THE JOURNAL OF BIOLOGICAL CHEMISTRY
Vol. 272, No. 6, Issue of February 7, pp. 3773–3779, 1997

Printed in U.S.A.

Specific Interaction between Casein Kinase 2 and the Nucleolar Protein Nopp140*

(Received for publication, October 16, 1996)

Dongxia Li‡, U. Thomas Meier§, Grazyna Dobrowolska‡, and Edwin G. Krebs‡¶

From the ‡Department of Pharmacology, University of Washington, Seattle, Washington 98195 and the §Department of Anatomy and Structural Biology, Albert Einstein College of Medicine, Bronx, New York 10461

Casein kinase 2 (CK2) is a multifunctional second messenger-independent protein serine/threonine kinase that phosphorylates many different proteins. To understand the function and regulation of this enzyme, biochemical methods were used to search for CK2-interacting proteins. Using immobilized glutathione *S*-transferase fusion proteins of CK2, the nucleolar protein Nopp140 was identified as a CK2-associated protein. It was found that Nopp140 binds primarily to the CK2 regulatory subunit, β. The possible *in vivo* association of Nopp140 with CK2 was also suggested from a coimmunoprecipitation experiment in which Nopp140 was detected in immunoprecipitates of CK2 prepared from cell extracts. Further studies using an overlay technique with radiolabeled CK2 as a probe revealed a direct CK2-Nopp140 interaction. Using deletion mutants of CK2β subunits, the binding region of the CK2β subunit to Nopp140 has been mapped. It was found that the NH_2-terminal 20 amino acids of CK2β are involved. Since Nopp140 has been identified as a nuclear localization sequence-binding protein and has been shown to shuttle between the cytoplasm and the nucleus, the finding of a CK2-Nopp140 interaction could shed light on our understanding of the function and regulation of CK2 and Nopp140.

Protein phosphorylation is known to be a very important means of cellular regulation, and in recent years much information about protein kinases and phosphatases, especially those involved in the mitogen-activated protein kinase pathway, has been obtained (for review, see Refs. 1–3). Little is known, however, about the function and regulation of one particular protein kinase, casein kinase 2 (CK2),[1] although increasing data suggest that it may be an important mediator in mitogenic signal transduction (for review, see Ref. 4).

CK2 is a multifunctional, second messenger-independent eukaryotic protein serine/threonine kinase present in the nucleus and cytoplasm of all eukaryotic cells. The holoenzyme form is generally composed of catalytic subunits α, α′, and a regulatory subunit β combined so as to form $\alpha_2\beta_2$, $\alpha\alpha'\beta_2$, or $\alpha'_2\beta_2$ heterotetramers. The α and α′ subunits are catalytically active, whereas the β subunit is inactive but can stimulate the catalytic activity of α and α′ under certain circumstances. Furthermore, the β subunit stabilizes the α and α′ subunits and can facilitate substrate recognition (4). CK2 is widely expressed, and its sequence is highly conserved throughout evolution, indicating that the enzyme may have a critical role in cell function. In fact, it has been shown in *Saccharomyces cerevisiae* that the simultaneous disruption of genes encoding CK2 α and α′ subunits is lethal (5). CK2 phosphorylates a large number of proteins including enzymes involved in nucleic acid synthesis, transcription and protein synthesis factors, structural proteins, and signal transduction proteins, suggesting a global role in the regulation of cellular processes (6). Several recent studies demonstrate that CK2 is important for cell growth and division. First, it has been shown that CK2 activity is required for progression of the cell cycle (7, 8) and that enzyme levels are elevated in many rapidly proliferating cells and tumor cells (4). Second, CK2 reportedly associates with several growth-related proteins including p53 (9), c-Raf (10), and c-Mos.[2] Thus it may modulate cellular responses to growth factor stimulation.[2] Most strikingly, overexpression of CK2α in transgenic mice caused a high predisposition for lymphoma development, and coexpression of the CK2α transgene with c-*myc* resulted in the rapid development of murine perinatal leukemia associated with disruption of lymphoid cell functions (12).

In spite of the above mentioned studies, it is still not entirely clear exactly what physiological roles CK2 may have and how the activity of this enzyme is regulated *in vivo*. CK2 has a vast number of the potential substrates. It is generally considered constitutively active (13) and is not known to respond to any second messenger molecules. These features makes it difficult to study the function and regulation of this enzyme (for review, see Refs. 4, 6, 14, and 15). Therefore, efforts have been made to search for proteins that interact with CK2 for a greater understanding of its role. For example, the nucleolar protein nucleolin was identified as a CK2-associating protein in previous work (16–18).

In this report, we provide evidence that CK2 and the nucleolar protein Nopp140 associate as a molecular complex *in vitro* and probably *in vivo*. These studies were performed using immobilized GST fusion proteins of CK2 subunits and a ^{32}P-radiolabeled CK2 overlay technique and by coimmunoprecipitation of the two proteins from cell extracts. The region of the β subunit of CK2 which binds to Nopp140 was also mapped. The possible roles of CK2 in the regulation of Nopp140 as well as in rRNA synthesis and ribosomal protein transport are supported by the results of this study.

EXPERIMENTAL PROCEDURES

Materials—Nucleotide oligomers used as polymerase chain reaction primers were synthesized by Drs. Y. F. Lee and P. S. H. Chou (Biopolymer Facility, Department of Immunology, University of Washington) and by Integrated DNA Technologies, Inc. BL-21 (DE3) pLysS compe-

* This work was supported by National Institutes of Health Grants DK42528 (to E. G. K.) and GM50725 (to U. T. M). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

¶ To whom correspondence should be addressed. Tel.: 206-543-8500; Fax: 206-543-0858.

[1] The abbreviations used are: CK2, casein kinase 2; GST, glutathione *S*-transferase; PAGE, polyacrylamide gel electrophoresis; DSD, CK2 peptide substrate RRRDDDSDDD.

[2] M. Chen, D. Li, E. G. Krebs, and J. A. Cooper, submitted for publication.

tent cells were purchased from Novogen. [γ-^{32}P]ATP was obtained from Amersham. The PD-10 gel filtration column and glutathione-Sepharose 4B beads were purchased from Pharmacia Biotech Inc. All other chemical reagents were purchased from Sigma.

Recombinant CK2 and Nopp140—Recombinant CK2 subunits α and α' and holoenzymes $\alpha_2\beta_2$ and $\alpha'_2\beta_2$ were expressed and purified from baculovirus-infected Sf-9 cells as described elsewhere.[3] Recombinant Nopp140 was expressed in *Escherichia coli* BL-21 (DE3) cells transformed with pET8c/Nopp140 (Nopp140 bacterial expression vector). Since the overexpressed Nopp140 protein was predominantly insoluble and segregated into inclusion bodies, it was denatured and renatured by solubilization in 6 M urea followed by extensive dilution and then purified using a hydroxylapatite column (19).

Antibodies—Polyclonal antibodies of Nopp140 were raised in rabbits against a synthetic peptide of Nopp140 (20). Polyclonal antibodies against CK2 subunits α, α', and β were prepared in this laboratory (21).

Cell Culture and Preparation of Cell Lysates—3T3 L1 cells were grown in Dulbecco's modified Eagle's medium containing 10% fetal calf serum until confluence. The cells were washed by phosphate-buffered saline, harvested in lysis buffer (50 mM Tris-Cl, pH 7.5, 50 mM NaF, 0.25 M NaCl, 0.1% Triton X-100, 0.25 M sucrose, 2 mM EDTA, 10 μg/ml leupeptin, 2 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride), and sonicated twice for 10 s. After centrifugation for 30 min at 13,000 rpm, the supernatants were used as cell lysates.

Construction of CK2 Deletion Mutants—Deletion mutants of GST-CK2β were constructed by truncating the NH_2-terminal (Δ1–20, Δ1–40, Δ1–80, Δ1–120, Δ1–141) and COOH-terminal (Δ161–215) amino acids of CK2β to give mutants GST-β_{21-215}, GST-β_{41-215}, GST-β_{81-215}, GST-$\beta_{121-215}$, GST-$\beta_{142-215}$, and GST-β_{1-160}. The cDNA for each deletion mutant was obtained by polymerase chain reaction amplification of human CK2β in Bluescript KS plasmid (22). For NH_2-terminal deletion mutations, the sense polymerase chain reaction primers were: 5′-GCGGATCCTTCTTCTGTGAAGTGGATG-3′ (GST-β_{21-215}); 5′-GCGGATCCGAGCAGGTCCCTCACTATC-3′ (GST-β_{41-215}); 5′-GCGGATCCGGATTGATCCACGCCCGCT-3′ (GST-β_{81-215}); 5′-GCGGATCCCCCATTGGCCTTTCAGACA-3′ (GST-$\beta_{121-215}$); and 5′-GCGGATCCGATGTGTACACACCCAAGT-3′ (GST-$\beta_{142-215}$). The T7 24-mer primer was used as the antisense primers for all of the NH_2-terminal deletion mutants of CK2β. For the COOH-terminal deletion mutant, GST-β_{1-160}, the sense primer was 5′-GCGGATCCAGCAGCTCAGAGGAGGTGT-3′, and the antisense primer was 5′-GCGGATCCTCAGCCGAAGTAGGCGCCATCC-3′. After polymerase chain reaction, the DNA fragments were purified, digested with *Bam*HI, and ligated into pGEX-2T vector (Pharmacia).

Expression of GST-CK2 Fusion Proteins and Their Deletion Mutants—GST-CK2α, GST-CK2α', and GST-CK2β and its deletion mutants were expressed in and purified from *E. coli* and immobilized on glutathione-Sepharose resin (18). The immobilized protein were eluted using an elution buffer containing 10 mM reduced glutathione in 50 mM Tris-HCl, pH 8.0. To remove the GST tag from GST-CK2β, a thrombin cleavage procedure developed by Pharmacia was employed. GST fusion proteins of CK2 holoenzyme $\alpha_2\beta_2$ and $\alpha'_2\beta_2$ were generated by mixing an equal amount of the immobilized GST-CK2α with CK2β, or GST-CK2α' with CK2β.

Binding Assays and Immunoprecipitation of CK2—3T3 L1 cell lysates (200 μl) were incubated at 4 °C either with 35 μl (50%; v/v) of glutathione beads immobilized with GST-CK2 fusion proteins (approximately 2 μg) for 4 h or with protein A-Sepharose conjugated with a mixture of antiserum against each CK2 subunit (18) for 2 h. The beads were then washed four times with an immunoprecipitation buffer (20 mM Tris-Cl, pH 7.5, 150 mM NaCl, 2 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, 1 μg/ml leupeptin, 1 mM dithiothreitol, 0.025% Triton X-100) by centrifugation. Bound proteins were extracted using Laemmli sample buffer and analyzed by SDS-PAGE, followed by immunoblotting with antibodies. Alternatively, the proteins bound to the immobilized CK2 were phosphorylated by GST-CK2 fusion proteins using a procedure described previously (18).

Immunoprecipitation of Nopp140—Approximately 10,000,000 BRL cells were washed twice and scraped into ice-cold phosphate-buffered saline. After pelleting, the cells were lysed in 0.5 ml of lysis buffer (50 mM Tris, pH 7.4; 1% Triton X-100; 0.2% SDS; 150 mM NaCl; 2 mM phenylmethylsulfonyl fluoride; and 1 μg/ml each leupeptin, antipain, chymostatin, and pepstatin A) by tip sonication (five times for 30 s on ice). The cell lysate was clarified for 10 min in a microcentrifuge and incubated with 5 μg of anti-Nopp140 peptide IgGs in the absence and presence of 5 μM free competing peptide for 1 h at room temperature. The antibody-antigen complexes were adsorbed to 5 μl of packed protein A-Sepharose beads (Pharmacia) for an additional 1-h incubation at room temperature. The beads were washed four times with 1 ml of wash buffer (50 mM Tris, pH 7.4, 0.1% Triton X-100, 0.02% SDS, 150 mM NaCl), and the antibody-antigen complexes were eluted with Laemmli sample buffer and analyzed by SDS-PAGE (19).

^{32}P-Labeled CK2 Overlay Method—Recombinant CK2 holoenzyme $\alpha_2\beta_2$ (obtained from Sf-9 cells) was radiolabeled by autophosphorylation and then subjected to a PD-10 column (Pharmacia) to remove [γ-^{32}P]ATP and other inorganic chemicals (18). Bacterially expressed pure Nopp140 was subjected to 8% SDS-PAGE and transferred onto a polyvinylidene difluoride membrane. The membrane was preincubated in Blotto blocking buffer (5% milk in 20 mM phosphate, pH 7.4, 0.15 mM NaCl) overnight and then overlaid with ^{32}P-labeled CK2 probe (in fresh Blotto) for 4 h at room temperature. After extensive washing, the associated CK2 band was detected by autoradiography. Bovine serum albumin (1–2 μg) was also loaded on the same SDS-polyacrylamide gel as a negative control.

Phosphorylation of Nopp140—Each of the recombinant CK2s: CK2α, CK2α', $\alpha_2\beta_2$, and $\alpha'_2\beta_2$ (30–120 ng), was incubated at 30 °C with 0.5 μg of recombinant Nopp140 in 25 μl of phosphorylation buffer (20 mM Tris-Cl, pH 7.5, 20 mM $MgCl_2$, and 0.1 mM [γ-^{32}P]ATP (2,000 cpm/pmol ATP)). After 30 min, the reaction was stopped by adding 8 μl of 4 × sample buffer, and the proteins were resolved by SDS-PAGE. The protein phosphorylation was detected by autoradiography.

CK2 Activity Assay—To test whether CK2-Nopp140 association would affect the catalytic activity of CK2, CK2 activity was assayed by a routine as well as a modified procedure. In the routine procedure, the assay was carried out in the same way as described previously (23, 24) in the presence and absence of Nopp140 in the reaction solution. In the modified procedure, CK2 holoenzyme was preincubated with [γ-^{32}P]ATP in a reaction buffer (50 mM Tris-Cl, pH 7.5, 10 mM $MgCl_2$, 0.1 mM [γ-^{32}P]ATP (2,000 cpm/pmol)), with and without Nopp140 protein (0.075 mg/ml), for 20 min at 30 °C. Then, CK2 substrate peptide RRRDDDSDDD (DSD; final concentration 0.1 mM) was added to the reaction mixture and incubated for another 10 min. Aliquots of reaction mixture was spotted onto P81 paper, washed, and assayed as described previously (23, 24).

To examine the activation of CK2α by deletion mutants of CK2β, CK2 assays were conducted using baculovirus-expressed CK2α (approximately 5 ng for each reaction) and bacterially expressed and purified GST-CK2β mutants. CK2α was premixed with GST-CK2β mutants for 10 min at room temperature prior to the assay to allow proper folding of the holoenzyme. An excess amount of CK2β compared with CK2α was used in this experiment to give maximal stimulation (23, 24).

RESULTS

Identification of a CK2-binding Protein as Nopp140—GST-CK2 holoenzymes were reconstituted *in vitro* by incubating bacterially expressed, immobilized GST-CK2α or GST-CK2α' with CK2β (obtained by thrombin cleavage of GST tag from GST-CK2β) and immobilized on glutathione-Sepharose resin. These forms were then incubated with 3T3 L1 cell lysates for 4 h at 4 °C. After extensive washing, phosphorylation reactions were initiated by adding a buffer containing [γ-^{32}P]ATP and $MgCl_2$ to the beads (18) and stopped using Laemmli sample buffer. The phosphorylated CK2-interacting proteins were analyzed by SDS-PAGE and autoradiography (Fig. 1). Several CK2-associated phosphoproteins were detected from the GST-CK2 resin, which had been incubated with cell lysates (Fig. 1, *lanes 1* and *2*) compared with the control experiment (*lane 3*), which shows only the autophosphorylation of the CK2 holoenzyme. Among them, one protein of 140 kDa was the most highly phosphorylated and bound to both forms of the CK2 holoenzyme.

One possible candidate for the highly phosphorylated protein p140 was a nucleolar protein, Nopp140, known to be a very good CK2 substrate and known to migrate on SDS gels with a similar molecular weight (20). A polyclonal antibody against protein Nopp140 was used to test this possibility. A binding experiment using GST-CK2 fusion proteins as described above was conducted, except that the phosphorylation step was omit-

[3] G. Dobrowolska, F. Lozeman, D. Li, and E. G. Krebs, manuscript in preparation.

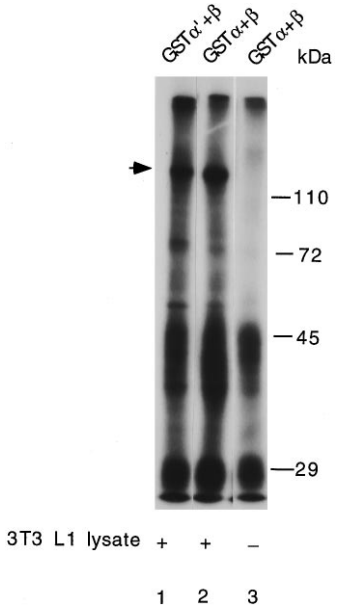

FIG. 1. **A highly phosphorylated protein p140 is associated with GST-CK2 fusion proteins.** 3T3 L1 cell lysates were incubated with immobilized GST-CK2 fusion proteins: GST-CK2α' + CK2β (*lane 1*) and GST-CK2α + CK2β (*lane 2*). After extensive washing, bound proteins were subjected to phosphorylating conditions using [γ-^{32}P]ATP as the phosphate donor. The phosphorylated CK2-bound proteins were detected by autoradiography. As a control, immobilized fusion protein GST-CK2α + CK2β (*lane 3*) was subjected to autophosphorylation and loaded on the same gel.

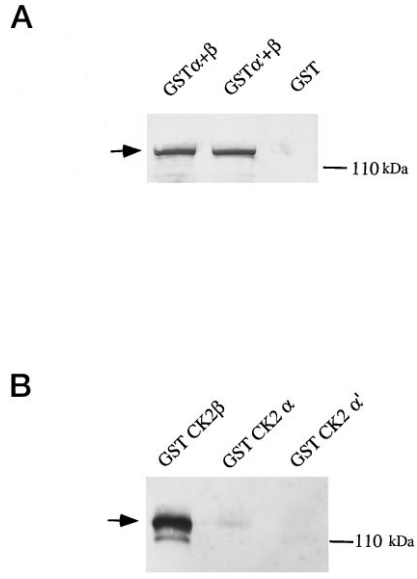

FIG. 2. **Nopp140 binds to CK2.** 3T3 L1 cell lysates were incubated with 2 μg of immobilized GST fusion proteins of holoenzyme CK2: *panel A*, from *left* to *right*, GST-α + β, GST-α' + β, and GST; and with each subunit, *panel B*, from *left* to *right*, GST-β, GST-α, and GST-α'. After extensive washing, the bound proteins were separated by SDS-PAGE, transferred to membrane, and analyzed by Nopp140 immunoblotting.

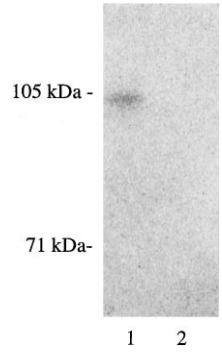

FIG. 3. **CK2 associates directly with Nopp140.** Purified bacterially expressed Nopp140 (0.5 μg, *lane 1*) and bovine serum albumin (1 μg, *lane 2*) were loaded on the SDS electrophoresis gel and transferred onto a polyvinylidene difluoride membrane. The membrane was overlaid with radiolabeled CK2 probe and subjected to autoradiography.

ted, and the CK2 interacting proteins were transferred electrophoretically to a polyvinylidene difluoride membrane and examined by Nopp140 immunoblotting. As illustrated in Fig. 2*A*, anti-Nopp140 antiserum recognized a protein of 140 kDa which bound to both GST-CK2 holoenzymes, GSTα + CK2β and GSTα' + CK2β (Fig. 2*A*, *left* and *center lanes*), but not to GST (*right lane*), suggesting a specific interaction between CK2 and Nopp140.

To investigate which subunits of CK2 bind to Nopp140, immobilized GST fusion proteins of each CK2 subunit, GST-CK2α, GST-CK2α', and GST-CK2β, were used for the type of binding experiment described above. A strong CK2β-Nopp140 interaction was detected when less than 2 μg of GST-CK2β was used (Fig. 2*B*, *left lane*), whereas no interaction was observed between Nopp140 and 2 μg of GST-CK2α, or Nopp140 and 2 μg of GST-CK2α' (*center* and *right lanes*), suggesting that the CK2 holoenzyme-Nopp140 association most probably occurred through the β subunit of CK2. However, with a higher amount (between 5 and 10 μg) of GST-CK2α, detectable affinity toward Nopp140 was seen; GST-CK2α' was associated very weakly with Nopp140 (data not shown).

CK2 Binds to Nopp140 Directly—To clarify further whether the CK2-Nopp140 interaction is a direct association, a radioactive CK2 overlay experiment was carried out using ^{32}P-labeled purified CK2 protein as a probe (18). Bacterially expressed pure Nopp140 protein was immobilized on the membrane as described (see "Experimental Procedures"). As demonstrated in Fig. 3, *lane 1*, Nopp140 did bind to the radiolabeled CK2 probe, whereas a control protein, bovine serum albumin (*lane 2*, 1 μg of bovine serum albumin), did not bind. This indicates that the CK2-Nopp140 interaction is specific and direct. It is noteworthy that in this experiment, a dephosphorylated form of Nopp140 was used because the purified Nopp140 was expressed in bacteria, giving a band of 100 kDa on the gel, whereas in intact cells, most of the Nopp140 is in a highly phosphorylated form (20). Taken together, the experiments of Figs. 2 and 3 make it seem very likely that the CK2-Nopp140 association is not dependent on the phosphorylation state of Nopp140.

Coimmunoprecipitation of CK2 and Nopp140—The association of CK2 and Nopp140 was also studied by coimmunoprecipitation of the two proteins from cell lysates. Immunoprecipitation of endogenous Nopp140 was performed in 3T3 L1 cell lysates (data not shown) and BRL cell lysates (Fig. 4*A*), and in both cases CK2 was detected in the immunoprecipitates of Nopp140 when anti-CK2α antiserum was used for immunoblotting analysis. In addition to CK2, NAP57, a Nopp140-binding protein (25), was also coprecipitated with Nopp140 (Fig. 4*A*). The specific precipitation of Nopp140 and coprecipitation of NAP57 and CK2α are present only in the absence of competing peptide (*−lanes*). It was found that a significant amount of CK2 was complexed with Nopp140 in the coprecipitation experiment; as determined by immunoblotting, it was estimated that more than half of the total cellular CK2 was removed from the supernatants by precipitation with anti-Nopp140 antibodies (data not shown).

In a parallel experiment, immunoprecipitation of endogenous CK2 from 3T3 L1 cell lysates was performed using a

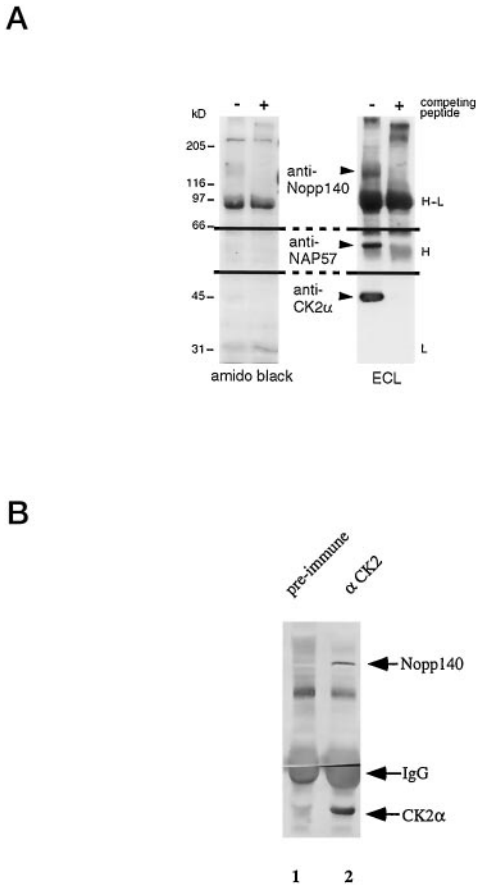

FIG. 4. **Coimmunoprecipitation of CK2 and Nopp140.** *Panel A*, coimmunoprecipitation of CK2 with anti-Nopp140 peptide antibodies. *Left*, Amido Black stain of proteins immunoprecipitated under nondenaturing conditions from whole cell lysates after separation by SDS-PAGE and transfer to nitrocellulose. BRL cell lysates were incubated with anti-Nopp140 peptide antibodies in the absence (*−lane*) and presence (*+lane*) of synthetic Nopp140 peptide against which the antibodies were raised. *Right*, immunodetection of precipitated proteins after incubation of the nitrocellulose with anti-Nopp140 (*top*), anti-NAP57 (*middle*), and anti-CK2α antibodies (*bottom*). The migrating position of the IgG heavy (*H*) and light (*L*) chains are indicated in the *right* margin. *Panel B*, coimmunoprecipitation of Nopp140 with anti-CK2 antibodies. CK2 antisera were used to immunoprecipitate CK2 and its binding proteins from total lysates of 3T3 L1 cells (*lane 2*). A mixture of preimmune sera was used as control (*lane 1*). After 10% SDS-PAGE, the proteins were transferred to the membrane. Nopp140 and CK2 were detected by Nopp140 antiserum (*top*) and by anti-CK2α antiserum (*bottom*).

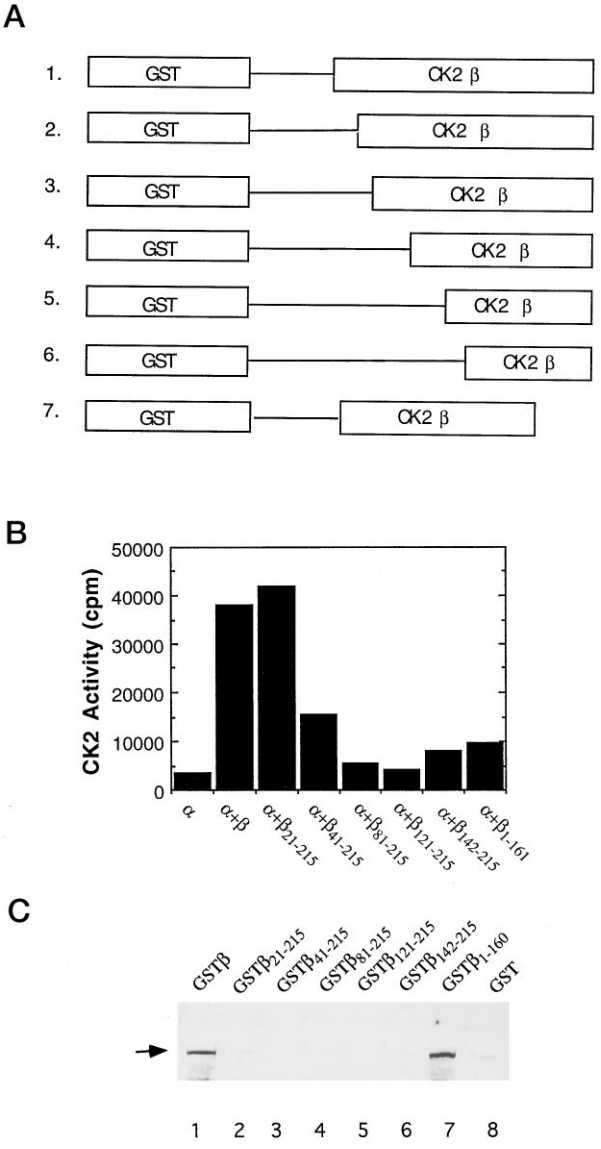

FIG. 5. **Mapping of the region of CK2β bound to Nopp140.** *Panel A*, GST-CK2β deletion mutants. *1*, GST-β; *2*, GST-β_{21-215}; *3*, GST-β_{41-215}; *4*, GST-β_{81-215}; *5*, GST-$\beta_{121-215}$; *6*, GST-$\beta_{142-215}$; *7*, GST-β_{1-160}. *Panel B*, activation of CK2α by GST-CK2β mutants. The activity of CK2α was determined in the presence of CK2β mutants as described (see "Experimental Procedures"). *Panel C*, mapping of the region of CK2β bound to Nopp140. 3T3 L1 cell lysates were incubated with immobilized deletion mutants of GST-CK2β (from *left* to *right*): GST-β (wild type), GST-β_{21-215}, GST-β_{41-215}, GST-β_{81-215}, GST-$\beta_{121-215}$, GST-$\beta_{142-215}$, GST-β_{1-160}, and GST. After extensive washing, the bound proteins were subjected to SDS-PAGE and analyzed by Nopp140 immunoblotting.

mixture of anti-CK2α, anti-CK2α′, and anti-CK2β antiserum. The presence of protein Nopp140 was examined by immunoblotting of the CK2 immunoprecipitate with a polyclonal anti-Nopp140 antiserum (20). As shown in Fig. 4*B*, Nopp140 was detected in the immunoprecipitate of CK2 (*lane 2*) but was not detected in the preimmune sera immunoprecipitate (*lane 1*), indicating a specific interaction of these two proteins. Together, coimmunoprecipitation of CK2 and Nopp140 suggested an *in vivo* association of these two proteins.

Expression of CK2β Deletion Mutants as GST Fusion Proteins—GST-CK2β deletion mutants were prepared for mapping the region of CK2β subunit which associates with Nopp140. The constructs used are shown in Fig. 5*A*. Expression of these deletion constructs produced NH_2-terminal and COOH-terminal truncated GST-CK2β proteins. To characterize whether these deletion mutants could stimulate the activity of CK2α as the wild type β does, the enzymatic activity of the recombinant α subunit (from Sf-9 cells) was measured in the presence of an excess amount (five times more) of either wild type β subunit or the deletion mutants of β. As illustrated in Fig. 5*B*, at 30 °C under our assay condition (with 0.1 M NaCl in final reaction mixture), CK2α showed very low activity because of the inhibition of NaCl (Fig. 5*B*, *far left lane*). Addition of the wild type GST-β protein greatly stimulated the catalytic activity of the α subunit (*second lane*). However, among all of the deletion mutants of β, only the mutant with deletion of the 20 NH_2-terminal amino acids, GST-β_{21-215}, could stimulate the activity of CK2α to the same extent as the wild type β (*third lane*). Deletion of amino acids 1–40 (GST-β_{41-215}) greatly decreased the ability of β to activate CK2α (*fourth lane*), and further deletion up to first 80 amino acids (GST-β_{81-215}) almost totally abolished the stimulation to α (*fifth lane*). This suggests that deletion of amino acids 21–80 causes a major structural change in the subunit. According to Kusk *et al.* (26), from their study in

the yeast two-hybrid system, amino acids 20–60 are needed for strong β-β interaction; so it is very possible that a tetrameric structure is needed to obtain full CK2 activity. Consistent with the data reported previously (27, 28), the COOH terminus of the β subunit was important for activating the α subunit because it is responsible for the α-β interaction; deletion of the COOH-terminal amino acids 160–215 caused a big decrease in the activation of the α subunit by β. Further investigation is under way to make a more thorough analysis of structure-function relationships in CK2β.

Mapping of the Binding Region of CK2β to Nopp140—The deletion mutants of CK2β were used in the *in vitro* CK2-Nopp140 binding studies. After incubating the immobilized GST-CK2β deletion mutants with 3T3 L1 cell lysates, the bound proteins were eluted and analyzed by SDS-PAGE and Nopp140 immunoblotting. Nopp140 was not detected in any of the eluates of the NH_2-terminal deletion mutants (Fig. 5*C*, *lanes 2–6*) but was detected in the eluate from the COOH-terminal deletion mutant GST-CK2β_{1-160} (Fig. 5*C*, *lane 7*). Since GST-CK2β_{21-215} is the smallest NH_2-terminal deletion, the lack of its binding ability to Nopp140 indicated that the first 20 NH_2-terminal amino acids are most probably involved in the CK2-Nopp140 interaction.

Phosphorylation of Nopp140 by CK2—To understand how the CK2β subunit might affect the specificity of CK2 toward Nopp140 as a substrate, bacterially expressed Nopp140 was subjected to phosphorylation by baculovirus-expressed CK2 catalytic subunits α and α′ and by holoenzymes $\alpha_2\beta_2$ and $\alpha'_2\beta_2$. The phosphorylation reactions were carried out in a buffer without NaCl in order to have maximal α or α′ catalytic activity (α and α′ are inhibited by NaCl). When a high concentration of CK2 (120 ng) was used, Nopp140 was found to be phosphorylated efficiently by all of the forms of CK2 after incubation in a phosphorylation buffer for 10 min at 30 °C; phosphorylation caused a gel mobility shift from 100 to 140 kDa (data not shown). However, when a lower amount of CK2 was used (approximately 30 ng), each form of holoenzyme, $\alpha_2\beta_2$ and $\alpha'_2\beta_2$, showed much higher specific activity toward Nopp140 than the monomeric active subunits, α and α′, although their relative activities toward a CK2 substrate peptide, DSD, had already been deliberately adjusted to the same level by dilution. Phosphorylation of Nopp140 by $\alpha'_2\beta_2$ and $\alpha_2\beta_2$ for 20 min at 30 °C resulted in a massive incorporation of ^{32}P and a significant gel mobility shift (Fig. 6*A*, *lanes 1* and *2*), whereas phosphorylation by α and α′ under the same condition was much weaker and did not shift the band significantly (Fig. 6*A*, *lanes 3* and *4*). From the densitometric reading, phosphorylation of Nopp140 by the holoenzymes CK2 was 4-fold higher than by the monomeric enzymes (Fig. 6*B*), indicating that Nopp140 is a much better substrate for the holoenzyme CK2 than for the monomeric α and α′ subunit.

Effect of CK2-Nopp140 Association on CK2 Activity Using a Different Substrate—To examine the impact of the CK2-Nopp140 association on the enzymatic activity of CK2, CK2 was assayed in the presence or absence of Nopp140 using purified Sf-9 cell-expressed CK2 and bacterially expressed Nopp140. CK2 peptide substrate DSD was used for the assay. Besides the routine assay method (23, 24), a modified method in which Nopp140 was first phosphorylated for 20 min by CK2 followed by adding the DSD peptide to start the reaction, was also used to decrease the competition that might be caused by Nopp140 as an alternative substrate. In each case, no significant change of CK2 activity was detected when Nopp140 was present in the reaction mixture. This occurred even though it was possible that in the experiment using the alternative method Nopp140 phosphorylation may not have been absolutely complete at the point at which DSD was added. Also, a slightly lower [^{32}P]ATP concentration would be present due to its utilization for Nopp140 phosphorylation. These factors would have been expected to decrease activity in the DSD phosphorylation reaction.

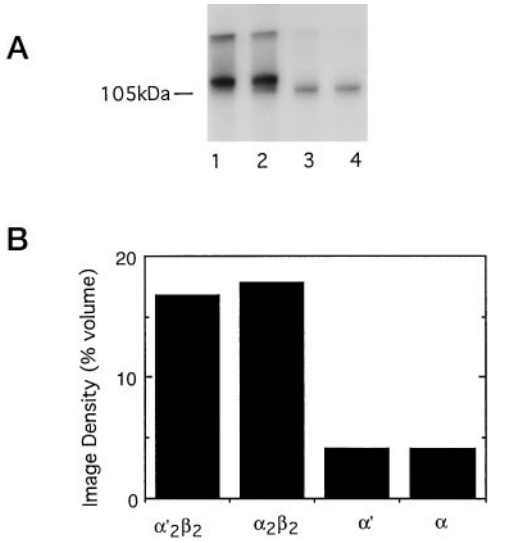

FIG. 6. **Phosphorylation of Nopp140 by CK2.** *Panel A*, recombinant Nopp140 (0.5 μg Nopp140 for each reaction) was subjected to phosphorylation by CK2, $\alpha'_2\beta_2$ (*lane 1*), $\alpha_2\beta_2$ (*lane 2*), α′ (*lane 3*), and α (*lane 4*). After incubation for 20 min at 30 °C, the reaction was stopped by adding sample buffer, and the proteins were resolved by SDS-PAGE. Nopp140 phosphorylation was analyzed by autoradiography. *Panel B*, image density from *panel A*.

DISCUSSION

In this study we have identified a nucleolar protein, Nopp140, as a CK2-associated protein. The interaction of the two proteins was shown to be direct and not dependent on the phosphorylation state of Nopp140. Furthermore, a possible *in vivo* interaction of CK2 and Nopp140 was suggested by the coimmunoprecipitation of the two proteins from cell lysates.

Nopp140 was first isolated as a nuclear localization sequence-binding protein (29). Immunostaining and immunoelectron microscopy revealed that Nopp140 is a nucleolar protein that shuttles between the cytoplasm and the nucleolus. A possible role of Nopp140 as a chaperone for import into or export from the nucleolus was suggested (20). Having 49 phosphorylation consensus sites for CK2, and upon their phosphorylation an additional 33, Nopp140 can be highly phosphorylated by CK2 in intact cells (20), giving an apparent molecular mass of 140 kDa on SDS-PAGE. Only phosphorylated Nopp140 binds to the nuclear localization sequence-containing peptide (20). However, a precise understanding of the function of Nopp140 and its phosphorylation by CK2 is not available.

Our data showed that CK2 most probably interacts with Nopp140 through its β subunit, although some binding of the protein with the α subunit of CK2 was seen. One conceivable role of the CK2-Nopp140 interaction could be increasing the substrate specificity for Nopp140 phosphorylation. To address this point, monomeric forms of the enzyme, α and α′, and the holoenzymes, $\alpha_2\beta_2$ and $\alpha'_2\beta_2$, were used for the phosphorylation of Nopp140. It was found that Nopp140 was a much better substrate for the holoenzyme form of CK2. Until now, with nearly all substrates including the routinely used CK2 substrate peptide DSD, the holoenzyme form of CK2 always exhibits a higher activity than the monomeric α or α′ subunit. An approximately 3–5-fold stimulation of CK2α activity by CK2β has normally been observed when DSD peptide is used in the assay (18, 30). In our experiment in which we compare the

ability of the α and α' subunits to phosphorylate Nopp140 with that of the holoenzyme forms, we deliberately used a lower amount of the holoenzyme CK2 so as to give it the same catalytic activity as CK2α and CK2α' toward the DSD peptide substrate. Under this condition, the holoenzymes still phosphorylated Nopp140 at four times the rate of either free α or α'. If normalized to the molarity level, an approximately 20-fold difference in phosphorylation could be estimated for the free catalytic subunit (α or α') compared with holoenzyme CK2. This large difference could be partly due to the association between CK2β and Nopp140, thus favoring substrate recognition. However, because CK2 can also associate with the phosphorylated form of Nopp140, this specific CK2-Nopp140 interaction may also be correlated with other cellular functions of CK2 and Nopp140.

CK2 has been shown to be a major nuclear protein (31). Its growth-related accumulation in the nucleolus has also been observed (32, 33). The finding of the association of CK2 with Nopp140 suggests that CK2 may play an important role in the nucleolus. It has been shown that many nuclear localization sequence-interacting nucleolar proteins, including nucleolin, B23, Nopp140, and its associated protein NAP57 (25), are good substrates of CK2 and can migrate back and forth between the nucleus and cytoplasm (for review, see Ref. 34). These nucleolar proteins all have NH_2-terminal domains containing stretches of acidic and serine residues with numerous CK2 phosphorylation sites, and the nuclear localization sequence binding ability of the protein seems to be dependent on their phosphorylation (for review, see Ref. 35). Recently, a major nucleolar protein, nucleolin, was also shown to be able to associate with CK2 *in vitro* and probably *in vivo* (16–18). It is possible that in addition to the phosphorylation, the association between CK2 and nucleolar proteins may represent another way of regulating the latter. The association of Nopp140 with CK2, together with phosphorylation, could affect its function in ribosomal protein transport.

Interestingly, it was found recently that Nopp140 appears to be a growth-inhibiting protein, *e.g.* when rat Nopp140 was overexpressed in yeast, growth impairment was observed (19). Also, SRP40, a yeast homolog of Nopp140, was identified by a genetic screen for genes that cause growth arrest when overexpressed (36). Indeed, when deletion and overexpression of SRP40 were conducted in yeast, deletion caused only minor growth impairment, but its overexpression resulted in a severe growth defect (19). It would be of interest to know if the growth inhibitory function of Nopp140 is mediated both by phosphorylation and its association with CK2.

Using deletion mutants of CK2β, the region at which CK2β binds to Nopp140 was mapped to the first 20 NH_2-terminal amino acids, a domain containing the autophosphorylation sites of CK2 Ser-2 or Ser-3 (21). Since bacterially expressed GST-CK2β was used in the binding assay of these studies, the subunit should have been in its dephosphorylated form. On the other hand, in the overlay experiments, CK2 was radiolabeled by the autophosphorylation reaction in which Ser-2 and/or Ser-3 of the CK2β subunit would be phosphorylated. The CK2 bound to the membrane which was detected would have been phosphorylated. This indicates that both the dephosphorylated and the phosphorylated forms of CK2β can interact with Nopp140. From CK2 activity data (Fig. 5*B*), deletion of the 20 NH_2-terminal amino acids gives a mutant that will still activate CK2α as well as the wild type β does. It is reasonable to assume that CK2 is still in its active form even when it is complexed with Nopp140, and this was indeed what we observed when CK2 activity toward the peptide substrate was measured in the presence of Nopp140.

One interesting observation is that when higher amounts of GST fusion proteins of CK2α and CK2α' were used in the binding assay, CK2α did exhibit affinity for Nopp140. By contrast, CK2α' bound very weakly to Nopp140. This CK2α-Nopp140 interaction may have occurred indirectly through a separate protein, since the association was not detected with a lower amount of GSTα. If CK2α and CK2α' have a different affinity for the hypothetical protein, this could cause the observed difference in their binding to Nopp140. One question that could be raised is whether α and α' subunits exhibit redundancy in vertebrates as they do in yeast (5). It is known that CK2α and CK2α' are encoded by different genes (22) and that they are structurally very homologous (85% homology) with major differences only in the COOH terminus (4). It has been shown that the α subunit can act as a transcription factor to control β gene expression, whereas α' cannot (37). Also, the α subunit phosphorylates the β subunit more efficiently than α'.[3] It would be interesting to know whether the two catalytic subunits are associated with different proteins in the cell and have different functions.

So far, a number of proteins have been found which interact with CK2, some with the catalytic α or α' subunit, and some with the β subunit. For example, proteins that are reported to be able to interact with CK2α are the transcription factor ATF1 (38), nucleolin (16–18), and the heat shock protein HSP 90 (39). Proteins that are shown to interact with CK2β are p53 (9), DNA topoisomerase II (11), c-Mos,[2] and Nopp140. Currently, it is not clear whether the binding properties of the different subunits are related to the regulation of CK2.

Acknowledgments—We thank Lynda Munar for assistance with the cell culture and Drs. Yim Foon Lee and Patrick S. H. Chou for DNA oligomer synthesis.

REFERENCES

1. Guan, K. L. (1994) *Cell. Signalling* **6,** 581–589
2. Seger, R., and Krebs, E. G. (1995) *FASEB J.* **9,** 726–735
3. Cobb, M. H., and Goldsmith, E. J. (1995) *J. Biol. Chem.* **270,** 14843–14846
4. Issinger, O.-G. (1993) *Pharmacol &. Ther.* **59,** 1–30
5. Padmanabha, R., Chen-Wu, J. L.-P., Hanna, D. E., and Glover, C. V. C. (1990) *Mol. Cell. Biol.* **10,** 4089–4099
6. Allende, J. E., and Allende, C. C. (1995) *FASEB J.* **9,** 313–323
7. Pepperkok, R., Lorenz, P., Ansorge, W., and Pyerin, W. (1994) *J. Biol. Chem.* **269** 6986–6991
8. Hanna, D. E., Rethinaswamy, A., and Glover, C. V. C. (1995) *J. Biol. Chem.* **270,** 25905–25914
9. Filhol, O., Baudier, J., Delphin, C., Loue-Mackenbach, P., Chambaz, E. M., and Cochet, C. (1992) *J. Biol. Chem.* **267,** 20577–20583
10. Janosch, P., Schellerer, M., Seitz, T., Reim, P., Eulitz, M., Brielmeier, M., Kolch, W., Sedivy, J. M., and Mischak, H. (1996) *J. Biol. Chem.* **271,** 13868–13874
11. Bojanowski, K., Filhol, O., Cochet, C., Chambaz, E. M., and Larsen, A. K. (1993) *J. Biol. Chem.* **268,** 22920–22926
12. Seldin, D. C., and Leder, P. (1995) *Science* **267,** 894–897
13. Litchfield, D., Dobrowolska, G., and Krebs, E. G. (1994) *Cell. & Mol. Biol. Res.* **40,** 373–381
14. Tuazon, P. T., and Traugh, J. A. (1991) *Adv. Second Messenger Phosphoprotein Res.* **23,** 123–264
15. Pinna, L. A. (1990) *Biochim. Biophys. Acta* **1054,** 267–284
16. Caizergues-Ferrer, M., Belenguer, P., Lapeyre, B., Amalric, F., Wallace, M. O., and Olson, M. O. J. (1987) *Biochemistry* **26,** 7876–7883
17. Jin, Y. J., and Burakoff, S. J. (1993) *Proc. Natl. Acad. Sci. U. S. A.* **90,** 7769–7773
18. Li, D., Dobrowolska, G., and Krebs, E. G. (1996) *J. Biol. Chem.* **271,** 15662–15668
19. Meier, U. T. (1996) *J. Biol. Chem.* **271,** 19376–19384
20. Meier, U. T., and Blobel, G. (1992) *Cell* **70,** 127–138
21. Litchfield, D. W., Lozeman, F. J., Cicirelli, M. F., Harrylock, M., Ericsson, L. H., Piening, C. J., and Krebs, E. G. (1991) *J. Biol. Chem.* **266,** 20380–20389
22. Lozeman, F. J., Litchfield, D. W., Piening, C., Takio, K., Walsh, K. A., and Krebs, E. G. (1990) *Biochemistry* **29,** 8436–8447
23. Kuenzel, E. A., Mulligan, J. A., Sommercorn, J., and Krebs, E. G. (1987) *J. Biol. Chem.* **262,** 9136–9140
24. Litchfield, D. W., Lozeman, F. J., Piening, C., Sommercorn, J., Takio, K., Walsh, K. A., and Krebs, E. G. (1990) *J. Biol. Chem.* **265,** 7638–7644
25. Meier, U. T., and Blobel, G. (1994) *J. Cell Biol.* **127,** 1505–1514
26. Kusk, M., Bendixen, C., Duno, M., Westergarrd, O., and Thomsen, B. (1995) *J. Mol. Biol.* **253,** 703–711
27. Boldyreff, B., Meggio, F., Pinna, L., and Issinger, O.-G. (1994) *Cell. Mol. Biol. Res.* **40,** 391–399

28. Marin, O., Meggio, F., Boldyreff, B., Issinger, O., and Pinna, L. A. (1995) *FEBS Lett.* **363,** 111–114
29. Meier, U. T., and Blobel, G. (1990) *J. Cell Biol.* **111,** 2235–2245
30. Sarno, S., Boldyreff, B., Marin, O., Guerra, B., Meggio, F., Issinger, O.-G., and Pinna, L. A. (1995) *Biochim. Biophys. Res. Commun.* **206,** 171–179
31. Krek, W., Maridor, E., and Nigg, E. A. (1992) *J. Cell Biol.* **116,** 43–55
32. Belenguer, P., Baldin, V., Mathieu, C., Bensaid, M., Bouche, G., and Amalric, F. (1989) *Nucleic Acids Res.* **17,** 6626–6638
33. Pfaff, M., and Anderer, F. A. (1988) *Biochim. Biophys. Acta* **969,** 100–109
34. Shaw, P. J., and Jordan, E. G. (1995) *Annu. Rev. Cell Dev. Biol.* **11,** 93–121
35. Xue, Z., and Melese, T. (1994) *Trends Cell Biol.* **4,** 414–417
36. Espinet, C., De La Torre, M. A., Aldea, M., and Herrero, E. (1995) *Yeast* **11,** 25–32
37. Pyerin, W. (1994) *Adv. Enzyme Regul.* **34,** 225–246
38. Wada, T., Kakagi, T., Yamaguchi, Y., Kawase, H., Hiramoto, M., Ferdous, A., Takayama, M., Lee, K. A. W., Hurst, H. C., and Handa, H. (1996) *Nucleic Acids Res.* **24,** 876–884
39. Miyata, Y., and Yahara, I. (1995) *Biochemistry* **34,** 8123–8129