Cyclic GMP-dependent Protein Kinase Regulates CCAAT Enhancer-binding Protein β Functions through Inhibition of Glycogen Synthase Kinase-3*

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The CCAAT enhancer-binding protein (C/EBPβ) plays an important role in the regulation of gene expression during cell proliferation, differentiation, and apoptosis. We previously showed that C/EBPβ participates in cGMP-regulated transcription of c- fos in osteoblasts (Chen, Y., Zhuang, S., Cassenaer, S., Casteel, D. E., Gudi, T., Boss, G. R., and Pilz, R. B. (2003) Mol. Cell. Biol. 23, 4066–4082). In the present work, we show that cGMP/cGMP-dependent protein kinase (PKG) induced dephosphorylation and activation of C/EBPβ by inhibiting glycogen synthase kinase-3β (GSK-3β). Phosphorylation of GSK-3β on Ser9 negatively regulates the enzyme activity, and we found that PKG phosphorylated this site both in vitro and in vivo; the in vivo phosphorylation occurred rapidly and preceded C/EBPβ dephosphorylation. Previous studies with GSK-3 inhibitors suggest that GSK-3β is a C/EBPβ kinase in resting cells. We determined that GSK-3β phosphorylated C/EBPβ in vitro on Thr189, Ser185, Ser181, and Ser177; C/EBPβ was phosphorylated on these same sites in intact, unstimulated osteoblasts, and phosphorylation was decreased in cGMP-treated cells. Mutation of the GSK-3 phosphorylation sites in C/EBPβ prevented C/EBPβ phosphorylation in resting cells, enhanced C/EBPβ DNA binding, and led to increased target gene transactivation, mimicking the stimulatory effects of cGMP on C/EBPβ. cGMP regulation of C/EBPβ was disrupted by a mutant GSK-3β(Ala) resistant to cGMP/PKG phosphorylation and inhibition. We conclude that cGMP increases the DNA binding potential of C/EBPβ by preventing the negative effects of GSK-3β phosphorylation.

Cyclic GMP is synthesized by cytosolic guanylate cyclases in response to nitric oxide (NO)2 stimulation and by receptor guanylate cyclases in response to binding of natriuretic peptides (1). The latter can act in an autocrine/paracrine fashion (e.g. C-type natriuretic peptide or PKG II develop dwarfism as a result of impaired bone development, whereas transgenic mice overexpressing C-type natriuretic peptide demonstrate marked skeletal overgrowth (4–6). In postnatal bone, NO moderates anabolic processes associated with mechanical loading, sex hormones, and fracture healing, with some studies demonstrating that the NO effects are mediated by cGMP (7–10). Thus, cGMP signaling is important for both bone development and homeostasis.

Regulation of gene expression by cGMP has been recognized relatively recently, with gene expression profiling contributing to the rapidly growing list of cGMP-regulated genes (11). In a variety of cultured cells and primary tissues, NO donors, natriuretic peptides, or membrane-permeable cGMP analogs induce rapid increases in c-fos, junB, and/or c-fos mRNA expression (11). We and others have shown that cGMP and calcium synergistically stimulate c-fos promoter activity in osteoblasts and neuronal cells and that this effect is mediated more efficiently by PKG II than PKG I (8–15). We found that the cGMP/calcium transcriptional synergism required cooperation between the transcription factors CCAAT enhancer-binding protein β (C/EBPβ, also known as NF-IL6 or LAP) and cAMP-response element-binding protein (CREB), with cGMP and calcium modulating the phosphorylation states of C/EBPβ and CREB, respectively (13).

C/EBPβ participates in multiple cellular functions, including cell proliferation, differentiation, tumorigenesis, and apoptosis (16–20). Like CREB, C/EBPβ is a leucine zipper transcription factor regulated by phosphorylation (19–25). C/EBPβ regulates multiple genes important for osteoblast functions, including c-fos, cyclooxygenase 2, and osteocalcin (13, 26–28). C/EBPβ also plays a key role in bone development, promoting differentiation of mesenchymal cells into osteoblasts (29); C/EBPβ-deficient mice have abnormal bone growth plate architecture with hypocalcification and increased apoptosis (30). Although C/EBPβ binds preferentially to CAAT enhancer elements, it also binds to the CAMP-response element (CRE) and can directly interact with CREB (13, 17). We found that C/EBPβ phosphorylation was decreased in cGMP-treated cells, whereas C/EBPβ-dependent transcriptional activity was increased, suggesting regulation of C/EBPβ function by cGMP-dependent dephosphorylation, involving cGMP inhibition of a C/EBPβ kinase and/or activation of a phosphatase (13).

Glycogen synthase kinase-3 (GSK-3) is a ubiquitously expressed serine/threonine protein kinase active in unstimulated cells; GSK-3 activity can be suppressed through phosphorylation of an N-terminal serine residue (Ser21 and Ser9 in the GSK-3α and -3β isoforms, respectively) (31, 32). GSK-3 regulates multiple cellular functions, including transcription; e.g. GSK-3 regulates the transcription factors c-Jun and C/EBPα, inhibiting c-Jun DNA binding and inducing a conformational
change in C/EBPα (the effects of GSK-3 phosphorylation on C/EBPα functions were not examined) (32–34). GSK-3β activity is suppressed by several signaling pathways; insulin activation of the phosphatidylinositol 3-kinase (PI3K)/Akt pathway, cAMP activation of cAMP-dependent protein kinase (PKA), or Ras activation of extracellular signal-regulated kinases (Erk-1/2) leads to Ser8 phosphorylation of GSK-3β and inhibition of GSK-3β activity (31, 32, 35, 36). We hypothesized that GSK-3 might be responsible for constitutive C/EBPβ phosphorylation in resting osteoblasts and that down-regulation of GSK-3 activity by cGMP/PKG could explain the decreased C/EBPβ phosphorylation in cGMP-treated cells. Our results support this hypothesis and establish a novel pathway for C/EBPβ regulation that includes cGMP, PKG, II, and GSK-3β.

EXPERIMENTAL PROCEDURES

Plasmids and Site-directed Mutagenesis—The expression vector encoding PKG II (pRC/CMV-GKII) was from S. Lohmann (37). Wild type and mutant GSK-3β (Ala9) were from J. Woodgett (32); a cDNA encoding rat C/EBPβ was from L. Sealy (38). Bacterial expression vectors for His-tagged C/EBPβ were constructed in pSET-B (Novagen); mammalian expression vectors for Myc epitope-tagged C/EBPβ were constructed in pXJ40-Myc (gift of Z. S. Zhao and E. Manser (39)). Site-directed mutagenesis was performed using the QuikChange™ mutagenesis kit according to the manufacturer’s protocol (Stratagene). All mutations were verified by sequencing the full cDNA. The reporter construct pCRE-Luc (containing four copies of a consensus CRE site) and pRSET-B (Novagen) were constructed in pRSET-B (Novagen); a cDNA encoding PKG II (pRC/CMV-GKII) was from L. Sealy (38).

Cell Culture and Transfections—Rat UMR106 osteosarcoma cells and human lung fibroblast WI-38 cells were from the American Type Culture Collection. All cells were routinely cultured in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum. UMR106 cells were transfected with Lipofectamine™ Plus (Invitrogen) or pXJ40-Myc (gift of Z. S. Zhao and E. Manser (39)). Site-directed mutagenesis was performed using the QuikChange™ mutagenesis kit according to the manufacturer’s protocol (Stratagene). All mutations were verified by sequencing the full cDNA. The reporter construct pCRE-Luc (containing four copies of a consensus CRE site) and pRSET-B (Novagen) were described previously (13).

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Antibodies and Western Blots—SDS-PAGE and Western blotting were performed as described previously (13). Antibodies specific for GSK-3β, phospho-GSK-3β(Ser9), phospho-GSK-3α/β(Ser21), Akt, phospho-Akt(Ser473), and phospho-Akt(Thr308) were from Cell Signalling Technology. Antibodies specific for C/EBPβ (mouse and rabbit), and for the Myc epitope tag were from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). The PKG II-specific antibody was a gift from Dr. S. Lohmann (37). For the analysis of GSK-3 and Akt phosphorylation, cells were transferred to serum-free medium overnight and exposed to fresh serum-free Dulbecco’s modified Eagle’s medium for 30 min prior to drug treatment for the indicated times.

Recombinant Proteins and in Vitro Phosphorylation Studies—Recombinant GSK-3β purified from bacteria was from New England Biolabs. PKG II purified from baculovirus-infected insect cells was from Alexis Biochemicals. His-tagged wild type and mutant C/EBPβ proteins were purified from bacterial inclusion bodies after solubilization in 6 M urea as described previously (34, 40), with minor modifications; nickel affinity chromatography was performed in the presence of urea, and the proteins were slowly renatured on the column with a linear urea gradient (from 6 to 0 M) over 12 h, prior to elution with imidazole. The proteins were purified to homogeneity as judged by SDS-PAGE/Coomassie Blue staining and were DNA binding–competent.

In vitro phosphorylation of GSK-3β was performed in a total volume of 10 μl, with 0.3 pmol of PKG II incubated at 30 °C with variable amounts of GSK-3β in kinase assay buffer (20 mM Tris-HCl, 10 mM MgCl2, 5 mM dithiothreitol, pH 7.5) and 5 μM [γ-32P]ATP in the presence or absence of 10 μM cGMP for the indicated time. For Western blots with anti-phospho-GSK3β(Ser9) antibody, reactions were performed using unlabeled ATP. For in vitro phosphorylation of C/EBPβ, 100 pmol of His-tagged wild type or mutant C/EBPβ was incubated with 1 pmol of either GSK-3β or purified catalytic subunit of PKA (gift of S. Taylor) in kinase assay buffer with 5 μM [γ-32P]ATP at 30 °C for 30 min. Proteins were separated by SDS-PAGE, transferred to Immobilon-P™, and analyzed by autoradiography. The phosphorylated C/EBPβ band was cut out, digested with trypsin, and analyzed by two-dimensional phosphopeptide mapping using high voltage electrophoresis in the first dimension and thin layer chromatography in the second dimension as described (40).

Some in vitro phosphorylation experiments were performed with Myc epitope-tagged wild type and mutant C/EBPβ proteins expressed in UMR106 cells. Cells were transfected with 1 μg of C/EBPβ expression vector per 6-well dish, C/EBPβ was isolated by immunoprecipitation with anti-Myc antibody, and precipitates were washed in kinase assay buffer and incubated with purified GSK-3β and [γ-32P]ATP.

Alkaline Phosphatase Treatment of C/EBPβ—UMR106 cells were transfected with 20 ng of expression vector encoding Myc epitope-tagged wild type and mutant C/EBPβ per 6-well dish. Anti-Myc immunoprecipitates were isolated and incubated at 37 °C for 30 min with either buffer alone (50 mM Tris-HCl, 100 mM NaCl, 10 mM MgCl2; 1 mM dithiothreitol, pH 7.9) or buffer plus 5 units of calf intestinal phosphatase (New England Biolabs), in the presence or absence of the phosphatase inhibitors β-glycerolphosphate (10 mM), NaF (10 mM), and imidazole (2 mM).

In Vivo Phosphorylation of C/EBPβ—UMR106 cells were either left untransfected or transfected in 6-well dishes with 50 ng of PKG II and 20 ng of wild type C/EBPβ, mutant C/EBPβ, or pkXJ40-Myc empty vector as indicated. Forty h after transfection, cells were transferred to phosphate-free Dulbecco’s modified Eagle’s medium for 1 h and labeled for 4 h with 32P-O4 (100 μCi/6-well dish), with 250 μCi 8-CPT-cGMP added to some cultures for the last 1 h. Cell lysates were subjected to immunoprecipitation with either a C/EBPβ-specific antibody (see Fig. 3) or anti-Myc antibody (see Fig. 4), and immunoprecipitates were analyzed by SDS-PAGE, electroblotting, and autoradiography. The amount of C/EBPβ present in the immunoprecipitates was determined by blotting with an anti-C/EBPβ antibody.

Electrophoretic Mobility Shift Assays—Nuclear extracts were incubated with 5'-end-labeled double-stranded oligodeoxynucleotide (oligo-dN) probes encoding a canonical CRE or C/EBP binding sequence and analyzed by nondenaturing PAGE and autoradiography as described previously (13). The CRE oligo-dNT (5'-AGAGATTGCCTGACGTCAAGAGAGCTAG-3') and the canonical C/EBP binding site (5'-TGGCAATTGGCAGGAAATTCTGA-3') were from Promega Life Sciences and Santa Cruz Biotechnology, respectively. (The CRE and C/EBP consensus sequences are underlined.) For supershift assays, nuclear extracts were incubated for 20 min at 4 °C with specific antibodies prior to the addition of probe (13).

Data Presentation—Results presented in bar graphs represent the mean ± S.D. of at least three independent experiments performed in duplicate. Autoradiographs and Western blots demonstrate a representative experiment performed at least three times with similar results.
RESULTS

cGMP Induces PKG II-dependent Phosphorylation of GSK-3β on Ser9—Early passage UMR106 rat osteoblasts express both PKG I and PKG II, and c-fos mRNA is synergistically induced by cGMP and calcium (13). This synergistic effect is dependent on PKG activity, is enhanced more by transfection of PKG II than PKG I, and requires cooperation between C/EBPβ and CREB, with cGMP and calcium regulating C/EBPβ and CREB activity, respectively (13). In C6 glialoma cells, cGMP induces dephosphorylation of C/EBPβ, suggesting that cGMP/PKG regulate the activity of a C/EBPβ kinase or phosphatase (13). GSK-3 is a candidate kinase, because GSK-3 is active in unstimulated cells, its activity is down-regulated by multiple signal transduction pathways, and GSK-3 can phosphorylate C/EBPs and CREB activity, respectively (13). In C6 glioma cells, cGMP induces dephosphorylation of C/EBPβ, suggesting that cGMP/PKG regulate the activity of a C/EBPβ kinase or phosphatase (13). GSK-3 is a candidate kinase, because GSK-3 is active in unstimulated cells, its activity is down-regulated by multiple signal transduction pathways, and GSK-3 can phosphorylate C/EBPβ and CREB activity, respectively (13). In C6 glioma cells, cGMP induces dephosphorylation of C/EBPβ, suggesting that cGMP/PKG regulate the activity of a C/EBPβ kinase or phosphatase (13). GSK-3 is a candidate kinase, because GSK-3 is active in unstimulated cells, its activity is down-regulated by multiple signal transduction pathways, and GSK-3 can phosphorylate C/EBPβ and CREB activity, respectively (13).

To determine the potential role of GSK-3 in mediating the effects of cGMP on C/EBPβ, we examined whether PKG activation induces GSK-3 phosphorylation on a regulatory site that inhibits GSK-3 activity (i.e. Ser9 on GSK-3β) (31, 32).

Early passage UMR106 cells were incubated in serum-free medium and treated for 20 or 60 min with the membrane-permeable cGMP analog 8-CPT-cGMP, and the phosphorylation state of GSK-3β was assessed by Western blotting using an antibody specific for GSK-3β phosphorylated on Ser9. 8-CPT-cGMP increased GSK-3 Ser9 phosphorylation to a similar degree as serum (Fig. 1, A, upper panel, compare lanes 2 and 3, cells treated with cGMP, and lane 5, serum-stimulated cells, with lanes 1 and 4, untreated cells). Specificity of the anti-phospho-Ser9 antibody was demonstrated using purified GSK-3β phosphorylated by PKA in vitro (data not shown, but Fig. 2B, discussed below, shows similar results). Equal loading of the blot was shown by reprobing with an antibody recognizing GSK-3β irrespective of its phosphorylation state (Fig. 1A, middle). UMR106 cells express low levels of GSK-3α, preventing assessment of the cGMP effect on GSK-3α phosphorylation.

Since cGMP can activate the PI3K/Akt pathway in some cell types and Akt can directly phosphorylate GSK-3β on Ser9 (31, 42–44), we examined the effect of cGMP on Akt phosphorylation on Ser473 and Thr308, two phosphorylation events associated with Akt activation (31). cGMP had no effect on Akt Ser473 phosphorylation, whereas serum stimulation increased Akt phosphorylation on this site (Fig. 1A, bottom; similar results were obtained with anti-phospho-Thr308 antibodies). We previously demonstrated that 8-CPT-cGMP does not cross-activate PKA or stimulate Erk-1/2 kinase activity in UMR106 cells (13). Therefore, the effect of cGMP on GSK-3β phosphorylation does not appear to be due to cGMP regulation of pathways known to regulate GSK-3 (i.e. PI3K/Akt, PKA, and Erk-1/2/RSK) (31, 32, 35).

As occurs in other cell types (45), we found that PKG expression progressively declined in UMR106 cells after prolonged passage in culture; total PKG activity, representing PKG I and II, was 220 ± 25 pmol/min/mg protein in early passage cells and <20 pmol/min/mg protein in late passage cells. In late passage UMR106 cells, 8-CPT-cGMP did not increase GSK-3β Ser9 phosphorylation in either untransfected cells or cells transfected with an empty vector, but transfecting cells with a PKG II expression vector restored the effect of cGMP on GSK-3 phosphorylation (Fig. 1B, compare top left panel, cells transfected with empty vector, with top right panel, cells transfected with PKG II expression vector; total GSK-3 levels and PKG II expression are shown in the second and the lowest panel, respectively). cGMP induced GSK-3β phosphorylation within 5 min, and the effect persisted for several hours, but cGMP did not change Akt phosphorylation (Fig. 1B; phospho-Akt levels are shown in the third panel). Similar results were obtained in PKG-deficient C6 rat glioma cells transfected with PKG II expression vector versus empty vector (data not shown). These results indicate that the effect of cGMP on GSK-3 phosphorylation is mediated by PKG II and independent of Akt.

To determine whether the effect of cGMP on GSK-3 phosphorylation occurred in other cells expressing endogenous PKG II, we examined human embryonal W138 fibroblasts. Treating W138 cells with 8-CPT-cGMP induced a rapid and sustained phosphorylation of GSK-3β Ser9 with no detectable change in Akt Ser473 phosphorylation (Fig. 1C). Thus, GSK-3β phosphorylation on Ser9, a site that negatively regulates kinase activity, was increased by cGMP in several cell types, including osteoblasts, glial cells, and embryonal lung fibroblasts.

Purified PKG II Directly Phosphorylates GSK-3β Ser9 in Vitro—Since 8-CPT-cGMP induced GSK-3β Ser9 phosphorylation in intact cells without activation of Akt, PKA, or Erk-1/2, we asked whether PKG II can directly phosphorylate this site in vitro. Incubating purified PKG II with recombinant GSK-3β in the presence of [γ-32P]ATP and cGMP led to high levels of 32P incorporation into GSK-3β; in the absence of cGMP, lower GSK-3 phosphorylation was observed, consistent with low basal PKG II activity (Fig. 2A, compare lanes 3 and 4, GSK-3β incubated with PKG II in the presence and absence of cGMP, respectively, with lane 1, GSK-β alone, and lane 2, PKG II alone; note PKG II autophosphorylation). A similar experiment, performed with unlabeled ATP and with the reaction products analyzed by Western blotting using...
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FIGURE 2. GSK-3β phosphorylation by PKG II in vitro. A, purified GSK-3β (lanes 1, 3, and 4) and/or purified PKG II (lanes 2–4) were incubated in the presence of [γ-32P]ATP for 30 min as described under “Experimental Procedures”; to some samples, 10 μM cGMP was added (lanes 2 and 3). Reaction products were analyzed by SDS-PAGE/autoradiography; autophosphorylated PKG II is visible on the top. B, purified GSK-3β (lanes 1–3) and PKG II (lanes 2 and 3) were incubated in the presence of unlabeled ATP; 10 μM cGMP was added to the sample in lane 2. Reaction products were analyzed by SDS-PAGE/Western blotting using antibodies specific for GSK-3β phosphorylated on Ser9 (top), or total GSK-3β (bottom). C, variable amounts of GSK-3β were incubated with PKG II and [γ-32P]ATP in the presence of 10 μM cGMP for 5 min. Reaction products were analyzed by SDS-PAGE/autoradiography (shown as an inset, with 0.07, 0.13, 0.25, 0.5, 1, 2.5, and 5 pmol of GSK-3β in lanes 1–7). The bands representing phosphorylated GSK-3β were excised, and the amount of 32PO4 in each band was quantitated by scintillation counting and comparison to a standard curve (generated by counting variable amounts of [γ-32P]ATP). A Lineweaver-Burk plot was generated using the mean ± S.D. of three independent experiments, yielding a Kₘ of 0.15 μM.

Characterization of GSK-3β as a C/EBPβ Kinase—Both C/EBPα and C/EBPβ contain a highly conserved serine/threonine-rich region adjacent to the C-terminal basic leucine zipper domain (Fig. 4A; the amino acid sequence of the serine-rich region in rat, mouse, and human C/EBPβ is 100% conserved). Ross et al. (34) observed C/EBPα phosphorylation in resting cells, which was decreased in cells treated with lithium, a GSK-3 inhibitor. They also showed GSK-3 phosphorylation of C/EBPα in vitro and mapped phosphorylation to three sites that constitute a typical GSK-3 consensus sequence (S/T)XXX(S/T), where X represents any amino acid; Fig. 4A). C/EBPβ immunoprecipitated from mammalian cells was also phosphorylated by GSK-3 in vitro, but the phosphorylation sites were not determined (41). Therefore, we decided to analyze C/EBPβ phosphorylation by GSK-3 in further detail.

Recombinant C/EBPβ purified from bacteria was incubated with [γ-32P]ATP in the presence or absence of purified GSK-3β or PKA catalytic subunit (Fig. 4A), with the latter serving as a positive control (Fig. 3A). Maximal 32PO4 incorporation into C/EBPβ was higher in the presence of GSK-3β than PKA, and the GSK-3β-phosphorylated protein migrated with a slightly higher apparent molecular weight than the PKA-phosphorylated protein. A two-dimensional phosphopeptide map of C/EBPβ phosphorylated by GSK-3 in vitro produced several confluent spots with low mobility in both dimensions (Fig. 3B, left). The latter map is similar to published data and thus served as a control for complete digestion of C/EBPβ (40). Using site-directed mutagenesis, PKA has been shown to phosphorylate rat C/EBPβ at Ser105 and Ser240 (in the left panel of Fig. 3B, phosphopeptides eliminated by mutation of Ser105 and Ser240 are marked with x and y, respectively) (40). GSK-3, therefore, does not appear to target Ser105 or Ser240 in vitro, although Ser105 together with Ser101 forms a potential GSK-3 consensus sequence (47).

To examine C/EBPβ phosphorylation in intact cells, UMR106 cells were incubated with [γ-32P]ATP for 4 h, and some cultures received 8-CPT-cGMP for 1 h prior to isolating C/EBPβ by immunoprecipitation. As previously shown in C6 glioma cells (13), cGMP treatment of UMR106 cells decreased 32PO4 incorporation into C/EBPβ by about 50% (Fig. 3C, compare lanes 2 and 3, cells cultured in the absence or presence of 8-CPT-cGMP; lane 1 shows cells subjected to immunoprecipitation with control IgG). To increase the recovery of C/EBPβ, we performed similar experiments in UMR106 cells transfected with small amounts of expression vectors encoding C/EBPβ and PKG II. As occurred with the endogenous protein, 32PO4 incorporation into transfected C/EBPβ was decreased in cGMP-treated cells (Fig. 3C, compare lanes 4 and 5). Unless stated otherwise, the amounts of transfected C/EBPβ and PKG II were titrated not to exceed endogenous protein levels by more than 2–3-fold. When the in vitro phosphorylated C/EBPβ from untreated UMR106 cells was subjected to two-dimensional phosphopeptide mapping, the majority of the radioactivity was recovered in several confluent spots with low mobility in both dimensions (arrow in Fig. 3B, right panel), similar to the spots obtained with C/EBPβ phosphorylated by GSK-3 in vitro (Fig. 3B, compare middle and right panels). Phosphopeptide maps obtained with C/EBPβ isolated from cGMP-treated cells showed decreased intensity of these confluent low mobility spots. Similar results were obtained in C6 glioma cells (not shown). Since GSK-3 is active in unstimulated cells (31, 32), these results suggest that phosphorylation of C/EBPβ in untreated cells may be due to GSK-3 and that...
C/EBPβ phosphorylation may be decreased in cGMP-treated cells because of GSK-3 inhibition. This conclusion is supported by the observation that C/EBPβ phosphorylation in intact cells is reduced when GSK-3 is inhibited by valproic acid or lithium (13, 41).

Mapping GSK-3β Phosphorylation Sites in the Serine-Rich Region of C/EBPβ—Since the serine-rich region of C/EBPβ contains a GSK-3 consensus motif that is conserved with the GSK-3 phosphorylation sites described in C/EBPα (Fig. 4A), we performed site-directed mutagenesis of C/EBPβ mutating all of the likely phosphoacceptor sites in this region to alanine residues. Wild type and mutant C/EBPβ containing Ala189, Ala185, Ala181, or Ala177 were purified from bacteria and incubated with GSK-3 in the presence of [γ-32P]ATP (Fig. 4B). Compared with wild type protein, 32PO4 incorporation into the C/EBPβ mutants Ala189 and Ala185 was severely reduced, whereas incorporation into mutants Ala181 and Ala177 was moderately decreased (Fig. 4B, top). Similar amounts of wild type and mutant proteins were present in the assay (Fig. 4B, bottom), shows a Coomassie stain of the unphosphorylated proteins). Thus, GSK-3 phosphorylates multiple sites in C/EBPβ. Thr189 and Ser185 appear to be preferred sites, and mutation of either site appears to impair phosphorylation of neighboring site(s). This may be because GSK-3 prefers serine/threonine residues immediately N-terminal to a proline, and GSK-3 is a “hierarchical” kinase whose substrate affinity is enhanced when the substrate is prephosphorylated at the +4-position of the consensus sequence (S/T)XXX(S/T)+ (32, 48). Some substrates require phosphorylation by a “priming” kinase at the +4-position, but others do not, and when sequential overlapping GSK-3 sites are present as in C/EBPβ, GSK-3 can act as its own priming kinase (48).

Purification of C/EBPβ from bacteria requires a denaturation/renaturation step, which could lead to variable amounts of unfolded proteins, which might be differentially recognized by kinases. We therefore performed additional in vitro phosphorylation experiments with C/EBPβ mutants expressed in mammalian cells. Myc epitope-tagged versions of wild type and mutant C/EBPβ were expressed at high levels in UMR106 cells, isolated by immunoprecipitation with anti-epitope antibody, and incubated with [γ-32P]ATP in the presence of purified GSK-3β. The pattern of 32PO4 incorporation into C/EBPβ mutants containing single amino acid substitutions (i.e. Ala189, Ala185, Ala181, or Ala177) was similar to the pattern observed with the bacterially expressed proteins (Fig. 4C, upper panel, compare lane 1, wild type protein, with lanes 2–5, single mutant proteins). These results confirm that Thr189 and Ser185 of C/EBPβ are preferred targets for GSK-3 in vitro. We also examined C/EBPβ mutants containing three or four alanine substitutions; we found minimal GSK-3β phosphorylation of the quadruple mutant containing Ala189, Ala185, Ala181, and Ala177 (M(A)4 in lane 6 of Fig. 4C) and reduced 32PO4 incorporation in the triple mutant containing Ala189, Ala181, and Ala177 (M(A)3 in lane 7 of Fig. 4C).
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All mutant proteins were expressed and immunoprecipitated at levels comparable with the wild type protein (Fig. 4C, bottom; lane 8 shows an immunoprecipitate obtained from cells transfected with empty vector). Thus, mutation of all four potential phosphoacceptor sites in the GSK-3 consensus sequence of C/EBPβ almost completely eliminated GSK-3 phosphorylation of the protein, and the triple mutant showed that GSK-3 can phosphorylate Thr189 in vitro.

To determine whether the same sites are targeted by GSK-3 in vivo, UMR106 cells expressing low levels of epitope-tagged C/EBPβ proteins were incubated with 32PO₄ and some cells were treated with cGMP. As described above, 32PO₄ incorporation into wild type C/EBPβ was lower in cGMP-treated compared with untreated cells, but minimal 32PO₄ incorporation into C/EBPβ M(A₄) occurred in either condition (Fig. 4D, upper panel, compare lanes 4 and 5, cells transfected with C/EBPβ M(A₄), with lanes 2 and 3, cells transfected with wild type C/EBPβ; the Western blot in the lower panel shows that similar amounts of wild type and mutant protein were immunoprecipitated). Thus, mutation of the four serine/threonine residues constituting a GSK-3 consensus sequence in C/EBPβ almost completely eliminated in vivo phosphorylation in UMR106 cells cultured in the presence or absence of cGMP, supporting the notion that GSK-3 is the main kinase phosphorylating C/EBPβ in resting cells.

Compared with wild type C/EBPβ isolated from UMR106 cells, C/EBPβ M(A₄) migrated slightly faster on SDS-PAGE (Fig. 4, D and E). To determine the effect of C/EBPβ phosphorylation on the protein’s migration, wild type and mutant C/EBPβ M(A₄) isolated from resting UMR106 cells were incubated with calf intestinal alkaline phosphatase. Dephosphorylation of wild type C/EBPβ by this nonspecific phosphatase caused most of the protein to shift to a faster migrating species that co-migrated with the M(A₄) mutant; phosphatase treatment did not affect migration of the mutant protein (Fig. 4E, compare lanes 1 and 2 with lanes 3 and 4; proteins incubated in the absence or presence of alkaline phosphatase, respectively; in lanes 5 and 6, protein phosphatase inhibitors were included as a control). Thus, mutation of C/EBPβ M(A₄) is similar to migration of completely dephosphorylated wild type C/EBPβ. The fact that cGMP-induced dephosphorylation of wild type C/EBPβ is only partial and not associated with a detectable gel shift is discussed below.

Effect of C/EBPβ Phosphorylation on Transcriptional Activation of a Target Gene—We previously showed that C/EBPβ cooperates with CREB to stimulate transcription from CRE-containing promoters and that the transactivation potential of C/EBPβ at the CRE is regulated by cGMP (13). To determine whether the effect of cGMP is mediated by GSK-3 phosphorylation of C/EBPβ, we performed two sets of experiments. First, we compared the wild type and phosphorylation-deficient mutant C/EBPβ M(A₄) with respect to their ability to transactivate a CRE-driven luciferase reporter gene. UMR106 cells were co-transfected with pCRE-Luc and increasing amounts of expression vector encoding either wild type or mutant C/EBPβ, with some cells treated with 8-CPT-cGMP (Fig. 5A). As described previously (13), transfection of wild type C/EBPβ led to transactivation of the CRE-dependent reporter gene, and at each level of C/EBPβ expression, cGMP further enhanced reporter gene activity (Fig. 5A). As described previously (13), transfection of wild type C/EBPβ led to transactivation of the CRE-dependent reporter gene, and at each level of C/EBPβ expression, cGMP further enhanced reporter gene activity (Fig. 5A). As described previously (13), transfection of wild type C/EBPβ led to transactivation of the CRE-dependent reporter gene, and at each level of C/EBPβ expression, cGMP further enhanced reporter gene activity (Fig. 5A). As described previously (13), transfection of wild type C/EBPβ led to transactivation of the CRE-dependent reporter gene, and at each level of C/EBPβ expression, cGMP further enhanced reporter gene activity (Fig. 5A). As described previously (13), transfection of wild type C/EBPβ led to transactivation of the CRE-dependent reporter gene, and at each level of C/EBPβ expression, cGMP further enhanced reporter gene activity (Fig. 5A).

FIGURE 4. Analysis of C/EBPβ phosphorylation site mutants. A, the domain structure of C/EBP proteins. AD, activation domain; SRR, serine-rich region; DBD, DNA binding domain; LZD, leucine zipper domain (38). The serine-rich region of rat C/EBPβ (amino acid residues 171–194), including a putative GSK-3 consensus sequence (S/T)X(S/T)X(S/T)X(S/T) is enlarged; a homologous region of C/EBPα is shown below, which contains three sites phosphorylated by GSK-3 (in boldface type) (34). The sequence of the C/EBPβ serine-rich region is identical in the rat, mouse, and human protein (Thr189 in rat C/EBPβ corresponds to Thr188 in the mouse and Thr195 in the human protein). B, wild type (WT; lane 1) and mutant C/EBPβ proteins with the indicated amino acid substitutions (lanes 2–5) were purified from bacteria, and equal amounts of protein were incubated with purified GSK-3β and [γ-32P]ATP as described under “Experimental Procedures.” Phosphorylation products were analyzed by SDS-PAGE and autoradiography (top). Unphosphorylated proteins were analyzed by SDS-PAGE and Coomassie Blue staining (bottom). C, Myc epitope-tagged wild type (lane 1) and mutant C/EBPβ constructs (lanes 2–7) were expressed in transfected UMR106 cells and isolated by immunoprecipitation with anti-epitope antibody. Immunoprecipitates were incubated with purified GSK-3β and [γ-32P]ATP as described under “Experimental Procedures,” and reaction products were analyzed by SDS-PAGE/electroblotting/autoradiography (top). The membrane was later probed with a C/EBPβ-specific antibody, demonstrating comparable amounts of C/EBPβ proteins present in the immunoprecipitates (bottom). Lanes 2–5, mutant C/EBPβ constructs with the single amino acid substitutions indicated in lanes 2–5 of B; lane 6 shows the quadruple mutant C/EBPβ M(A₄), containing Ala177, Ala181, Ala193, and Ala195, and lane 7 shows a triple mutant, C/EBPβ M(A₄), containing Ala177, Ala193, and Ala195. Lane 8 shows antiepitope antibody immunoprecipitates obtained from cells transfected with empty vector. D, UMR106 cells were transfected with empty vector (lane 1), Myc epitope-tagged wild type (lanes 2 and 3), or mutant C/EBPβ M(A₄), (lanes 4 and 5); cells were labeled with 32PO₄ and some cultures were treated with 8-CPT-cGMP (lanes 3 and 5) as described in Fig. 38. Cell lysates were subjected to immunoprecipitation using anti-epitope antibody, and immunoprecipitates were analyzed by SDS-PAGE/electroblotting/autoradiography (top), or Western blotting with an anti-C/EBPβ antibody (bottom). E, wild type (lanes 1, 3, and 5) and mutant C/EBPβ M(A₄) (lanes 2, 4, and 6) were isolated from transfected, untreated UMR106 cells as described in C. The immunoprecipitated C/EBPβ proteins were incubated with buffer alone (lanes 1 and 2), with purified alkaline phosphatase (AP), or with alkaline phosphatase plus phosphatase inhibitor mixture (AP+Inh) as described under “Experimental Procedures.” Reaction products were analyzed by SDS-PAGE and Western blotting using a C/EBPβ-specific antibody. Migration of phosphorylated and dephosphorylated C/EBPβ is indicated by two arrows.
UMR106 cells were transfected with pCRE-Luc (a luciferase reporter gene under control of a minimal, CRE-containing promoter), pRSV-βGal (a control vector expressing β-galactosidase), and PKG II in 12-well dishes; cells were co-transfected with empty vector (E.V.) or the indicated amounts of expression vector encoding wild type (WT) or phospho-regulation-deficient mutant C/EBPβ M(A)δ. Cultures were either left untreated (gray bars) or treated with 250 μM 8-CPT-cGMP for 8 h (black bars). Luciferase activity was normalized to β-galactosidase activity, and the luciferase/β-galactosidase ratio measured in untreated cells transfected with empty vector was assigned a value of 1. B, cell extracts from one of the experiments shown in A were analyzed by Western blotting using a C/EBPβ-specific antibody. Lane 1, empty vector; lanes 2–4 and lanes 5–7 show 5, 10, or 20 ng of vector encoding wild type or mutant C/EBPβ M(A)δ, respectively. C, cells were transfected with pCRE-Luc, pRSV-βGal, and PKG II, and some cultures were treated with 8-CPT-cGMP as described in A, but cells were co-transfected with 5 ng of wild type C/EBPβ and either wild type or mutant GSK-3β (Alaδ) as indicated. The luciferase/β-galactosidase ratio measured in untreated cells co-transfected with wild type GSK-3β was assigned a value of 1.

Effect of cGMP, leading to enhanced transactivation of a target gene, but rendered the protein insensitive to cGMP.

Next, we examined C/EBPβ-dependent transcription in cells expressing the constitutively active GSK-3β (Alaδ) mutant (32). UMR106 cells were co-transfected with pCRE-Luc, wild type C/EBPβ, and either wild type or mutant GSK-3β (Alaδ); some cells received 8-CPT-cGMP (Fig. 5C). In cells co-transfected with wild type GSK-3β, cGMP stimulated C/EBPβ-dependent reporter gene activity 2-fold, but co-transfection of the constitutively active mutant GSK-3β (Alaδ) prevented the transcriptional effect of cGMP (p < 0.05 for the comparison between cGMP-treated cells transfected with wild type versus mutant GSK-3β). These findings are consistent with the interpretation that cGMP targets GSK-3 and that the effect of cGMP on C/EBPβ-mediated transactivation is explained by GSK-3 inhibition and dephosphorylation of C/EBPβ in cGMP-treated cells. These results support our earlier finding that inhibition of GSK-3 with valproate mimics the effect of cGMP on C/EBPβ transactivation (13).

Effect of C/EBPβ Phosphorylation on Its DNA Binding Activity—To examine the DNA binding activity of the phosphorylation-deficient mutant C/EBPβ M(A)δ, we performed electrophoretic mobility shift assays with double-stranded oligo-dNT probes encoding either a canonical CRE sequence (top) or a canonical C/EBPβ binding site (middle). Bottom, nuclear extracts were analyzed by Western blotting using a Myc epitope-specific antibody.

FIGURE 5. Transactivation potential of wild type and mutant C/EBPβ M(A)δ. A, UMR106 cells were transfected with pCRE-Luc (a luciferase reporter gene under control of a minimal, CRE-containing promoter), pRSV-βGal (a control vector expressing β-galactosidase), and PKG II in 12-well dishes; cells were co-transfected with empty vector (E.V.) or the indicated amounts of expression vector encoding wild type (WT) or phospho-regulation-deficient mutant C/EBPβ M(A)δ. Cultures were either left untreated (gray bars) or treated with 250 μM 8-CPT-cGMP for 8 h (black bars). Luciferase activity was normalized to β-galactosidase activity, and the luciferase/β-galactosidase ratio measured in untreated cells transfected with empty vector was assigned a value of 1. B, cell extracts from one of the experiments shown in A were analyzed by Western blotting using a C/EBPβ-specific antibody. Lane 1, empty vector; lanes 2–4 and lanes 5–7 show 5, 10, or 20 ng of vector encoding wild type or mutant C/EBPβ M(A)δ, respectively. C, cells were transfected with pCRE-Luc, pRSV-βGal, and PKG II, and some cultures were treated with 8-CPT-cGMP as described in A, but cells were co-transfected with 5 ng of wild type C/EBPβ and either wild type or mutant GSK-3β (Alaδ) as indicated. The luciferase/β-galactosidase ratio measured in untreated cells co-transfected with wild type GSK-3β was assigned a value of 1.

FIGURE 6. DNA binding activity of wild type and mutant C/EBPβ M(A)δ. A, equal amounts of nuclear extract protein from UMR106 cells transfected with empty vector (lanes 2 and 5), Myc epitope-tagged wild type C/EBPβ (WT; lanes 1, 3, and 6), or phospho-regulation-deficient mutant C/EBPβ M(A)δ (lanes 4 and 7) were incubated with a radioactively labeled oligo-dNT probe encoding a consensus CRE as described under “Experimental Procedures.” Some samples were preincubated with a 50-fold excess of unlabeled oligo-dNT (lane 1, + comp.) or with a C/EBPβ-specific antibody (lanes 5–7). Specific protein-DNA complexes generated by wild type and mutant C/EBPβ are indicated by the arrows, and supershifted complexes are indicated by an asterisk. A shorter exposure of lanes 2–4 is shown in the right panel. Nuclear extracts contained equal amounts of wild type and mutant C/EBPβ protein by Western blot analysis with an anti-Myc antibody (not shown). B, UMR106 cells were transfected with empty vector (lane 1), Myc epitope-tagged wild type (lanes 2 and 3), or mutant C/EBPβ M(A)δ (lanes 4 and 5), and some cells were treated with 250 μM 8-CPT-cGMP (lanes 3 and 5) for 1 h prior to harvesting. Electrophoretic mobility shift assays were performed with radioactively labeled oligo-dNT probes encoding either a canonical CRE sequence (top) or a canonical C/EBPβ binding site (middle). Bottom, nuclear extracts were analyzed by Western blotting using a Myc epitope-specific antibody.

Effect of C/EBPβ Phosphorylation on Its DNA Binding Activity—To examine the DNA binding activity of the phosphorylation-deficient mutant C/EBPβ M(A)δ, we performed electrophoretic mobility shift assays with double-stranded oligo-dNT probes encoding either a CRE or a canonical C/EBPβ binding sequence. With the CRE probe, nuclear extracts from UMR106 cells transfected with wild type or mutant C/EBPβ M(A)δ produced a distinct protein-DNA complex not present in cells transfected with empty vector (Fig. 6A, compare lanes 3 and 4, cells transfected with wild type or mutant C/EBPβ, with lane 2, cells transfected with empty vector; the right panel shows a shorter exposure). The protein-DNA complex formed by mutant C/EBPβ M(A)δ was more intense and demonstrated altered mobility compared with the complex formed by wild type C/EBPβ. Preincubation of nuclear extracts with excess unlabeled oligo-dNT probe resulted in competition of all specific protein-DNA complexes (Fig. 6A, lane 1, shows results for wild type protein, but similar results were obtained with the mutant protein). The addition of excess unlabeled oligo-dNT with an unrelated sequence (e.g. SP1 consensus sequence) had no effect on formation of the protein-DNA complexes (data not shown). Preincubation of nuclear extracts with a C/EBPβ-specific antibody resulted in disappearance of the protein-DNA complexes formed by the transfected proteins and appear-
ance of "supershifted" complexes (Fig. 6A, lanes 6 and 7; supershifted complexes are marked with an asterisk). Thus, although the protein-DNA complex formed by mutant C/EBPβ M(A)4 detracts differently from the complex formed by the wild type protein on non-denaturing PAGE, it was nevertheless recognized by the C/EBPβ-specific antibody. In UMR106 cells transfected with wild type C/EBPβ, the amount of C/EBPβ-specific complexes formed with the CRE probe increased in cells treated with 8-CPT-cGMP, compared with untreated cells (Fig. 6B, top, compare lanes 2 and 3, cells cultured in the absence and presence of cGMP). In contrast, the mutant C/EBPβ M(A)4 demonstrated increased DNA binding in the absence of cGMP that was insensitive to C/EBPβ (Fig. 6B, top, lanes 4 and 5). Wild type and mutant C/EBPβ were expressed at the same level (Fig. 6B, lower panel). These results are consistent with increased transcriptional activation of a CRE-dependent reporter gene by the mutant C/EBPβ M(A)4 (Fig. 5). Similarly, binding of wild type C/EBPβ protein to an oligo-DN probe encoding a canonical C/EBP binding sequence was enhanced when cells were treated with cGMP, and the mutant C/EBPβ M(A)4 bound more effectively than wild type, but binding of the mutant protein was not modulated by cGMP (Fig. 6B, middle, compare lanes 4 and 5, mutant C/EBPβ M(A)4 with lanes 2 and 3, wild type C/EBPβ; the lower panel shows expression of both proteins on a Western blot). Thus, mutation of the C/EBPβ phosphorylation sites in C/EBPβ increases DNA binding to both CRE and canonical C/EBP binding sequences but renders DNA binding insensitive to cGMP regulation. Consistent with these results, alkaline phosphatase treatment of nuclear extracts increases the C/EBPβ DNA binding (41). Thus, increased DNA binding of C/EBPβ in cGMP-treated cells can be explained by dephosphorylation of C/EBPβ, related to down-regulation of C/EBP activity by cGMP-induced GSK-3 Ser9 phosphorylation (Fig. 1).

**DISCUSSION**

We previously found that cGMP modulates the activity and phosphorylation state of C/EBPβ in osteoblasts and neuronal cells (13). In the present work, we found that GSK-3β constitutively phosphorylates C/EBPβ on specific sites in resting cells, thereby restraining C/EBPβ DNA binding and transactivation potential. The effects of cGMP on C/EBPβ can be explained by inhibition of GSK-3 activity, resulting in dephosphorylation of C/EBPβ, leading to increased DNA binding and transactivation of target genes.

**PKG Phosphorylation of GSK-3β on Ser9**—GSK-3β is abundant and highly active in unstimulated cells, and activation of several signal transduction pathways, including PI3K/Akt, cAMP/PKA, or Ras/Erk-1/2, decreases GSK-3 activity through phosphorylation of Ser9 in GSK-3α or Ser3 in GSK-3β (31, 32). GSK-3α and -3β are thought to have overlapping functions and are typically coordinately regulated, although their expression varies among different cell types, and UMR106 cells express predominantly GSK-3β (31, 32). We found that cGMP induced GSK-3β Ser9 phosphorylation to a similar extent as serum stimulation; the cGMP effect was not explained by activation of Akt, or Erk-1/2 but was dependent on PKG activity. In neuronal and endothelial cells, cGMP can activate the PI3K/Akt pathway, whereas in platelets, cGMP inhibits PI3K activity (43, 44, 49, 50); we found no significant effect of cGMP on Akt phosphorylation in osteoblasts and fibroblasts, suggesting that the effect of cGMP on PI3K/Akt is cell type-specific. C/EBPβ-induced GSK-3β Ser9 phosphorylation occurred in UMR106 osteoblasts, C6 glioma cells, and WI38 embryonal fibroblasts; it occurred in cells expressing endogenous PKG and was absent in PKG-deficient cells but was restored by transfection of PKG II into these cells. PKG II phosphorylated Ser9 of GSK-3β in vitro with a Kₘ similar to the Kₘ for cystic fibrosis transmembrane conductance regulator, an established PKG II substrate, and lower than the Kₘ reported for PKA phosphorylation of GSK-3β (35, 46).

**GSK-3 as a C/EBPβ Kinase**—Our results and those from other laboratories (41) indicate that C/EBPβ is a physiological GSK-3 substrate and that basal C/EBPβ phosphorylation is due to GSK-3 activity. We found that the phosphopeptide map of C/EBPβ phosphorylated in vitro, in resting UMR106 osteoblasts and C6 glioma cells, was similar to the map of C/EBPβ phosphorylated by GSK-3 in vitro. Similarly, C/EBPβ is phosphorylated in unstimulated NIH 3T3 fibroblasts and human macrophages on a tryptic peptide that includes the GSK-3 consensus sequence described in Fig. 4 (51, 52). Thus, C/EBPβ phosphorylation on these sites may be quite ubiquitous, as is the expression and activity of GSK-3 (32). Mutation of phosphosacceptor sites within the GSK-3 consensus sequence of the C/EBPβ serine-rich region abolished C/EBPβ phosphorylation in intact cells as well as in vitro phosphorylation by GSK-3. Finally, dephosphorylation of C/EBPβ occurred in intact cells when GSK-3β was inhibited by cGMP/PKG II or valproate, and C/EBPβ dephosphorylation induced by cGMP or valproate led to increased DNA binding and target gene activation by the transcription factor (see Ref. 13 for results with the GSK-3 inhibitor valproate). In experiments where we transfected wild type or mutant C/EBPβ, we were careful to transfet only low levels of C/EBPβ proteins, and cGMP treatment of UMR106 cells reduced phosphorylation of endogenous C/EBPβ to a similar degree as transfected C/EBPβ, suggesting that the level of transfected C/EBPβ did not exceed the regulatory capacity of the cells.

**C/EBPβ Dephosphorylation after Inhibition of GSK-3β**—In C6 glioma cells, we showed that cGMP-induced dephosphorylation of C/EBPβ required PKG activity (13), as did cGMP-induced phosphorylation of GSK-3 on the inhibitory Ser9 site. C/EBPβ dephosphorylation was detectable at 15 min, was maximal at 1 h, and persisted for >2 h (13). The kinetics of cGMP-induced GSK-3β Ser9 phosphorylation were similar in C6 and UMR106 cells and consistent with GSK-3 inhibition causing C/EBPβ dephosphorylation, because GSK-3β phosphorylation was detectable at 5 min, peaked at 20 min, and persisted for several hours. C/EBPβ dephosphorylation implies the activity of protein phosphatase(s), and we cannot exclude the possibility that cGMP may also increase the activity of C/EBPβ phosphatase(s), in addition to decreasing GSK-3 activity. Two major serine/threonine protein phosphatases, PP1 and PP2B (calcineurin), however, are negatively regulated by cGMP/PKG (53–55). Future work will be aimed at identifying the protein phosphatase(s) responsible for dephosphorylating C/EBPβ on the sites targeted by GSK-3. The finding that cGMP induced only partial (~50%) dephosphorylation of C/EBPβ without a detectable gel shift of the protein suggests that the four GSK-3 phosphorylation sites may turn over with different kinetics, perhaps due to differential phosphatase sensitivities, leading to C/EBPβ-induced dephosphorylation of some but not all sites.

Dephosphorylation of C/EBPβ due to inhibition of GSK-3 activity was also observed by Piwien-Pilipuk et al. (41), who showed that growth hormone induces C/EBPβ dephosphorylation in 3T3-F442A preadipocytes and increases DNA binding through activation of PI3K and Akt, leading to Akt-mediated Ser9 phosphorylation and GSK-3 inhibition. These results and ours suggest that C/EBPβ is an in vivo substrate of GSK-3 and that inhibition of GSK-3 activity leads to C/EBPβ dephosphorylation and activation. Similarly, insulin stimulates PI3K and Akt activity and induces dephosphorylation of C/EBPα through phosphorylation of GSK-3 in 3T3-L1 adipocytes (34). Other transcription factors targeted by GSK-3 are c-Jun, c-Myc, NF-κB, and the adipocyte determination- and differentiation-dependent factor 1, most of which are negatively regulated by GSK-3 phosphorylation (32, 33, 56).
In conclusion, we found that GSK-3 phosphorylated C/EBPβ in resting cells on several sites in the serine-rich region, apparently restraining its DNA binding; cGMP increased C/EBPβ DNA binding and transactivation of target genes by inhibiting GSK-3 activity and inducing C/EBPβ dephosphorylation on these negatively acting phosphorylation sites.

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