopp140 antibodies and immunoblotting using anti-CK2α antibodies or anti-hNopp140 antibodies. When proteins in the cell extract were precipitated with anti-hNopp140 antibodies in the absence or presence of InsP₆, equal amounts of hNopp140 were observed (Fig. 5B, lower panel). However, the amount of CK2α co-precipitating with hNopp140 decreased to 20–30% of the control in the presence of 25 μM InsP₆ (Fig. 5B, upper panel). These results indicate that InsP₆ stimulated the activity of CK2 by blocking the interaction between hNopp140 and CK2α.

**DISCUSSION**

In this study we have shown that hNopp140 can bind to and negatively regulate CK2. Moreover, InsP₆ was shown to stimulate hNopp140 repressed CK2 by blocking the interaction between hNopp140 and CK2. Previously, Li et al. (29) showed that mouse Nopp140 in extracts of 3T3 cells bound primarily to the regulatory subunit of CK2 and that a CK2α interacted only marginally with hNopp140. In the current study hNopp140 interacted with both subunits of CK2 depending on the phosphorylation state of hNopp140. CK2α interacted preferentially with the highly phosphorylated form of hNopp140 and CK2β with the unphosphorylated form. The weak binding affinity between mouse Nopp140 and CK2α in 3T3 cell extracts could be due to a low level of phosphorylation of mouse Nopp140. Alternatively, mouse and human Nopp140 might have different binding affinities for CK2 subunits depending on their level of phosphorylation. Binding of hNopp140 to CK2 significantly reduced the catalytic activity of CK2 and the degree of hNopp140 inhibition of CK2 depended on the substrate. Phosphorylation of PCTP-like protein or α-casein by CK2 was effectively inhibited by hNopp140 (Fig. 1), suggesting that the binding of hNopp140 may restrict the substrate proteins to access to the active site of CK2. On the other hand, it was reported that the phosphorylation of the acidic peptide DSD (amino acid sequence RRRDDDDDDD) by CK2 was only marginally affected by hNopp140 (29). The reduced inhibitory effect of hNopp140 on CK2-dependent phosphorylation of peptide substrate may be due to the highly flexible conformation, which could be more easily accessible to the active site. Thus, hNopp140 appears to restrict the access of a certain set of substrate proteins such as α-casein or PCTP-like protein (24) to the CK2 active site but fails to affect the accessibility of small or flexible substrates such as the DSD peptide.

The interaction between hNopp140 and CK2 was modulated by InsP₆. Inositol polyphosphates such as InsP₆ are found in mammalian cells in micromolar concentrations (30, 31). Although the biological functions of inositol polyphosphates are poorly understood, they are implicated in several aspects of cell regulation such as vesicle trafficking (32), DNA repair (33), and chromatin remodeling (34). Furthermore, the cellular concentration of InsP₆ fluctuates at least 3-fold during the cell cycle (35), implying that InsP₆ has an important role in cell cycle progression. When the cellular levels of InsP₆ increase during cell division, CK2 may dissociate from hNopp140 and become fully activated. The increased level of InsP₆ in mitotic cells (36) and the high level of CK2 activity in proliferating cells support the coordinated regulation of CK2 by InsP₆ and hNopp140 during cell growth.

Tobin and co-workers (24) demonstrated the presence of an InsP₆-dependent negative regulator of CK2. In their investigation, InsP₆ only slightly affected the catalytic activity of purified CK2 but stimulated CK2 activity 2–3-fold when the enzyme had been repressed by unidentified heat stable cellular factor(s) (24). These properties of the unknown negative regulator are well in agreement with those of hNopp140, including its high thermostability (25). Our data suggest that CK2 forms a complex with hNopp140 at low concentrations of InsP₆ and the activity of hNopp140-bound CK2 is reduced 4–5-fold compared with that of free CK2. Even in this repressed state, however, flexible substrates or small peptides can be efficiently phosphorylated by CK2. This state of CK2 may be responsible for the basal and constitutive activities observed in resting cells. We also examined whether hNopp140 could negatively regulate the CK2-dependent phosphorylation of proteins other than PCTP-like protein using geminin, a regulator of eukaryotic DNA replication (27) and a substrate of CK2 (28). Unlike PCTP-like protein, the phosphorylation of geminin by CK2 was only slightly inhibited by hNopp140 (Fig. 1), although the slightly reduced phosphorylation was alleviated by InsP₆ (Fig. 4), implying that the degree of repressed CK2 activity by hNopp140 depends on the nature of repressed substrate proteins. It should be noted that inositol pyrophosphates such as InsP₇ could specifically phosphorylate hNopp140 in a non-enzymatic way (37). This property of hNopp140 along with the stimulatory effect of InsP₆ on the activity of CK2 repressed by hNopp140 suggested a coordinated regulation mechanism of inositol polyphosphates on the cellular function of hNopp140.

Recently, the APC protein was identified as a negative regulator of CK2. APC binds CK2 and inhibits the catalytic activity of CK2 (20). Co-transfection experiments with fragments of APC and CK2 revealed that a small region (amino acids 2086–2394) at the C terminus of APC, which consists of 2843 amino acids, strongly inhibited CK2. Although the CK2 regulatory regions of APC and hNopp140 are both negative regulators of CK2, they showed no apparent sequence homology (data not shown). It is noticeable, however, that both hNopp140 and the CK2 regulatory region of APC have a high percentage of lysine and serine residues, ~15–17%. This characteristic may underlie their binding to CK2. However, the APC fragment effectively inhibited the phosphorylation of peptide substrates, suggesting that APC regulation of CK2 differs from that of hNopp140.

In summary, we have identified hNopp140 as an InsP₆-dependent negative regulator of CK2. The coordinated regulation of CK2 by InsP₆ and hNopp140 could control the range of CK2 activity from basal to highly activated level. This regulatory mechanism controls phosphorylation of a subgroup of CK2 substrates rather than all possible CK2 substrates. Identification of CK2 substrates whose phosphorylation is regulated by hNopp140 will elucidate the significance of hNopp140 on its regulation of CK2.
REFERENCES
