The Identification of Phosphorylation Sites of pp32 and Biochemical Purification of a Cellular pp32-kinase†

Rui Hong,‡ Todd Macfarlan,‡ Sara N. Kutney,‡ Sang-beom Seo,‡ Yuki Mukai,§ Felix Yelin,§ Gary R. Pasternack,|| and Debabrata Chakravarti*,‡

Department of Pharmacology, University of Pennsylvania School of Medicine, Philadelphia, Pennsylvania 19104-6160, University of Pennsylvania School of Arts and Sciences, Philadelphia, Pennsylvania 19104-6160, and Department of Pathology, Johns Hopkins University School of Medicine, Baltimore, Maryland 21205

Received March 29, 2004; Revised Manuscript Received June 3, 2004

ABSTRACT: The versatile phosphoprotein pp32 is involved in important physiological processes, including cell proliferation, apoptosis, mRNA transport, and transcription. We have previously reported that pp32, through histone masking, inhibits histone acetylation and transcriptional activation by histone acetyltransferases. However, how pp32 itself is regulated remained largely unknown. Although pp32 is a phosphoprotein, neither the phosphorylation sites nor the cellular kinase has been identified. In this report, utilizing an in vitro kinase assay and a biochemical purification scheme, we identify casein kinase II as a cellular pp32-kinase. Our deletion and site-specific mutagenesis studies identify serines 158 and 204 as the sites of phosphorylation. Generation and utilization of antibodies with higher affinity for phospho-pp32 demonstrate that pp32 is indeed phosphorylated in vivo at these two sites. Mutagenesis studies on pp32 suggest a role for serines 158 and 204 in its function. The identification of the pp32 kinase and the sites of pp32 phosphorylation as well as the generation of antibodies with higher affinity for phospho-pp32 should now provide key information and tools for future studies on pp32 regulation.

The multi-functional nuclear phosphoprotein of 32 kDa (pp32) is 249 amino acids long and a member of a highly acidic family of nuclear proteins that also includes PHAPII, LANP, and APRIL (*1*−*5*). The C-terminal one-third of pp32 is rich in acidic amino acids harboring approximately 70% glutamic acid and aspartic acid residues. pp32 was originally identified as a protein selectively expressed in normal self-renewing cells but was later found to be expressed in neoplastic tissues (*6*). pp32 functions as a tumor suppressor in cell culture models, where its overexpression inhibits the transformation induced by a wide variety of oncogenic pairs, including ras + myc, ras + jun, ras + E1A, and ras + mutated p53 (*2*). Isoforms of pp32 encoded by separate but closely related genes are highly expressed in neoplastic cell lines and prostate cancer cells and are believed to be tumorigenic (*6*−*8*).

Following the original discovery, pp32 has also been identified in a variety of biological and functional assays. For example, pp32 was purified as an associating protein with the HLA-DR-α chain, possibly functioning in the intracellular signaling pathway of HLA class II molecules (*1*). pp32 was discovered to have protein phosphatase 2A (pp2A) inhibitory activity (*9*). pp32 was also identified as one of the ligand proteins for the mRNA binding protein HuR, which stabilizes mRNAs containing AU-rich elements. The binding of pp32 to HuR is critical for nuclear-cytoplasmic shuttling of HuR (*10*, *11*). More recently, pp32 was biochemically purified as a pro-apoptotic factor that promotes the activation of caspase-9 upon apoptosome formation during mitochondria mediated apoptosis, thus providing a molecular mechanism for the function of pp32 as a tumor suppressor (*12*).

Our laboratory previously identified pp32 as one subunit of a cellular protein complex called INHAT (*inh*ibitor of *a*cetyl*t*ransferases) that binds to histones and inhibits the histone acetyltransferase (HAT) activity of several transcriptional co-activators, including p300/CBP and PCAF in vitro, a process we referred to as "histone masking". INHAT and each of its subunits can also inhibit HAT-dependent transcriptional activation in vivo, including transcriptional activation mediated by the liganded retinoic acid receptor (*13*, *14*). The HAT-inhibitory/histone binding function was mapped at the C-terminal part of the pp32 protein. Finally, recent studies from our laboratory show that pp32 associates with histone deacetylase activity, preferentially binds to hypoacetylated over hyperacetylated histones, and plays a signaling role in integrating histone hypoacetylation to gene inactivation (*15*). These studies together implicate pp32 as a critical multi-tasking protein, regulating chromatin and cell-based processes (*16*).

Despite these well-studied physiologically important functions of pp32, its regulation remains poorly understood. The level of pp32 expression is constant in cultured cell lines and it is not subject to active proteosome-mediated protein

† This work was supported by grants (DK65148 and DK57079) from the National Institutes of Health, USA (to D. C.).

* To whom correspondence should be addressed. 810 BRBII/III, 421 Curie Blvd., Department of Pharmacology, University of Pennsylvania School of Medicine, Philadelphia, PA 19104-6160. Tel.: 215-573-8470. Fax: 215-573-9004. E-mail: debu@pharm.med.upenn.edu.

‡ University of Pennsylvania School of Medicine.

§ University of Pennsylvania School of Arts and Sciences.

|| Johns Hopkins University School of Medicine.

10.1021/bi0493968 CCC: $27.50
Published on Web 07/16/2004

degradation (R. H. and D. C., unpublished data). pp32 was originally identified and subsequently cloned as a nuclear phosphoprotein that is phosphorylated at serines in vivo and can serve as an in vitro substrate for casein kinase II (CKII)[1] (*17*). However, it remained to be determined whether CKII is indeed a cellular pp32 kinase. Additionally, the sites and role of pp32-phosphorylation in its activity remained unknown. Protein phosphorylation, as a reversible covalent modification, has been shown to play regulatory roles in numerous cellular pathways. Phosphorylation of transcriptional coregulators has been shown to regulate their activities. For example, phosphorylation of histone deacetylase (HDAC) 1 promotes its corepressor complex formation and enzymatic activity (*18*, *19*). The C-terminal binding protein 1 (CtBP), a ubiquitous corepressor implicated in development and oncogenesis, is negatively regulated by the p21-activated kinase 1 (Pak1) (*20*). In yeast, the Hog1 kinase phosphorylates Sko1 transcriptional repressor and converts Sko1 to a transcriptional activator (*21*). Recently, Ubeda and Habener showed that CKII phosphorylates the transcription factor CHOP and inhibits its transcriptional activation function (*22*). On the basis of the above discussions, it is conceivable that phosphorylation/de-phosphorylation of pp32 could also play an important regulatory role in diverse functions of pp32. However, such studies could not be initiated, as no cellular kinase responsible for pp32 serine phosphorylation or the precise sites of phosphorylation on pp32 has been identified. By utilizing a kinase assay based approach, we set out to biochemically identify the cellular kinase responsible for pp32 phosphorylation, map the site(s) of phosphorylation, and probe the in vivo phosphorylation status of pp32.

In this report, utilizing a kinase assay-driven biochemical purification, we identified casein kinase II (CKII) from HeLa cell nuclear extract as a cellular pp32 kinase. Using deletion and mutagenesis studies, we have identified serines 158 and 204 as the sites of phosphorylation by CKII. We have further generated antibodies against phosphorylated forms of pp32 and showed that pp32 is indeed phosphorylated at these sites in vivo. In addition, mutagenesis studies suggest a role for serines 158 and/or 204 in pp32-function.

EXPERIMENTAL PROCEDURES

Materials and Constructs. Heparin-benzalkonium was from Sigma (H7280). DRB (5,6-Dichloro-1-β-D-ribofuranosylbenzimidazole) and staurosporine were from CalBiochem. Pure CKII enzyme was from Promega or New England BioLabs. Histone tail peptides and streptavidin-sepharose beads were from Upstate Biotechnology.

Site-directed mutagenesis of pp32 was carried out utilizing the Quickchange Site Directed Mutagenesis Kit according to the manufacturer's instruction (Stratagene). Radiolabeled proteins were made using in vitro transcription and translation method (TNT, Promega) in the presence of ^{35}S-methionine (10 mCi/mL, > 1000 Ci/mmol, Amersham Biosciences).

Cell Culture and Transfection. Bulk HeLa S3 cells were obtained from the National Cell Culture Center, MN. HeLa S3 were grown in DMEM medium supplemented with 100 U/mL penicillin/streptomycin and 10% fetal bovine serum. Cells were transfected using Fugene 6 (Roche) or Lipofectamine 2000 (Invitrogen) transfection agent, according to the manufacturers' instructions.

In Vitro Kinase Assay. Chromatographic fractions or purified kinase and substrate proteins were incubated in kinase assay buffer (50 mM Tris-HCl, pH 7.5, 10 mM $MgSO_4$) in the presence of [γ-^{32}P]-ATP (NEN Easytides, 3000 Ci/mmol, 10 μCi/μL) at 30 °C for 10 min. The reaction was stopped by the addition of SDS−PAGE loading buffer. Proteins were then resolved on SDS−PAGE and radiolabeled proteins visualized with phosphorimager.

Purification of the pp32 Kinase from HeLa Cell Nuclear Extract. All purification procedures were performed at 4 °C, unless specified otherwise. Chromatographic steps on HiTrap SP (Pharmacia), HiTrap Q (Pharmacia), and Concanavalin A (ConA) Sepharose column (Pharmacia) were carried out on an automatic fast protein liquid chromatography (FPLC) station (Pharmacia). Purification steps on Phenyl Sepharose (Pharmacia) and GST-pp32 affinity column were carried out on a hand-packed column in a gravity-driven manner. All columns were equilibrated and washed in buffer DEAE100 (20 mM Tris-HCl, pH7.4, 0.1 mM EDTA, 2% glycerol with 100 mM KCl), unless otherwise specified. Elution buffers have the same contents except the concentration of KCl: 500 mM in DEAE500 and 1M in DEAE1000.

HeLa cell nuclear extract was prepared as described in ref *13*. A 280-mg sample of the extract was applied onto a HiTrap SP column followed by a ConA Sepharose chromatography. The flow-through fraction was collected and loaded directly onto a HiTrap Q column. The bound material was eluted with a 25−50% gradient of DEAE1000. The fractions eluted were assayed for kinase activity using GST-pp32 or INHAT complex as substrate where indicated. Peak active fractions were pooled and concentrated using Centricon (Millipore) with an appropriate buffer change and then loaded onto a Phenyl Sepharose column equilibrated with DE-AE500. After they were washed, the bound materials were eluted with DEAE without KCl. Fractions with kinase activity were incubated with GST-pp32 immobilized onto glutathione-sepharose beads. The beads-bound material was separated on SDS−PAGE, and the gel was stained with silver reagents (BioRad). Bands specific to the active fractions were cut out and subjected to protein identification by MALDI-TOF Mass Spectrometry (Proteomic Mass-Spectrometry Laboratory at University of Massachusetts Medical School).

In-gel Kinase Assay. In-gel kinase assays were carried out according to a protocol from the laboratory of Andrew Murray at Harvard University (http://www.bio.com/protocolstools/prnprot.jhtml?id=911) with the following modifications: The experimental gel contained 0.5 mg/mL pp32 peptide (corresponding to its amino acid 150−167). The control gel contained 0.5 mg/mL BSA.

Phosphatase Treatment. Proteins were incubated with Calf Intestine Phosphatase (CIP, New England BioLabs) in manufacturer-supplied buffer at 37 °C for 1 h. The reaction was stopped by the addition of SDS−PAGE loading buffer.

Immunoprecipitation and Immunoblotting. Cell extract was pre-cleared with control IgG and protein A/G agarose beads for 1 h at 4 °C. The supernatant was incubated with the indicated antibody for 4 h or overnight at 4 °C. Then protein

[1] Abbreviations: DRB, 5,6-Dichloro-1-β-D-ribofuranosylbenzimidazole; CKII, casein kinase II.; MALDI-TOF, Matrix-Assisted Laser Desorption Ionization − Time-of-Flight; HAT, histone acetyltransferase; SDS-PAGE, SDS-polyacrylamide gel electrophoresis; INHAT, inhibitors of acetyltransferases; HDAC, histone deacetylase; ConA, Concanavalin A.

A/G agarose beads were added and incubation continued for another hour. The beads-bound protein complex was extensively washed and resolved by SDS−PAGE. Immunoblotting was performed using chemiluminescence detection reagent (PerkinElmer Western Lightning Western Blot Chemiluminescence Reagent *Plus*) according to the manufacturer's protocol.

Antibodies acquired commercially were anti-CKIIα/α′ (BD Biosciences) and α-pp32 (Santa Cruz).

Generation and Characterization of Antiphospho-pp32 Antibodies. The immunogenic peptides were chemically synthesized with phospho-serine corresponding to Ser158 or Ser204 of pp32 (Protein Facility, Baylor Medical Center): Peptide 158P (amino acid 151−165/C-DDKEAPDS(phospho)DAEGYVE) and peptide 204P (amino acid 197−211/C-EGEEEDVS(phospho)GEEEEDE). The cysteine residue was added at the N-termini of the peptides for conjugation purposes. Peptides were conjugated to immunogenic carrier protein keyhole limpet hemocyanin (KLH) (Pierce, Imject Maleimide Activated Immunogen Conjugation Kit with mcKLH and BSA). The conjugated peptides were used to immunize rabbits separately, and rabbit serum was harvested in a commercial facility (Covance). For antibody purification, peptide 158P and 204P were reduced and conjugated separately to agarose matrix according to the manufacturer's instruction (Pierce, Reduce-Imm Reducing Kit and SulfoLink kit). The immobilized peptides were then packed into columns for affinity purification. Serum raised against the corresponding peptide was loaded onto the affinity column equilibrated in PBS. Each column was washed extensively with PBS before elution with 0.1 M Glycine (pH 2.5). 1 M Tris-HCl, pH8.0 was immediately added to eluted fractions to neutralize the pH. Antibodies were then concentrated and tested in immunoblotting.

HAT Assay. HAT assays were performed essentially as described previously (*14*). Briefly, 1 pmol of recombinant p300 was incubated for 30 min at room temperature with 375−500 ng of histone H4 in the presence of increasing amounts of GST-pp32 in buffer containing 50 mM Tris-HCl, pH 7.5, 10% glycerol, 0.1 mM EDTA, 0.1 mM PMSF, 200 mM KCl, 10 mM sodium butyrate, 0.6 mM DTT, and 0.15 μL of ^{14}C-acetyl CoA (50 μCi/ml, 1000 pmol/μL, Amersham Biosciences). Prior to addition to HAT assay reaction, GST-pp32 was incubated with 200 μM ATP and kinase fraction where indicated and spiked with radiolabeled ATP to ensure phosphorylation of pp32. Reaction products were subjected to SDS-PAGE, and analyzed by phosphorimager.

HDAC Assay. ^{3}H-labeled acetylated histones were prepared from HeLa cells essentially as described (*23*). The recombinant GST fusion proteins where indicated were incubated with HeLa cell nuclear extracts and collected by glutathione-sepharose beads. Retained protein complex was extensively washed and assayed for the retention of HDAC activity by incubating the pelleted beads with 10 000 cpm of ^{3}H-labeled acetylated HeLa cell histones in a total volume of 200 μL of HDAC assay buffer (20 mM Tris-HCl, pH 8.0, 150 mM NaCl, 10% glycerol) at 37 °C for 2 h. The beads were collected and separated from the aqueous reaction mixtures. Reactions were stopped with 50 μL of stop solution (1 M HCl, 0.16 M acetic acid) and extracted with 600 μL of ethyl acetate. Released ^{3}H-acetic acid was measured by scintillation counting of the organic phase (*24*).

RESULTS

Identification of the Kinase and Phosphorylation Sites of pp32. pp32 had been previously shown to be phosphorylated at serine residues in vivo, although the precise sites of phosphorylation, the cellular kinase, and the function of phosphorylation were not known (*17*). Therefore, it became necessary to purify the pp32-kinase and identify the site(s) of phosphorylation. Since pp32 is primarily nuclear (*14*), we prepared nuclear extracts from HeLa cells and fractionated the extract (the complete purification scheme is shown in Figure 1A) for a kinase activity specific for pp32, utilizing an in vitro kinase assay shown in Figure 1B. In this assay, we incubated alternate fractions eluted from a chromatographic column (in Figure 1B, pooled active Q-sepharose fraction was used) with purified INHAT complex in the presence of [γ-^{32}P]-ATP. Radiolabeled protein(s) were then identified by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) followed by phosphorimager analysis (Figure 1B). pp32, but not the other two INHAT subunits, TAF−Iα or TAF−Iβ (also known as the SET protein) (*25*), was phosphorylated when the purified INHAT complex was used as a substrate (lane 1). The specificity of the pp32-kinase was further demonstrated by the lack of phosphorylation of other nonspecific substrates including histone H1 that was phosphorylated by PKA (lane 2). To confirm the identity of the phosphorylated band as pp32, we utilized bacterially expressed and purified recombinant GST fusion proteins as substrates. The kinase phosphorylated recombinant GST-pp32 (lane 6) but not GST (lane 4) or other recombinant INHAT subunits such as GST-TAF-Iβ (lane 5). These results together indicate that pp32 present in the purified INHAT complex as well as the recombinant pp32 can be specifically phosphorylated by a putative kinase that we refer to as the pp32-kinase. Utilizing this unbiased assay and the purification scheme shown in Figure 1A (see also Figure 2), we purified the pp32-kinase and used the purified kinase to identify the sites of phosphorylation.

In our previous work, we have generated N- and C-terminal deletion mutants of pp32 (*14*). We thus utilized pp32 deletion mutants to broadly map the phosphorylation sites. A recombinant pp32 deletion mutant (GST-pp32-ΔC2) spanning the first 150 amino acids was not phosphorylated by the pp32-kinase activity in vitro, although recombinant wild-type human pp32 was robustly phosphorylated under the same experimental conditions (Figure 1C, compare lanes 2 and 3). These results indicated that the pp32-kinase might target the carboxyl-terminal of pp32. A scanning of kinase substrate motifs reveals potential phosphorylation sites within this region for kinases, including Casein kinase II, ATM, DNA PK, FGFR kinase, insulin receptor kinase, Nck, etc., with CKII predicted as the most probable kinase. Since pp32 was previously reported to be phosphorylated at serine residues, we focused on the serine residues of pp32. A close examination of the pp32 sequence revealed that while 9 serine residues map in the first 150 amino acids of pp32, the carboxyl-terminal amino acids from 150 to 249 contain only two serine residues at positions 158 and 204 of human pp32 (*2*). To determine whether serines 158 and 204 are indeed targets of the pp32-kinase, we employed a quick-change mutagenesis procedure to generate both alanine (Ala, A) and aspartic acid (Asp, D) substitution at either 158

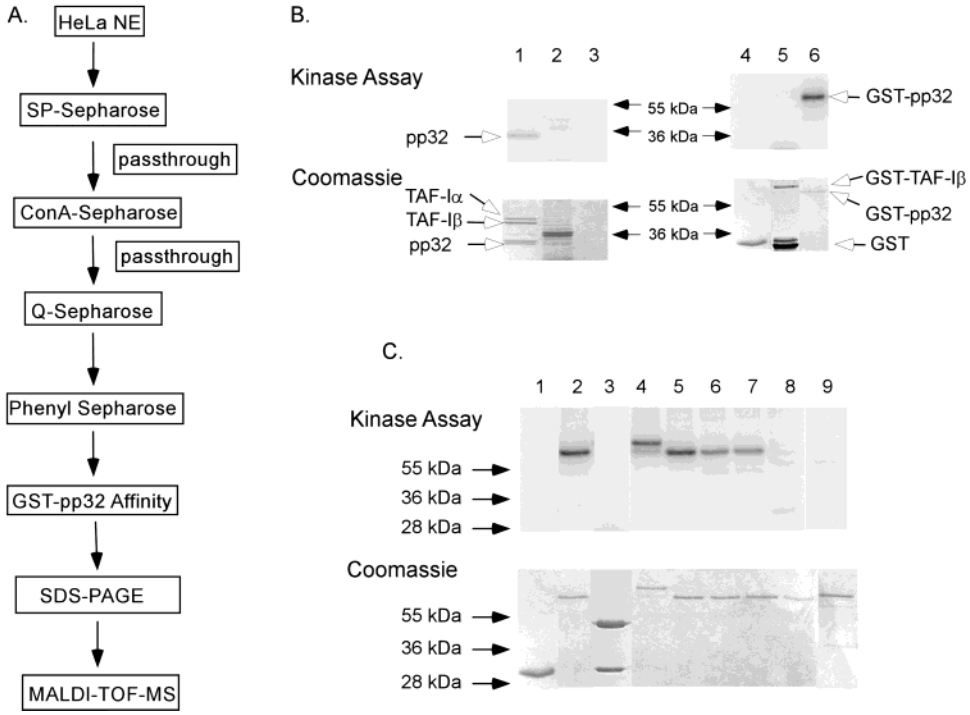

FIGURE 1: Identification of a cellular pp32-kinase and phosphorylation sites of pp32. (A) Purification scheme for pp32-kinase from HeLa cell nuclear extract is shown. ConA, Concanavalin A. (B) A partially purified kinase specifically phosphorylates pp32. In vitro kinase assays were performed using active Q fractions in the presence of [γ-^{32}P]-ATP and INHAT (lane 1), histone H1 (lane 2), no substrate (lane 3), GST (lane 4), GST-TAF−Iβ (lane 5), and GST-pp32 (lane 6) as substrates. The reaction mixtures were then resolved on SDS-PAGE and visualized by phosphorimager. The upper panel shows the phosphorimage and the lower panel shows the Coomassie staining of the substrate proteins used in the assays. The kinase activity specifically phosphorylates pp32. (C) Mapping of the phosphorylation site(s). Kinase assays similar to (B) were carried out using the following substrates: GST (lane 1), GST-pp32 (lane 2), GST-pp32-ΔC2 (lane 3), GST-pp32-S158A (lane 4), GST-pp32-S158D (lane 5), GST-pp32-S204A (lane 6), GST-pp32-S204D (lane 7), GST-pp32-S158/204A (lane 8) and GST-pp32-S158/204D (lane 9). The upper panel shows the phosphorimage and the lower panel shows the Coomassie staining of the substrate proteins used in the assays.

(S158A, S158D) or 204 (S204A, S204D) or both sites (S158/204A, S158/204D). The recombinant constructs were sequenced to verify the sites of mutation and also to determine the absence of other undesired mutations within pp32 cDNA.

To determine site specificity of phosphorylation, pp32 mutants were expressed as GST-fusions, purified, and analyzed for their ability to serve as substrates of the putative pp32-kinase. Single mutation of either Ser158 or Ser204 to alanine did not prevent phosphorylation, indicating that the kinase can phosphorylate either site (Figure 1C, lanes 4 and 6). To further investigate whether phosphorylation of one site is dependent on the phosphorylation of the other site, we generated and analyzed the aspartic acid mutants, since aspartic acid may mimic phosphorylated form of serine. The observation that there is no qualitative significant difference between the phosphorylation of Ala and Asp mutants suggests that there is no hierarchical preference and that these sites on pp32 may be independently phosphorylated by the kinase (Figure 1C, lanes 4−7). As expected, double Ala or Asp mutants were not phosphorylated by the kinase (Figure 1C, lanes 8 and 9). Coomassie staining of gels showed that equal amounts of the GST fusions of pp32 point mutants were utilized in the assay. Loss of phosphorylation in the double mutants is also consistent with the previous observation that pp32 is phosphorylated at serine residues, although it is possible that mutation of serines 158 and 204 altered the protein conformation, which led to loss of phosphorylation of other sites. Together, these results indicate that we have partially purified a pp32-specific kinase from HeLa cell nuclear extracts and identified serine residues 158 and 204 at its C-terminus as sites of phosphorylation.

Further Purification of the pp32 Kinase. We further purified the pp32-kinase following the kinase activity utilizing the purification scheme shown in Figure 1A (Table 1). Meanwhile, to gain more information about this kinase, especially its catalytic subunit, we performed in-gel kinase assays with the pooled Q fractions. This assay had been effectively utilized in identifying the catalytic subunits of protein kinases such as the histone kinase Snf1 (*26*). In our in-gel kinase assay, we utilized a pp32 peptide corresponding to its amino acid 150−167 as the substrate embedded in the gel. As a negative control, a gel with BSA embedded as substrate was also subjected to the same procedure, to monitor any nonspecific phosphorylation or autophosphorylation. Bands specifically present in the active Q fraction on the pp32 peptide gel should correspond to the potential pp32-kinase catalytic subunit. As shown in Figure 2A, a doublet with a size around 40 kDa was strongly detected in the active (lane 1) but not in inactive fraction (lane 2) or in BSA-gel. The enzymatic activity of these fractions are shown in Figure 2A, right side. This assay also indicates that the potential pp32-kinase (~40 kDa) can phosphorylate pp32 peptide without forming a complex with other proteins. The protein profile of the active fractions at different stages of purification is shown in Figure 2B. After GST-pp32 affinity purification step, one protein specifically bound to GST-pp32 with a size of about 40 kDa (Figure 2B, lane 7, marked with "*"). The in gel kinase assay (Figure 2A) and the retention

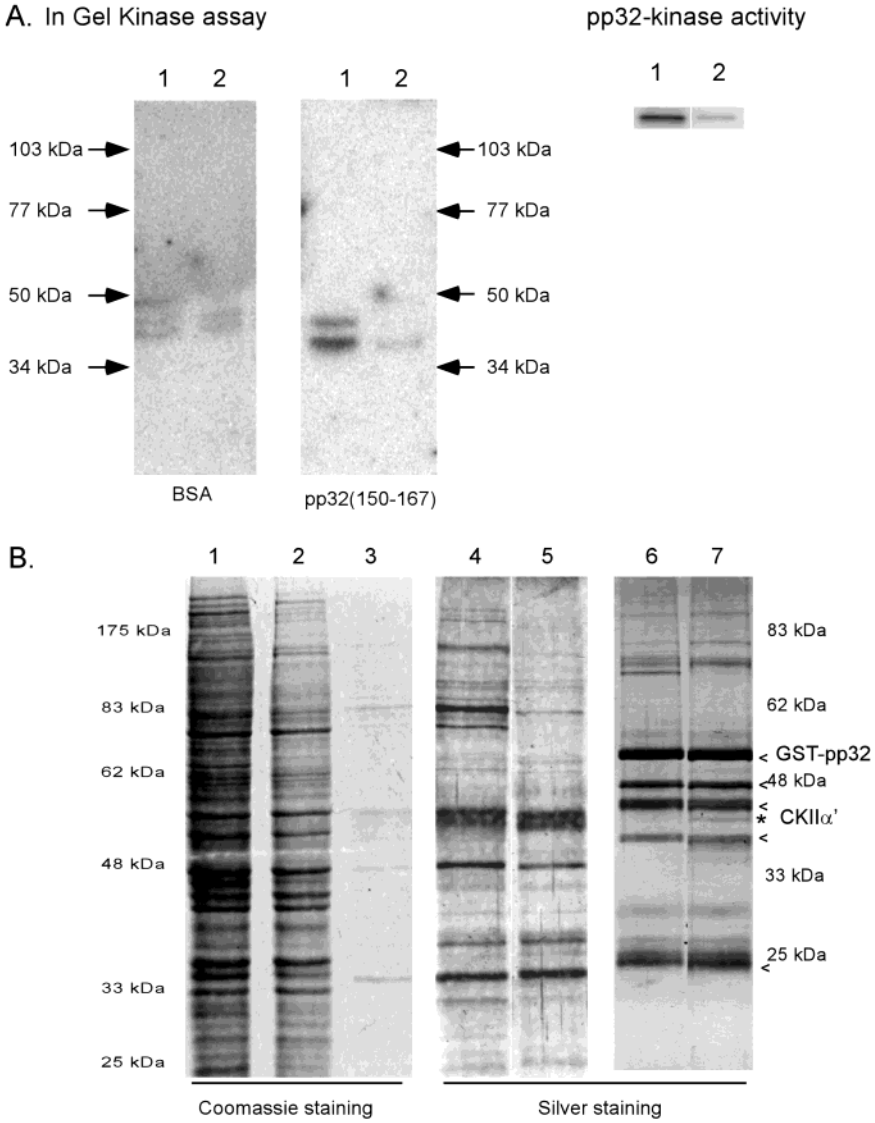

FIGURE 2: Identification of the catalytic subunit of the pp32-kinase. (A) An in-gel kinase assay demonstrates that an ~40 kDa protein is the putative catalytic subunit of the pp32-kinase. Active (lane 1) or inactive (lane 2) Q fractions were resolved on SDS-PAGE embedded with BSA (left panel) or pp32 peptide (amino acid 150−167) (right panel). Proteins were denatured and renatured on the gel and then subjected to an in-gel kinase assay in the presence of [γ-^{32}P]-ATP. Bands only present in the autoradiograph of the active fraction (lane 1) on the pp32 peptide gel represent the position of the putative catalytic subunit of the pp32-kinase. The kinase activities of the two Q fractions are shown on the right side. (B) Protein profile after each purification step. The Coomassie or silver staining of the protein samples for HeLa cell nuclear extract (lane 1), the pass-through from ConA-SP Sepharose column (lane 2), pooled active fractions from Q Sepharose column (lanes 3 and 4) and Phenyl Sepharose column (lane 5), GST-pp32 only (lane 6), and GST-pp32 with bound proteins (lane 7) are shown. Bands marked with "<" indicate possible degradation products of GST-pp32. Band marked with "*" represents the protein that specifically bound to GST-pp32 during this affinity purification step. This protein (*) was identified as α′ subunit of casein kinase II by MALDI-TOF mass spectrometry.

of a ~40 kDa protein in the final GST-pp32 affinity column (Figure 2B) are consistent with each other and together indicate that we have purified the putative catalytic subunit of the pp32-kinase. The protein band of ~40 kDa was therefore excised from a preparative silver-stained gel and subjected to MALDI-TOF mass spectrometric sequencing. This band was identified as the α′ subunit of casein kinase II (CKII).

Casein Kinase II is a Cellular pp32-Kinase. On the basis of the identification of CKIIα′ by MALDI-TOF mass spectrometry, further analyses were carried out to determine whether CKII is the pp32-kinase. First, the amino acid sequence surrounding Ser158 (*S*DAE) and Ser204 (*S*GEE) matched the consensus substrate sequence of CKII: S/T-XXE. Second, using anti-CKII antibodies, we detected all three CKII subunits α, α′ and β in the active Q fractions, consistent with their pp32-kinase activity (data not shown). α and α′ subunits are the catalytic subunits of CKII, with a size of around 40 kDa, consistent with the 40 kDa doublet we detected in our in-gel kinase assay (Figure 2A). Third, we utilized commercially available pure CKII enzyme in the pp32-phosphorylation assay. In this assay, like the pp32-kinase preparation (Figure 3A, upper panel), CKII specifically phosphorylated GST-pp32 (Figure 3A, lower panel, lane 2) or pp32 of the INHAT complex (lane 4) but failed to phosphorylate pp32 S158/204D mutant (lane 3).

CKII is a ubiquitously expressed Ser/Thr protein kinase. Different from most other Ser/Thr kinases, CKII is not sensitive to the general kinase inhibitor staurosporine, and it can use GTP as well as ATP as phosphate donor. To further

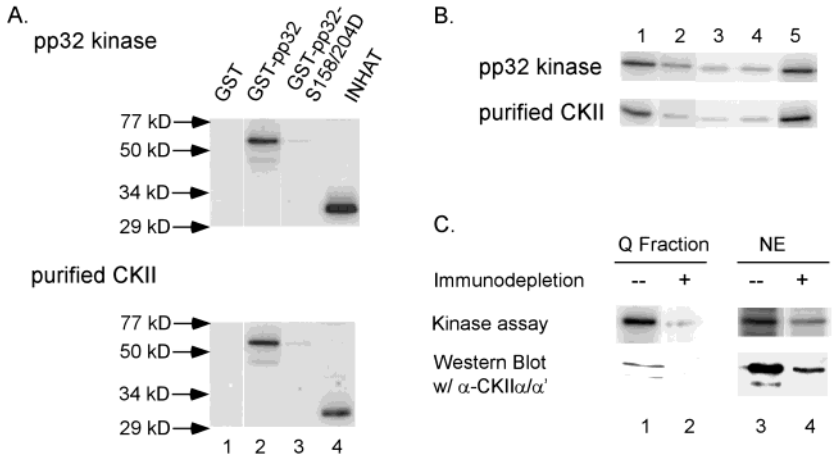

FIGURE 3: CKII is the pp32-kinase. (A) Substrate and site specificity of the pp32-kinase and pure CKII. Kinase assays were performed with partially purified pp32-kinase Q fraction (upper panel) or commercially available CKII (1.2 units) (lower panel) with GST (lane 1), GST-pp32 (lane 2), GST-pp32-S158/204D (lane 3), or purified INHAT complex (lane 4) as substrates in the presence of [γ-^{32}P]-ATP. Proteins were then resolved on SDS-PAGE and visualized by phosphorimager. (B) Inhibitors of CKII also inhibit the pp32-kinase. Kinase assays as in (A) were performed with recombinant GST-pp32 as substrate in the absence (lane 1) or presence of different kinase inhibitors: 50 μM DRB (lane 2), 10 μg/mL heparin (lane 3), 100 μM GTP (lane 4), and 0.5 ng/mL staurosporine (lane 5). pp32-kinase is sensitive to inhibitors of CKII. C. Immunodepletion of CKII α/α' reduces pp32-kinase activity. The active Q fraction (lanes 1 and 2) or HeLa cell nuclear extract (lanes 3 and 4) was immunodepleted (3−5 cycles) with anti-CKII α/α' antibody (lane 2 and lane 4). The supernatant was subjected to in vitro kinase assay using GST-pp32 as a substrate and visualized by phosphorimager (upper panel). The supernatant used in the assays was also subjected to immunoblotting using anti-CKII α/α' antibody (lower panel). CKII is the predominant cellular pp32-kinase.

Table 1: Purification of the pp32 Kinase: 280 mg of HeLa Cell Nuclear Extract Were Used as the Starting Material for the Purification

fractions	protein amount (mg)	total activity recovered (units[a])	specific activity (units[a]/mg)	fold purification
1. SP-ConA Sepharose	224	N. D.[b]	N. D.	N. D.
2. Q-Sepharose	5	4.0×10^3	8.0×10^2	N. D.
3. Phenyl Sepharose	0.5	2.4×10^3	4.8×10^3	6.0
4. GST-pp32 affinity	<0.5 ug	1.7×10^2	3.4×10^5	4.3×10^2

[a] Fractions were assayed with [γ-^{32}P]-ATP in the absence or presence of saturating amount of GST-pp32. The reaction mixture was then separated on SDS-PAGE, and the phosphorylation of GST-pp32 was monitored and quantitated with phosphorimager analysis with a known amount of pure CKII as the control. The unit of pp32 kinase activity was defined as the amount of the kinase needed to transfer 1 picomole of phosphate per minute at 30 °C, using GST-pp32 as the substrate. [b] N. D., activity not determined during the early stages of purification.

demonstrate that the phosphorylation of pp32 is due to the activity of CKII, we performed the pp32-phosphorylation assays in the presence of CKII inhibitors, heparin or DRB (5,6-dichloro-1-β-D-ribofuranosylbenzimidazole). As a control, we also included staurosporine that inhibits PKA-mediated phosphorylation. As shown in Figure 3B, both pure CKII and the pp32 kinase were sensitive to DRB (lane 2) and heparin (lane 3) but not to staurosporine (lane 5). When the kinase assays were performed in the presence of excess amount of unlabeled GTP, the incorporation of ^{32}P from ATP into the substrate was reduced for CKII as well as for the pp32 kinase (lane 4). Therefore, the pp32 kinase behaved similarly to the pure CKII in inhibitor assays. These data further indicate that the phosphorylation of pp32 is indeed due to the activity of CKII.

The identity of the cellular pp32 kinase as CKII was further confirmed by immunodepletion studies with the Q fraction containing peak pp32 kinase activity or total HeLa cell nuclear extract. In this experiment, anti-CKIIα/α' antibody was added to the sample and collected with protein A agarose beads. After multiple rounds of immunodepletion, the supernatant was subjected to in vitro pp32-phosphorylation assays as well as immunoblotting with anti-CKIIα/α' antibody. For the Q fraction, after successful removal of CKIIα/α', over 90% of the kinase activity was depleted from the fraction (Figure 3C, compare lane 1 with lane 2). For HeLa cell nuclear extract, partial removal of CKIIα/α' was also accompanied by a significantly reduced kinase activity (Figure 3C, compare lane 3 with lane 4). Taken together, our data strongly indicate that CKII represents the predominant, if not all, pp32-kinase activity in the HeLa cell nuclear extract.

pp32 is Phosphorylated in Vivo. After demonstrating that CKII is the major kinase phosphorylating pp32 on Ser158 and Ser204 in vitro, it was critical to examine whether pp32 is phosphorylated in vivo. For this purpose, we generated phospho-specific antibodies in rabbits using phospho-serine 158 or 204 peptide of pp32. We further affinity-purified these antibodies using immobilized peptides and characterized two antibodies that recognize phosphorylated pp32: ab585, specific for pp32 phospho-serine 158; and ab583, specific for pp32 phospho-serine 204. For the characterization of these antibodies, GST-pp32 was incubated with an active Q fraction in the absence or presence of unlabeled ATP. The proteins were subjected to immunoblotting using ab585 or ab583. ab585 showed about 2−3-fold higher affinity toward phosphorylated GST-pp32 compared to nonphosphorylated GST-pp32 (Figure 4A, compare lane 1 with lane 2). Similar experiments with ab583 demonstrated that ab583 is an anti-pp32 antibody with more than 10-fold higher affinity toward the phosphorylated form of GST-pp32 (Figure 4B, compare lane 1 with lane 2). As a control, a commercially available pp32 antibody (Santa Cruz Biotechnology) α-pp32 recognized pp32 independent of phosphorylation status (Figure 4C, compare lane 1 with lane 2). These results together indicate that these antibodies can be used to study pp32 phosphorylation in vivo.

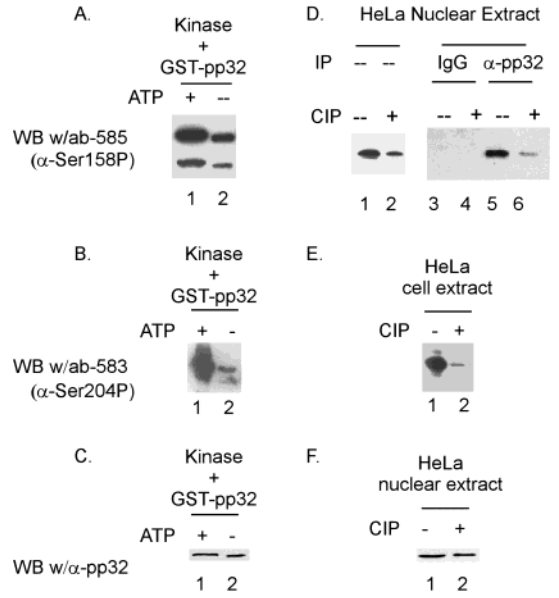

FIGURE 4: Generation and use of pp32 antibodies demonstrate that pp32 is phosphorylated in vivo. (A) ab585 recognizes phosphorylated pp32 with higher affinity. GST-pp32 was incubated with the pp32-kinase in the presence (lane 1) or absence (lane 2) of unlabeled ATP. After kinase reaction, proteins were subjected to SDS-PAGE and immunoblotting using ab585. (B) ab583 has significantly higher affinity toward phosphorylated pp32. Kinase and immunoblot assays as in (A) were performed, except that ab583 was used in immunoblotting. (C) Commercial α-pp32 antibody recognizes pp32 independent of its phosphorylation status. Kinase and immunoblot assays as in (A) were performed, except that a commercially available pp32 antibody (α-pp32) was used. (D) pp32 is phosphorylated in vivo at Ser158. HeLa cell nuclear extract (lanes 1 and 2) or its immuno-precipitates with control IgG (lanes 3 and 4) or commercially available α-pp32 antibody (lanes 5 and 6) was subjected to CIP treatment (lanes 2, 4, and 6) and then to immunoblotting using ab585. (E) pp32 is phosphorylated in vivo at Ser204. HeLa cell extract was subjected to CIP treatment (lane 2) and then to immunoblotting using ab583. (F) α-pp32 antibody recognizes HeLa pp32 independent of its phosphorylation status. HeLa cell nuclear extract was subjected to CIP treatment (lane 2) and then to immunoblotting using α -pp32 antibody. Use of ab583, ab585, and α-pp32 antibodies establishes that pp32 is phosphorylated in vivo at Ser158 and Ser204.

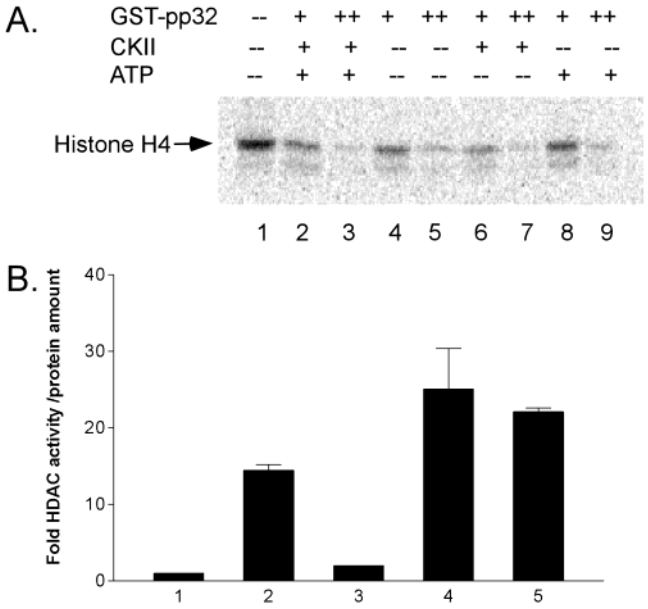

FIGURE 5: Altered function of pp32 mutants. (A) Analysis of CKII-mediated phosphorylation on the HAT inhibitory activity of pp32 in vitro. Recombinant p300 was incubated with histone H4 and ^{14}C-labeled acetyl-CoA in the presence of increasing amounts of GST-pp32 alone (lanes 4 and 5) or GST-pp32 that was previously incubated with the kinase (lanes 6 and 7), ATP (lanes 8 and 9), or both (lanes 2 and 3). After reaction, proteins were resolved on SDS-PAGE and acetylation of histones was visualized by phosphorimager. Phosphorylation of GST-pp32 was confirmed (data not shown). CKII-mediated phosphorylation has minimal or no effect on the "histone masking" activity of pp32. (B) Analysis of histone deacetylase activity retention by pp32 mutants. GST (lane 1), GST-pp32 (lane 2), GST-pp32-ΔC2 (lane 3), GST-pp32-S158/204A (lane 4) and GST-pp32-S158/204D (lane 5) were incubated with HeLa cell nuclear extracts and collected by glutathione-sepharose beads. The beads-bound HDAC activity was measured as described in Experimental Procedures. HDAC activities were normalized against the amounts of the protein used with the activity pulled down by the GST protein set at 1. Mutants of pp32 retain relatively higher HDAC activity.

To test whether pp32 is in vivo phosphorylated, nuclear extract from HeLa cells grown under normal conditions (10% FBS and 5% CO_2) was treated with CIP (Calf Intestine Phosphatase) followed by immunoblotting with these three antibodies. CIP treatment greatly reduced the level of pp32 recognized by ab585 (Figure 4D, compare lane 1 with lane 2), suggesting that pp32 is phosphorylated at Ser158 in vivo. CIP treatment was also performed on the immunoprecipitates of control IgG (lanes 3 and 4) or α-pp32 antibody (lanes 5 and 6) from HeLa cell nuclear extract followed by immunoblotting using ab585. The results also indicate that pp32 is phosphorylated at Ser158 in vivo (Figure 4D, compare lane 5 with lane 6). Similar experiments were also performed using ab583, indicating that pp32 is also phosphorylated at Ser204 in vivo (Figure 4E, compare lane 1 with lane 2). As a control, the total amount of pp32 (detected by α-pp32) did not change upon CIP treatment (Figure 4F, compare lane 1 with lane 2). These results demonstrate that pp32 is phosphorylated in vivo at Ser158 and Ser204.

Altered Function of pp32-Mutants. After identifying the pp32-kinase and verifying that pp32 is phosphorylated in vivo on Ser 158 and 204 as determined by immunoblotting, we further examined the potential role of phosphorylation and serines 158 and 204 on the functions of pp32. We have previously shown that pp32, like other subunits of INHAT, associates with histones and prevents them from serving as substrates for histone acetyltransferases p300/CBP and PCAF, a process we termed "histone masking" (*13*). The histone binding and HAT inhibitory activity of pp32 resides in the C-terminus of the protein (*14*). Because specific phosphorylation of pp32 also occurs on the serine residues at the C-terminus, we examined whether phosphorylation may play a regulatory role in pp32's ability to bind histones and function as a HAT inhibitor. For this purpose, HAT assays were performed utilizing GST-pp32 that was phosphorylated in vitro by CKII in the presence of ATP. The incorporation of phosphate group into GST-pp32 was confirmed in a parallel experiment (data not shown). Both unphosphorylated and in vitro phosphorylated GST-pp32 inhibited acetylation of histone H4 by p300 in a similar dose dependent manner (Figure 5A, compare lanes 2 and 3 to lanes 4 and 5). CKII and ATP alone had no effect (lanes 6−9). These results show that the HAT inhibitory activity of pp32 is not altered by its CKII-mediated phosphorylation in vitro.

We have recently shown that pp32 can associate with histone deacetylase (HDAC) activity and repress transcription when targeted to promoters in living cells (*15*). We therefore examined whether the HDAC activity is differentially recruited by wild-type pp32 and its ΔC2, S158/204A, and S158/204D mutants. For this purpose, approximately equal amounts of recombinant GST fusion of pp32 and its mutants S158/204A and S158/204D were incubated separately with HeLa cell nuclear extract and collected with glutathione-sepharose beads. The beads-bound proteins were then subjected to HDAC assays. Data are presented as fold HDAC activity normalized against protein amount with the value for GST set as 1 (Figure 5B). Compared to GST alone, GST-pp32 pulled down 15−20-fold more HDAC activity from HeLa cell nuclear extract (compare lane 1 and lane 2). Deletion of the C-terminus of pp32 in the ΔC2 mutant resulted in a loss of the associated HDAC activity (lane 3). Additionally, both GST-pp32-S158/204A and GST-pp32-S158/204D consistently pulled down 20−30% percent more HDAC activity than GST-pp32 wild type (compare lanes 4 or 5 to lane 2), suggesting a role for serines 158 and/or 204 in the HDAC-association function of pp32.

DISCUSSION

CKII (casein kinase II) is a ubiquitously expressed and constitutively active Ser/Thr protein kinase. More than 300 substrates have been identified for this kinase in vitro, although much less is known about its physiological function with many of the substrates (*27*). The presence of numerous CKII substrates in the cells as well as the involvement of CKII in many general processes such as transcription and translation (*28, 29*) also poses challenges to in vivo studies on CKII and its individual substrate. In this work, we show that pp32, a subunit of the INHAT complex, is phosphorylated in vitro by a biochemically purified pp32 kinase that contained CKII as its catalytic subunit. Our results identify Ser158 and Ser204 as the likely sites of phosphorylation. Based on our work, we propose that CKII could be the predominant cellular kinase that phosphorylates pp32 on serines 158 and 204. First, utilization of a kinase assay-driven biochemical fractionation led to the purification and subsequent characterization of CKII as the pp32 kinase. Second, the sequences surrounding Ser158 and Ser204 match perfectly to the CKII consensus target site. Accordingly, mutation of these sites to Ala or Asp decreased (single mutation) or abolished (double mutation) phosphorylation, indicating that they indeed served as the CKII target sites. It's interesting to note that these two sites are independently phosphorylated by CKII. This is in contrast to situations where phosphorylation of one site precedes and influences the phosphorylation of the other site (*30, 31*). Third, the generation and use of phospho-specific antibodies suggest that pp32 is phosphorylated in vivo at these two sites. Together with the immunodepletion studies, our results suggest that CKII likely represents the predominant if not the only, pp32 kinase activity in HeLa cell nuclear extract. Our phospho-specific antibodies should serve as useful tools to identify the existence of other pp32-kinases, should they exist.

CKII is a constitutively active protein kinase, which probably contributes significantly to constitutive phosphorylation of endogenous pp32. Consequently, it is possible that events leading to constitutive phosphorylation of pp32 may have minor or minimal effect on its function. For example, we observed that in vitro phosphorylation of recombinant pp32 by CKII had no effects on its histone binding and histone acetyltransferase inhibitory activity. However, it appears that CKII target sites (serines 158 and 204) may have a modulatory role in the pp32-function. Our data from HDAC activity association assays suggest that serines 158 and 204 or their phosphorylation may modulate pp32-associated HDAC activity and its transcriptional repression function. Further study is needed to validate this possibility.

CKII has been shown to be involved in processes of cell survival and neoplasia (*27, 32*). For example, CKII functions as an anti-apoptotic protein by phosphorylating the pro-apoptotic protein Bid, thus rendering it resistant to cleavage by caspase 8 in cell death pathways induced by Fas ligand (*33*). In UV-triggered cell death pathway, CKII phosphorylates IκB in a UV-inducible manner. The inhibition of this phosphorylation prevents the degradation of IκB and increases cell death (*34*). pp32 has recently been shown to have pro-apoptotic function upon UV induction of apoptosis (*12*). Because both proteins are involved in cell growth and death and pp32 is phosphorylated by CKII, phosphorylation of pp32 by CKII could play a role in apoptosis-related pathways.

pp32 is primarily localized in the nucleus, although minor cytoplasmic localization was also reported (*3, 10*). A putative nuclear targeting sequence was found at the C-terminus of the protein buried in the polyacidic region, close to the phosphorylation sites of pp32 (*3*). Therefore, another possible function for pp32 phosphorylation may be to regulate its subcellular localization, as exemplified by CtBP, whose cellular distribution is regulated through phosphorylation by Pak1 (*20*).

In summary, pp32 is an important multitasking protein involved in critical physiological pathways. Our discovery that pp32 is indeed phosphorylated in vivo at Ser158 and Ser204 and CKII is likely the cellular pp32 kinase, coupled with the generation of antibodies with higher affinity for phospho-pp32, should provide key information and tools necessary for future studies on the role of phosphorylation in pp32 function. Since pp32 is constitutively phosphorylated on serines 158 and 204 by CKII in living cells, cellular phosphatase activities may play a role in regulating various functions of pp32. Our study provides the framework for identification of pp32 phosphatase(s) and the in vivo roles of phosphorylation/dephosphorylation in regulating the pp32-function.

ACKNOWLEDGMENT

We thank Drs. Marc Schwartz and Shelley Berger of Wistar Institute for antibody purification protocol, and National Cell Culture Center, MN for large-scale culture of HeLa cells.

REFERENCES

1. Vaesen, M., Barnikol-Watanabe, S., Gotz, H., Awni, L. A., Cole, T., Zimmermann, B., Kratzin, H. D., and Hilschmann, N. (1994) Purification and characterization of two putative HLA class II associated proteins: PHAPI and PHAPII, *Biol. Chem. Hoppe-Seyler 375*, 113−126.

2. Chen, T. H., Brody, J. R., Romantsev, F. E., Yu, J. G., Kayler, A. E., Voneiff, E., Kuhajda, F. P., and Pasternack, G. R. (1996) Structure of pp32, an acidic nuclear protein which inhibits oncogene-induced formation of transformed foci, *Mol. Biol. Cell 7*, 2045−2056.
3. Matsuoka, K., Taoka, M., Satozawa, N., Nakayama, H., Ichimura, T., Takahashi, N., Yamakuni, T., Song, S. Y., and Isobe, T. (1994) A nuclear factor containing the leucine-rich repeats expressed in murine cerebellar neurons, *Proc. Natl. Acad. Sci. U. S .A. 91*, 9670−9674.
4. Mencinger, M., Panagopoulos, I., Contreras, J. A., Mitelman, F., and Aman, P. (1998) Expression analysis and chromosomal mapping of a novel human gene, APRIL, encoding an acidic protein rich in leucines, *Biochim. Biophys. Acta 1395*, 176−180.
5. Ulitzur, N., Humbert, M., and Pfeffer, S. R. (1997) Mapmodulin: a possible modulator of the interaction of microtubule-associated proteins with microtubules, *Proc. Natl. Acad. Sci. U. S. A. 94*, 5084−5089.
6. Walensky, L. D., Coffey, D. S., Chen, T. H., Wu, T. C., and Pasternack, G. R. (1993) A novel M(r) 32 000 nuclear phosphoprotein is selectively expressed in cells competent for self-renewal, *Cancer Res. 53*, 4720−4726.
7. Kadkol, S. S., Brody, J. R., Epstein, J. I., Kuhajda, F. P., and Pasternack, G. R. (1998) Novel nuclear phosphoprotein pp32 is highly expressed in intermediate- and high-grade prostate cancer, *Prostate 34*, 231−237.
8. Kadkol, S. S., Brody, J. R., Pevsner, J., Bai, J., and Pasternack, G. R. (1999) Modulation of oncogenic potential by alternative gene use in human prostate cancer, *Nat. Med. 5*, 275−279.
9. Li, M., Makkinje, A., and Damuni, Z. (1996) Molecular identification of I1PP2A, a novel potent heat-stable inhibitor protein of protein phosphatase 2A, *Biochemistry 35*, 6998−7002.
10. Brennan, C. M., Gallouzi, I. E., and Steitz, J. A. (2000) Protein ligands to HuR modulate its interaction with target mRNAs in vivo, *J. Cell. Biol. 151*, 1−14.
11. Gallouzi, I. E., and Steitz, J. A. (2001) Delineation of mRNA export pathways by the use of cell-permeable peptides, *Science 294*, 1895−1901.
12. Jiang, X., Kim, H. E., Shu, H., Zhao, Y., Zhang, H., Kofron, J., Donnelly, J., Burns, D., Ng, S. C., Rosenberg, S., and Wang, X. (2003) Distinctive roles of PHAP proteins and prothymosin-alpha in a death regulatory pathway, *Science 299*, 223−226.
13. Seo, S. B., McNamara, P., Heo, S., Turner, A., Lane, W. S., and Chakravarti, D. (2001) Regulation of histone acetylation and transcription by INHAT, a human cellular complex containing the set oncoprotein, *Cell 104*, 119−130.
14. Seo, S. B., Macfarlan, T., McNamara, P., Hong, R., Mukai, Y., Heo, S., and Chakravarti, D. (2002) Regulation of histone acetylation and transcription by nuclear protein pp32, a subunit of the INHAT complex, *J. Biol. Chem. 277*, 14005−14010.
15. Kutney, S. N., Hong, R., Macfarlan, T., and Chakravarti, D. (2004) A signaling role of histone binding proteins and INHAT subunits pp32 and Set/TAF−Ibeta in integrating chromatin hypoacetylation and transcriptional repression, *J. Biol. Chem. 279*, 30850−30855.
16. Chakravarti, D., and Hong, R. (2003) SET-ting the stage for life and death, *Cell 112*, 589−591.
17. Malek, S. N., Katumuluwa, A. I., and Pasternack, G. R. (1990) Identification and preliminary characterization of two related proliferation-associated nuclear phosphoproteins, *J. Biol. Chem. 265*, 13400−13409.
18. Galasinski, S. C., Resing, K. A., Goodrich, J. A., and Ahn, N. G. (2002) Phosphatase inhibition leads to histone deacetylases 1 and 2 phosphorylation and disruption of corepressor interactions, *J. Biol. Chem. 277*, 19618−19626.
19. Pflum, M. K., Tong, J. K., Lane, W. S., and Schreiber, S. L. (2001) Histone deacetylase 1 phosphorylation promotes enzymatic activity and complex formation, *J. Biol. Chem. 276*, 47733−47741.
20. Barnes, C. J., Vadlamudi, R. K., Mishra, S. K., Jacobson, R. H., Li, F., and Kumar, R. (2003) Functional inactivation of a transcriptional corepressor by a signaling kinase, *Nat. Struct. Biol. 10*, 622−628.
21. Proft, M., and Struhl, K. (2002) Hog1 kinase converts the Sko1-Cyc8-Tup1 repressor complex into an activator that recruits SAGA and SWI/SNF in response to osmotic stress, *Mol. Cell 9*, 1307−1317.
22. Ubeda, M., and Habener, J. F. (2003) CHOP Transcription Factor Phosphorylation by Casein Kinase 2 Inhibits Transcriptional Activation, *J. Biol. Chem. 278*, 40514−40520.
23. Carmen, A. A., Rundlett, S. E., and Grunstein, M. (1996) HDA1 and HDA3 are components of a yeast histone deacetylase (HDA) complex, *J. Biol. Chem. 271*, 15837−15844.
24. Guenther, M. G., Yu, J., Kao, G. D., Yen, T. J., and Lazar, M. A. (2002) Assembly of the SMRT-histone deacetylase 3 repression complex requires the TCP-1 ring complex, *Genes Dev. 16*, 3130−3135.
25. Nagata, K., Kawase, H., Handa, H., Yano, K., Yamasaki, M., Ishimi, Y., Okuda, A., Kikuchi, A., and Matsumoto, K. (1995) Replication factor encoded by a putative oncogene, SET, associated with myeloid leukemogenesis, *Proc. Natl. Acad. Sci. U. S. A. 92*, 4279−4283.
26. Lo, W. S., Duggan, L., Tolga, N. C., Emre, Belotserkovskya, R., Lane, W. S., Shiekhattar, R., and Berger, S. L. (2001) Snf1: A histone kinase that works in concert with the histone acetyltransferase Gcn5 to regulate transcription, *Science 293*, 1142−1146.
27. Meggio, F., and Pinna, L. A. (2003) One-thousand-and-one substrates of protein kinase CK2?, *FASEB J. 17*, 349−368.
28. Ballesta, J. P., Rodriguez-Gabriel, M. A., Bou, G., Briones, E., Zambrano, R., and Remacha, M. (1999) Phosphorylation of the yeast ribosomal stalk. Functional effects and enzymes involved in the process, *FEMS Microbiol. Rev. 23*, 537−550.
29. Krogan, N. J., Kim, M., Ahn, S. H., Zhong, G., Kobor, M. S., Cagney, G., Emili, A., Shilatifard, A., Buratowski, S., and Greenblatt, J. F. (2002) RNA polymerase II elongation factors of *Saccharomyces cerevisiae*: A targeted proteomics approach, *Mol. Cell. Biol. 22*, 6979−6992.
30. Fiol, C. J., Mahrenholz, A. M., Wang, Y., Roeske, R. W., and Roach, P. J. (1987) Formation of protein kinase recognition sites by covalent modification of the substrate. Molecular mechanism for the synergistic action of casein kinase II and glycogen synthase kinase 3, *J. Biol. Chem. 262*, 14042−14048.
31. Fiol, C. J., Wang, A., Roeske, R. W., and Roach, P. J. (1990) Ordered multisite protein phosphorylation. Analysis of glycogen synthase kinase 3 action using model peptide substrates, *J. Biol. Chem. 265*, 6061−6065.
32. Ahmed, K., Gerber, D. A., and Cochet, C. (2002) Joining the cell survival squad: An emerging role for protein kinase CK2, *Trends Cell Biol. 12*, 226−230.
33. Desagher, S., Osen-Sand, A., Montessuit, S., Magnenat, E., Vilbois, F., Hochmann, A., Journot, L., Antonsson, B., and Martinou, J. C. (2001) Phosphorylation of bid by casein kinases I and II regulates its cleavage by caspase 8, *Mol. Cell. 8*, 601−611.
34. Kato, T., Jr., Delhase, M., Hoffmann, A., and Karin, M. (2003) CK2 Is a C-Terminal IkappaB Kinase Responsible for NF-kappaB Activation during the UV Response, *Mol. Cell 12*, 829−839.

BI0493968