Although the activation domains within early growth response gene protein 1 (Egr-1) have been mapped, little is known of the kinases which phosphorylate Egr-1 and how phosphorylation correlates with the transcriptional activity of Egr-1. In this study we report that casein kinase II (CKII) co-immunoprecipitates with Egr-1 from NIH 3T3 cell lysates. The association of Egr-1 and CKII requires the C terminus of Egr-1 and CKII phosphorolizes Egr-1 in vitro. The in vitro phosphorylation of Egr-1 by CKII and that induced by serum in vivo was compared by examining the CNBr-digested fragments of the phosphorylated Egr-1. CKII strongly phosphorolizes fragments 7 and 10 which cover part of the activation/nuclear localization and DNA binding domains of Egr-1. CKII also phosphorolizes, albeit weakly, fragments 5 and 8 which cover part of activation domain and the entire repression domain of Egr-1, respectively. Strong phosphorylation on fragment 10 as well as fragment 5 was also observed in Egr-1 immunoprecipitated from serum-induced, 32P-labeled cells. CKII phosphorylation of Egr-1 resulted in a decrease of its DNA binding as well as its transcriptional activities.

Expression and Purification of Recombinant Egr-1—Recombinant Egr-1 was expressed in Escherichia coli (DH5α) and bound to glutathione-Sepharose 4B beads (Pharmacia Biotech, Uppsala, Sweden) as described by Smith and Johnson (23) except that bacteria were grown to a density of 4 x 10^9 cells/ml.

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**EXPERIMENTAL PROCEDURES**

Reagents—588, an antibody to the last 14 amino acids at the C terminus of Egr-1, was purchased from Santa Cruz Biotechnology (Santa Cruz, CA). 5232 (10) and 1133 are polyclonal antibodies generated against full-length Egr-1 fusion proteins. Anti-Egr-1 monoclonal antibody 4F18 was a gift from Amgen Inc. (Thousand Oaks, CA). Anti-mouse and anti-rabbit IgG conjugated to horseradish peroxidase were purchased from Sigma. A monoclonal antibody to the CKII α-subunit was purchased from Boehringer Mannheim (Mannheim, Germany) and the CKII polyclonal antibody against its α-subunit was purchased from Upstate Biotechnology Inc. (Lake Placid, NY). The pCMVCKII α-subunit plasmid was a gift from Dr. Steven Sloan (Howard Hughes Medical Institute, University of Pennsylvania School of Medicine, Philadelphia).

Construciton of Expression Vector for GST-Egr-1 Fusion Proteins—A near full-length GST-Egr-1 fusion construct was obtained by digesting pCMV-Egr-1 (21) with NcoI to give a 1.92-kb fragment that was cloned into the pGEX-KG (22) NcoI site. The resulting plasmid was named pGEX-NCO 1.9 and its protein product designated F-1. The Egr-1 deletion N-1 was produced by digesting pCMV-Egr-1-TTL (21), which contains a stop codon at nucleotide position 768 of the egr-1 cDNA, with NcoI and inserting it into the NcoI site of pGEX-KG such that a protein encoding only the first 171 amino acids was expressed. This plasmid was called pGEX-Egr-1-TTL. The Egr-1 deletions C-2 and C-1 were made by digesting pCMV-Egr-1-TTL with Rsal (nucleic acids 1221-2253) and PvuII (779-2060) and using the corresponding DNA fragments into the blunt XbaI site in pGEX-KG to produce pGEX-RSA 1.0 and pGEX-PVU 1.2, respectively.

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an A540 of 0.5 before inducing the fusion protein production with 0.2 mM of isopropyl β-D-thiogalactopyranoside for 3 h. GST fusion proteins thus expressed were bound to glutathione-Sepharose beads and stored in phosphate-buffered saline (PBS) with 0.2% sodium azide at 4°C and were used for phosphorylation and binding experiments.

Cytoplasmic and Nuclear Extract Preparation—Confluent NIH 3T3 cells were induced with 20% serum for 1 h. Cytoplasmic and nuclear extracts were prepared as described elsewhere (24, 25) with phosphatase and protease inhibitors added to all buffers at the following concentrations: 50 mM NaF, 100 mM sodium orthovanadate, 2 μg/ml leupeptin, 2 μg/ml aprotinin, 1 μg/ml pepstatin A, and 1 mM PMSF. Cytoplasmic extracts were kept for 5 h as described previously (10) to allow the low speed centrifugation of homogenized cells, and nuclear extracts were produced by extracting nuclear proteins with a high salt buffer.

Immunoprecipitation and Western Blots—Immunoprecipitation was performed with 0.5 mg of nuclear protein in a buffer containing 1% Triton X-100, 1% sodium deoxycylolate, 150 mM NaCl, 50 mM Tris-HCl, pH 7.4, 1 mM PMSF, and the protease and phosphatase inhibitors as listed above. Samples were preclotted with protein A-agarose beads prior to immunoprecipitation with the appropriate antibody. Immunocomplexes bound to protein A-agarose beads were washed in immunoprecipitation buffer four times and once in PBS. The protein samples were separated by 7.5% SDS-PAGE after boiling in Laemmli (26) buffer. Western blots were performed as described by Towbin et al. (27). After the transfer, nitrocellulose membranes were blocked overnight at 4°C with 5% skim milk in PBST (PBS and 0.1% Tween 20) and incubated with the appropriate primary and secondary antibodies in PBST containing 1% skim milk for 1 h each. The bands were visualized either by using a chromogenic reaction (0.6 mg/ml 3′-diaminobenzidine, 0.03% hydrogen peroxide, 50 mM Tris-HCl, pH 7.5, and 0.02% cobalt chloride) or by using the Amersham ECL kit (Amersham International Plc, UK).

Cloning and Expression of CNBr Fragments—The DNA fragments corresponding to the CNBr peptides of Egr-1 were amplified from pUC-Egr-1 by the polymerase chain reaction (28), using oligonucleotides complementary to 5′ and 3′ ends of each fragment as primers (Table I) and cloned downstream of the T3, T7 promoters in PCRScript (Stratagene, La Jolla, CA) or the SP6 promoter in pSP65 (Promega, Madison, WI). The oligonucleotides had an EcoRI site at the 5′ end and a SalI site at the 3′ end to facilitate cloning into the vectors mentioned, although blunt end ligation was also used. The oligonucleotides were designed with an extra ATG codon at the 5′ oligonucleotide to ensure ease of visualization of the corresponding peptides on an autoradiogram in the presence of [35S]methionine using the TNT reticulocyte lysate kit (Promega). The products were produced by analysis with 16.5% SDS-PAGE by the procedure of Schägger and Von Jagow (29) with a minor modification as described in the Sigma Technical Bulletin no. MWM-100.

[35P]P]orthophosphate Labeling and CNBr Mapping—Cells in 150-mm dishes were labeled for 5 h as described previously (10). The media were exchanged for fresh media containing either 10 μCi of [35S]methionine using the TNT reticulocyte lysate kit (Promega). The visualization of the corresponding peptides on synthesis in the presence with an extra ATG codon at the 5′ end and a SalI end to facilitate cloning into the vectors mentioned, although blunt end ligation was also used. The oligonucleotides were designed with an extra ATG codon at the 5′ oligonucleotide to ensure ease of visualization of the corresponding peptides on an autoradiogram in the presence of [35S]methionine using the TNT reticulocyte lysate kit (Promega). The products were produced by analysis with 16.5% SDS-PAGE by the procedure of Schägger and Von Jagow (29) with a minor modification as described in the Sigma Technical Bulletin no. MWM-100.

Mobility Shift Assay—The assays were carried out as described previously (25) except that the binding buffer contained 1 mM EDTA, 0.05% Nonidet P-40, 20 mM HEPES, 12% glycerol, 10 mM NaF, 10 mM MgCl2, 10 μM ZnCl2, and 1 mM dithiothreitol. GST-Egr-1 fusion protein, F-1 used in this assay was eluted from glutathione beads with 20 mM reduced glutathione in 20 mM Tris-HCl, pH 8.0.

In Vitro Kinase Assay and Phosphomonoester Acid Analysis—The glutathione-Sepharose beads containing GST-Egr-1 fusion proteins (50 μl) were resuspended in 100 μl of CKII assay buffer (200 mM NaCl, 10 mM MgCl2, and 25 mM Tris-HCl, pH 7.4, 10 μM ATP) in the presence of 2–5 μCi of [γ-32P]ATP. The kinase reaction was started by adding 1 unit of CKII (Promega) and incubating samples at 37°C for 30 min. The beads were washed with the Triton buffer (1% Triton X-100, 50 mM Tris-HCl, pH 8.0, 150 mM NaCl, 1 mM PMSF) four times and separated by 10% SDS-PAGE, dried, and exposed to x-ray film. For phosphomonoester acid analysis the gel was blotted onto polyvinylidene difluoride membrane, and the Egr-1 band was excised. Phosphomonoester acid analysis was performed as described by Boyle et al. (30) and Kamps (31).

In Vitro Binding Assay—The assay was essentially similar to that described by Jinn and Burakoff (17). CKII (50 units) was allowed to autoprophosphorylate in the presence of [γ-32P]ATP, and the reaction was stopped by adding 50 mM EDTA. The volume was increased to 1.0 ml with Triton buffer. The labeled kinase was preclotted with Sepharose beads containing GST alone (50 μl) for 1 h at 4°C. The supernatant was removed after centrifugation at 5000 rpm for 5 min and divided into five tubes containing the three Egr-1 deletions, an almost full-length Egr-1, and GST alone (50 μl each). The volume was again increased to 1.0 ml with Triton buffer, and the samples were mixed for 1 h at 4°C. The beads were then washed three times with Triton buffer and once with PBS (10 mM Tris-HCl, pH 8.0, 150 mM NaCl), resuspended in Laemmli buffer, boiled for 5 min, and analyzed by 12.5% SDS-PAGE and exposure to x-ray film.

DNA Transfection and Chloramphenicol Acetyltransferase Assays—The transfection experiment was performed as described previously (25). Cells were transfected with pCMV-Egr-1 (1–5, 50 μg) of protein extracts were assayed at 37°C in a total reaction volume of 30 μl in CKII assay buffer containing 1 mM synthetic substrate peptide RRREEETEEE (33), 100 mM ATP, and 5 μCi of [γ-32P]ATP. Controls consisted of assays done in the absence of substrate peptide and were subtracted as background. Reactions were terminated by adding an equal volume of 0.01 mM ATP and 0.4 mM HCl prior to chloroacetic acid precipitation. The samples (15 μl) were spotted on Whatman P81 paper and washed in 0.5% H3PO4, three times for 10 min each, and the samples were counted.

RESULTS

CKII Co-purifies with Cellular Egr-1 and Associates With It in Vitro—A kinase activity was found to be associated with Egr-1 immunoprecipitated from nuclear extracts of serum induced NIH 3T3 and MRC5 cells. This activity was determined to be similar to that of CKII based on its ability to utilize GTP during phosphorylation and its inhibition by heparin (data not shown). To further investigate this, Egr-1 was immunoprecipitated from nuclear extracts by a monoclonal Egr-1 antibody. Conversely, CKII was also precipitated from the same nuclear

| Fragment no. | Oligonucleotide sequence |
|--------------|-------------------------|
| 5            | GGGAATTACGATGTCGACTTACCC |
| 6            | GGGAATTACGATGTCGACTTACCC |
| 7            | GGGAATTACGATGTCGACTTACCC |
| 8            | GGGAATTACGATGTCGACTTACCC |
| 9            | GGGAATTACGATGTCGACTTACCC |
| 10           | GGGAATTACGATGTCGACTTACCC |
CKII Binds Egr-1

The immunoprecipitated samples were resolved by SDS-PAGE, blotted onto nitrocellulose and probed with a polyclonal antibody to the CKII α-subunit. The polyclonal antibody recognizes the CKII α-subunit immunoprecipitated by the monoclonal CKII antibody (Fig. 1A, lane 1) as well as that co-immunoprecipitated with Egr-1 (lane 2). CKII was also co-immunoprecipitated with other Egr-1 polyclonal antibodies 588 and 5232, but not with preimmune serum (data not shown). The converse was also true when CKII was immunoprecipitated from cytoplasmic and nuclear extracts with a monoclonal CKII antibody, and the immunoblot was probed with an anti-Egr-1 polyclonal antibody. Egr-1 was found to be associated with CKII immunoprecipitated from nuclear extract (Fig. 1B, lane 2), but not from the cytoplasmic sample (Fig. 1B, lane 1).

The association of Egr-1 and CKII in vitro and the localization of CKII binding domain on Egr-1 was further investigated. A near full-length GST-Egr-1 fusion protein (amino acids 29–533) as well as three Egr-1 deletions coupled to GST were made (Fig. 1C). All the fusion proteins appeared as multiple bands on SDS-PAGE, stained with Coomassie Blue (Fig. 1D). This could be due to the degradation of Egr-1 products or could also be due to the multiple initiation sites. The near full-length GST-Egr-1 fusion protein, F-1, migrates as an about 100-kDa protein on SDS-PAGE, although theoretically it is only 82 kDa. This anomalous behavior is observed with cellular Egr-1 as well which runs at 80 kDa instead of its predicted molecular mass of 56 kDa (11). The three Egr-1 deletions coupled to GST, C-2 (shorter C terminus fragment from amino acids 322–533), C-1 (longer C terminus Egr-1 fragment from amino acids 175–533), and N-1 (N terminus fragment of Egr-1 from amino acids 29–171) each had apparent molecular masses that matched their calculated molecular masses of 51, 66, and 43 kDa, respectively, as shown in Fig. 1, C and D.

Purified CKII can be autophosphorylated on its β-subunit, and (γ-32P)ATP was used to tag CKII by autophosphorylation. The labeled CKII was incubated with the various GST-Egr-1 fusion proteins attached to glutathione-Sepharose beads. As shown in Fig. 1E, the association of CKII with Egr-1 was indicated by the presence of the 25-kDa phosphorylated β-subunit band with F-1, but not with GST alone. It was also found that CKII associated with C-2 as well as with C-1, but to a lesser extent. However, no association occurred with N-1. These results indicated that the CKII association domain within Egr-1 lies between amino acids 322 and 519. This region contains the three zinc fingers as well as the 5′ and 3′ nuclear localization signals as identified by Gashler et al. (13) and Russo et al. (14). The last 14 amino acids at the C terminus of Egr-1 are unlikely to be involved in CKII binding as immunoprecipitation with 588 (an anti-Egr-1 antibody raised against the last 14 amino acids) did not disrupt the interaction between the two proteins (results not shown).

CKII Phosphorylates Egr-1 in Vitro—In order to ascertain whether CKII phosphorylates Egr-1 in vitro, F-1 was incubated with CKII and [γ-32P]ATP-labeled ATP. As shown in Fig. 2A, F-1 (lane 1) but not GST alone (lane 2) was phosphorylated by CKII. Phosphoamino acid analysis was performed on the phosphorylated F-1, and as shown in Fig. 2B the phosphorylation occurs more on serine than on threonine residues. To test this in vivo, Egr-1 was immunoprecipitated from platelet-derived growth factor-induced, [32P]orthophosphate-labeled, NIH 3T3 cells, and in this case strong phosphorylation on serine and weak phosphorylation on threonine residues were observed (Fig. 2C).

The specificity of Egr-1 phosphorylation by CKII was investigated. Phosphorylation of the F-1 fusion protein by CKII was performed with different concentrations of the kinase. Increasing the amount of CKII resulted in a corresponding increase in the phosphorylation of Egr-1 (Fig. 3A, lanes 1–5). It has been reported that CKII phosphorylates a specific substrate, RRREEE (33) and can also phosphorylate consensus sequences SXXD/E, SXXD/E, or SXXS/E (34). The phosphoryla-
Egr-1 containing an S at the onadensitometer and the relativedensities were plotted. The numbers

B

7.5% SDS-PAGE and the gel was fixed, dried, and exposed to x-ray film. The 32P-labeled Egr-1 band was excised from the membrane and subjected to two-dimensional phosphoamino acid analysis on thin layer chromatography plates as described under “Experimental Procedures.” The plates were exposed to x-ray film, and in the resulting autoradiograph the position of amino acid standards are indicated by dotted lines and labeled by the one-letter amino acid code. C, phosphoamino acid analysis of in vivo phosphorylated Egr-1. Confluent NIH 3T3 cells were labeled with 32P-orthophosphate for 4 h and induced with platelet-derived growth factor (75 ng/ml) for 2 h. The cell extract was immunoprecipitated by an anti-Egr-1 polyclonal antibody, and the labeled Egr-1 was subjected to phosphoamino acid analysis as described in B.

DNA was then cloned in vectors containing a SP6, T7, or T3 promoter. The [35S]methionine-labeled CNBr peptides were then synthesized in vitro with a rabbit reticulocyte lysate system and resolved by 16.5% SDS-PAGE. The peptides thus produced were in close agreement to their predicted molecular mass indicated in Fig. 4A (data not shown).

In parallel to the above experiment, F-1 was phosphorylated by CKII, digested by CNBr, and resolved by 16.5% SDS-PAGE. Fragment 10 was strongly phosphorylated and fragment 7 moderately phosphorylated as compared to the other fragments (Fig. 4B, lane 1, 2-h exposure). The phosphorylation on fragment 5 and 8 was also observed on longer exposure of the autoradiograph (Fig. 4B, lane 2, 6-h exposure), whereas fragment 6 is detected only on overnight exposure (not shown). Some of the high molecular mass bands as shown by asterisks in Fig. 4B are due to incomplete CNBr digestion of Egr-1. CKII phosphorylation sites on Egr-1 in vitro were compared with those found in vivo from serum-induced, 32P-labeled NIH 3T3 cells. Egr-1 was immunoprecipitated from these cells and digested with CNBr. Fragments 5 and 10 were strongly phosphorylated, whereas fragment 7 was moderately phosphorylated in vivo (Fig. 4B, lane 3). Phosphorylation of fragments 6 and 8 was also observed only on longer exposure of the gel. Hence, serum-induced phosphorylation of fragments 5, 7, and 10 could be due to endogenous CKII, but the differential levels of phosphorylation on these fragments suggest that CKII is not the only kinase that phosphorylates Egr-1 in vivo.

To further delineate the phosphorylation sites, CKII in vitro phosphorylated Egr-1 was digested with trypsin. Small peptides were separated on HPLC, and the fractions containing 32P-labeled peptides were subjected to microsequencing. The results indicate that these trypsin-generated peptides are parts of fragments 5, 6, 7, and 8 (see Fig. 4A (c)). The labeled peptides contain CKII sites (34) which are S101XDXD in fragment 5, T145XDXE in fragment 6, S194XDXD in fragment 7, and S299XDXD in fragment 8, all of which are potential sites for CKII phosphorylation. There are three CKII recognition sites (S376XDXD, T389XDXE, and T516XXDXD) in fragment 10. T389 and T516 are unlikely to be responsible for the strong phosphorylation of fragment 10 as phosphorylation on threonine is much weaker than that on serine. Thus S376 is likely to be the major phosphorylation site by CKII within fragment 10. The reason S376XDXD could not be detected by this method, despite heavy phosphorylation in fragment 10, is because trypsin digestion
resulted in a peptide around S376 of only four amino acids (NFS376R) which are not resolved by HPLC. We further confirmed phosphorylation of fragments 6, 8, and 10 by sequencing these peptides from excised gel sections (data not shown). Identification of the exact sites of CKII phosphorylation would require a site-directed mutational approach.

Effect of CKII Phosphorylation on Egr-1 DNA Binding Activity—The three zinc fingers of Egr-1 are located from amino acids 332–416. As fragment 10 contains the second and third zinc fingers, it is likely that phosphorylation on this fragment alters its DNA binding activity. To study the effect of CKII phosphorylation on Egr-1 DNA binding activity, GST-Egr-1 fusion protein (F-1) was phosphorylated with CKII prior to carrying out the DNA binding reaction in a gel mobility shift assay. The phosphorylation of Egr-1 by CKII resulted in a decrease in its DNA binding activity in a stoichiometric manner (Fig. 5, lanes 2–6). This decrease in DNA binding activity can be blocked competitively in a dose-dependent manner by the increasing concentrations of either CKII specific substrate peptide (Fig. 5, lanes 7–10) or by the FX peptide which has SXD as the CKII phosphorylation site (lanes 11–14).

CKII Affects the Transcriptional Activity of Egr-1 in NIH 3T3 Cells—In order to study the effect of CKII phosphorylation on Egr-1 transcriptional activity in vivo, NIH 3T3 cells were cotransfected with a chloramphenicol acetyltransferase reporter plasmid comprised of three Egr-1 binding sites, an Egr-1 expression plasmid, pCMV-Egr-1, and a CKII expression plasmid, pCMVCKII (containing the CKII α-subunit). The CKII activity in untransfected and transfected cells was determined to ensure that the pCMVCKII plasmid produced active CKII in NIH 3T3 cells. A 1.6-fold increase in CKII activity was observed on transfecting cells with 2 μg of pCMVCKII α-subunit (data not shown), which was in close agreement with previously reported results in COS-1 cells (32).

Transfection of cells with increasing concentrations of pCMV-Egr-1 results in an increase in transcriptional activity reflected by an increase in chloramphenicol acetyltransferase activity (Fig. 6A, top panel). However, when the increasing concentrations (1, 2, and 5 μg) of CKII plasmids were co-transfected with egr-1, a corresponding decrease in egr-1 transcriptional activity was observed (Fig. 6A, lower panels).

Nuclear extracts were prepared from cells transfected with or without pCMVCKII in the presence of pCMV-Egr-1 and gel mobility shift analysis was performed using the Egr-1 binding site as a probe. A decrease in DNA binding activity was found when CKII was co-transfected with pCMV-Egr-1 (Fig. 6B, panel i, lane 2). Western analysis of these extracts showed that there was no change in the amount of Egr-1 protein present in
CKII phosphorylation of Egr-1 affects its transcriptional activity. A, expression of the CKII α-subunit reduces Egr-1 transcriptional activity. Chloramphenicol acetyltransferase assays were performed on NIH 3T3 cells transfected with increasing concentrations (shown at the bottom) of pCMV-Egr-1 alone (top panel) or with increasing concentrations of pCMV-CKII α-subunit (1, 2, and 5 μg, shown at the left of the panels). TLC plates were exposed to x-ray film and the resulting autoradiograph is shown. B, a decrease in transcription is related to a decrease in DNA binding activity. Cells were transfected with (10 μg) of pCMV-Egr-1 with (5 μg) or without pCMVCKII. Nuclear extracts were prepared from the transfected cells, subjected to gel mobility shift analysis as described under “Experimental Procedures,” and are shown in an autoradiograph (panel i). The same amount (10 μg) of nuclear protein used for gel mobility shift analysis was separated by SDS-PAGE and subjected to Western blot analysis with an anti-Egr-1 antibody, and the signal was detected by enhanced chemiluminescence (panel ii). C, increased phosphorylation of transfected egr-1 gene product by co-transfection with CKII expression plasmid. Panel i, NIH 3T3 cells were transfected with pCMV-Egr-1 in the presence (5 μg) or absence (0 μg) of pCMVCKII and labeled with [32P]orthophosphate. Cells were lysed with radioimmune precipitation buffer and immunoprecipitated by an anti-Egr-1 antibody (1133). The precipitates were separated by 7.5% SDS-PAGE, transferred to a nitrocellulose membrane, and exposed to x-ray film, and the resulting autoradiograph is shown. Panel ii, Western blot of the same membrane probed with an anti-Egr-1 antibody, the location of which was revealed by enhanced chemiluminescence.

Fig. 6. CKII phosphorylation of Egr-1 affects its transcriptional activity. A, expression of the CKII α-subunit reduces Egr-1 transcriptional activity. Chloramphenicol acetyltransferase assays were performed on NIH 3T3 cells transfected with increasing concentrations (shown at the bottom) of pCMV-Egr-1 alone (top panel) or with increasing concentrations of pCMV-CKII α-subunit (1, 2, and 5 μg, shown at the left of the panels). TLC plates were exposed to x-ray film and the resulting autoradiograph is shown. B, a decrease in transcription is related to a decrease in DNA binding activity. Cells were transfected with (10 μg) of pCMV-Egr-1 with (5 μg) or without pCMVCKII. Nuclear extracts were prepared from the transfected cells, subjected to gel mobility shift analysis as described under “Experimental Procedures,” and are shown in an autoradiograph (panel i). The same amount (10 μg) of nuclear protein used for gel mobility shift analysis was separated by SDS-PAGE and subjected to Western blot analysis with an anti-Egr-1 antibody, and the signal was detected by enhanced chemiluminescence (panel ii). C, increased phosphorylation of transfected egr-1 gene product by co-transfection with CKII expression plasmid. Panel i, NIH 3T3 cells were transfected with pCMV-Egr-1 in the presence (5 μg) or absence (0 μg) of pCMVCKII and labeled with [32P]orthophosphate. Cells were lysed with radioimmune precipitation buffer and immunoprecipitated by an anti-Egr-1 antibody (1133). The precipitates were separated by 7.5% SDS-PAGE, transferred to a nitrocellulose membrane, and exposed to x-ray film, and the resulting autoradiograph is shown. Panel ii, Western blot of the same membrane probed with an anti-Egr-1 antibody, the location of which was revealed by enhanced chemiluminescence.

To show that this decrease is a result of phosphorylation of the Egr-1 protein by CKII, transfected cells were labeled with [32P]orthophosphate. Egr-1 was immunoprecipitated with Egr-1 antibody, resolved by 7.5% SDS-PAGE, and transferred to a nitrocellulose membrane, and autoradiography was performed. A 2-fold increase in Egr-1 phosphorylation was observed when cells were co-transfected with pCMV-Egr-1 in the presence of pCMVCKII as compared to that in the absence of pCMVCKII (Fig. 6C, panel i). Western blot analysis was performed on the same nitrocellulose membrane to confirm that there was no difference in the amount of Egr-1 protein (Fig. 6C, panel ii).

DISCUSSION

To activate or repress transcription, transcription factors must locate to the nucleus, bind DNA, and interact with the basal transcription apparatus (35). Accordingly, extracellular signals that regulate transcription factor activity may affect one or more of these processes. Most commonly regulation is achieved by phosphorylation of the transcription factor(s) which in turn modulate either its trans-activating activity or DNA binding activity (36). Although Egr-1, a transcription factor, has been shown to play an important role in proliferating and differentiating cells, little is known of its function in relation to its phosphorylation status. In this report we show that Egr-1 is phosphorylated by the protein kinase CKII and that this has a negative effect on its DNA binding and transcription activities.

Although the CKII phosphorylation pattern of Egr-1 does not exactly coincide with that of serum-induced Egr-1, it is possible that CKII is one of several kinases which phosphorylate and regulate Egr-1 function. As shown in Fig. 4A, the CKII phospho-

phorylation sites on Egr-1 cover the regions of the protein important for its transcriptional activation (fragment 7), repression binding (fragment 8), nuclear import, and DNA binding (fragment 10). The CKII sites present on these fragments are of S/TXDXE, S/TXDXE, and S/TXXDXE types (34). The observation that CKII attenuates Egr-1 DNA binding activity appears to be contradictory to earlier reports that Egr-1 DNA binding activity (25) is elevated after cell stimulation with growth factors. This could be due to a change in conformation arising from phosphorylation by other kinases beside CKII after serum induction. Furthermore, strong phosphorylation in the activation domain (fragment 5) of Egr-1 in vivo after serum induction might lead to an increase in its transcriptional activity.

It has been reported that the CKII α-subunit alone is sufficient to increase CKII activity in vivo for several reasons. First, the α-subunit alone is moderately active without the β subunit (37, 38). Second, the α-subunit in nuclei is not bound to β but is complexed to other nuclear components (39). Third, the β-subunit is synthesized in large excess over the α-subunit and only a small fraction of it contributes to the tetrameric holoenzyme complex (40). In our experiments, co-transfection of pCMV-

CKII α and pCMV-Egr-1 results in a decrease in transcriptional activity in vivo which is possibly due to a decrease in DNA binding activity caused by CKII phosphorylation of the DNA binding fragment.

Besides phosphorylating Egr-1, the association of CKII and Egr-1 could also induce further protein-protein interactions. As CKII is a tetrameric protein, it is possible that by anchoring different proteins to its four subunits it could promote interactions between diverse proteins. DNA topoisomerase II, which associates with CKII, is believed to participate in DNA transcription (as well as replication and recombination) as part of a multienzyme complex (41). CKII could thus be the building block for such a multienzyme complex which might possibly include a transcription factor. On the other hand, CKII has also been found to associate with the proteasome complex resulting in its degradation (42). Hence, CKII association with Egr-1 could be a prelude to degradation. This would correlate well with the fact that CKII phosphorylation of Egr-1 leads to a decrease in DNA binding and transcriptional activities. It is thus possible that Egr-1 transcriptional activity might be affected via its link with CKII by other CKII associated proteins.

Our data show that CKII associates with Egr-1 and regulates its DNA binding and transcriptional activities by phosphorylation. Egr-1 is not the only DNA-binding protein that is inhibited by CKII. Other such proteins are c-jun (19), c-Myb (43), and serum response factor (44). It is now necessary to study Egr-1 regulation in the context of CKII. A map of CNBr fragments of Egr-1 has been generated and the fragments phosphorylated by CKII have been identified. This will facilitate not only the further study of Egr-1 phosphorylation by CKII as well as other kinases but also aid in determining the phosphorylated residues on Egr-1 and lead to a better understanding of Egr-1 function in regulating cell growth and differentiation.

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