A Secondary Phosphorylation of CREB\textsuperscript{341} at Ser\textsuperscript{129} Is Required for the cAMP-mediated Control of Gene Expression

A ROLE FOR GLYCOGEN SYNTHASE KINASE-3 IN THE CONTROL OF GENE EXPRESSION*

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The cAMP-dependent protein kinase (PKA) phosphorylates CREB\textsuperscript{327} at a single serine residue, Ser\textsuperscript{119}, respectively. Phosphorylation at this site creates the sequence motif SXXX(S/P), a consensus site of the glycogen synthase kinase-3 (GSK-3) enzyme (Fiol, C., J. M., Mahrenholz, A. M., Wang, Y., Roeseke, R. W., and Roach, P. J. (1987) \textit{J. Biol. Chem.} \textbf{262}, 14042–14048). We examined the phosphorylation of CREB at the SXXX(S/P) consensus site and its role in CREB transactivation to cAMP induction. Neither isoform of the GSK-3 enzyme (GSK-3\textalpha\ or \textbeta) utilizes CREB as its substrate unless CREB is already phosphorylated at Ser\textsuperscript{119}. A 13-amino acid peptide containing the sequence surrounding Ser\textsuperscript{119} was phosphorylated by GSK-3, at Ser\textsuperscript{119}, only after the primary phosphorylation of the peptide by PKA (at Ser\textsuperscript{119}), suggesting that Ser\textsuperscript{119} is a GSK-3 phosphoacceptor site. Mutant CREB\textsuperscript{327} proteins containing Ser\textrightarrow Ala substitutions confirmed Ser\textsuperscript{119} as the only GSK-3 phosphorylation site. Transfection assays of wild type and mutant Gal4-CREB fusion proteins in PC12 cells demonstrated that Ser\textrightarrow Ala substitution of residue 129 of CREB\textsuperscript{41} impairs the transcriptional response to cAMP induction. Analogous mutation in CREB\textsuperscript{327} results in 70% decrease in its transactivation response to cAMP. In undifferentiated F9 cells, which are refractory to cAMP induction, transfected GSK-3\textbeta kinase induces a 60-fold increase in cyclic AMP response element-dependent transcription, mediated via the endogenous CREB protein. We propose that the hierarchical phosphorylation at the PKA and GSK-3 sites of CREB are essential for cAMP control of CREB.

A functional role for protein phosphorylation in regulating nuclear processes has been predicted for many years, and recently, phosphorylation of several transcription factors, including CREB, AP-1/C-Jun and c-Fos, c-Myb, c-Myc, and ADR-1, has been reported (reviewed in Ref. 2). The nuclear factor CREB interacts specifically with the cyclic AMP response element (CRE) in genes controlled by the cyclic AMP-mediated pathways of signal transduction (3, 4). Phosphorylation of CREB by cyclic AMP-dependent protein kinase was initially thought to be necessary and sufficient for the activation of transcription by cAMP (5). In either of two forms of CREB (CREB\textsuperscript{327} and CREB\textsuperscript{341}) that result from alternative splicing, a mutation of the PKA phosphorylation site Ser\textsuperscript{119} to an alanine or an aspartic residue prevented activation by PKA \textit{in vitro} and abolished the cAMP control of transcription \textit{in vivo} (6). A phosphate group in the transactivating region is therefore essential for a transcriptionally active protein. Later, measurements \textit{in vivo} of the activities of deletion and point-mutated chimeric fusion proteins of CREB\textsuperscript{327} showed that Ser\textsuperscript{119} was necessary but not sufficient for the full cAMP-inducible transactivation functions of CREB (7). It was suggested that phosphorylation within a domain termed the P-box (a 46-amino acid sequence, residues 92–137) was required for the full response to cAMP. This domain includes consensus phosphorylation sites for protein kinase C, casein kinase II, and the well established PKA site (Ser\textsuperscript{119}). It is now recognized from the study of protein phosphorylation in general that multiple phosphorylation of proteins is the norm rather than the exception (8). Furthermore, in several instances the multiple phosphorylations do not occur independently (1, 9) but follow an obligate order in what has been termed hierarchical phosphorylation (9) and involves “primary” and “secondary” kinases. The mechanistic basis for the phenomenon is that the secondary kinases appear to require prior phosphorylation of the substrate by primary kinases. The best studied example of this synergism is the phosphorylation of the metabolic enzyme glycogen synthase (1) by GSK-3. GSK-3 preferentially phosphorylates substrates where primary kinases have produced the primary sequence motif SXXX(S/P). This motif is found in other GSK-3 substrates such as ATP citrate lyase (10) and the G subunit of type I protein phosphatases (11). The role of GSK-3 as the activator of the ATP-Mg\textsuperscript{2+}-dependent phosphatase is also well studied and involves a synergistic phosphorylation of the inhibitor 2 protein. However, in this instance the secondary site is 13 residues N-terminal to the primary CK-II site (12).

The DNAs encoding two isoforms of GSK-3 termed GSK-3\textalpha (51 kDa) and GSK-3\textbeta (47 kDa) have been isolated from a rat brain library by Woogdett (13). These two proteins share 85% homology at the amino acid level. It is becoming clear that

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\(\dagger\) The abbreviations used are: CRE, cyclic AMP response element; PKA, cAMP-dependent protein kinase; GSK-3, glycogen synthase kinase-3; CAT, chloramphenicol acetyltransferase.
there is a family of related GSK-3-like enzymes. A third mammalian form, DAL-1, has been identified by polymerase chain reaction. Two yeast enzymes, MCK-I and MDS-1, appear to recognize the SXXXS(P) sequence motif. MCK-I has 45% sequence identity with GSK-3 within the catalytic domain, and MDS-1 has 57% sequence identity over a 296-amino acid overlap with GSK-3α from rats. Zeste white/Shaggy has been shown to be the Drosophila melanogaster homologue of GSK-3β, with 88% homology within the catalytic domains and similar specificity for the known GSK-3 protein substrates. It is therefore reasonable to expect that Shaggy will participate in hierarchical phosphorylations, although it has not been directly demonstrated. In transgenic flies, it has been shown that GSK-3β can substitute for Shaggy (15). Shaggy plays a crucial role in embryogenesis, and it is speculated that it may be involved in the regulation of transcription factors involved in the expression of genes leading to embryonic segmentation.

It was apparent from inspection of the CREB amino acid sequence that phosphorylation by PKA at Ser133 generates a GSK-3 site at Ser1155/129 with the potential for hierarchical phosphorylation of the CREB protein by PKA and GSK-3. A synthetic peptide, based on the transactivation domain of CREB, is a substrate for GSK-3 (16). In this study, recombinant CREB proteins are shown to be substrates for either isoform of GSK-3 only after primary phosphorylation by PKA. The functional significance of this secondary phosphorylation site is examined only after primary phosphorylation by PKA. The functional phosphorylation of the CREB protein by PKA and GSK-3.

Expression and Purification of Recombinant CREB—Wild type or mutant forms of CREBβ7841 at Ser129 proteins were expressed in bacteria via the T7/B21 Lys (SE/D3) system (23, 24). Purification of the recombinant protein was carried out as described in Colbran et al. (25).

Construction of Ser→ Ala CREB Mutants—Site-directed Ser→Ala substitutions were constructed at the GSK-3 consensus phosphorylation sites, Ser257 and Ser34, of CREBβ7841 at Ser129 and Ser34, respectively. The site-directed mutagenesis protocol of O'Connor and Eckstein (26). The introduced mutation was verified by dideoxy sequencing.

CREB-dependent Assay System—The indicator plasmid contains the somatostatin promoter (−750 to +50) fused to the chloramphenicol acetyltransferase (CAT) reporter gene (27). The CREB site at nucleotide position −48 has been replaced by the insertion of five Gal4 DNA binding sites. Specifically, the PslI site of the pBS STα construct, described in Andriamialiso et al. (27), was used to insert the synthetic Gal4 DNA binding site. The resulting indicator plasmid is pBS STαGal4-CAT. The CREB expression vector is used is described in Berkowitz and Gilman (28) and was kindly provided by Dr. M. Gilman (Cold Spring Harbor Laboratory). In this vector, the Gal4-1-32 DNA binding domain is fused at the NH2-terminal end of CREB. The resulting Gal4-CREB fusion proteins are functional, as demonstrated in earlier studies (28).

Transfection of PC12 Cells—Expressor plasmid (5 μg) was transfected with indicator plasmid (30 μg) in PC12 cells by the CaPO4 precipitation method using the Life Technologies, Inc. transfection kit. PC12 cells were grown in Dulbecco’s modified Eagle’s medium containing heat-inactivated horse serum (10%) and heat-inactivated fetal bovine serum (5%) on 100-mm tissue culture dishes coated with rat collagen. PC12 cells were transfected at passage 18. 48 h following introduction of the DNA, the cells were harvested, and extracts were prepared in the lysis buffer described in Ref. 29, which yields higher CAT extract activity. Cellular extract (35 μg) was assayed for CAT enzyme activity for 30 min at 37 °C as previously described (30). In vivo metabolic labeling of transfected PC12 cells, with [35S]methionine, was carried out by employing the protocol described by Lee et al. (17). Immunoprecipitation reactions with Gal4 antibodies were carried out as described (17). The Gal4 antibody was kindly provided by Dr. M. Pashine.

Transfection of Undifferentiated F9 Cells—Undifferentiated F9 cells were maintained as monolayer cultures on 100-mm dishes, coated with 0.1% gelatin, in Dulbecco’s modified Eagle’s medium supplemented with 15% heat-inactivated fetal bovine serum. Subconfluent (90%) monolayers were passaged 1:30 the day before the transfection. Transfections were carried out via the CaPO4 coprecipitation method, employing the Life Technologies, Inc. transfection kit. The Ga4 programmed transfection system utilizes a variety of indicator plasmids. The Gal4-CAT, 5 μg of expresor Gal4-CREBβ7841 at Ser129, and 10 μg of CMV4-GSK-3β plasmid DNA. The amount of total DNA transfected was kept constant by addition of CMV4 vector DNA. The cells were harvested 48 h post-transfection following 20 μm forskolin stimulation. Cell extracts were prepared in the lysis buffer described in Ref. 29 and assayed for CAT enzyme activity for 2 h at 37 °C as described earlier (30).

RESULTS

In Vitro Phosphorylation of CREB—To examine if the transactivation factor CREB is a substrate for the GSK-3 protein
kine, recombinant CREB<sup>327</sup> protein was used in <i>in vitro</i> enzymatic reactions employing PKA and either of the two GSK-3 isoforms, GSK-3α or GSK-3β. The CREB<sup>327</sup> proteins were phosphorylated <i>in vitro</i> by PKA to a stoichiometry of 0.6 (Fig. 1, A and B, lanes 3). A substitution of Ser<sup>119</sup> by an alanine in CREB abolished PKA phosphorylation, confirming that Ser<sup>119</sup> is the only phosphorylation site for cAMP-dependent protein kinase (Fig. 1, A and B, lanes 5). GSK-3α (Fig. 1A, lane 2) or GSK-3β (Fig. 1B, lane 2) alone did not phosphorylate CREB. However, if CREB was previously phosphorylated by PKA at Ser<sup>119</sup>, GSK-3α or GSK-3β could stoichiometrically introduce another phosphate (Fig. 1, A and B, respectively, lanes 4). The Ser<sup>119</sup> mutant was unable to act as a substrate for either isofrom of GSK-3 (lane 6). Thus, the <i>in vitro</i> phosphorylation reactions shown in Fig. 1 demonstrate that CREB can be phosphorylated by either isofrom of GSK-3 but only after primary phosphorylation by PKA.

Mapping of Phosphorylation Sites in CREB—To map the GSK-3 phosphorylation sites in CREB, a peptide was synthesized with sequence as shown in Fig. 2. The CREB peptide was stoichiometrically phosphorylated by cyclic AMP-dependent protein kinase (lane 3), but it was not phosphorylated by GSK-3 (lane 2). Once the CREB peptide had been phosphorylated by PKA, it became a substrate for both GSK-3α and GSK-3β reproducing the synergistic phosphorylation of the CREB protein consistent with previous results (16). Stoichiometric phosphorylation by GSK-3α of the monophosphopeptide produced by PKA is shown in lane 4 of Fig. 2. CREB peptide phosphorylated by PKA alone or in combination with GSK-3 was reacted with ethanethiol and subjected to sequence analysis. The sequencing pattern shown in Fig. 3 is consistent with a phosphopeptide being present only on Ser<sup>119</sup> after phosphorylation with PKA (panel B) and with phosphate also present at Ser<sup>115</sup>/129 after phosphorylation with GSK-3 (panel C). Thus, we were confident that we had mapped the GSK-3 site to Ser<sup>115</sup>/129 of CREB.

To confirm the identification of the phosphorylation site in the intact native CREB protein, we carried out site-directed mutagenesis of Ser<sup>115/129</sup> to Ala, as shown in Table I. Ser<sup>115/129</sup> is the putative phosphorylation site for GSK-3, based on the peptide phosphorylation studies. The mutant proteins were expressed in bacteria, purified, and utilized for <i>in vitro</i> phosphorylation reactions (Fig. 4). Controls for these experiments include the wild type CREB<sup>327</sup> protein (lanes 1–4) and the CREB proteins that contain Ser<sup>119</sup>→Ala substitutions at the PKA phosphorylation site (lanes 5 and 6). Lane 7 in panel A shows that the mutant CREB<sup>327</sup> with the Ala<sup>115</sup> substitution is a substrate for PKA with a stoichiometry essentially equal to that of wild type CREB<sup>327</sup> shown in lane 3. Unlike the native CREB<sup>327</sup> (lane 4), mutant CREB did not become a substrate for either GSK-3α (panel A, lane 8) or GSK-3β (data not shown) after phosphorylation by PKA. This result confirms that Ser<sup>115</sup> was the site for GSK-3 phosphorylation <i>in vitro</i>. The same results were obtained with mutant CREB<sup>341</sup> protein containing Ser<sup>129</sup>→Ala substitution, employing GSK-3β (panel B, lane 8) or GSK-3α (data not shown).

Transactivation Properties of GSK-3 Site Mutant CREB<sup>327/341</sup> Proteins—The role of this secondary phosphorylation reaction of CREB in its transactivation response to cAMP was examined by <i>in vivo</i> functional assays in the well documented cAMP-responsive PC12 cell line (17). The transactivation activity of the wild type CREB<sup>327/341</sup> protein was compared with the activity of the CREB protein mutants containing Ser→Ala substitutions at the GSK-3 phosphorylation site.

The assay system used has been previously reported by Berkowitz and Gilman (28), Lee et al. (7) and Sheng et al. (31). The DNA binding domain of CREB is reprogrammed, via fusion to the heterologous Gal4<sup>147</sup> DNA binding domain. A schematic diagram of the vector is shown in Fig. 5A. A diagram of the
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**TABLE I**

*Mutagenesis of CREB327-341 Proteins*

| CREB327     | CREB341     |
|-------------|-------------|
| Ser115→Ala115 | Ser115→Ala115 |
| Ser129→Ala129 | Ser133→Ala133 |

The constructed site-directed Ser→Ala mutants are listed below.

**FIG. 3.** Determination of phosphorylation sites in CREB peptide. Phosphorylated residues identified by amino acid sequence analysis, after modification of the phosphoserines with ethanethiol, is shown. A, elution profile of the phenylthiohydantoin-serine of the unphosphorylated peptide. B, disappearance of phenylthiohydantoin-serine and appearance of S-ethylcysteine (S-ET-Cys) at cycle 11, which corresponds to phosphorylation of Ser115. The sequenced peptide is phosphorylated by GSK-3 and CAMP-dependent protein kinases. Automated Edman degradation was performed in a Porton instrument 1090 microsequencer. DTT, dithiothreitol; DPTU, diphenylthiourea.

**FIG. 4.** Mapping of the GSK-3 site in the CREB proteins. Purified recombinant wild type CREB327 and Gal4-CREB341, respectively, in response to forskolin. As expected, the Ser→Ala substitutions within the PKA phosphorylation site in CREB341 (lane 3) and CREB327 (lane 4) are transcriptionally inactive in response to cAMP, in agreement with earlier studies (6, 27). The Ser→Ala substitution within the GSK-3 phosphorylation site in CREB341 (lane 5) also renders the CREB protein transcriptionally inactive. However, in CREB327, the Ser115→Ala mutation at the GSK-3 site reduces its transcriptional response to cAMP induction, on average, to 30% of its wild type activity. The histogram in Fig. 5C shows the quantitation of three independent transfection assays in PC12 cells.

The in vivo synthesis and stability of the transfected Gal4-CREB proteins was confirmed by in vivo metabolic labeling studies, employing [35S]methionine as shown in Fig. 6A and B. The radiolabeled proteins were immunoprecipitated with Gal4-specific antibodies. Because we have encountered difficulties in transfecting the PC12 cell line (Fig. 6A), we carried out additional in vivo labeling assays in HeLa cells (Fig. 6B). The results of the immunoprecipitation reactions confirm the expression of the CREB341 proteins as shown in lanes 1 and 2. The control immunoprecipitation reaction shown in Fig. 6B, lane 3, employing extracts of 35S-labeled cells transfected with vector lacking the Gal4-CREB cDNA insert, confirms the specificity of the Gal4 antibody and supports that the immunoprecipitated proteins shown in Fig. 6 correspond to the Gal4-CREB fusion.

**Transfection of GSK-3β Encoding Plasmids in UF9 Cells**—The undifferentiated F9 cell line is refractory to cAMP induction of the CRE-dependent promoters of somatostatin and vasointestinal peptide (19), although the known positive effectors CREB and PKA are present and functional (19). Overexpression of exogenous CREB and PKA compensates for the...
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FIG. 6. Immunoprecipitations of in vivo 32S-labeled Gal4-CREB proteins. A, immunoprecipitations of 32S-labeled extracts prepared from PC12 cells transfected with wild type Gal4-CREB341 expressor (lane 1), GSK-3 mutant Gal4-CREB341 (lane 2), and GSK-3 mutant Gal4-CREB297 (lane 3). B, immunoprecipitation of 32S-labeled extracts prepared from HeLa cells transfected with wild type Gal4-CREB297 expressor (lane 1), GSK-3 mutant Gal4-CREB297 (lane 2), and vector DNA lacking the Gal4-CREB341 insert. Analysis of the immunoprecipitates in A and B is by SDS-polyacrylamide gel electrophoresis and autoradiography. Autoradiography was carried out at −80 °C for 3 days. Sizes of molecular mass markers are shown; arrow denotes the specific Gal4-CREB band.

endogenous positive-acting factors of the cAMP transduction pathway.

We have employed the UF9 cell line to examine if GSK-3 kinase activity is an additional positive factor required for the cAMP transduction pathway. We have carried out transfection assays of the mammalian expression vector CMV4, encoding the GSK-3β kinase, to assess the effect on transcription directed from the CRE/CREB-dependent promoter of the rat somatostatin gene.

Initially, we employed the Gal4-CREB-dependent assay system described earlier in Fig. 5. The pBSxSST(Gal4)/CAT indicator plasmid and the CREB expressor, described by Berkowitz and Gilman (28), were used in the CREB-dependent assay system. B, functional assays of CREB and its mutants in PC12 cells. Transient transfections of PC12 cells were carried out with 10 µg of indicator and 5 µg of expressor DNA in the presence of 20 µM forskolin. Cells were harvested 48 h later, and extracts were prepared. 25 µg of cellular extract was assayed at 37 °C for 30 min. Expressor DNA is as follows: lane 1, wild type Gal4-CREB341; lane 2, wild type Gal4-CREB297; lane 3, PKA mutant Gal4-CREB441; lane 4, PKA mutant Gal4-CREB297; lane 5, GSK-3 mutant Gal4-CREB441; lane 6, GSK-3 mutant Gal4-CREB297; and lane 7, Gal4-147 DNA binding domain. Percent chloramphenicol conversion to the acetylated form is shown above each lane. C, histograms show the quantitation of three independent transfection assays in PC12 cells. Percent CAT activity is plotted against each expressor plasmid tested. Each bar is one independent assay. Groups 1–7 correspond to the expressor plasmid tested as follows: 1, wild type Gal4-CREB341; 2, wild type Gal4-CREB297; 3, PKA mutant Gal4-CREB441; 4, PKA mutant Gal4-CREB297; 5, GSK-3 mutant Gal4-CREB441; 6, GSK-3 mutant Gal4-CREB297; and 7, Gal4-147 DNA binding domain.

lack of CRE-dependent induction by the endogenous cAMP effector molecules (6). This observation has been interpreted to mean (19) that negative regulators block the activity of the
in UF9 cells, employing the Gal4-CREB assay system as a function of cells. Expressor plasmids are shown in Fig. 5A. Transfections contained 8 μg of pBxSST(Gal4)/CAT indicator, 5 μg of Gal4-CREB327/341 expressor, and where indicated, 10 μg of CMV4-GSK-3β plasmid DNA or CMV4 vector DNA. The transfected cells were treated with 20 μM forskolin for 48 h. Schematic diagrams of the indicator and where indicated, 10 pg of CMV4-GSK-3β plasmid DNA or CMV4 vector DNA. The transfected cells were treated with 20 μM forskolin for 48 h.

GSK-3β is a positive effector of the CAMP transduction pathway. Transfection of the GSK-3β expression vector results in a 60-fold induction in transcription of the CRE-dependent promoter, employing the available endogenous CREB. This transcriptional induction requires the presence of the catalytic subunit of PKA or 20 μM forskolin in UF9 cells (Fig. 8A). An approximately 50-fold induction in the transcriptional activity of the somatostatin promoter mediated via the endogenous CREB is observed in the presence of cotransfected GSK-3β kinase. The cotransfected GSK-3β kinase requires the presence of the catalytic subunit of PKA for CREB transactivation in agreement with the well documented mode of action of this class of enzymes. In agreement with earlier studies (6, 19), overexpression of the catalytic subunit of PKA without overexpression of exogenous CREB is not sufficient for CRE-dependent transcriptional induction in UF9 cells. The results shown in Fig. 8 support the positive effector role of GSK-3 type kinases in the CAMP transduction pathway.

**DISCUSSION**

This study provides evidence in vitro and in vivo to support a role for hierarchical phosphorylation in the regulation of the transactivation properties of CREB. The amino acid residues 92–137 are critical for the transcriptional activation of CREB in response to CAMP induction (7). This domain of CREB is serine-rich and has been shown to be multiply phosphorylated in addition to the phosphorylation by PKA. We therefore tested the possibility of hierarchical phosphorylation reactions occurring within CREB92/137 in the regulation of its transcriptional response to CAMP. A synthetic CREB peptide and site-directed CREB mutants were used to map the secondary cAMP-dependent phosphorylation to Ser105/106 within the activation domain of CREB92/137. The in vitro data strongly suggested that the primary kinase is PKA and the secondary kinase is one or both isoforms of GSK-3, though it is not certain which kinase(s) carry out the sequential phosphorylations in vivo. Our results from the in vitro transfection assays in PC12 cells are consistent with the conclusion that phosphorylation by PKA is essential for the activation of CREB327/341. However, the PKA phosphorylation is not sufficient since we demonstrated that ablation of the secondary phosphorylation site of CREB327/341, Ser105/106, impaired the transactivating function of the protein. Furthermore, analysis of the UF9 cell system directly demonstrates that GSK-3β is a positive effector of the CAMP transduction pathway. Transfection of the GSK-3β expression vector results in a 50-fold induction in transcription of the CRE-dependent promoter, employing the available endogenous CREB. This transcriptional induction requires the presence of the catalytic PKA subunit. The results of these two types of in vivo experiments in PC12 cells (Fig. 5) and UF9 cells (Fig. 8) are interpreted to mean that two phosphorylations must occur to generate a fully activated form of CREB. This is consistent with the proposal that PKA phosphorylation is not sufficient for full CREB activation. Overexpression of exogenous CREB and PKA is required for detectable CRE-dependent transcription in UF9 cells, which compensate for the partial activation state of CREB.

The in vivo data support the concept that a secondary phosphorylation at Ser105/106 is an important component of the trans-
activation response of CREB and suggest a mechanism for inte-
geration of signals from different signal transduction
pathways. Evidence for CREB being involved in the cross-talk between different signaling pathways has appeared (31). There
are reports of CREB41 functioning as a Ca2+-regulated tran-
scription factor through the phosphorylation of Ser139 (31). It is
also possible that a secondary phosphorylation, dependent on
primary phosphorylation of Ser139, mediates the response to
Ca2+ signal since mutation of Ser139 would destroy the phos-
phorylation at the secondary site as well.

At the moment, it is not clear what may regulate the activity
of GSK-3 in the cell. One possibility is that GSK-3 activity is
constant and dependent on the level of substrate maintained by
primary kinases responding to several different signal trans-
duction pathways. There is evidence that suggests that GSK-3
must be phosphorylated on a tyrosine residue to be in an active
state (32). Another report suggests that one form of GSK-3
GSK-3β may be a target for protein kinase C phosphorylation
resulting in down-regulation (33). It has been proposed that a
down-regulation of GSK-3 activity by protein kinase C in
response to phorbol esters results in a reduced level of c-Jun
phosphorylation and subsequent stimulation of c-Jun binding
to DNA. Though there is no evidence for inactivation of CREB
upon 12-O-tetradecanoylphorbol-13-acetate treatment, such
down-regulation of GSK-3β activity would also imply a possible
role for protein kinase C inactivation of CREB41 via a decrease
in phosphorylation of Ser139 by GSK-3. Based on these models,
GSK-3 would appear to have opposing roles in the expression of
CRE or TRE containing genes. It is important to note that
presently it is not known which cellular isoform of GSK-3 car-
ries out the phosphorylation of Ser139, and GSK-3α appears not
to be down-regulated by protein kinase C phosphorylation. This
is consistent with the observation that 12-O-tetradecanoyl-
phorbol-13-acetate treatment does not influence CRE-mediated
stimulation of transcription (7). More recent reports (34, 35)
have shown that phosphorylation of GSK-3α or GSK-3β by
mitogen-activated protein kinase-activated protein kinase-1
(34) and p70 S6 kinase (35) results in almost complete inactiva-
tion. Thus, GSK-3 has been implied as a target for inactiva-
tion through the insulin response pathway. This inactivation by
insulin would provide a mechanism to antagonize cAMP-dep-
endent gene expression. A secondary phosphorylation of
CREB41 at Ser139 potentiated by the primary phosphorylation of
Ser139 by PKA appears to be necessary to fully evoke the
change in transcriptional function of CREB41. Another ex-
ample in which GSK-3 has been shown to act synergistically
with cAMP-dependent protein kinase is in the phosphorylation of
two sites in the glycogen binding subunit of type 1 protein
phosphatase (11). Judging from these two examples, one can
postulate that the sequence SRR/G/P/S is a consensus for a
coupled pair of PKA/GSK-3 sites. At present, the only enzymes
known to have the appropriate specificity, for the sequence
motif SXXXS(P) are enzymes designated GSK-3 as previously
discussed. This work strongly suggests a role for GSK-3 or a
related family of acidotropic kinases in the control of transcrip-
tional events through hierarchical protein phosphorylations, in
particular the expression of genes responding to changes in
cAMP levels.

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