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16. The S-10 and S-17 lymphoid colonies were obtained by the addition of stromal cell conditioned medium to Whitlock-Witte cultures (*7*). Factors from the S-17 cell line potentiate the growth of colonies in which cells progress up to various stages of development. Some of the cells in S-10 pre-B colonies express surface immunoglobulin M (*8*).
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19 November 1991; accepted 27 January 1992

Calcium-Regulated Phosphorylation Within the Leucine Zipper of C/EBPβ

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Alterations in intracellular calcium levels activate several signal transduction pathways resulting in distinct patterns of gene expression. Here, a pathway for calcium-mediated signals is demonstrated that involves C/EBPβ, a member of the bZip family of transcription factors. In pituitary cells C/EBPβ was phosphorylated in response to increased intracellular calcium concentrations as a consequence of the activation of a calcium-calmodulin–dependent protein kinase. Phosphorylation of serine at position 276 within the leucine zipper of C/EBPβ appeared to confer calcium-regulated transcriptional stimulation of a promoter that contained binding sites for C/EBPβ.

Many regulatory molecules that function by binding to plasma membrane receptors cause changes in the intracellular Ca^{2+} concentration (*1*). This second messenger can modulate the expression of target genes by effecting changes in the phosphorylation status of specific transcription factors. Many Ca^{2+}-mediated changes in gene expression have been attributed to the phosphorylation of transcription factors by protein kinase C (*2*). However, fluctuations in intracellular Ca^{2+} concentrations can also activate Ca^{2+}-calmodulin–dependent kinases (*3*, *4*), the most studied of which is the multifunctional Ca^{2+}-calmodulin–dependent protein kinase II (CaMKII) (*5*) isozymes, which are expressed in most tissues (*6*).

In transient transfections of a pituitary cell line (G/C), the DNA sequence motif 5′-AAATGTAGTCTTATGCAATACACTTGTAGTCTTGCAACA-3′ rendered a reporter gene responsive to a constitutively active mutant of the brain-specific α-subunit of CaMKII (*4*). Critical positions within this CaMKII responsive element (CaMRE) coincide with binding sites for the nuclear factor C/EBP (*7*) (Fig. 1A). We hypothesized that CaMKII might exert its stimulation either directly or indirectly through a member of the C/EBP family of transcription factors (*8*). Members of this family belong to the bZip class of transcription factors. These factors contain a basic DNA-binding region adjacent to a leucine zipper dimerization domain and can form homodimers or heterodimers with other bZip proteins (*9*–*12*).

Electrophoretic mobility shift experiments with a ^{32}P-labeled CaMRE and G/C nuclear extract yielded four complexes (C1 to C4) (*4*) (Fig. 1B), all of which displayed the same methylation interference pattern (*13*) (Fig. 1A). This pattern was also identical to the one obtained with bacterially produced recombinant C/EBPα. Antisera to known members (α, β, and δ) of the C/EBP family (*9*, *10*) were used to establish the identity of the CaMRE binding activity in G/C nuclear extracts (Fig. 1B). Only the antiserum to C/EBPβ reacted specifically with the complexes formed between G/C nuclear extract and the CaMRE. Whereas low concentrations of antiserum caused the formation of new complexes with lower mobility, higher concentrations of the C/EBPβ-specific antiserum resulted largely in the elimination of the G/C cell–specific complexes (Fig. 1C). The observation that complexes C1 to C4 were equally affected by the C/EBPβ-specific antiserum suggests that C/EBPβ is present in all of these complexes and, as the major CaMRE binding protein in G/C cells, is a potential target for CaMKII.

To assess the ability of C/EBPβ to be phosphorylated, we performed immunoprecipitations on nuclear extracts prepared from G/C cells labeled in vivo with [^{32}P]orthophosphate (*14*) (Fig. 2A). The amount of phosphorylated C/EBPβ was 2.5-fold greater in untreated G/C cells than in cells treated with the CaMKII inhibitor KN-62. KN-62 competitively inhibits the binding of calmodulin to CaMKII by interacting with the calmodulin binding site and thus inactivates the enzyme (*15*). The amount of phosphorylation of C/EBPβ was

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elevated 3.5-fold in response to the Ca^{2+} ionophore A23187 when compared to untreated cells, and 9-fold when compared to KN-62–treated cells. The concentration of C/EBPβ protein was quantitated by [^{35}S]methionine labeling and was equivalent in control and treated cells. Because no A23187-dependent increase in the rate of phosphorylation was observed when the cells were pretreated with KN-62 (Fig. 2A), it seems likely that most of the Ca^{2+}-dependent phosphorylation of C/EBPβ is a result of Ca^{2+}-dependent activation of CaMKII or related kinases.

We used bacterially expressed C/EBPβ to test whether CaMKII could phosphorylate C/EBPβ in vitro (Fig. 2B) (*16*). Indeed, C/EBPβ was as efficient a substrate for CaMKII as was the cyclic AMP (adenosine 3′,5′-monophosphate) response element binding protein (CREB), the only other transcription factor known to be phosphorylated by CaMKII (*3*). C/EBPα (*9*) was not a substrate of CaMKII (Fig. 2B). When CaMKII was replaced by the catalytic subunit of protein kinase A, C/EBPβ was phosphorylated one to two orders of magnitude less than was CREB, which is phosphorylated equally well by both kinases (*3*). Thus, in contrast to CREB, C/EBPβ is an efficient substrate for CaMKII, but not for protein kinase A. Thus, a potential activation of C/EBPβ in vivo by protein kinase A (*17*) is likely to involve an indirect mechanism.

As a means of mapping in vitro the phosphorylation sites for CaMKII, we performed a deletion analysis of C/EBPβ (*18*). Truncated versions of C/EBPβ that contained only the 139 or 264 NH_2-terminal amino acids were poor substrates for CaMKII, whereas mutants that contained either the 286 NH_2-terminal or the 86 COOH-terminal amino acids were phosphorylated by CaMKII as efficiently as was full-length C/EBPβ (Fig. 3A). Therefore, the major phosphorylation site for CaMKII was between amino acids 264 and 286 of C/EBPβ, a region of the protein that contained the leucine zipper dimerization domain. Closer inspection of the primary amino acid sequence revealed that this region is the only part of the protein with a consensus phosphorylation site for CaMKII [Arg-X-X-(Ser/Thr)] (*19*) (Fig. 3B). Mutation of the Ser^{276} to alanine reduced the in vitro phosphorylation of C/EBPβ to background amounts, whereas mutation of a serine residue in the NH_2-terminal portion of the protein (Ser^{100}) did not affect CaMKII-mediated phosphorylation (Fig. 3A). These studies implicate Ser^{276} of C/EBPβ as the major in vitro phosphorylation site for CaMKII.

We tested the ability of CaMKII to activate C/EBPβ in G/C cells in transient transfection assays (*20*) with a Ca^{2+}-independent, constitutively active variant of CaMKII (CaMKα1-290) (*4*) (Fig. 4). Expression of C/EBPβ resulted in a two- to threefold increase in expression of a luciferase reporter gene under the control of a

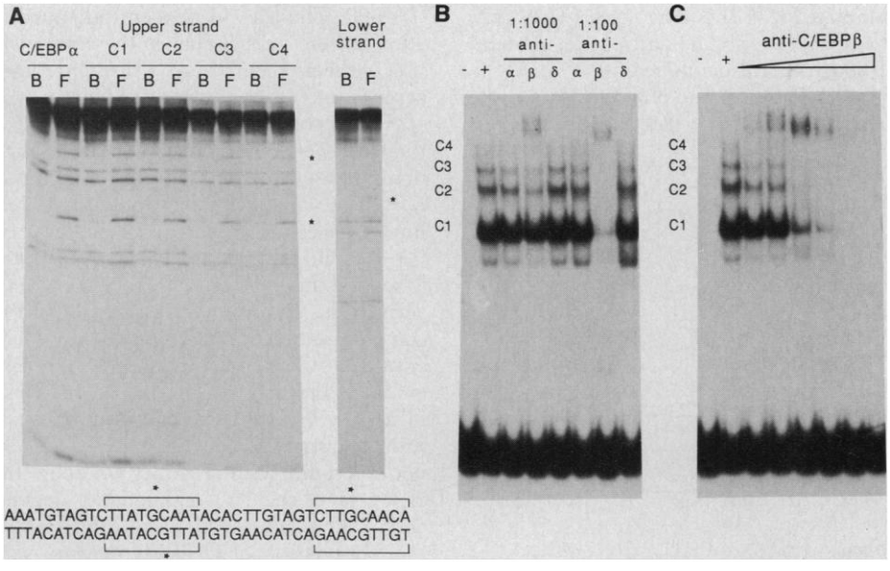

Fig. 1. Identification of C/EBPβ as the major CaMRE binding protein in G/C cells. (**A**) Methylation interference experiments on the complex formed between bacterially expressed C/EBPα and the CaMRE as well as on complexes C1 to C4 observed between G/C nuclear extracts and the CaMRE. Major groove contacts are marked by asterisks. The interference pattern for the lower strand is shown only for C1 and was identical for C1 to C4 and bacterially expressed C/EBPα. (**B** and **C**) Electrophoretic mobility shift experiments with a ^{32}P-labeled CaMRE in the presence of G/C nuclear extract and antisera to various members of the C/EBP family. Preimmune serum showed no effect on the mobility shift pattern (*22*). No cross-reactivity between the antisera could be detected. The supershift observed with the antiserum specific for C/EBPβ was reproduced with a second antiserum to C/EBPβ (*22*). In (B), the antisera (α, anti-C/EBPα; β, anti-C/EBPβ; δ, anti-C/EBPδ) were added at dilutions of 1:1000 and 1:100 as indicated. In (C), the antiserum to C/EBPβ was added at dilutions of 1:4000, 1:1000, 1:400, 1:100, and 1:40, respectively, as indicated by the triangle above the lanes.

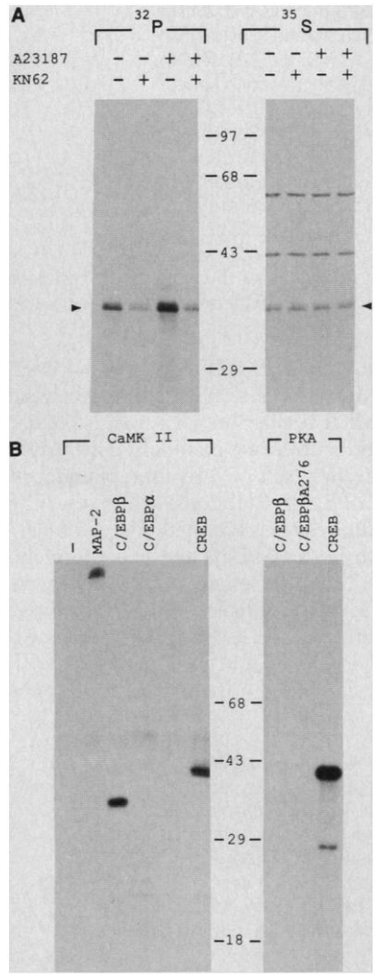

Fig. 2. Phosphorylation of C/EBPβ. (**A**) Immunoprecipitation of [^{32}P]orthophosphate- and [^{35}S]methionine-labeled C/EBPβ from G/C cells. G/C cells remained untreated or were exposed for 40 min to KN-62 (1.5 μM) and 30 min to A23187 (1 μM) as indicated above the lanes. (**B**) In vitro phosphorylation of bacterially expressed proteins by purified brain CaMKII and the catalytic subunit of protein kinase A (PKA). Reactions were performed in the presence of γ-[^{32}P]ATP. As in (A), labeled products were separated on SDS-polyacrylamide gels (10%) and were visualized by autoradiography. The amount of phosphorylation for C/EBPβ in vitro was compared to those of known kinase substrates (MAP-2, CREB) and to that of the related C/EBPα. In (A) and (B), the sizes of marker proteins (in kilodaltons) are indicated between the panels.

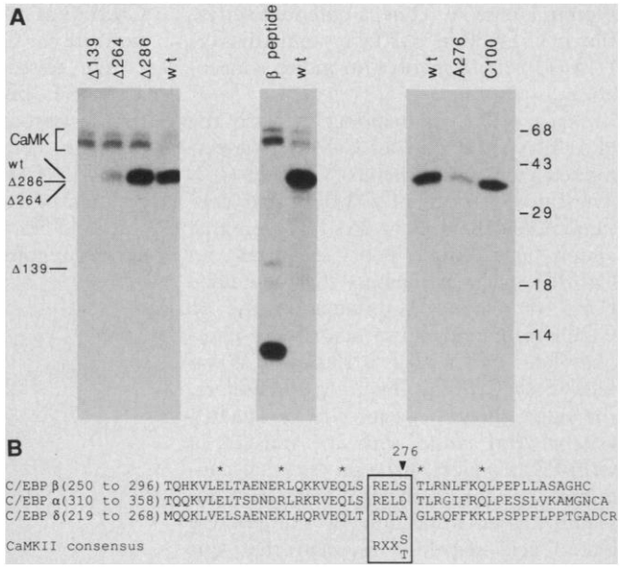

Fig. 3. Identification of phosphorylation sites on C/EBPβ. (**A**) In vitro phosphorylation by purified brain CaMKII. Truncated versions of C/EBPβ that contain the NH_2-terminal 139, 264, or 286 amino acids, respectively (Δ139, Δ264, or Δ286), a fragment that encompassed the 86 COOH-terminal amino acids of C/EBPβ (β peptide), and two C/EBPβ mutants with amino acid substitutions at position 100 (C100) or 276 (A276) were used to map the phosphorylation site for CaMKII on C/EBPβ to Ser^{276} (wt, wild-type C/EBPβ). Labeled products were separated on SDS-polyacrylamide gels (15%). (**B**) Localization of the phosphorylation site in the leucine zipper of C/EBPβ and comparison with similar regions from other C/EBP proteins (*10*). Numbers in parentheses indicate amino acid positions.

promoter that contained a multimerized CaMRE. Expression of constitutively active CaMKII resulted in a 10- to 12-fold stimulation of the same promoter. This may have been the result of activation of endogenous C/EBPβ, as mutations within the C/EBP binding sites abolished the stimulation. With both C/EBPβ and constitutively active CaMKII, expression of the reporter gene was stimulated 60-fold. This was not observed when C/EBPβ was expressed together with an inactive form of CaMKII (*4*) that contained a lysine to methionine substitution at residue 42 (M42 in Fig. 4). The CaMKII-mediated stimulation was specific for C/EBPβ because C/EBPα did not respond to CaMKII. The mutation of Ser^{276} to Ala caused C/EBPβ to lose its ability to be activated by CaMKII in transient transfection experiments (βA276 in Fig. 4). Responsiveness to CaMKII was abolished in a mutated C/EBPβ that consisted of only the COOH-terminal half of the protein, although it still contained Ser^{276} (βΔN in Fig. 4). Thus, for the phosphorylation site at Ser^{276} to exert its effect on gene expression, the major NH_2-terminal transactivation domain (*12, 21*) was necessary.

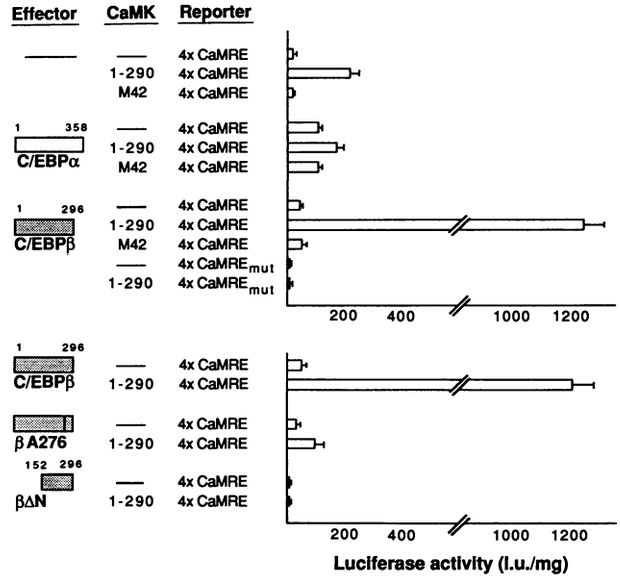

Fig. 4. Transient transfection experiments in G/C cells. The reporter plasmid contained four tandem copies of the CaMRE or a mutated version in front of the rat prolactin minimal promoter (from position −36 to +33), which drove transcription of a luciferase reporter gene. The effector plasmids contained the cDNAs of C/EBPα or C/EBPβ [in its wild-type version, with Ser^{276} mutated to alanine (βA276) or after deletion of the NH_2-terminus (βΔN)] under the control of the CMV promoter. Reporter and effector plasmids were cotransfected with vectors expressing a constitutively active (1-290) or inactive version (M42) of CaMKII (*4*) as indicated. Transcription from the reporter was determined by the luciferase activity of cellular extracts and was measured as the amount of light units per microgram of protein (l.u./μg). Results are the average of two to three independent experiments, each performed in triplicate ± standard error of the mean.

The mechanism by which the phosphorylation of Ser^{276} changes the activity of C/EBPβ is unknown. Phosphorylation of Ser^{276} did not detectably affect the DNA-binding affinity of C/EBPβ or its ability to form homodimers (*22*). Contrary to PC12 but similar to HeLa cells (*17*), C/EBPβ is localized predominantly in the nucleus of G/C cells, independent of whether the cells were treated with a Ca^{2+} ionophore (*4, 22*). Therefore, nuclear translocation does not seem to be a result of Ca^{2+}-dependent activation of C/EBPβ in G/C cells.

Although Ser^{276} is not part of the dimerization interface of the leucine zipper (*23*), phosphorylation of this residue might modify the conformation of the leucine zipper. Thus, phosphorylation might change the ratio of homo- to heterodimers within the cells or might result in a switch of dimerization partners in heteromeric complexes. The possibility of a change in heteromeric partners is intriguing, given the existence of a C/EBP-related protein that has a leucine zipper dimerization domain but lacks an adjacent functional DNA-binding region (*24*). Heterodimers between this protein and other C/EBP family members are incapable of DNA binding (*24*). Alternatively, phosphorylation of Ser^{276} might not be the final step in the activation of C/EBPβ. The Ca^{2+}-dependent phosphorylation at Ser^{276} could change the conformation of the protein to make a different target accessible to other unidentified kinases.

This report provides direct evidence for the existence of a distinct signal transduction pathway by which alterations in intracellular Ca^{2+} levels can be translated into changes in gene expression by way of Ca^{2+}-calmodulin–dependent protein kinases and independent of protein kinase C activation. Phosphorylation of C/EBPβ can be mediated by CaMKII. However, because other multisubstrate Ca^{2+}-calmodulin–dependent protein kinases exist in most cells, some of which may exhibit similar substrate specificities (*25*), we cannot determine with certainty whether one or several of the Ca^{2+}-calmodulin–dependent protein kinases are responsible for the phosphorylation of C/EBPβ in vivo. An activation mechanism similar to the one for C/EBPβ might also operate for immunoglobulin–EBP-1, another member of the C/EBP family, which on the basis of sequence analysis has similar potential phosphorylation sites (*26*). Because both C/EBPβ and most Ca^{2+}-calmodulin–dependent protein kinases are expressed in cells assuming differentiated phenotypes (*27*), this signal transduction pathway may be particularly used in terminally differentiating cells.

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28. We thank S. L. McKnight and M. S. Kapiloff for providing various reagents and expression plasmids and for discussions. We also thank H. Hidaka for supplying KN-62, S. Taylor for supplying purified protein kinase A catalytic subunit, and C. Nelson for maintenance and aid in tissue culture experiments. M.W. is a recipient of a postdoctoral fellowship from the Deutsche Forschungsgemeinschaft. M.G.R. is an investigator with the Howard Hughes Medical Institution. Supported by a grant from the National Institute of Diabetes and Digestive and Kidney Diseases.

3 January 1992; accepted 26 February 1992

Alternative Forms of Max as Enhancers or Suppressors of Myc-Ras Cotransformation

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Max is a basic-helix-loop-helix–leucine zipper protein capable of forming sequence-specific DNA binding complexes with Myc proteins. An alternatively spliced messenger RNA has been identified that encodes a form of Max truncated at the COOH-terminus. This ΔMax protein retained the ability to bind to the CACGTG motif in a complex with c-Myc but lacks the nuclear localization signal and the putative regulatory domain of Max. When tested in a *myc-ras* cotransformation assay in rat embryo fibroblasts, Max suppressed, whereas ΔMax enhanced, transformation. Thus, the *max* gene may encode both a negative and a positive regulator of c-Myc function.

Members of the *myc* gene family have been implicated in the control of normal cell proliferation as well as in neoplasia (*1*). A more direct role for *myc* genes in transformation is indicated by their ability to transform primary rat embryo fibroblasts in association with the c-Ha-*ras* oncogene (*2*). Adjacent basic-helix-loop-helix (bHLH) and leucine zipper (Zip) DNA binding and dimerization motifs (*3*) in the COOH-terminus of Myc proteins are similar to motifs found in several E-box–binding transcriptional regulators (*4*). The ability of Myc to bind to an E-box–containing core sequence CACGTG (*5*) is enhanced by heterodimerization with the bHLH-Zip protein Max (*6–8*).

We amplified *max*-specific sequences from human erythroleukemia cell (HEL) cDNA by the polymerase chain reaction (PCR) (*9*). Analysis of the PCR products revealed two separate bands of about 500 and 600 base pairs (bp). Sequence analysis indicated that the larger band contained a 101-bp insert in the middle of the coding sequence (Fig. 1A). Further PCR studies from human, mouse, and rat genomic DNA indicated that the additional sequence was derived from an alternatively spliced exon (Fig. 1B) that is conserved in evolution (*10*).

This alternative exon introduced an in-frame translation termination codon, which predicted the formation of a truncated Max polypeptide (ΔMax) that consisted of 103 amino acids. The 98 NH_2-terminal

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