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Nopp140 Is a Mediator of the Protein Kinase A Signaling Pathway That Activates the Acute Phase Response α_1-Acid Glycoprotein Gene*

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The acute phase response (APR) in liver during inflammation is one of the well known examples for elucidating the signaling pathways that lead to the combinatorial regulation of gene expression. The APR is exemplified by α_1-acid glycoprotein gene (*agp*) expression. A number of transcription factors, including CCAAT/enhancer-binding protein β (C/EBPβ), glucocorticoid receptor, cAMP-response element-binding protein (CREB), and Nopp140, are known to participate in its induction. The underlying mechanism of Nopp140 and other factors for regulating *agp* expression remains unclear. Here we demonstrate that protein kinase A (PKA)-dependent phosphorylation of Nopp140, together with C/EBPβ, induces *agp* gene expression synergistically. The cooperative activation of the *agp* gene by Nopp140 and forskolin is sensitive to inhibition by PKI. Results from biochemical and functional characterizations of Nopp140 mutants defective in PKA phosphorylation sites suggest that PKA-dependent Nopp140 phosphorylation is important for its role in *agp* gene activation. Furthermore, maximal activation of the *agp* gene by PKA-phosphorylated Nopp140 depends on the presence of CREB and C/EBPβ. The participation of CREB in the activation is, however, independent of its PKA-mediated phosphorylation. In summary, we demonstrate the existence of a novel Nopp140-mediated PKA signaling pathway that leads to the activation of *agp*, one of the major acute phase response genes.

α_1-Acid glycoprotein (AGP)[1] belongs to a member of acute phase response proteins (1, 2). Its gene expression is markedly increased in liver during acute inflammation or by treatment with IL-1, IL-6, glucocorticoids, or lipopolysaccharide (3–5). The induction is mainly attributed to regulations at both transcriptional and posttranscriptional levels (6, 7). We have previously demonstrated that both positive and negative factors are involved in transcriptional control of the *agp* gene (5, 8–12). Glucocorticoid receptor and C/EBPβ are strong positive factors that cooperatively mediate *agp* gene expression (13, 14), whereas nucleolin is a negative factor involved in its regulation (12).

cAMP-dependent eukaryotic gene transcription is critical for glucose homeostasis (15–17). Many cAMP-response genes possess regions corresponding to the consensus sequence called cAMP-responsive elements (CREs) (18, 19). CRE binding protein (CREB) was initially found to bind this element (20). Phosphorylation of CREB at Ser^{133} by PKA has been shown to mediate the expression of numerous genes (21–24). One key regulator of the *agp* gene, C/EBPβ, is also regulated by CREB during liver regeneration (25). Although it has been demonstrated that C/EBPβ is phosphorylated in response to cAMP (26), *in vitro* phosphorylation of C/EBPβ at Ser^{105} by PKA has no effect on its DNA binding activity (27). In addition to cytokines and other inducing agents, it has also been shown that AGP mRNA levels were coincidentally increased with cAMP levels in alveolar macrophages upon PGE2 treatment (28).

We have previously reported that the nucleolar phosphoprotein, Nopp140, is a coactivator of C/EBPβ-mediated *agp* gene expression (8, 11). Nopp140 was originally defined as a shuttle protein between nucleolus and cytoplasm (29). Its alternating positively and negatively charged repeat domains have also been described for targeting to the coiled bodies through p80 coilin interaction during *de novo* synthesis (30). Two classes of small nucleolar ribonuleoprotein particles and the largest subunit of RNA polymerase I can be coimmunoprecipitated with Nopp140 (31, 32). These data imply that Nopp140 may be involved in the nucleologenesis and rRNA gene transcription. Recent studies by Isaac *et al.* (33) indicate that overexpression of Nopp140 leads to the presence of a nuclear endoplasmic reticulum-like structure (R-rings) in COS cells. R-rings are the unique membrane cisternae distinct from nuclear envelope, nucleoli, or coiled bodies. Immunofluorescence staining showed that Nopp140 seems to redirect several of its associated proteins, like fibrillarin, NAP57, and p80 coilin to this structure (32). Despite these results, the functional roles of Nopp140 in regulation of gene expression remain to be determined. Although Nopp140 has been demonstrated as a casein kinase II (CKII)-interacting protein and to be phosphorylated by CKII *in vitro* (34), other potential kinases that regulate its biological activities remain unknown. Earlier evidence has shown that a phosphoprotein pp135 (*i.e.* Nopp140) as well as nucleolin undergo extensive phosphorylation when rats were treated with isoprenaline to stimulate PKA activity (35). Thus, Nopp140 may play some roles in cAMP-dependent signaling pathway.

In this study, we present data showing that Nopp140 can regulate *agp* gene expression in a PKA-dependent manner. We

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[1] The abbreviations used are: AGP, α_1-acid glycoprotein; PKA, cAMP-dependent protein kinase; CRE, cAMP-responsive element; CREB, cAMP-responsive element binding protein; C/EBPβ, CCAAT/enhancer-binding protein β; CKII, casein kinase II; IL, interleukin; LPS, lipopolysaccharide; TFIIB, transcription factor IIB; BHK, baby hamster kidney; CAT, chloramphenicol acetyltransferase; LC, liquid chromatography; MS, mass spectrometry; HPLC, high pressure liquid chromatography; GST, glutathione *S*-transferase.

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report that forskolin and Nopp140 activate the AGP promoter synergistically. Furthermore, Nopp140 can serve as a substrate for PKA *in vitro* and *in vivo*. Mutation of these phosphorylation sites reduces the synergistic activation of *agp* gene expression by Nopp140 and forskolin. Since the induction is promoter-specific and cis-element-dependent and is further activated in the presence of both CREB and C/EBPβ, these data suggest a novel regulatory mechanism exerted by Nopp140 to modulate *agp* gene transcription.

EXPERIMENTAL PROCEDURES

Plasmids—The wild type as well as the mutant AGP-CAT constructs, CMV-C/EBPβ (LAP), and CMV-LIP were described previously (9). CMV-Nopp140, pRSET-Nopp140, and GST-Nopp140 constructs were as described (11). Plasmids expressing CREB were constructed by inserting its cDNA into pRSET 2B vector (Invitrogen). cDNA of CKIIα was obtained by reverse transcription-PCR and cloned into pCRII TA vector (Invitrogen). The recombinant cDNA from pCRII was then subcloned into FLAG cloning plasmid. cDNA along with the FLAG sequence were subsequently excised and cloned into CMV plasmid (Promega). TFIIB expression plasmid was a generous gift from Dr. B. Emerson (The Salk Institute for Biological Studies, La Jolla, CA). The 3pBS-CAT reporter was obtained from Dr. Y. Lin (Academia Sinica, Taipei, Taiwan). FLAG-tagged Nopp140 (FLAG-Nopp140) was obtained by inserting the corresponding cDNA into the *Bam*HI/*Eco*RI sites of pCMV-Tag2B (Stratagene). The Nopp140 mutants (S113A, S627A, S628A, and S113A,S627A) were constructed by site-directed mutagenesis by a two-step PCR technique as described (36). To amplify the template for mutant construction, oligonucleotides 5′-C AAG CGA GCC GCT TTG CCT CAG-3′, 5′-G AAA AGG GCA GCT TCC CCT TT-3′, 5′-A AGG GCA TCT GCC CCT TTC CG-3′, and their corresponding reversed sequences were used as primers in combination with T7 and SP6, respectively. The Nopp140 (S113A,S627A) double mutant was then obtained using Nopp140 (S627A) plasmid as the template for PCR. These mutants were all confirmed by DNA sequencing. FLAG-Nopp140 (S627A) mutant was also constructed by inserting the mutant cDNA into pCMV-Tag2B vector. GST Nopp140 deletion mutants (BS, SS, and SR) were created by inserting *Bam*HI/*Sac*I, *Sac*I/*Sac*I, *Sac*I/*Eco*RI fill-in fragments of Nopp140 into the pGEX plasmids (Amersham Biosciences). The GST Nopp140 BSR construct was prepared by an in frame deletion of the internal *Sac*I/*Sac*I fragment from full-length GST Nopp140 vector.

Recombinant Proteins—Recombinant Nopp140, C/EBPβ (LAP), and CREB were expressed in *Escherichia coli* BL21 (DE3) pLysS and then purified over nickel-nitrilotriacetic acid-agarose resin (Qiagen). Recombinant TFIIB was also induced in the same *E. coli* strain and further purified through a phosphocellulose column. GST-Nopp140 BS, SS, SR, BS2, and BSR deletion clones were expressed in *E. coli* DH5α and immobilized onto glutathione-Sepharose affinity resin for kinase reaction.

Cell Culture, Transient Transfection, and Reporter Assays—Baby hamster kidney (BHK) and human embryonic kidney 293T cells were cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum (HyClone) in a 5% CO_2 incubator at 37 °C. Before transfection, the BHK or 293T cells were passaged onto 6-cm Petri dishes for growing to ~30–50% confluence. Transfection of cells was performed by calcium phosphate precipitation method (37). The amounts of CAT reporter and expression vectors for transfection were detailed in each figure legend. Transfection efficiency was normalized by co-transfection with 0.5 μg/plate of RSV-β-Gal. pCDNA3 plasmid was added to each reaction to adjust the total DNA to ~2.5 μg/plate. After the addition of DNA mixtures, the cells were incubated at 37 °C for about 24 h. To activate the PKA pathway of BHK cells, the cells were changed to Dulbecco's modified Eagle's medium containing 2% fetal bovine serum supplemented with 20 μM forskolin (Sigma) in Me_2SO. Cells were harvested 16–24 h after transfection. The whole cell lysates were used for β-galactosidase and CAT assays. The CAT activities were determined using an image analyzer (FujiX BAS1000). The relative CAT activity was normalized against β galactosidase activity. All transfection experiments were performed in duplicate and repeated at least twice.

Protein Extracts, Immunoprecipitation, and Western Blotting—FLAG-Nopp140-transfected cells were washed with phosphate-buffered saline buffer once and then treated with 50 μM forskolin in serum-free medium. After incubation for 30 min, protein lysates from forskolin-treated or untreated cells were prepared by direct lysis with 9 M urea to prevent the degradation of Nopp140 polypeptide. Subsequently, the protein extracts were diluted with binding buffer (20 mM HEPES, pH 7.9, 0.2 M NaCl, 1 mM EDTA, 1 mM EGTA, 0.25% Triton X-100, 1 mM NaF, 10 mM β-glycerophosphate, 0.1 mM Na_3VO_4, 1 mM phenylmethylsulfonyl fluoride, and 1 μg/ml leupeptin plus pepstatin) to 0.3 M urea concentration. Immunoprecipitation was performed with anti-FLAG (M2)-agarose beads (Sigma) at 4 °C, 2 h. Immunoprecipitates were collected by centrifugation and washed three times with the binding buffer, and a small aliquot was subjected to immunoblot analysis with monoclonal antibodies to FLAG and tubulin α (NeoMarkers) and polyclonal antibody to serine 133-phosphorylated CREB (Upstate Biotechnology, Inc., Lake Placid, NY). Western blot analysis was performed essentially as described previously (11) using the enhanced chemiluminescence kit (Pierce).

In Vitro Kinase Assay—Purified recombinant proteins (Nopp140, C/EBPβ, and CREB) and 1 μg of bovine serum albumin (New England Biolabs) were incubated with the kinase buffer (50 mM Tris-HCl, pH 7.5, 10 mM $MgCl_2$, 200 μM ATP, and 5 μCi of [γ-^{32}P]ATP) containing bovine PKA (Sigma). Purified Nopp140 was also incubated with kinase buffer (20 mM Tris-HCl, pH 7.5, 10 mM $MgCl_2$, 50 mM KCl, 200 μM ATP, and 5 μCi of [γ-^{32}P]ATP) containing CKII (New England Biolabs). In solid phase kinase reactions, GST-Nopp140 together with truncated mutants of the fusion protein were immobilized onto glutathione beads using TE buffer followed by washing twice with the same buffer. The beads were subsequently washed with the kinase buffer once before initiating the PKA reaction (30 °C for 30 min). The phosphorylated polypeptides were separated by SDS-PAGE and visualized by Coomassie Blue staining and autoradiography. The relative kinase activities were quantitated using image analyzer (FujiX).

Gel Mobility Shift Assay—Nuclear extracts of BHK and 293T transfected cells for the gel mobility shift assay were prepared according to the modified procedures detailed elsewhere (38). Briefly, nuclear pellets collected from hypotonic lysis procedures were extracted with the same buffer containing 0.25% or 0.1% Triton X-100 once. The extracted pellet was then used for preparation of nuclear extracts. The gel retardation assays were performed as described (5). The oligonucleotide of D motif (200 ng) was used as the probe and labeled with Klenow fragment in the presence of [α-^{32}P]dCTP. The probe (~1 ng) was incubated with 10 μg of nuclear extracts for 20 min in the presence of 0.5 μg of poly(dI-dC) (Sigma). For oligonucleotide competition assay, 50-fold molar excess of unlabeled oligonucleotide was incubated with the binding mixtures. For supershift assay, 1 μl of control antibody or monoclonal antibody against C/EBPβ was added later to the incubation. The relative shifted signals were quantitated using an image analyzer (FujiX).

Mass Spectrometry (LC/MS/MS) Analysis—GST-Nopp140 deletion constructs phosphorylated by PKA *in vitro* and FLAG-Nopp140 protein purified from 293T cells were separated by SDS-PAGE and stained with Coomassie Blue. The gel containing target polypeptides was excised and subjected to in-gel digestion with 50 ng of modified trypsin (Promega), as described previously (39). The enzyme digests were dried in a Speed-Vac (Savant) and kept at −20 °C until use. The sample was separated by capillary HPLC (ABI 140D HPLC; PerkinElmer Life Sciences) in-line coupled with the LCQ ion trap mass spectrometer (Finnigan). The mass spectra of the eluted peptides were collected by the "triple play" method (39). The acquired mass spectra were analyzed by a SEQUEST browser to correlate the MS/MS spectrum with the amino acid sequence of rat Nopp140 protein. Both an in-house program and the EXPLORE program (Finnigan) were used to identify the phosphopeptides as well as to evaluate the phosphorylation sites using the method by Tsay *et al.* (39).

RESULTS

Nopp140 and Forskolin Activate agp Gene Expression Synergistically—The cAMP/PKA pathway has been implicated in the gene expression of a number of acute phase proteins (40, 41). To explore whether the PKA pathway plays a role in regulation of the α_1-acid glycoprotein gene (*agp*), we performed transient transfection experiments. The CAT reporter plasmid containing the AGP promoter from −180 to +20 was transiently transfected into BHK cells. When the transfected cells were treated with 20 μM forskolin, the CAT activity increased (Fig. 1*A*). This activation was abrogated by coexpression of PKI, a specific inhibitor of PKA. In contrast, no such forskolin-mediated gene activation was observed when a reporter plasmid containing p53-binding elements was tested (data not

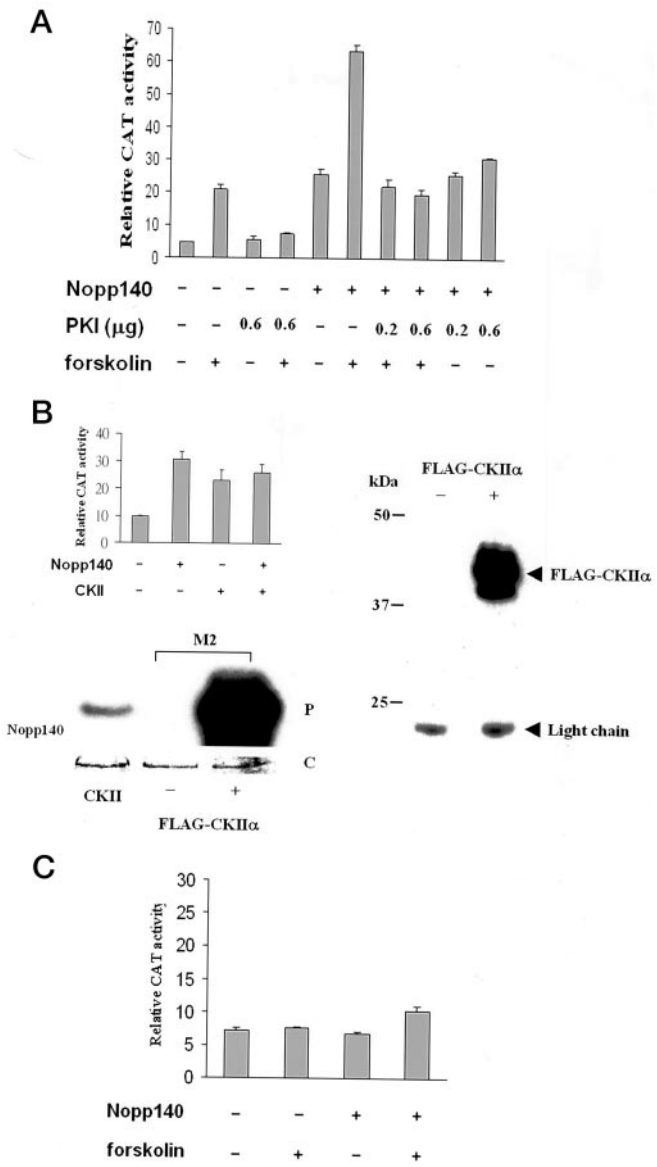

FIG. 1. **PKA-mediated Nopp140 phosphorylation affected *agp* gene activation.** *A*, BHK cells were co-transfected with AGP-CAT reporter plasmid and CMV-Nopp140 expression plasmid (0.5 μg) in the absence or presence of PKI. The amount of PKI plasmid used was indicated as 0.2 and 0.6 μg, respectively. Appropriate amounts of the vector pCDNA3 were added to each transfection mixture to maintain the total plasmid DNA at 2.6 μg/transfection. The transfected cells were either treated with Me_2SO or forskolin 16 h before harvest. The relative CAT activity was normalized with β-galactosidase activity. The activation was measured in quadruplicate assays. *B*, CMV-Nopp140 and FLAG-CKIIα (0.2-μg) expression vectors were co-transfected with AGP-CAT reporter into BHK cells. The lysates with (+) or without (−) FLAG-CKIIα co-transfection were immunoprecipitated by anti-FLAG (M2) beads and then performed solid-phase kinase assay using Nopp140 as a substrate (*lower left panel*). The Nopp140 phosphorylation was also determined by CKII kinase (2.0 units) as a control. *P*, autoradiogram signal; *C*, protein stained with Coomassie Blue. The corresponding FLAG-tagged protein was stained with anti-FLAG antibody (*right panel*). The lower band of this panel was antibody light chain. *C*, F9 cells were co-transfected with AGP-CAT reporter as well as Nopp140 as described above. The relative CAT activity was normalized with β-galactosidase activity and assayed at least twice.

shown). These results indicate that the AGP promoter could be specifically activated by the PKA signaling pathway.

To examine the possible involvement of Nopp140 in the forskolin-induced activation, we performed a transfection experiment with the Nopp140 expression vector and AGP-CAT in the presence of forskolin. When AGP-CAT was co-transfected with Nopp140, the CAT activity was activated (~4.5-fold, Fig. 1*A*). This activation was further augmented by the treatment with forskolin (12-fold, Fig. 1*A*). In addition, the synergistic effect could be reversed by an overexpression of PKI in the presence of forskolin. When we overexpressed both Nopp140 and PKI, the relative CAT activity appeared to be unaffected in the absence of forskolin treatment. To further confirm the specificity of PKA and Nopp140 co-activation of the *agp* gene, we performed co-transfection of the Nopp140 and CKIIα expression plasmid (a prominent kinase that phosphorylates Nopp140 *in vitro*). The further activation of the *agp* gene by Nopp140 was not observed by the addition of CKIIα (Fig. 1*B*, *upper left panel*). The kinase activity of transfected CKIIα was verified by immunoprecipitation with anti-FLAG (M2) beads and subsequent *in vitro* kinase assay with Nopp140 as the substrate (Fig. 1*B*, *lower left panel*). The level of FLAG-CKIIα was determined by Western blot analysis (Fig. 1*B*, *right panel*). This result suggests that the activity of CKII was not involved in the Nopp140-mediated activation of the AGP promoter. Furthermore, neither Nopp140 nor forskolin had synergistic activation effect on the p53-responsive promoter (*i.e.* 3pBS-CAT) (data not shown). When forskolin treatment was replaced by co-transfection of PKAc expression plasmid into BHK cells, the stimulation of AGP-CAT was also evident (data not shown). When we used the PKA-defective cell line F9 for the same assay, Nopp140-dependent activation was not observed (Fig. 1*C*). Together, these results indicate that functional synergism between Nopp140 and PKA is dependent on a specific promoter.

The observed synergistic activation of AGP-CAT by Nopp140 and forskolin raised the possibility that Nopp140 might be the target of PKA. This was verified by incubation of recombinant His tag Nopp140 with bovine PKA in the *in vitro* kinase assay. An intense phosphorylation signal was detected when wild-type Nopp140 was treated with PKA (Fig. 2*A*, *lane 6*). The transcription factor C/EBPβ and a general transcription factor TFIIB were also phosphorylated by PKA *in vitro*, albeit to a lesser extent (Fig. 2*A*, *lanes 5* and *7*). TFIIB is a general transcription factor that has been found to directly interact with Nopp140 *in vitro* (11). When the phosphorylation of Nopp140 and TFIIB were compared quantitatively, Nopp140 is a better substrate of PKA than TFIIB in terms of phosphorylation efficiency.

To further demonstrate that Nopp140 is an efficient substrate for PKA, the time course of Nopp140 phosphorylation by PKA has been assessed (Fig. 2*B*). The phosphorylations of CREB by PKA and of Nopp140 by CKII were included for comparison. Under the same assay conditions, bovine serum albumin was not efficiently phosphorylated by PKA. In contrast, the phosphorylation of CREB by PKA reached the plateau within 10 min, whereas the kinetics of Nopp140 phosphorylation by PKA and CKII behaves similarly (Fig. 2*B*, *upper* and *lower panels*). In terms of stoichiometry, Nopp140 appears to be a better substrate than CREB (Fig. 2*B*, *lower panel*). Nopp140 is phosphorylated to a higher level than CREB per molecule. The number of PKA phosphorylation sites of Nopp140 is at least twice as many as that of CREB, which contains a single site, Ser^{133} (21). Furthermore, the phosphorylation of Nopp140 by CKII is much better than its phosphorylation by PKA. This may reflect that the number of sites phosphorylated by CKII in Nopp140 is more than that by PKA. Taken together, these results demonstrate that forskolin and Nopp140 can activate AGP-CAT reporter synergistically. The synergism may be attributed to Nopp140 phosphorylation by PKA.

Identification of Nopp140 Residues Phosphorylated by PKA—To further explore the role of PKA-mediated phospho-

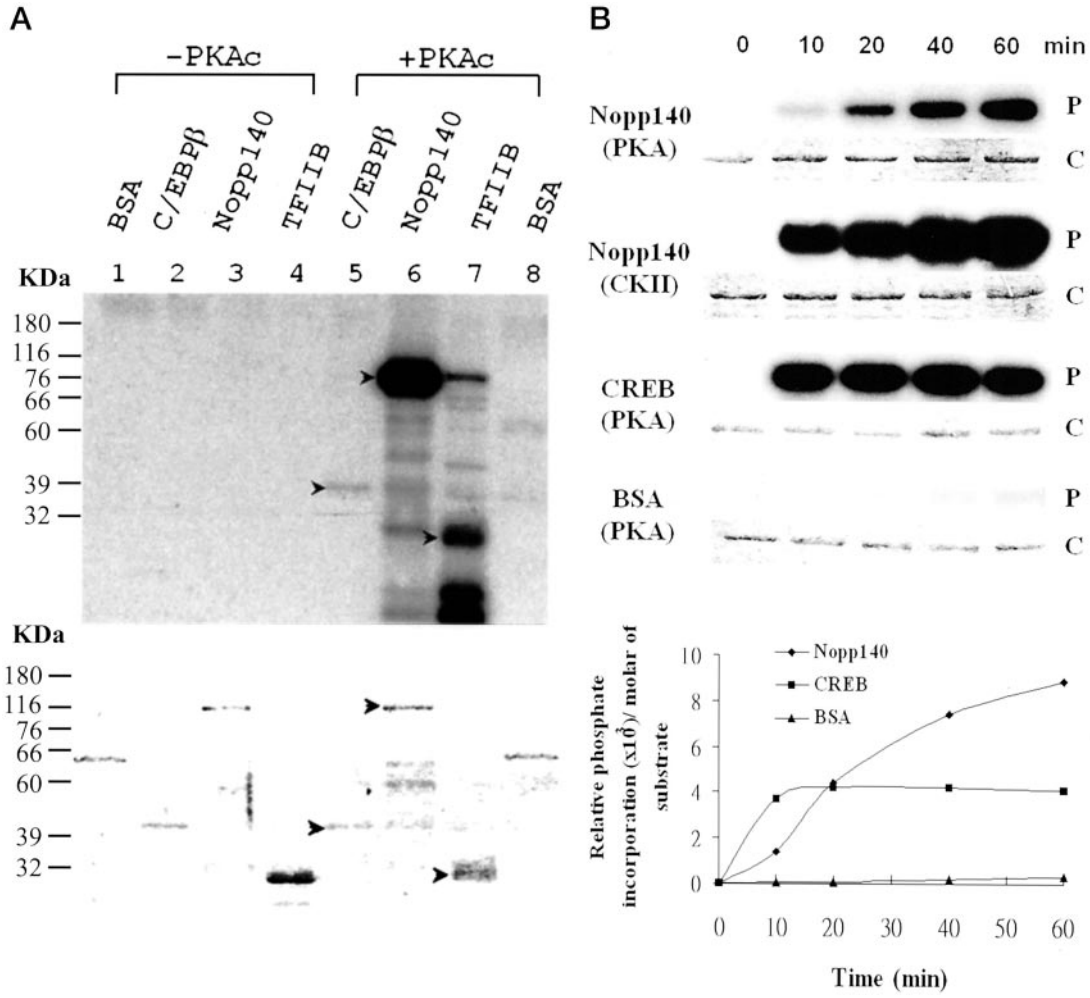

FIG. 2. **Nopp140 serves as a better substrate for PKA *in vitro*.** *A*, Nopp140 was phosphorylated by PKA *in vitro*. Recombinant C/EBPβ (*lanes 2* and *5*), Nopp140 (*lanes 3* and *6*), or TFIIB (*lanes 4* and *7*) was served as substrate for *in vitro* phosphorylation by PKAc (0.2 units). Control reactions in the absence of PKAc are shown in *lanes 1–4*. After reaction for 30 min in the presence of [γ-^{32}P]ATP at 37 °C, reactions were terminated by adding SDS sample buffer and resolved on SDS-PAGE. The gel was stained with Coomassie Brilliant Blue, destained, dried, and autoradiographed. Bovine serum albumin (*lanes 1* and *8*) was used as negative control. The same gel stained with Coomassie Blue was shown in the *lower panel*. The *arrowheads* indicate the stained positions corresponding to the radioactive bands. The molecular markers are shown on the *left side* of each *panel*. *B*, time course of phosphorylation of Nopp140 by PKA. The same amount (~0.5 μg) of Nopp140, CREB, and bovine serum albumin were phosphorylated separately by PKA *in vitro*. An identical amount of Nopp140 was also phosphorylated by CKII. The time course of phosphorylation is shown in the *upper panel*. The phosphorylation signals are presented (*P*), and the corresponding Coomassie Blue stains are shown (*C*). The relative incorporation of radioactive phosphate into Nopp140, CREB, and bovine serum albumin per molecule is quantitatively displayed in the *lower panel*.

rylation, we first employ an LC/MS/MS approach to map the phosphorylation sites of Nopp140. First, we constructed several GST-fused Nopp140 deletion constructs to locate the region(s) where phosphorylation may occur. These Nopp140 deletion constructs were immobilized to glutathione beads and then incubated with PKA. Recombinant proteins covering the regions spanning from amino acid 1 to 169 (GST-BS) and 371 to 704 (GST-SR), but not the control GST protein, were phosphorylated by PKA (Fig. 3*A*). Together, this result shows that at least two PKA phosphorylation sites are present in Nopp140. One was near the N-terminal region, whereas the other one was located at the C-terminal half. Consistent with PKA phosphorylation consensus sequence search (Fig. 3*B*, *underlined*), all four putative sites were exclusively located outside the region of GST-SS protein (Fig. 3*A*). Due to the low stability of the intact form of full-length GST-Nopp140 fusion protein during *in vitro* glutathione bead binding, these deletion constructs were used subsequently to map their phosphorylation sites.

Three GST-Nopp140 proteins, BS, BS2, and SR, were used as substrates for PKA. The phosphorylated fusion proteins were resolved by SDS-PAGE, stained with Coomassie Blue, and then excised for in-gel trypsin digestion. The tryptic peptides were analyzed by LC/MS/MS. Using the SEQUEST program for initial analysis, we had nearly 80–90% of peptide coverage (Fig. 3*B*, *boldface characters*). One candidate phosphopeptide of Nopp140 SR protein was initially screened out. It is singly phosphorylated ^{625}RASSPFRR^{632}. Inspection of the collision-induced dissociation spectrum showed that there existed two pairs of b_3 and y_5 ions with distinct sizes, b_3-y_5 and b_3*-y_5* (Fig. 3*C*). The presence of the former pair suggested a phosphorylated Ser^{627}, whereas the latter one suggested that Ser^{628} was phosphorylated. Compared with the relative abundance of two ion pairs, the Ser^{627} residue seems to the better site for PKA phosphorylation *in vitro*. Nevertheless, it appears that both Ser^{627} and Ser^{628} are phosphorylated by PKA.

We utilized the selected ion tracing method (39) for a more comprehensive study of the other phosphorylated peptides/residues. This analysis identified a second phosphopeptide from Nopp140 BS protein. This peptide is singly phosphorylated ^{111}RASLPQHAGK^{120}, whose collision-induced dissociation spectrum contains a dominant 524.0 *m/z* ion (Fig. 3*D*). The presence of this signature fragment ion indicated this peptide

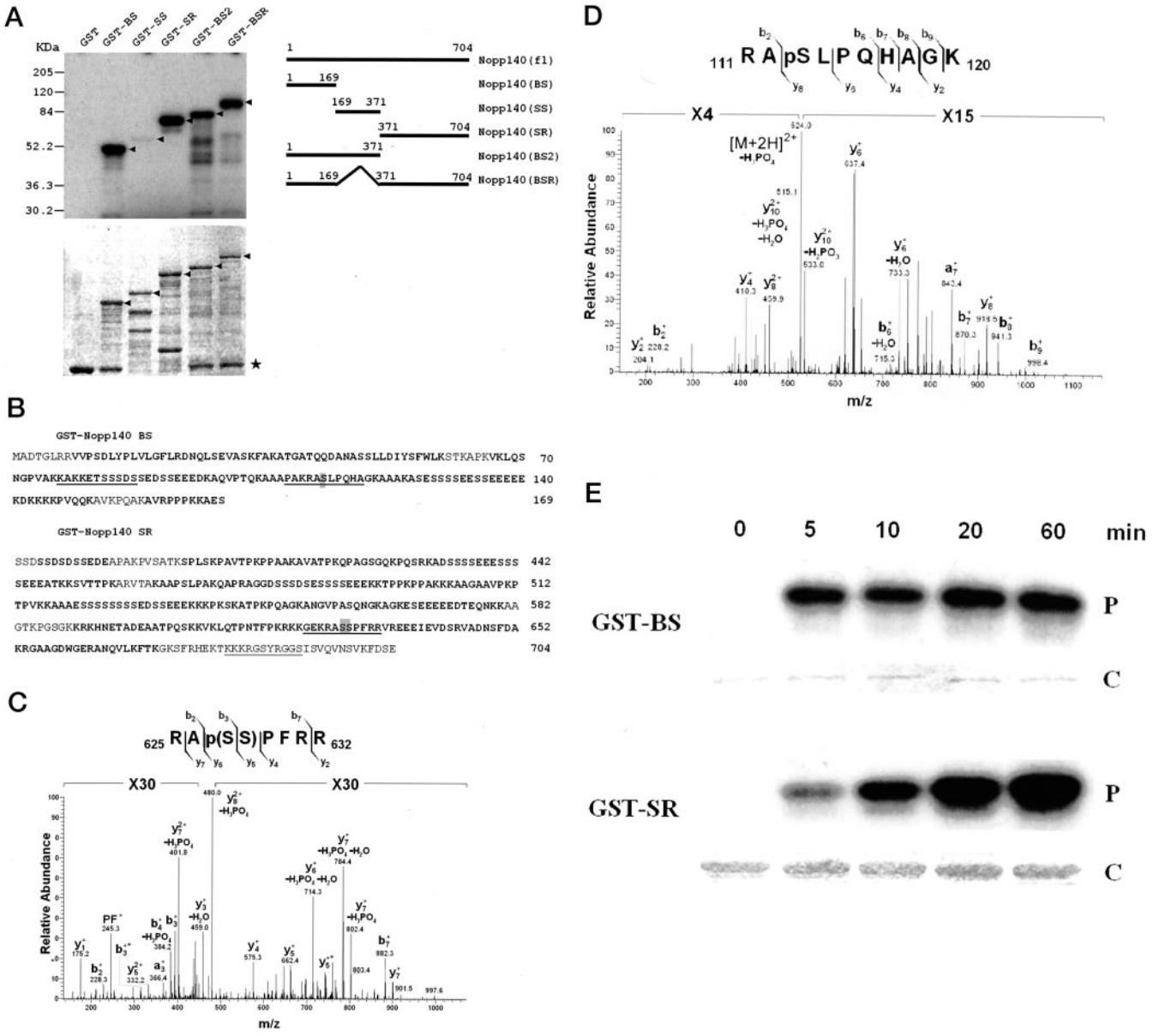

FIG. 3. **Determination of the *in vitro* PKA phosphorylated residues of Nopp140.** *A*, mapping of Nopp140 phosphorylation regions by PKA. *Right panel*, schematic representation of Nopp140 deletion mutants fused to the GST expression vector. Five GST-Nopp140 deletion proteins (BS, SS, SR, BS2, and BSR) were purified and bound to glutathione beads. Immobilized GST fusion proteins were then phosphorylated by bovine PKAc (Sigma) in the presence of [γ-^{32}P]ATP at 37 °C for 20 min. The reaction mixtures were subjected to SDS-PAGE, stained with Coomassie Blue, and autoradiographed. The Coomassie Blue-stained gel is shown in the *lower left panel*. The *arrowheads* indicate the positions of each recombinant protein corresponding to the radioactive labeled polypeptide. The *asterisk* indicates the position of GST protein. *B–D*, identification of *in vitro* PKA phosphorylation sites of Nopp140 by mass spectrometry. GST-Nopp140 BS and SR clones were used as substrates for trypsin digestion. *B*, the amino acid sequence derived from each recombinant protein is shown in the *top panel*. The *boldface characters* of the amino acid sequence represent the portion corresponding to the tryptic peptides, which could be selected out by the SEQUEST program and an ion tracing search. About 88% of the sequence for GST-BS and 82% for GST-SR were covered by such a search. *C* and *D*, the collision-induced dissociation spectra of the phosphopeptide ^{625}RASSPFRR^{632} and ^{111}RASLPQHAGK^{120} are shown in the *bottom panel*. Representative tandem mass spectra of *m/z* 480, 384.2, and 524 selected ions were used for identifying the phosphorylation sites of Ser^{627}, Ser^{628}, and Ser^{113}, respectively. The Nopp140 consensus phosphorylation sequences for PKA are *underlined* in the sequence of GST-BS and GST-SR. The corresponding residues are shown as a *shaded box* in the amino acid sequence. The spectra beside each signature peak are amplified for a better view by the indicated magnitude. *E*, characterization of time course phosphorylation of Nopp140 truncated mutants GST-BS and GST-SR by PKA *in vitro*. The immobilized GST deletion proteins (0.5–1 μg) were phosphorylated by PKA. The reaction times are shown in the *figure*. The representation of autoradiogram and Coomassie Blue gel staining were the same as shown in Fig. 2*B*.

was indeed phosphorylated. Since Ser^{113} is the only potential phosphorylation residue within the sequence, it should be an unambiguous phosphorylated site.

We also examined the phosphorylation of two Nopp140 deletion mutants, BS and SR (Fig. 3*E*). The time course studies of GST-BS and GST-SR by PKA indicated the kinetic behavior for BS is very similar to that of CREB, whereas SR is similar to Nopp140 (compare Fig. 2*B* and Fig. 3*E*). The results are consistent with the notion that there is one single PKA site in BS and at least two sites in SR, as identified by mass spectrum analysis.

We have also performed the LC/MS/MS analysis of Nopp140 phosphorylated by CKII. None of the peptides corresponding to Ser^{113} and Ser^{627}/Ser^{628} were phosphorylated (data not shown). This is a striking contrast to the phosphorylation of both peptides by PKA when analyzed by a parallel experiment.

In summary, at least three PKA specific sites of Nopp140 have been identified in our experiment.

Synergistic Activation of agp Gene Expression by Both Forskolin and Nopp140 Depends on Phosphorylation of Nopp140 by PKA—Whereas Nopp140 is a highly phosphorylated protein, its phosphorylation state may be altered in a cell cycle-dependent manner (42). To investigate whether forskolin stimulation could change the phosphorylation status at certain sites of Nopp140, we determined the *in vivo* phosphorylation sites and their relative extent of phosphorylation via the selected ion tracing approach. The detailed method has been described in our previous publication (39). We have shown that the ratio between phosphorylated and nonphosphorylated peptides can serve as an index on local phosphorylation state for a particular peptide. To obtain a sufficient amount of Nopp140 polypeptide for analysis, a FLAG-tagged recombinant Nopp140 plasmid was transiently transfected into 293T cells. Based on its normal nucleolar distribution in 293T cells (data not shown), FLAG-Nopp140 appears to behave like endogenous Nopp140 inside the cells. To assess the PKA activity upon forskolin treatment, anti-phospho-CREB antibody was used to detect the lysates of Nopp140-transfected cells. After 20–30-min treatment with 50 μM forskolin, the phosphorylation on CREB Ser^{133} has significantly increased (Fig. 4*A*). The phosphorylation level decreased under prolonged incubation. The relative protein amount of tubulin α as well as ectopically expressed FLAG-Nopp140 was not affected by this treatment (Fig. 4, *A* and *B*). These results also demonstrate that overexpression of FLAG-Nopp140 had no apparent effect on PKA-stimulated CREB phosphorylation.

FLAG-Nopp140 polypeptides immunoprecipitated from forskolin-treated and untreated cell lysates were subjected to SDS-PAGE separation and in-gel trypsin digestion, followed by LC/MS/MS analysis. We could recover nearly 60% of Nopp140 peptides including those covering all three PKA *in vitro* phosphorylation sites. SEQUEST and selected ion tracing programs identified at least four phosphorylation sites. Two of these four sites, Ser^{113} and Ser^{627}, were also the serine residues modified by PKA *in vitro*. There was no change in the ratio of phosphorylated to unphosphorylated Ser^{113} in cells with or without forskolin treatment. On the contrary, a substantially increased ratio of phosphorylated to unphosphorylated Ser^{627} was evident when cells were treated with forskolin (Fig. 4*C*). These results suggest that forskolin-induced kinase activity may have preferentially occurred at Ser^{627} of Nopp140, with a lesser role expected for the phosphorylation at Ser^{113}.

Based on the findings from *in vitro* and *in vivo* experiments, we performed functional assays of various PKA phosphorylation-deficient mutants of Nopp140 for their activation on AGP promoter. Four site-directed mutants, S113A, S627A, S628A, and S113A,S627A, were used for transfection into BHK cells. Overexpression of wild type Nopp140 showed a 4–6-fold increase in reporter activity (Fig. 4*D*). In contrast, the overexpression of mutant S113A or S627A impaired the activation of reporter. The decreased stimulatory effect of S628A was not as much as that of either S113A or S627A. The reduction in reporter activation was also shown by the S113A,S627A double mutant. The expression level of each site-directed mutant has been assessed in transfected 293T cells by Western blot analysis. Identical levels of proteins were detected in comparison with the endogenous tubulin α protein in each lysate sample (Fig. 4*E*). This result indicates that any variation in the transactivating activities of Nopp140 is not due to changes in protein levels. Although the Ser^{113} mutant was observed to affect the stimulatory activity on AGP-CAT, the phosphorylation level at Ser^{113} remained less responsive to forskolin than Ser^{627} based on the quantitative analysis of phosphorylated ion counts (Fig. 4*C*). Together, these results demonstrate that Ser^{627} of Nopp140 may be crucial for the PKA dependent co-activation of *agp* gene in BHK cells in response to forskolin.

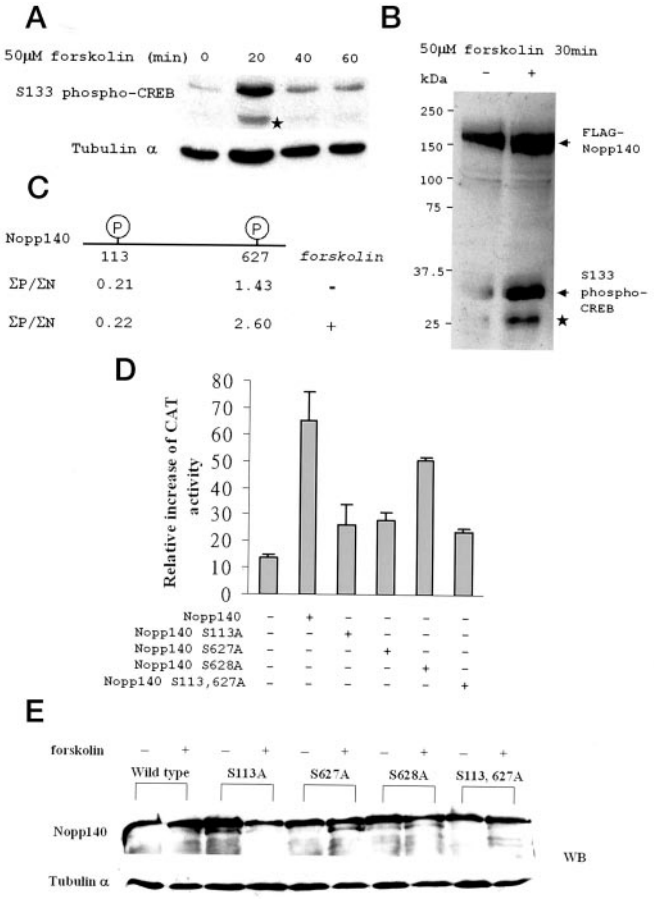

FIG. 4. **Determination and characterization of the forskolin-induced phosphorylation sites on Nopp140.** *A*, 293T cells treated with 50 μM forskolin were harvested at the indicated time points. Western blot analysis was performed with anti-phospho-CREB as well as anti-tubulin α antibodies. *B*, FLAG-Nopp140 expression vector was transiently transfected into 293T cells. After culturing for 2 days, the cells were treated with Me_2SO or forskolin for 30 min. The lysates were subjected to Western blot analysis and then probed with anti-FLAG (M2) and anti-phospho-CREB antibodies. The *asterisks* in *A* and *B* indicate the position of an additional forskolin-inducible phosphoprotein that can be recognized by anti-phospho-CREB antibody, possibly CREM. *C*, FLAG-Nopp140 purified from the above cell lysates by immunoprecipitation with M2 beads was subjected to LC/MS/MS analysis. $\Sigma P/\Sigma N$ represents the ratio of phosphorylated *versus* unphosphorylated ion counts for peptides containing the site of Ser^{113} or Ser^{627}. *D*, the PKA phosphorylation sites are important for Nopp140-induced *agp* gene expression by forskolin. CMV expression vectors encoding Nopp140 (wild type) and three mutants (S267A, S268A, and S113A,S627A) were transfected into BHK cells together with AGP-CAT plasmid. The transfected cells were cultured in the presence or absence of forskolin as described in the legend to Fig. 1*A*. The relative CAT activities were shown as the net amount by subtracting the forskolin-treated activity from untreated activity. The result is the average of four independent experiments, and the S.D. values are indicated with *error bars*. *E*, immunoblot of cell lysates from transfected cells with or without forskolin treatment was shown by anti-murine Nopp140 antibody and control (anti-tubulin α) antibody.

Cis Element Involved in Nopp140-mediated agp Gene Co-activation by Forskolin—Our previous results showed that Nopp140 served as a co-activator for C/EBPβ-induced expression of the *agp* gene (11). Above, we demonstrated that the synergistic stimulation of *agp* gene expression by Nopp140 and PKA is specific for the AGP promoter. To identify the potential motif(s) involved in the activation by Nopp140 and forskolin,

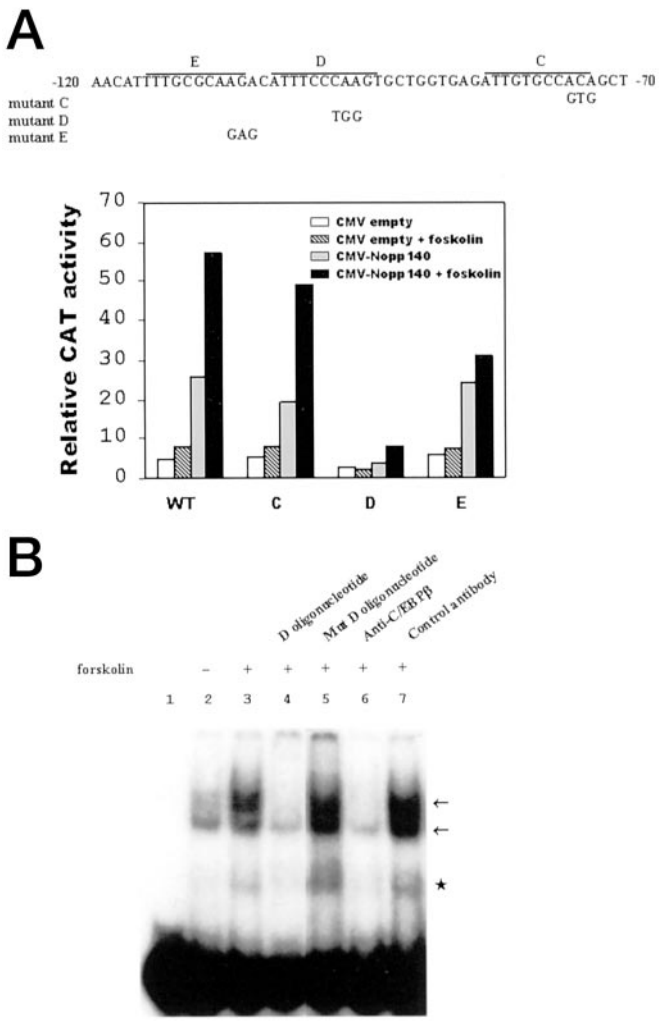

FIG. 5. **Activation of *agp* gene expression by both Nopp140 and forskolin is dependent on C/EBPβ specific cis-element in the AGP promoter.** *A*, the wild type (*WT*) and mutant C, D, and E of AGP promoter are shown in the *upper panel*. BHK cells were transiently co-transfected with 1 μg of CAT reporter plasmid of wild type or each mutant with Nopp140 expression plasmid or empty vector. Each combination of transfected cells was treated with forskolin or Me_2SO as described in the legend to Fig. 1*A*. Relative activities are shown as the average of two independent experiments. *B*, gel mobility shift assay of D element binding complexes. The nuclear extracts of BHK cells treated (*lanes 3–7*) or untreated (*lane 2*) with 50 μM forskolin for 2 h were prepared for binding assays. Oligonucleotide D was used as the probe. A 50-fold molar excess of the unlabeled oligonucleotide D (*lane 4*) or mutated D (*lane 5*) was added to the incubation reaction for the competition experiment. Anti-C/EBPβ (*lane 6*) or control (*lane 7*) monoclonal antibody was added subsequently for supershift assay. *Lane 1* represents the control reaction of probe alone. The *arrows* indicate the two major gel shift complexes, whereas an *asterisk* represents the minor signal.

we performed experiments using mutants of the AGP promoter (9). Among these motifs, at least three (C, D, and E motifs; Fig. 5*A*, *upper panel*) were demonstrated to be involved in both C/EBPβ and Nopp140 activation (11). Mutation of the C motif (−73 to −83) had no effect on Nopp140-mediated coactivation by forskolin treatment (Fig. 5*A*, *lower panel*). However, the D motif mutant apparently reduced the forskolin-dependent stimulation irrespective of co-transfection with Nopp140. In addition, mutation of the E motif appeared to affect the Nopp140-dependent forskolin stimulation rather than the basal activity (Fig. 5*A*). These results suggest that D and E motifs of AGP promoter may be critical for the forskolin stimulation of *agp* gene expression.

When oligonucleotide probe from the D motif was used for electrophoretic mobility shift assay, prominent retarded signals were stronger from nuclear extract of forskolin-treated than untreated cells (Fig. 5*B*, *lanes 2* and *3*). Two retarded complexes could be competed by unlabeled wild-type oligonucleotide (50-fold molar excess) (Fig. 5*B*, *lane 4*, *arrows*). However, these complexes were not competed by mutated D oligonucleotide (Fig. 5*B*, *lane 5*). Interestingly, the upper, but not the lower, complex was susceptible to competition by the E motif oligonucleotide (data not shown). When we used antibody for supershift assay, the upper complex could be disrupted by anti-C/EBPβ antibody but not by control antibody (Fig. 5*B*, *lanes 6* and *7*). These results suggest that there may be at least two classes of forskolin-induced complexes based on their specificity toward D elements. It is noteworthy that the complex with probably equal affinity toward D and E elements may also contain C/EBPβ.

Activation of agp Gene by Functional Interaction of PKA-phosphorylated Nopp140, C/EBPβ, and CREB—To further determine how PKA-phosphorylated Nopp140 participates in the activation of the *agp* gene, we performed co-transfection experiments using expression vectors of C/EBPβ and CREB. When CREB was overexpressed in the BHK cells, the extent of forskolin-induced activation of AGP-CAT was similar to that transfected with Nopp140 (Fig. 6*A*, compare *lanes 7* and *8* with *lanes 3* and *4*). To examine whether this activation is dependent on C/EBPβ, we performed an experiment using a dominant negative form of C/EBPβ (*i.e.* LIP) (43) for testing the co-activation. We found that this CREB-mediated stimulation could be abolished when cells were co-transfected with a 5-fold excess of LIP expression plasmid (*lane 9*). These results suggest that CREB-mediated forskolin stimulation of *agp* expression is probably through a C/EBPβ-dependent pathway. When we co-transfected both Nopp140 and CREB into BHK cells, the relative CAT activity appeared to be further activated (3–4-fold). Consistent with the effect of LIP on CREB, the synergistic activation of Nopp140 and CREB was also found to be repressed by LIP (*lanes 9* and *12*).

To determine whether the PKA-phosphorylated Nopp140 is important for the CREB-Nopp140-mediated activation of the *agp* gene, we performed co-transfection of Nopp140 (S627A) mutant and CREB expression plasmids into BHK cells. There was no synergistic activation of AGP-CAT by the combination of CREB and Nopp140 (S627A) (*lanes 13* and *14*). This result indicates that Ser^{627} phosphorylation of Nopp140 may be crucial in its functional interaction with CREB. To further investigate whether PKA phosphorylation of CREB is involved in this activation, we used the CREB (S133A) mutant in place of the wild-type CREB in the co-transfection experiment. The synergistic activation by Nopp140 and CREB (S133A) was the same as the one by both Nopp140 and wild-type CREB (compare *lanes 10* and *11* with *lanes 17* and *18*). This is an indication that phosphorylation of Nopp140, but not CREB, is key to the synergistic activation of the *agp* gene. We next investigated the role of C/EBPβ in the functional interaction among PKA, Nopp140, and CREB and observed that C/EBPβ did activate *agp* gene expression in the presence of CREB and Nopp140 in a forskolin-dependent manner (*lanes 4*, *8*, *11*, and *20*).

To examine whether Nopp140 and CREB-mediated induction of *agp* gene expression in response to forskolin is also dependent on the C, D, or E motif, three motif mutant reporters described previously (Fig. 5*A*) were used in the reporter assay. The synergistic activation of the *agp* gene by forskolin, CREB, and Nopp140 was observed when the C or E mutant reporter was tested (Fig. 6*B*). However, mutation of the D motif seems to specifically interfere with the activation effect of either CREB

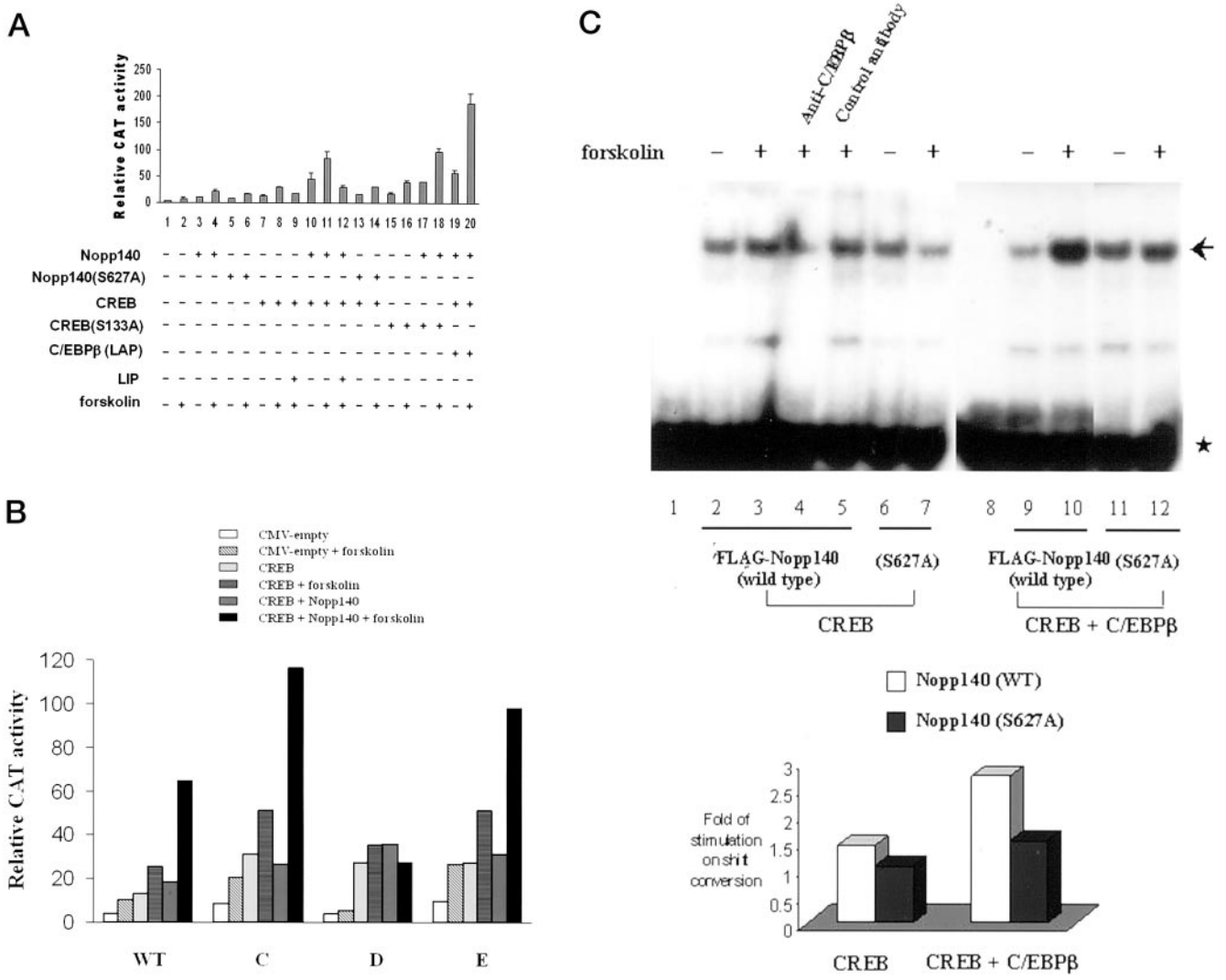

FIG. 6. **Activation of the *agp* gene by forskolin-induced phosphorylated Nopp140 depends on CREB and C/EBPβ.** *A*, BHK cells were transiently transfected with AGP-CAT reporter plasmid and 200 ng of CMV-CREB or CMV-CREB (S133A) or 1 μg of CMV-Nopp140 or CMV-Nopp140 (S627A) expression plasmid in combination with 20 ng of CMV-C/EBPβ (LAP) or 100 ng of CMV-C/EBPβ (LIP) as indicated. The transfected cells were treated with forskolin or Me_2SO control as previously described. The S.D. of data was generated from results of duplicate experiments. *B*, the reporters of wild type and AGP promoter mutants were transiently transfected into BHK cells for CAT activity assay. CMV-CREB in combination with or without CMV-Nopp140 was co-transfected as indicated. The transfected cells were then treated with forskolin or Me_2SO at 16–24 h post-transfection. The relative activity is shown as the average of two independent experiments. *C*, 293T cells were co-transfected with wild type or S627A mutant of FLAG-Nopp140 as well as CREB and C/EBPβ (LAP) similar to the functional reporter assays detailed in *A*. The transfected cells were treated with 50 μM forskolin about 1.5 h before harvest. Gel mobility shift assay was performed using nuclear extracts from forskolin-treated and untreated cells. After incubation at room temperature for 20 min, anti-C/EBPβ (*lanes 4*) or control (*lanes 5*) monoclonal antibody was added, and incubation continued for 10 min. *Lanes 1* and *8* are the probe-alone reactions. The *arrow* indicates the shifted complex, and the *asterisk* represents the free probe. The *bar graph* in the *lower panel* summarizes the quantitative comparisons (*i.e.* stimulation -fold) of the levels of the forskolin-stimulated shifted complex formation between wild type and Nopp140 (S627A) in the presence of overexpressed CREB or CREB/C/EBPβ.

alone or CREB plus Nopp140 under forskolin treatment. These results suggest that the functional interaction of Nopp140 and CREB requires the presence of the D motif. They also lead to the above notion that the coactivation effect of Nopp140 in forskolin-induced cells is motif-specific.

To characterize the biochemical nature of the promoter activation involving PKA-phosphorylated Nopp140, CREB, and C/EBPβ, we performed electrophoretic mobility shift assays using the D motif as probe. When nuclear extracts prepared from 293T cells overexpressing Nopp140 and CREB were incubated with the D motif probe, the shifted signal seemed to be slightly increased in response to forskolin (*lanes 2* and *3*). However, nuclear extracts from cells expressing Nopp140 (S627A) and CREB showed no such difference in the shifted complex in the presence or the absence of forskolin (*lanes 6* and *7*). To demonstrate more clearly, the relative stimulation on shifted complex was quantitatively displayed and shown in the *lower panel* of Fig. 6*C*. These results are consistent with those from the functional reporter assays. Moreover, the complex formed was specifically impaired when anti-C/EBPβ, but not the nonspecific control antibody, was included in the incubation mixture (Fig. 6*C*, *lanes 4* and *5*). This result indicates that C/EBPβ is present in the retarded complex derived from cells expressing wild type Nopp140 and CREB and treated with forskolin. To further test whether C/EBPβ was indeed present in the complex formation, C/EBPβ was co-transfected with wild type or mutant Nopp140 and CREB. Likewise, the shifted complex was significantly increased by forskolin treatment when wild type was used but was not altered when the mutant Nopp140 was used (Fig. 6*B*, *lanes 9–12*). We also found that

anti-C/EBPβ antibody also specifically disrupted the complex formation (data not shown). The result supports the observation on the co-transfection experiment in the absence of C/EBPβ. The relative extent of stimulation was more dramatic in the presence than in the absence of C/EBPβ. The difference of forskolin-induced response between the wild type and mutant of Nopp140 was quite striking (*lower right plot*). This result also suggests that C/EBPβ is important for the Nopp140-mediated PKA signaling pathway.

DISCUSSION

A wide array of factors are involved in the regulation of gene expression during APR. Among them, those proteins that play dual or multiple roles are particularly intriguing. Nopp140 and nucleolin are among the dual/multifunction proteins that exert their regulatory effects on *agp* gene expression (11, 12). In the present study, we reported the novel results of synergistic activation of a prominent APR gene, *agp*, expression by PKA, and Nopp140. We further demonstrated that this synergistic stimulation is the result of specific phosphorylation of Nopp140 by PKA. The PKA phosphorylation sites of Nopp140 protein were unequivocally identified by LC/MS/MS. Thus, the multifunctional Nopp140 protein is once again shown to serve as a transcription co-activator in the context of PKA signaling pathway.

Nopp140 has been known as one of the most highly phosphorylated proteins in cells (29). CKII was reported to specifically interact with and phosphorylate Nopp140 mainly in its acidic repeats region (34). However, only PKA, and not CKII, is shown to be involved in the Nopp140-mediated activation of the *agp* gene (Fig. 1, *A* and *B*). Nopp140 *per se* could specifically activate the *agp* gene by PKA. The fact that both Ser^{113} and Ser^{627} of Nopp140 are phosphorylated to a low level in the absence of forskolin treatment *in vivo* (Fig. 4*C*) supports our previous results on the activation of the *agp* gene by Nopp140 without forskolin treatment (11). Forskolin treatment specifically increases the level of phosphorylation of Ser^{627} but not Ser^{113}. The basal level of phosphorylation at Ser^{113} and Ser^{627} sites may be mediated by other forskolin-independent kinase(s) onto a minor population of Nopp140. Ser^{113} appears to have a lower basal level of phosphorylation than S627 (Fig. 4*C*). To examine whether the phosphorylation of Nopp140 by CKII affects the subsequent phosphorylation by PKA, we used the full-length recombinant Nopp140 as a substrate for *in vitro* kinase assay. We found that the CKII-pretreated Nopp140 has no effect on PKA-dependent phosphorylation. To test the possibility of phosphorylation at the Ser^{113} site, the N-terminal truncated construct GST-BS was used as substrate. We also obtained similar results (data not shown). Thus, prior phosphorylation of Nopp140 by CKII has no effect on the phosphorylation by PKA *in vitro*. Together, the results suggest that the basal level of phosphorylation at both sites is probably not due to the phosphorylation by CKII.

The identification of *in vitro* PKA phosphorylation sites of the Nopp140 deletion construct has indeed pinpointed Ser^{113} as a PKA target. However, the level of Ser^{113} phosphorylation is not enhanced when PKA is activated, implying that Ser^{113} phosphorylation is not related to PKA activity. An explanation for the discrepancy between *in vivo* and *in vitro* data is that Ser^{113} may be not accessible by PKA *in vivo* but a good substrate *in vitro*. This is partly supported by the fact that Ser^{113} is located in a specific acidic-basic rich sequence that is implicated in the functions of other general transcription factors like TFIIB (11) through protein-protein interaction (44). It is likely that this region interacts with other proteins that do not allow PKA-like enzymes to act on Ser^{113}. On the other hand, the deleterious effect of the S113A mutant in the reporter assay could be accounted for by the fact that the protein conformation in the region surrounding the Ser^{113} site is crucial for the transcriptional activation of target gene by Nopp140. Thus, this may be an explanation why the mutant S113A could impair the effect of Nopp140 upon activation of the *agp* gene although this residue was unresponsive to forskolin *in vivo* (Fig. 4*D*).

The synergistic activation of the *agp* gene by Nopp140 and forskolin occurs at the transcriptional level, because it is not only gene-specific (*i.e.* the p53 promoter is not their target) but also motif-specific (*i.e.* D and E motifs but not the C motif of the AGP promoter). Although the genes responsive to forskolin stimulation often possess the CRE (23), we have not found any CRE consensus sequence in the proximal responsive region of the AGP promoter. Our results clearly demonstrated that CREB or a PKA phosphorylation-deficient mutant CREB (S133A) plays a role in the activation of *agp* by PKA-dependent phosphorylation of Nopp140. Although the activation of the *agp* gene by both CREB and Nopp140 is not dependent on the phosphorylation of CREB by PKA, CREB nonetheless is a crucial component (Fig. 6*A*). CREB's involvement in this functional interaction remains unclear. In addition, we could not find any difference in subcellular localization of transfected Nopp140 and any change in relative abundance of endogenous Nopp140 in lysates treated with forskolin (data not shown). Previous reports have demonstrated that forskolin stimulation can induce the translocation of a human homologue of C/EBPβ, NF-IL6, to the nucleus to activate c-*fos* expression (45). *In vitro* study also showed that PKA could phosphorylate C/EBPβ and has no effect on its DNA binding affinity (27). The motif involved in the activation of the *agp* gene by Nopp140 and PKA (*i.e.* D motif in the AGP promoter) is also the motif recognized by C/EBPβ (8); thus, C/EBPβ is likely to cooperate with PKA-phosphorylated Nopp140 in activating the *agp* gene. This conclusion is strengthened by the fact that LIP, a dominant repressor of C/EBPβ, could disrupt the synergistic interaction of Nopp140, CREB, and forskolin. However, our present results clearly demonstrated that PKA phosphorylation of Nopp140 alone is required for the functional interaction among CREB, C/EBPβ, and Nopp140. Whether additional PKA phosphorylation of C/EBPβ is essential will be addressed in the future studies. Since Nopp140 is not a DNA-binding protein but mediates the DNA motif-dependent induction of the *agp* gene, the phosphorylation of Nopp140 by PKA may facilitate the assembly of a multiprotein complex that results in this activation. We have examined the interaction between C/EBPβ and PKA-phosphorylated Nopp140 and shown it not to be affected *in vitro* (data not shown). Despite that, C/EBPβ should be a component of the multiprotein complex responsible for its binding to the specific DNA motifs.

The physiological function of C/EBPβ in regulating hormone-induced PKA signaling of gluconeogenic gene expression is supported by several reports (26, 46, 47). Although CREB has also been known to mediate heptic gluconeogenesis directly or indirectly through the control of genes containing CREs or the glucocorticoid response element (40, 48), our present results demonstrate that a novel PKA- and Nopp140-mediated signaling pathway may also be involved in a similar physiological process upon *agp* gene expression. This mode of regulation may be independent of or in conjunction with the PKA-mediated phosphorylation of the CREB pathway. The distinction between these two pathways is that the PKA/CREB pathway is dependent on CRE, whereas the PKA/Nopp140 is dependent on a certain C/EBP-binding motif. The genes that may be regulated by the convergence of these two pathways remain to be identified. Furthermore, our results demonstrated that both

C/EBPβ and CREB are involved in the activation of the *agp* gene, albeit no PKA phosphorylation of CREB was required (Fig. 6*A*). However, CREB may be the target of signaling pathways other than PKA. Exactly how the CREB is involved in the synergistic action on Nopp140, PKA, or C/EBPβ remains to be elucidated. From the physiological view, the cAMP-dependent pathway is important for the modulation of expression of certain hepatic enzymes (17, 48). The phosphorylation of Nopp140 may play some roles in hepatic tissues in response to cAMP elevation to up-regulate the expression of these gluconeogenic enzymes.

In another aspect, production of acute phase reactants in adipose tissues under hyperglycemia conditions was reported (49). Hyperglycemia-induced expression of α1-acid glycoprotein during the differentiation of 3T3-L1 cells involves C/EBPβ and other factors. Whether PKA-mediated phosphorylation of Nopp140 takes part in the expression of α1-acid glycoprotein during adipocyte differentiation is an intriguing possibility.

In summary, the results of this study demonstrate that Nopp140 is the target of the PKA pathway, leading to the activation of *agp* gene expression. Signaling pathways leading to C/EBPβ activation have been studied extensively (17, 50–53), whereas the mechanism that dictates the activation and regulation by Nopp140 is less well understood. In our previous studies, we demonstrated that C/EBPβ and Nopp140 stimulate *agp* expression synergistically (11). Here we present data on the activation of Nopp140 by PKA and its subsequent stimulation of *agp* expression via a functional, cooperative interaction with C/EBPβ. Our findings may provide an example of a novel signaling pathway to modulate C/EBPβ-dependent gene activation. However, the underlying molecular mechanism of the activation of the *agp* gene by phosphorylated Nopp140 remains to be further investigated.

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REFERENCES

1. Baumann, H., and Gauldie, J. (1994) *Immunol. Today* **15,** 74–80
2. Fournier, T., Medjoubi-N, N., and Porquet, D. (2000) *Biochim. Biophys. Acta* **1482,** 157–171
3. Akira, S., and Kishimoto, T. (1992) *Immunol. Rev.* **127,** 25–50
4. Alam, T., An, M. R., Mifflin, R. C., Hsieh, C. C., Ge, X., and Papaconstantinou, J. (1993) *J. Biol. Chem.* **268,** 15681–15688
5. Chang, C. J., Chen, T. T., Lei, H. Y., Chen, D. S., and Lee, S. C. (1990) *Mol. Cell. Biol.* **12,** 6642–6653
6. Kulkarni, A. B., Reinke, R., and Feigelson, P. (1985) *J. Biol. Chem.* **260,** 15386–15389
7. Vannice, J. L., Taylor, J. M., and Ringold, G. M. (1984) *Proc. Natl. Acad. Sci. U. S. A.* **81,** 4241–4245
8. Lee, Y. M., Miau, L. H., Chang, C. J., and Lee, S. C. (1996) *Mol. Cell. Biol.* **16,** 4257–4263
9. Lee, Y. M., Tsai, W. H., Lai, M. Y., Chen, D. S., and Lee, S. C. (1993) *Mol. Cell. Biol.* **13,** 432–442
10. Miau, L. H., Chang, C. J., Shen, B. J., Tsai, W. H., and Lee, S. C. (1998) *J. Biol. Chem.* **273,** 10784–10791
11. Miau, L. H., Chang, C. J., Tsai, W. H., and Lee, S. C. (1997) *Mol. Cell. Biol.* **17,** 230–239
12. Yang, T. H., Tsai, W. H., Lee, Y. M., Lei, H. Y., Lai, M. Y., Chen, D. S., Yeh, N. H., and Lee, S. C. (1994) *Mol. Cell. Biol.* **9,** 6068–6074
13. Chang, C. J., Chen, Y. L., and Lee, S. C. (1998) *Mol. Cell. Biol.* **18,** 5880–5887
14. Savoldi, G., Fenaroli, A., Ferrari, F., Rigaud, G., Albertini, A., and Di Lorenzo, D. (1997) *DNA Cell Biol.* **16,** 1467–1476
15. Higuchi, T., Watanabe, Y., and Yamamoto, M. (2002) *Mol. Cell. Biol.* **22,** 1–11
16. Kawaguchi, T., Takenoshita, M., Kabashima, T., and Uyeda, K. (2001) *Proc. Natl. Acad. Sci. U. S. A.* **98,** 13710–13715
17. Roesler, W. J. (2000) *Mol. Cell. Endocrinol.* **162,** 1–7
18. Deutsch, P. J., Hoeffler, J. P., Jameson, J. L., Lin, J. C., and Habener, J. F. (1988) *J. Biol. Chem.* **263,** 18466–18472
19. Montminy, M. R., Sevarino, K. A., Wagner, J. A., Mandel, G., and Goodman, R. H. (1986) *Proc. Natl. Acad. Sci. U. S. A.* **83,** 6682–6686
20. Montminy, M. R., and Bilezikjian, L. M. (1987) *Nature* **328,** 175–178
21. Gonzalez, G. A., and Montminy, M. R. (1989) *Cell* **59,** 675–680
22. Sassone-Corsi, P. (1998) *Int. J. Biochem. Cell Biol.* **30,** 27–38
23. Shaywitz, A. J., and Greenberg, M. E. (1999) *Annu. Rev. Biochem.* **68,** 821–861
24. Tyson, D. R., Swarthout, J. T., and Partridge, N. C. (1999) *Endocrinology* **140,** 1255–1261
25. Niehof, M., Manns, M. P., and Trautwein, C. (1997) *Mol. Cell. Biol.* **17,** 3600–3613
26. Tae, H. J., Zhang, S., and Kim, K. H. (1995) *J. Biol. Chem.* **270,** 21487–21494
27. Trautwein, C., van der Geer, P., Karin, M., Hunter, T., and Chojkier, M. (1994) *J. Clin. Invest.* **93,** 2554–2561
28. Fournier, T., Riches, D. W., Wiston, B. W., Rose, D. M., Scott, K. Y., Noble, P. W., Lake, F. R., and Henson, P. M. (1995) *J. Immunol.* **155,** 2123–2133
29. Meier, U. T., and Blobel, G. (1992) *Cell* **70,** 127–138
30. Isaac, C., Yang, Y., and Meier, U. T. (1998) *J. Cell Biol.* **142,** 319–329
31. Chen, H. K., Pai, C. Y., Huang, J. Y., and Yeh, N. H. (1999) *Mol. Cell. Biol.* **19,** 8536–8546
32. Yang, Y., Isaac, C., Wang, C., Dragon, F., Pogacic, V., and Meier, U. T. (2000) *Mol. Biol. Cell* **11,** 567–577
33. Isaac, C., Pollard, J. W., and Meier, U. T. (2001) *J. Cell Sci.* **114,** 4253–4264
34. Li, D., Meier, U. T., Dobrowolska, G., and Krebs, E. G. (1997) *J. Biol. Chem.* **272,** 3773–3779
35. Hoffmann, J., and Schwoch, G. (1989) *Biochem. J.* **263,** 785–793
36. Dascher, C., and Balch, W. E. (1994) *J. Biol. Chem.* **269,** 1437–1448
37. Chen, C., and Okayama, H. (1987) *Mol. Cell. Biol.* **7,** 2745–2752
38. Schreiber, E., Matthias, P., Muller, M. M., and Schaffner, W. (1989) *Nucleic Acids Res.* **17,** 6419
39. Tsay, Y. G., Wang, Y. H., Chiu, C. M., Shen, B. J., and Lee, S. C. (2000) *Anal. Biochem.* **287,** 55–64
40. Daniel, P. B., Walker, W. H., and Habener, J. F. (1998) *Annu. Rev. Nutr.* **18,** 353–383
41. Pelletier, N., Boudreau, F., Yu, S. J., Zannoni, S., Boulanger, V., and Asselin, C. (1998) *FEBS Lett.* **439,** 275–280
42. Pai, C. Y., Chen, H. K., Sheu, H. L., and Yeh, N. H. (1995) *J. Cell Sci.* **108,** 1911–1920
43. Welm, A. L., Mackey, S. L., Timchenko, L. T., Darlington, G. J., and Timchenko, N. A. (2000) *J. Biol. Chem.* **275,** 27406–227413
44. Smith, C. A., Bates, P., Gonzalez, R. R., Gu, B., and Deluca, N. A. (1993) *J. Virol.* **67,** 4676–4687
45. Metz, R., and Ziff, E. (1991) *Genes Dev.* **10,** 1754–1766
46. Arizmendi, C., Liu, S., Croniger, C., Poli, V., and Friedman, J. E. (1999) *J. Biol. Chem.* **274,** 13033–13040
47. Liu, X., Wall, Q. T., Taylor, L., and Curthoys, N. P. (2001) *Am. J. Physiol.* **281,** F649–F657
48. Herzig, S., Long, F., Jhala, U. S., Hedrick, S., Quinn, R., Bauer, A., Rudolph, D., Schutz, G., Yoon, C., Puigserver, P., Spiegelman, B., and Montminy, M. (2001) *Nature* **413,** 179–183
49. Lin, Y., Rajala, M. W., Berger, J. P., Moller, D. E., Barzilai, N., and Scherer, P. E. (2001) *J. Biol. Chem.* **276,** 42077–42083
50. Koj, A. (1996) *Biochim. Biophys. Acta* **1317,** 84–94
51. Buck, M., Poli, V., Hunter, T., and Chojkier, M. (2001) *Mol. Cell* **8,** 807–816
52. Buck, M., Poli, V., van der Geer, P., Chojkier, M., and Hunter, T. (1999) *Mol. Cell* **4,** 1087–1092
53. Hanlon, M., Sturgill, T. W., and Sealy, L. (2001) *J. Biol. Chem.* **276,** 38449–38456