The Rel-associated pp40 protein prevents DNA binding of Rel and NF- κ B: relationship with I κ B β and regulation by phosphorylation

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The product of proto-oncogene Rel associates with a number of cellular proteins. We have studied the effect of one of them, a phosphoprotein of 40 kD (pp40), on the DNA-binding activity of the Rel protein. We demonstrate that purified pp40 not only inhibits the binding of Rel, but also NF- κ B (p50-p65) heterocomplex to DNA. Additionally, I κ B β , but not I κ B α , also prevented the binding of Rel to the κ B site. I κ B β and pp40 are related proteins because (1) they share a number of common tryptic peptides, (2) their inhibitory effect on DNA binding can be abolished by preincubation with pp40-specific antiserum, and (3) labeled I κ B β can be immunoprecipitated with pp40 antibodies. pp40 is part of the Rel complex present in the cytoplasm and nuclear extracts of WEHI-231 cells. The activity of pp40 to inhibit the DNA binding of Rel and NF- κ B is modulated by phosphorylation.

[**Key Words:** Rel; DNA-binding activity; pp40 protein; NF- κ B; I κ B β]

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The proto-oncogene Rel (c-Rel) is the cellular cognate of v-Rel, which is the transforming gene of the acutely leukemogenic retrovirus REV-T (Wong and Lai 1981; Stephens et al. 1983; Wilhelmsen et al. 1984; Garson and Kang 1986; Gilmore and Temin 1986; Herzog and Bose 1986; Rice et al. 1986; Simek and Rice 1988a,b). Recently it has been demonstrated that both v-Rel and c-Rel proteins bind to the κ B site, which was first identified as a 10-bp sequence in the enhancer element of the murine κ -light-chain gene (Sen and Baltimore 1986; Ballard et al. 1990; Kieran et al. 1990; Inoue et al. 1991). c-Rel can activate the transcription of genes linked to the κ B site (Inoue et al. 1991); however, v-Rel on the other hand, suppresses the transcription of genes containing the κ B site (Ballard et al. 1990; Inoue et al. 1991). One explanation for the inability of overexpressed c-Rel to cause cellular transformation is the proposition that v-Rel subverts the transcription of genes required for normal cellular functions.

The discovery that the p50 subunit of the transcription factor NF- κ B is structurally related to the Rel proteins prompted us to ask whether the two proteins function in a similar manner (Ghosh et al. 1990; Kieran et al. 1990). The NF- κ B complex appears to be a heterodimer that contains 50- and 65-kD subunits capable of binding to decameric κ B motifs (Kawakami et al. 1988; Baeuerle

and Baltimore 1989; Urban and Baeuerle 1990, 1991). This complex is maintained in an inactive form in the cytoplasm by virtue of its association with a family of inhibitor proteins referred to as I κ B (Baeuerle and Baltimore 1988a; Zabel and Baeuerle 1990). Upon treatment of cytoplasmic extracts with ionic detergents, the DNA-binding activity of the inactive complex is unmasked (Baeuerle and Baltimore 1988b). The proposed model envisages that NF- κ B is initially located in the cytoplasm in a complex with I κ B. Upon activation of the complex by various inducers, probably by phosphorylation of I κ B, the NF- κ B heterodimer is released and migrates to the nucleus, where it binds to its cognate DNA-binding site and activates transcription (for review, see Baeuerle 1991; Baeuerle and Baltimore 1991).

Interestingly, both v-Rel and c-Rel proteins have been found to associate with a number of cellular proteins. Immunoprecipitation of extracts from a variety of avian cells with anti-Rel antibodies (viral or cellular) coprecipitate other cellular proteins with a wide range of molecular masses; 125, 115, 68–75, and 40 kD (Simek and Rice 1988a,b; Tung et al. 1988a,b; Morrison et al. 1989; Davis et al. 1990a,b; Lim et al. 1990). The p68–p75 is the c-Rel protein (Simek and Rice 1988b; Davis et al. 1990a,b; Lim et al. 1990), and p115 may represent the unprocessed form of the p50 subunit of the NF- κ B dimer. The 40-kD

protein is phosphorylated (pp40) and can be detected in both the cytoplasm and the nucleus of v-Rel-transformed cells (Davis et al. 1990a,b). Because of the similarity in size of the pp40 and I κ B proteins (37–43 kD) and structural homologies in the DNA-binding domain of Rel and NF- κ B 50-kD protein, we explored the possibility that pp40 also regulates the binding of Rel proteins to their cognate DNA-binding sites.

In this report we show that (1) pp40 and I κ B β interfere with the binding of both Rel and NF- κ B (p50–p65 heterodimer) to the κ B site, (2) antiserum specific for pp40 removes the inhibitory activity of pp40 and I κ B β , (3) pp40 and I κ B β are structurally related, (4) pp40 is part of the Rel complex present in the cytoplasmic and nuclear extracts of WEHI-231 cells, and (5) the hypophosphorylated form of pp40 prevents DNA binding of Rel, but dephosphorylated (p40) or hyperphosphorylated (pp40*) forms do not manifest any inhibitory effect.

Results

pp40 inhibits the binding of Rel and NF- κ B to DNA

To determine whether pp40 prevents the binding of the Rel proteins to the κ B DNA-binding motif, purified pp40 was prepared from REV-T-transformed chicken lymphoid cells for use in electrophoretic mobility-shift as-

says (EMSAs). Proteins immunoprecipitated from extracts of REV-T-transformed avian cells with antiserum against pp40 and the purity of pp40 preparations in the following experiments are shown in Figure 1A (lanes 1–3; Davis et al. 1990a). As demonstrated previously, both v-Rel and c-Rel proteins bind to the κ B-site (Ballard et al. 1990; Inoue et al. 1991; Fig. 1B, lanes 1 and 3). The multiple DNA complexes observed with Rel synthesized in wheat germ extracts is a likely consequence of the heterogeneity of Rel protein products resulting from numerous internal initiation sites located in the 5' end of Rel mRNA (Bull et al. 1989; J. Inoue and L. Kerr, unpubl.). The heterogeneity is not observed when bacterially expressed Rel (BactRel) is used (see Fig. 2). When either v-Rel or c-Rel proteins was incubated with the labeled κ B-binding site in the presence of purified pp40, no DNA binding was detected (Fig. 1B, lanes 2 and 4). If pp40 was preincubated with pp40 antiserum (Davis et al. 1990a) prior to the addition of c-Rel protein, no inhibition of DNA binding by pp40 was observed (Fig. 1B, lane 5), suggesting the specificity of the interactions. The specificity of the antiserum to pp40 has been demonstrated previously (Davis et al. 1990a). pp40 is not a general inhibitor of protein–DNA binding, because pp40 did not interfere with the binding of the c-Jun homodimer with the AP-1 site (Fig. 1C, lane 3). Additionally, the binding of c-Fos/c-Jun heterodimers to the AP-1 site or

Figure 1. Purified pp40 inhibits the Rel association with κ B DNA. (A) SDS-PAGE analysis of proteins immunoprecipitated from REV-T-transformed cells and purified pp40. Cell culture of REV-T-transformed non-virus-producing chicken lymphoid cell line, RECC-UTC41, and metabolic labeling was as described previously (Davis et al. 1990). Immunoprecipitation of [35 S]methionine-labeled cell extracts was performed as described previously (Lim et al. 1990) with low RIPA buffer (containing no ionic detergent). (Lane 1) An immunoprecipitation with anti-pp40 serum; (lane 2) with antiserum specific for pp59^{vRel}; (lane 3) a photograph of a Coomassie-stained SDS-PAGE analysis of the 40-kD M_r phosphoprotein eluted in the 1 M MgCl₂ fraction from the final S-200 column in the large-scale purification of pp40 (see Materials and methods). (B) pp40 inhibits the c-Rel and v-Rel association with κ B DNA. EMSA analysis of in vitro-translated c-Rel and v-Rel in the presence and absence of pp40. Two microliters of a

50- μ l total wheat germ translate of c-Rel or v-Rel mRNA was incubated with (+) or without (-) 7.5 ng of pp40 for 45 min on ice prior to the addition of an excess of κ B probe (0.5 ng, 3×10^5 cpm) with incubation at ambient temperature for 40 min. Where indicated, the pp40 was preincubated for 10 min in either the presence (+, lane 5) or absence (-, lane 4) of anti-pp40 serum (3 μ l). Polyacrylamide gel analysis was as described in Materials and methods. The autoradiogram was exposed at -70°C for 8 hr. (C) pp40 has no significant effect on Jun/AP-1 association. The preparation of in vitro-translated c-Jun protein was as described previously (Kerr et al. 1990). c-Jun (3 μ l) was preincubated in the absence (lane 2) and presence (lane 3) of pp40 (7.5 ng) on ice for 30 min in TM buffer [100 mM Tris-HCl (pH 7.9), 25 mM MgCl₂, 2 mM EDTA, 2 mM DTT, 40% glycerol, and 200 mM KCl]. Radiolabeled TRE oligonucleotide (30,000 cpm) (Kerr et al. 1988) was added, and the reactions were incubated at ambient temperature for 45 min prior to analysis on a 4% nondenaturing polyacrylamide gel. The autoradiogram was exposed for 6.5 hr at -70°C .

cAMP responsive element-binding (CREB) protein to the cAMP-response element (CRE) was also not influenced by pp40 (data not shown). Thus, pp40 selectively prevents the binding of the Rel protein to the κ B site in a manner analogous to I κ B and NF- κ B.

pp40 is related to I κ B β but not I κ B α

Because pp40 inhibits DNA binding by Rel proteins, the ability of pp40 to inhibit the binding of the NF- κ B dimer to the κ B site was evaluated. Figure 2A shows purified NF- κ B composed of the p50 and p65 subunits (lane 1), I κ B α (lane 2), and I κ B β (lane 3) used in the following experiments. Figure 2B (lane 2) shows that the NF- κ B complex bound to DNA and that this binding was completely inhibited by the addition of purified pp40 (Fig. 2B, lane 5). The broad pattern of migration of the purified NF- κ B–DNA complex is likely because of heterogeneity of p50 and p65 homodimers, and heterodimer complexes that associate with κ B sequences (Ghosh et al. 1990; Kieran et al. 1990; Nolan et al. 1991). Binding of the NF- κ B complex to DNA was also compromised in the presence of purified I κ B α (lane 3) or I κ B β (lane 4) as described previously (Baeuerle and Baltimore 1988a,b; Zabel and Baeuerle 1990). Inhibition by I κ B α and I κ B β of binding of NF- κ B complexes to their cognate DNA was specific because they do not influence binding of Jun homodimers to AP1 sites (Fig. 2D, lanes 3 and 4). Interestingly, I κ B β also inhibited the binding of c-Rel to the κ B site (Fig. 2B, lane 8), similar to that observed with pp40 (lane 9). I κ B α , which completely blocked the binding of the NF- κ B complex to DNA (lane 3), had no effect on the binding of Rel to the κ B site (Fig. 2B, lane 7).

The functional and antigenic relationship of Rel-associated pp40 and I κ B β was determined with antibodies raised against avian pp40 (Davis et al. 1990a,b). Figure 2C shows that incubation of the reaction mixture containing BactRel and pp40 with pp40-specific antibodies prevented the inhibition of DNA binding (lane 7). Preimmune sera had no effect on the interaction of pp40 with Rel (lane 6). Binding of Rel to DNA in the presence of I κ B β is also restored by the presence of pp40 antibodies (Fig. 2C, lanes 3 and 4). Antibodies to pp40 also reversed the binding inhibition of NF- κ B by I κ B β , but not by I κ B α (L. Kerr, unpubl.). To explore further the relationship between pp40 and I κ B α , we phosphorylated purified I κ B β in vitro with casein kinase II (CKII) (Fig. 2E, lane 1). Figure 2E (lane 2) shows that labeled I κ B β can be immunoprecipitated with anti-pp40 serum. The slower migrating protein in lane 1 is the autophosphorylated CKII, which is not immunoprecipitated with pp40-specific antiserum. A 40-kD protein can also be identified in murine WEHI-231 and 70Z/3 cells, and in human Jurkat cells by Western blotting with pp40-specific antiserum (data not shown).

Results shown above suggest that pp40 obtained from avian cells is functionally and immunologically related to I κ B β isolated from human placenta. To confirm that the two proteins may also be structurally related, we performed tryptic peptide analysis of in vitro-phospho-

rylated pp40 and I κ B β . A comparison of the two-dimensional tryptic peptide maps of pp40 and I κ B β (Fig. 3A,B) shows that these two proteins share a number of common peptides. A mixture of the tryptic peptides of the two proteins (Fig. 3C) demonstrates that spots b, c, f, g, and h are shared between the two proteins. Considering that the avian and mammalian species diverged >200 million years ago, the identification of a number of common tryptic peptides suggests that the two proteins have been well conserved during evolution. Consistent with the functional data, I κ B β and I κ B α have completely unique patterns of tryptic peptide maps (Fig. 3B,D) indicating that these two proteins are unrelated.

pp40 associates with the amino terminus of Rel

Immunoprecipitation experiments have suggested previously that pp40 is associated with the amino-terminal region of v-Rel and c-Rel (Simek and Rice 1988b; Davis et al. 1990a,b). To delineate the domain in the Rel protein involved in the association with pp40, we generated a series of deletion mutants in the c-Rel cDNA, which were transcribed with T3 polymerase, and the resulting RNA translated in vitro in the presence of [³⁵S]methionine. The labeled Rel mutant proteins were then mixed with pp40 and immunoprecipitated with pp40-specific antibodies. The results obtained with 10 mutants are summarized in Figure 4. The pp40 binds to the amino-terminal half of c-Rel because the mutant encoding only the carboxy-terminal amino acids 275–588 (c-Rel 3') (Bull et al. 1990) does not precipitate with pp40 antibodies. Of the remaining 9 mutants, only 1, where the amino-terminal 51 amino acids were deleted, was unable to associate with pp40, even though the truncated protein was synthesized in vitro in quantities similar to those observed with the full-length Rel protein. We propose that interactions between Rel and pp40 occur at the amino terminus of Rel involving at least the first 50 amino acids.

pp40 is a component of the κ B binding-protein complexes

To confirm and extend the data obtained with the Rel–pp40 complexes in vitro, we undertook studies to determine whether pp40 is a part of the complex interacting with κ B sites in murine cells. Cytoplasmic and nuclear extracts from WEHI-231 cells (a murine B-cell lymphoma, IgM-nonsecreting cell line) were analyzed by EMSA. WEHI-231 cells contain a constitutive κ B-specific DNA-binding activity in both cytoplasmic fractions (Fig. 5A, lane 1) and nuclear extracts (Fig. 5A, lane 4). Also, we have shown previously that in WEHI-231 cells both the cytoplasmic fractions and nuclear extracts contain c-Rel in the slowest migrating complex (upper complex) as displayed by decreased mobility (supershift) of the DNA–protein complex in the presence of antibodies raised against peptides to either the amino-terminal (5075 antibody) or carboxy-terminal (5071 antibody) por-

Figure 2. The effect of IκBα, IκBβ, and pp40 on the binding potential of NF-κB and bacterial c-Rel. (A) SDS-PAGE analysis of NF-κB and bacterial c-Rel. (Lane 1) A photograph of a Coomassie-stained SDS-PAGE analysis of affinity-purified p50-p65 NF-κB heterodimer complex (Zabel et al. 1991); (lane 2) a photograph of a Coomassie-stained SDS-PAGE analysis of p37, IκBα (Zabel et al. 1991) and Baeuerle 1990; (lane 3) a photograph of the SDS-PAGE analysis of purified IκBβ (P. Baeuerle, in prep.). (B) Contrast of the effect of the inhibitor on NF-κB and c-Rel. Radiolabeled κB oligonucleotides (~0.5 ng) were incubated with affinity-purified NF-κB (0.3 μl) or bacteria-derived c-Rel (BactRel) (3 μl), which had been pretreated (10 min on ice) in either the absence (-) or presence (+) of purified IκBα (4 μl), IκBβ (3 μl) or pp40 (3 μl) or pp40 (3 μl) or pp40 (3 μl). Reactions were incubated at ambient temperature for 30 min prior to non-denaturing polyacrylamide gel analysis. Autoradiograph was exposed at -70°C for 16 hr. (C) Anti-pp40 serum reverses the IκBβ and pp40 inhibition of BactRel association with κB DNA. *E. coli* c-Rel (BactRel) was preincubated in the absence (-) or presence (+) of IκBβ (4 μl, lane 3), pp40 (3 μl, lane 5), or IκBα (4 μl, lane 8). Where indicated, IκBβ, pp40, or IκBα was pretreated with anti-pp40 serum (2 μl) for 10 min at ambient temperature prior to the addition of poly(dI-C) and BactRel. pp40 (3 μl) was preincubated with preimmune serum (2 μl) for 10 min as described above (lane 6). Following preincubation, all samples received 3×10^5 cpm of radiolabeled κB oligonucleotide, followed by incubation at ambient temperature. EMSA analysis was performed as described previously. Representative autoradiogram was exposed at -70°C for 18 hr. (D) IκBα and IκBβ have no effect on c-Jun/AP-1 association. c-Jun (3 μl) was preincubated in the absence (-) and presence (+) of IκBα (4 μl, lane 3), and IκBβ (3 μl, lane 4) on ice for 10 min in TM buffer [100 mM Tris-HCl (pH 7.9), 25 mM MgCl₂, 2 mM EDTA, 2 mM DTT, 40% glycerol, and 200 mM HCl]. Radiolabeled TRE oligonucleotide (30,000 cpm) was added, and the reactions were incubated at ambient temperature for 45 min prior to analysis on a 4% non-denaturing polyacrylamide gel. The autoradiogram was exposed for 6.5 hr at -70°C. (E) Immunoprecipitation of ³²P-labeled IκBβ by anti-pp40 serum. Purified IκBβ (~5 ng) was phosphorylated in vitro with CKII (see Materials and Methods) in the presence of [γ-³²P]ATP. An aliquot of the reaction mixture (15,000 cpm) was analyzed directly (lane 1) while another portion was immunoprecipitated with anti-pp40 serum under nonionic wash immunoprecipitation conditions as described previously (lane 2). Phosphorylated products were analyzed with a 10% SDS-PAGE. The autoradiogram was exposed at 70°C for 2.5 hr.

Figure 3. Two-dimensional phosphotryptic analysis of ^{32}P -labeled pp40, -IkB β , and -IkB α . Purified pp40, IkB β , and IkB α were phosphorylated in vitro in the presence of $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ by purified cAMP-dependent PKA as described in Materials and Methods. Phosphorylated proteins were subjected to exhaustive digestion with trypsin and analyzed by two-dimensional mapping as described in Materials and Methods. (A) Four-day exposure, 1000 cpm of ^{32}P -labeled pp40; (B) 4-day exposure, 1000 cpm of ^{32}P -labeled IkB β ; (C) 3-day exposure, 1000 cpm of ^{32}P -labeled pp40 and 1000 cpm of ^{32}P -labeled IkB β ; (D) 4-day exposure, 1000 cpm of ^{32}P -labeled IkB α . Arrows indicate the directions of first-dimension electrophoresis (rightward arrow) and second-dimension chromatography (upward arrow); (O) The spot where samples were applied.

Figure 4. pp40 association domain in c-Rel. Wild-type and mutant c-Rel cDNAs were transcribed in vitro, and the resulting mRNA was translated in vitro in the presence of L-[³⁵S]methionine as described in Materials and methods. Equal amounts of protein (as determined by SDS-PAGE analysis of an aliquot from translation reaction) were incubated in the absence or presence of purified, unlabeled pp40 (7.5 ng) for 10 min at 30°C prior to immunoprecipitation with anti-pp40 serum. Mutants ΔRel 265-588, 311-588, 385-588, and 466-588 indicate that sequences between the indicated amino acids have been deleted. Mutant ΔBamHI 1-51 indicates that sequences between the indicated amino acids have been deleted. Mutants BamHI 51, 124, 289, and 326 indicate that a BamHI site (in-frame) was inserted at the indicated amino acid position. Mutants Rel 275-588' indicate that the resulting Rel protein contains amino acids 275-588 (Bull et al. 1989)

tion of c-Rel protein (Inoue et al. 1991). When both cytoplasmic fractions and nuclear extracts were incubated with anti-pp40-specific serum, a supershifted (highly retarded) complex and a faster-migrating κB-binding complex (Fig. 5A, lanes 2 and 5) were observed. No change in the gel-shift pattern was observed with the preimmune serum (lanes 3 and 6). One interpretation of these data is that the complex in Figure 5A (lane 1) contains a number of κB-binding proteins, including Rel. Additionally, it contains pp40 or pp40/IκBβ-like proteins that perhaps are associated with Rel. Upon addition of pp40-specific antiserum, the pp40-Rel complex supershifts and the resulting κB complex migrates faster because it has lost several proteins from the complex. In agreement with this explanation, the supershifted complex observed in Figure 5A (lanes 2 and 5) contains Rel as it is further retarded if incubated with anti-Rel serum [data is not shown for WEHI-231 extracts but is shown for 70Z/3 extract (Fig. 5B, lane 6)]. As reported previously, the supershifted complex in the cytoplasmic extracts has a slower mobility (lane 2) as compared with the nuclear extracts (lane 5), perhaps indicative of the presence of additional protein(s) (Inoue et al. 1991). The faster-migrating complex in Figure 5 (lanes 2 and 5) probably represents other κB proteins capable of binding to the κB site. It is worth noting that even though the nuclear extract has more intense κB-binding complex, the gel-shift pattern following treatment with pp40 antiserum appears to be relatively similar. These results (Fig. 5A) sug-

gest that pp40 is a component of the κB-binding Rel complex in both cytoplasmic fractions and nuclear extracts.

In contrast, in 70Z/3 cells (a murine pre-B-cell line) κB-binding activity is not expressed constitutively (Fig. 5B, lanes 2 and 3). As described previously, upon treatment of the cytoplasmic extract with deoxycholate (DOC), κB-binding activity can be detected (Fig. 5B, lane 5). Interestingly, the κB-binding activity is also detected if the 70Z/3 cell cytoplasmic extract is treated with pp40-specific antiserum (lane 4), and the complex shows a supershift as observed in Figure 5A (lanes 2 and 5). Thus, it appears that in unstimulated 70Z/3 cells, the κB-binding activity is masked, at least in part, due to its association with pp40 or IκBβ. We therefore investigated to what extent inhibitory factors released from cytoplasmic complexes following treatment with DOC retain the capacity to block binding of the NF-κB dimers. Figure 5C shows that the addition of increasing amounts of concentrated, dialyzed, flowthrough fractions from the κB-affinity resin were able to inhibit the binding of the NF-κB dimer to its cognate DNA-binding site (Fig. 5C, lanes 1, 2-4). The ability of the flowthrough fraction to inhibit NF-κB binding was partially removed if it was incubated first with pp40-specific antibodies (lane 5). Western blotting analysis demonstrated that the flowthrough fraction contained pp40 and/or pp40-related proteins (data not shown).

Phosphorylation regulates the inhibitory activity of pp40

There is a paradox in the ability of pp40 to influence DNA binding of Rel/NF-κB proteins to the κB site. On one hand, the addition of pp40 to Rel and NF-κB prevents DNA binding in vitro (Figs. 1 and 2); on the other hand, it is part of the complex in nuclear and cytoplasmic fractions of WEHI-231 cells, which bind to the κB site (Fig. 5A, lanes 1 and 4), because incubation with pp40 antiserum shows a supershift and increased mobility of the complex (Fig. 5A, lanes 2 and 5). Because IκB activity is apparently modulated by phosphorylation with protein kinase C (PKC) (Ghosh and Baltimore 1990) experiments were performed to demonstrate that the function of pp40 is regulated by its phosphorylation state. Purified pp40 was phosphorylated in vitro with either purified protein kinase A (PKA) (Fig. 6A, lane 2) or partially purified PKC (Fig. 6A, lane 5). In vitro phosphorylation by PKA is ATP dependent (Fig. 6A, lane 3) and is inhibited by HA 1004 (Hidaka et al. 1984), an inhibitor of PKA (lane 4). In addition to autophosphorylation of PKA subunits, the primary phosphorylated band is a hyperphosphorylated form of pp40, which was identified by immunoprecipitation with pp40-specific antiserum (Fig. 6A, lane 1). Similarly, we performed in vitro phosphorylation of pp40 with purified PKC in the presence of phosphatidylserine (PS; Fig. 6A, lane 5). Little or no labeled pp40 is detectable when phosphorylation is carried out in the absence of phosphatidylserine (Fig. 6A, lane 6) confirming that phosphorylation is due to PKC. The other labeled proteins in lane 5 represent autophosphorylation of PKC

Figure 5. The involvement of pp40 in cellular extracts. (A) The effect of anti-pp40 serum on WEHI-231 cell extract κ B-binding proteins. Equal amounts (2 μ g) of either WEHI-231 cytoplasmic (C) or nuclear (N) proteins were analyzed by standard EMSA (see Materials and methods) either in the presence (+) or absence (–) of anti-pp40 (3 μ l) or preimmune (3 μ l) serum. Extracts were preincubated in binding buffer containing poly[d(I-C)] for 45 min on ice prior to the addition of excess radiolabeled oligonucleotide (3×10^5 cpm), and the time of electrophoresis was extended to separate and visualize multiple complexes. Incubation proceeded at ambient temperature prior to analysis. A representative autoradiogram demonstrates an 8-hr exposure at -70°C and is a composite of three separate experiments. (B) Comparison of 70Z/3 cell extract treatment with either DOC or anti-pp40 serum. Equal amounts (10 μ g) of either unstimulated cytosolic (C) or nuclear (N) extract were utilized for κ B-binding analysis. Extracts were treated in vitro with DOC (0.8%)/NP-40(1%) for 10 min at ambient temperature prior to the addition of probe. Where indicated, the unstimulated cytosolic extract was treated with anti-pp40 serum (3 μ l) (lane 4) or anti-pp40 (3 μ l) and anti-c-Rel serum (5071 antibody 6 μ l) (lane 6), on ice for 40 min prior to the addition of radiolabeled probe (3×10^5), followed by incubation at ambient temperature for 40 min. (C) Soluble factors antigenically related to pp40 effect inhibition of NF- κ B. Unstimulated cytoplasmic extract (5 mg) was treated with DOC (0.8%)/NP-40 (1%) for 15 min at ambient temperature prior to separation by κ B/oligonucleotide/Sepharose affinity chromatography (see Materials and methods). The κ B-bound fraction (1 μ l) was used for EMSA as described previously in the presence (+) and absence (–) (lane 1) of increasing amounts (1, 5, and 10 μ l) of dialyzed and concentrated flowthrough fractions (lanes 2–4, respectively). Lane 5 contains 10 μ l of flowthrough fraction that was preincubated with anti-pp40 serum (3 μ l) for 10 min at ambient temperature prior to the addition of the κ B-bound fraction (1 μ l). Following a 45-min preincubation on ice, 3×10^5 cpm of κ B oligonucleotide probe was added prior to incubation of the samples at ambient temperature. Low-ionic polyacrylamide gel analyses was as described previously. The photograph represents an autoradiogram exposed for 16 hr at -70°C .

and some impurities present in the PKC preparations. Like PKA phosphorylation (Fig. 6A, lane 1), upon immunoprecipitation with pp40-specific antiserum, the labeled 40-kD band was identified (data not shown). When BactRel was mixed with PKA-hyperphosphorylated pp40 (pp40*), the DNA binding of Rel was not affected (Fig. 6B, lanes 2 and 4). In contrast, PKC-hyperphosphorylated pp40 (pp40*) still inhibited binding of BactRel to the κ B DNA (Fig. 6B, lane 5). The results suggest that PKA phosphorylation may regulate the ability of pp40 to inhibit the DNA binding of Rel protein in vitro. When the PKA-hyperphosphorylated pp40 (pp40*) was treated with potato acid phosphatase (PAP), partial DNA binding was observed (lane 6), suggesting that both the unphosphorylated and hyperphosphorylated forms of pp40 do not influence Rel DNA binding (see also Fig. 6C, lane 10).

We extended the results obtained with hyperphosphorylated pp40 and BactRel by using cytoplasmic extracts from WEHI-231 cells. The cytoplasmic κ B-binding activity from WEHI-231 cells contained pp40 as judged by the supershift induced with pp40-specific antiserum (Fig. 5A, lanes 1 and 2). The κ B-binding activity of the WEHI-231 extracts was inhibited if the extracts were incubated with pp40 (Fig. 6C, cf. lanes 3 and 4), suggesting that the

addition of exogenous pp40 has the ability to bind to the κ B-binding proteins (Rel or NF- κ B heteromer) in the extracts. Because the WEHI-231 cytoplasmic extracts contain pp40-like proteins, the simplest interpretation is that exogenous pp40 binds to the κ B-binding proteins more efficiently, possibly by displacing the bound inactive pp40-like proteins. Because PKA-hyperphosphorylated pp40 (pp40*) has no effect on the binding of Rel to DNA in vitro (Fig. 6B, lane 4), its effect on the κ B binding of WEHI-231 extracts was determined. The data show that PKA-hyperphosphorylated pp40 (pp40*) was unable to inhibit the κ B-binding activity in these extracts (Fig. 6C, cf. lanes 3 and 6). Hyperphosphorylation by PKA required ATP and was inhibited by HA 1004 (Fig. 6C, lanes 5 and 7). As with BactRel (Fig. 6B), relief of suppression of binding to DNA by pp40 was not observed if it was hyperphosphorylated by PKC (Fig. 6C, lanes 8 and 9). Furthermore, we asked whether the dephosphorylated form of pp40 would have any effect on binding of κ B proteins to DNA. In agreement with results shown in Figure 6B (lane 6) when pp40 was dephosphorylated by PAP, partial binding was restored [Fig. 6C, lane 10], and this effect was reversed if the phosphatase reaction was carried out in the presence of inorganic phosphate (Fig.

6C, lane 11). These data suggest that of the three possible phosphorylation states of the Rel-associated 40-kD protein, the hypophosphorylated form (pp40) inhibits Rel/NF- κ B DNA binding, whereas neither the dephosphorylated form (p40) nor the PKA-catalyzed hyperphosphorylated state (pp40*) influence DNA binding.

To confirm the notion that the hyperphosphorylated form of pp40 (pp40*) is unable to inhibit the binding of Rel/ κ B complexes to the κ B site, unstimulated 70Z/3 cell cytoplasmic fractions were phosphorylated *in vitro* with purified PKA and examined for DNA binding. As seen in Figure 6D (lane 3), there was an increase in DNA-binding activity following PKA treatment (cf. lanes 2 and 3), which was dependent on ATP (lane 4) and blocked by HA, an inhibitor of PKA (lane 5). Incubation with ATP alone (lane 7) leads to slight activation, probably due to endogenous kinase activity in the extracts. Presumably, the hypophosphorylated pp40, which prevents DNA binding, had been phosphorylated by PKA, which allows binding. Treatment of the extracts with DOC, however, showed a much increased DNA-binding activity (lane 6), suggesting that other modes of regulation may be involved in the activation of Rel/ κ B complex.

Discussion

The cell responds to external stimuli by turning on a plethora of early response genes, many of which act as transcription factors. Induction of these genes does not require protein synthesis, but their activities are usually modulated by post-transcriptional or post-translational mechanisms. The product of proto-oncogene Rel has been identified as a transcriptional factor that specifically binds to and activates transcription from genes linked to the κ B site (Ballard et al. 1990; Inoue et al. 1991). We have explored the role of the Rel-associated protein pp40 in the regulation of the function of Rel proteins.

DNA-binding activity of Rel is regulated

Both v-Rel and c-Rel proteins bind to κ B sites derived from either the human immunodeficiency virus long terminal repeat (HIV-LTR) or major histocompatibility complex (MHC) class I promoter (Ballard et al. 1990; Kieran et al. 1990; Inoue et al. 1991). *In vitro* Rel proteins can form either oligomers or heterodimers with the p50 subunit of NF- κ B, but it is not clear whether this formation occurs *in vivo* as well (Kieran et al. 1990; Inoue et al. 1991). Because of the structural and functional homologies between Rel and NF- κ B, it was prudent to undertake a study of the regulation of Rel by accessory proteins similar to that performed with NF- κ B. It has been well established that the NF- κ B complex exists in the cytoplasm in association with a 37-kD protein referred to as I κ B, which dissociates *in vitro* from the NF- κ B complex, upon phosphorylation by agents that activate PKC pathways (Baeuerle and Baltimore 1988a; Ghosh and Baltimore 1990; Zabel and Baeuerle 1990). Presumably, the p50-p65 dimer migrates to the nucleus and binds to κ B

sites. Thus, binding and translocation from the cytoplasm to the nucleus are thought to be regulated by the phosphorylation of an associated protein. Data shown in Figures 1 and 2 clearly demonstrate that Rel binding to κ B sites is completely inhibited in the presence of purified pp40, a Rel-associated protein (Davis et al. 1990a,b). Table 1 compares the properties of pp40 and I κ B proteins I κ B α and I κ B β . Despite the parallels between I κ B and pp40, there are some differences: (1) Although pp40 and I κ B β can bind directly to Rel and prevent DNA binding, I κ B α is specific for the p65 subunit of NF- κ B (Urban and Baeuerle 1990; Zabel and Baeuerle 1990); and (2) I κ B α has been postulated to be present in the cytoplasm in complex with NF- κ B and dissociates before nuclear translocation. pp40, on the other hand, has been found to be in both the cytoplasm and nucleus (Davis et al. 1990a) and, moreover, remains associated with Rel in the nuclear extracts (Fig. 5).

We are unable to quantitate the molar amounts of the Rel protein and pp40 protein required to abrogate DNA binding. In the *in vitro* translational experiments, the amount of c-Rel synthesized is very little, and in Rel synthesized in bacteria, the purification procedure involves denaturation and renaturation of the protein. Therefore, we do not know the exact quantity of the biologically active Rel protein. We are now in the process of purifying Rel without undergoing the denaturation and renaturation steps.

pp40 is I κ B β

The ability of pp40 to inhibit the binding of the NF- κ B dimer to the κ B site suggests that (1) it is the homolog of I κ B or (2) there is a family of pp40/I κ B-like proteins with common functions. However, there must be a degree of functional specificity of I κ B family members, because I κ B α , which has been shown to inhibit binding of the NF- κ B heterodimer to DNA has no effect on Rel binding to the κ B site (Fig. 2B, lanes 3 and 7). On the other hand, I κ B β can prevent binding of both Rel and NF- κ B complex to DNA (Fig. 2B, lanes 4 and 8). The binding of I κ B β to either Rel or the NF- κ B dimer can be prevented by anti-pp40 specific serum, suggesting functional and immunological similarities. Anti-pp40-serum had no effect on the function of I κ B α in preventing binding of NF- κ B to the κ B site (L. Kerr, unpubl.). The structural data (Fig. 3) obtained by tryptic peptide analysis suggest that pp40 and I κ B β are highly related and perhaps identical proteins.

The p50 component of the p50-p65 NF- κ B dimer is the carboxy-terminally truncated portion of a 105-kD protein (Ghosh et al. 1990; Kieran et al. 1990). The p50 protein binds to the κ B site and has been shown to share homology with the amino-terminal half of the Rel protein (Ghosh et al. 1990; Kieran et al. 1990). Interestingly, pp40 can bind to p50 but does not prevent its association to the κ B-binding site (L. Kerr, in prep.). Inability of either I κ B α or I κ B β to inhibit binding of p50 to DNA is presumably because these proteins require the presence of the p65 subunit. However, I κ B β can inhibit Rel bind-

Table 1. Properties of pp40 and I κ B proteins

Properties	pp40 chicken	I κ B β human	I κ B α rabbit or human
Size (kD)	40	43	37
Inhibition of Rel DNA-binding activity	+	+	–
Inhibition of NF- κ B DNA-binding activity	+	+	+
Neutralization of activity with pp40 antiserum	+	+	–
Inhibition of activity by phosphorylation			
PKA	+	+ ^a	– ^a
PKC	–	+ ^a	+ ^a
CKII	–	– ^a	– ^a

^aData are from unpublished observations.

ing to DNA without requiring p65. It is worth pointing out that the product of the carboxy-terminal half of the p105, the precursor of p50, can inhibit binding of p50 to DNA (L. Kerr, in prep.). The nature of the truncated carboxy-terminal polypeptide is not known but, presumably, could be another member of the I κ B/pp40 family of proteins.

Regulation by phosphorylation

The transcription of the proto-oncogene Rel is induced in response to signals that activate second messenger pathways (Bull et al. 1990). On the other hand, in a num-

ber of lymphoid cells, the c-Rel gene is constitutively expressed (Grumont and Gerondakis 1990). The Rel protein is probably nonfunctional because of its association with the hypophosphorylated form of the Rel-associated pp40. Hyperphosphorylation of pp40 (pp40*), on the other hand, does not prevent binding of Rel to its cognate DNA either in vitro or in vivo (Fig. 6). In the case of NF- κ B, phosphorylation of I κ B α by the PKC pathway activates the DNA binding of NF- κ B in vitro (Ghosh and Baltimore 1990). Unlike I κ B α , I κ B β can be regulated by phosphorylation with either PKA or PKC (Table 1). The hyperphosphorylated form of I κ B β , like pp40, is unable to inhibit DNA binding (L. Kerr, unpubl.). Interestingly, the phosphatase-treated pp40 is also unable to bind to Rel. Thus, unphosphorylated and hyperphosphorylated pp40 (pp40*) are unable to prevent DNA binding of Rel to its cognate DNA (Fig. 6). At present, we do not know whether the unphosphorylated or hyperphosphorylated form of pp40 associates with NF- κ B complexes upon activation. It is not known whether Rel–Rel dimers, Rel/p50 complex, or p50–p65 dimers are functional in the cell. It is therefore likely that the transcriptional function of Rel is regulated by both phosphorylation and choice of the partner to form functional dimers or oligomers.

Materials and methods

Cell culture

The murine B-lymphoma cell line WEHI-231 was cultured in Dulbecco's modified Eagle medium (DMEM) (GIBCO) supplemented with 10% heat-inactivated fetal bovine serum (Hyclone

Figure 6. Hyperphosphorylation of pp40 by PKA in vitro alters its ability to inhibit κ B-binding proteins. (A) In vitro phosphorylation of pp40 by PKA and PKC. Purified pp40 (10 ng) was phosphorylated by PKA in an in vitro kinase reaction (see Materials and methods) in the presence (+) (lane 2) and absence (–) (lane 3) of [γ -³²P]ATP. The protein kinase inhibitor HA was included (0.3 mM) in the kinase reaction mixture in lane 4. A parallel phosphorylation was performed with [γ -³²P]ATP incorporation in which the sample was then immunoprecipitated with anti-pp40 serum (lane 1). Similarly, pp40 was phosphorylated in vitro by PKC (see Materials and methods) with [γ -³²P]ATP in either the presence (+) (lane 5) or absence (–) (lane 6) of phosphatidylserine to demonstrate the phospholipid dependence of the PKC. Samples used in EMSA were phosphorylated in the presence of unlabeled ATP. SDS-PAGE (10%) analysis demonstrates the phosphorylated products. (B) Hyperphosphorylation of pp40 by PKA, but not PKC, prevents the pp40 inhibition of bacterial c-Rel. *E. coli* c-Rel (BactRel) was preincubated (30 min on ice) either in the absence (–) (lane 2) or presence (+) of pp40 (3 μ l, lane 3); pp40 phosphorylated in vitro by PKA (7.5 ng, lane 4); pp40 phosphorylated in vitro by PKC (7.5 ng, lane 5); or pp40 phosphorylated in vitro by PKA, dephosphorylated by PAP, and inactivated by inorganic phosphate (lane 6). In vitro phosphorylations with unlabeled ATP were as described in Materials and methods. Radiolabeled κ B-DNA was added, and incubation proceeded at ambient temperature for 45 min prior to analysis on a low-ionic strength polyacrylamide gel as before. Autoradiography was performed at –70°C for 16 hr. (C) Hyper- or dephosphorylation of pp40 prevents its inhibitory function. Lane 2 demonstrates that 7.5 ng of pp40 has no intrinsic ability to bind κ B sequences. WEHI-231 cytoplasmic extract (2 μ g) was preincubated (30 min on ice) in the absence (–) (lane 3) and presence (+) of 7.5 ng of pp40 (lane 4); pp40 treated with PKA in the absence of ATP (lane 5); pp40 phosphorylated by PKA (lane 6); and pp40 treated with PKA in the presence of HA (0.3 μ M) (lane 7). Similarly, WEHI-231 cytoplasmic extracts were incubated with pp40 treated with PKC in either the presence (+) (lane 8) or absence (–) (lane 9) of phosphatidylserine. To examine the hypophosphorylated state of pp40, 10 ng of pp40 was treated for 30 min at 30°C with 0.3 units of PAP in either the presence (+) (lane 11) or absence (–) (lane 10) of 20 mM sodium phosphate (P_i) to decrease the possibility of nonspecific phosphatase activities. EMSA was as described previously. The representative autoradiogram was exposed at –70°C for 8 hr. The experiment is representative of four separate phosphorylation trials with two different preparations of pp40 and two different PKA preparations. (D) PKA phosphorylation of 70Z/3 cell extracts releases κ B-binding activity. Unstimulated 70Z/3 cell cytosolic extract (10 μ g) was either untreated (lane 2); phosphorylated by PKA (lane 3) either in the absence of ATP (lane 4) or in the presence of HA as described previously (lane 5); or treated with DOC/NP-40 as described previously (lane 6) for 10 min at 30°C prior to analysis by EMSA. The autoradiogram was exposed at –70°C for 12 hr.

Laboratories, Inc.), 2 mM glutamine, 1 mM sodium pyruvate, and 50 μ M 2-mercaptoethanol at 37°C in an atmosphere containing 10% CO₂. The murine pre-B-cell line 70Z/3 was cultured in DMEM supplemented with 10% heat-inactivated fetal bovine serum at 37°C in an atmosphere containing 10% CO₂. All cultures were maintained in penicillin (25 U/ml) and streptomycin (25 μ g/ml) with removal of antibiotics prior to use in experiments. Cells were utilized in the exponential phase of growth with a density below 1×10^6 /ml.

Preparation of cellular extracts and EMSAs

Nuclear and cytoplasmic extracts from WEHI-231 and 70Z/3 cells were prepared by the method of Dignam et al. (1983). 70Z/3 cells were stimulated with 10 μ g/ml of *Escherichia coli* lipopolysaccharide (Sigma) and 10 μ g/ml of cycloheximide (Sigma) for 4 hr at 37°C prior to collection. EMSAs were performed as described previously (Inoue et al. 1991), with the following additions. Reactions were carried out in the presence of 75 mM NaCl, 15 mM Tris-HCl (pH 7.5), 1.5 mM EDTA, 1.5 mM DTT, 7.5% glycerol, 0.3% NP-40, and 20 μ g of bovine serum albumin (fraction V, Sigma) per reaction. The mouse κ -light-chain enhancer motif double-stranded oligonucleotide [AB site; 5'-AGCTTCAGAGGGGACTTCCGAGG-3' and 5'-TCGACCTCTCGGAAAGTCCCCTCTGA-3' described in Urban and Baeuerle (1990)] was phosphorylated with polynucleotide kinase in the presence of [γ -³²P]ATP (NEN-Dupont, >3000 Ci/mole) and purified by G-50 Sephadex spin column.

Purification of pp40

REV-T-transformed lymphoid cells (18–20 liters) were collected by centrifugation at 3500 rpm in a GSA rotor (Sorvall). The cells are washed in PBS and lysed in 25 mM Tris, 150 mM NaCl, 0.05% 2-mercaptoethanol, 5 mM EGTA, 5 mM EDTA, and 0.2 mM phenylmethylsulfonyl fluoride (PMSF) by mechanical shearing in a Virtishear tissue homogenizer (Virtis). A cytosolic extract is prepared by ultracentrifugation of the cell lysate (250,000g for 45 min). The cytosolic extract is then vacuum concentrated and subjected to gel filtration chromatography on a Sephacryl S-200 column (26 $\times$ 75 cm) equilibrated in 25 mM Tris (pH 7.5), 150 mM NaCl, 5 mM EGTA, 5 mM EDTA, and 0.05% 2-mercaptoethanol. The fractions containing pp59^{vRel} are combined and subjected to affinity chromatography utilizing a monoclonal antibody column specific for pp59^{vRel}. The affinity column is washed with 100 column volumes of the S-200 buffer and equilibrated to 10 mM sodium acetate (pH 6.0), 0.05% 2-mercaptoethanol, and 0.2 mM PMSF. The column is washed again with 100 column volumes of the S-200 column buffer. pp40 is specifically eluted from the column in 1 M MgCl₂, and the remaining bound proteins are eluted in 3.5 M MgCl₂.

Preparation of DNA affinity resin and chromatography

The previously described HIV-LTR enhancer κ B-motif double-stranded oligonucleotide (Inoue et al. 1991) was phosphorylated, ligated, and coupled to Sepharose CL-6B (Pharmacia) as described (Kadonaga and Tjian 1986). The efficiency of coupling was estimated to be >70% with [γ -³²P]ATP as a tracer. Cytosolic extract (5 mg) from unstimulated 70Z/3 cells was treated with DOC (0.8%) in the presence of NP-40 (1%) for 15 min at ambient temperature. The following procedures were performed at 4°C. The extract was diluted to 10 ml with buffer K [25 mM HEPES-K+ (pH 7.8), 12.5 mM MgCl₂, 1 mM dithiothreitol, and 20% glycerol] containing 0.1 M KCl. Bovine serum albumin (500

μ g; fraction V, Sigma) was added to reduce nonspecific binding. The diluted extract was passed over 1 ml of the κ B-affinity resin, which had been equilibrated in buffer K prior to the pouring of the column. The resin was washed four times with buffer K containing 0.1 M KCl, and the flowthrough fractions and washes were collected. The fractions were dialyzed for 18 hr at 4°C (to remove DOC) against three changes of a 500-fold excess of buffer D (Dignam et al. 1983) prior to a 20-fold concentration using a Centricon 3 microconcentrator (Amicon) as described. The proteins bound to the κ B resin were eluted in buffer K containing 1.0 M KCl, diluted to 0.1 M KCl with buffer K, and reappplied to a fresh affinity resin column. The κ B-bound proteins were eluted as before, dialyzed against buffer D, and concentrated (25-fold) as above.

pp40 association assay

Mutant and wild-type c-Rel cDNA plasmids were linearized by standard restriction enzyme digestion to yield a 5' overhang site. Linearized plasmid (5 μ g) was transcribed in vitro (Promega). mRNA (2 μ g) was translated in vitro (Promega) with rabbit reticulocyte lysate in the presence of [³⁵S]methionine (Expre³⁵S³⁵S-NEN, Dupont, >1200 Ci/mole). Aliquots (5 μ l) were removed for analysis by SDS-PAGE (Laemmli 1970). An approximate equal amount of protein (on the basis of the 5 μ l-sampled aliquot) from each sample was raised to 400 μ l in dilution buffer: 50 mM NaCl, 20 mM Tris-HCl (pH 7.5), and 0.1 mM PMSF. To one set from each mutant, 7.5 ng of purified pp40 was added prior to incubation of both sets at 30°C for 10 min. Anti-pp40 serum (3 μ l) was added and tubes were incubated end over end overnight at 4°C. Protein A-Sepharose (4 mg; Pharmacia) was added for 2 hr at ambient temperature with rocking. The protein A-Sepharose was pelleted by centrifugation at 5000g for 2 min. The pellet was washed four times in dilution buffer prior to dilution in 30 μ l of Laemmli sample buffer. Proteins were analyzed by SDS-PAGE, fixed, and impregnated with 2,5-diphenyloxazole (PPO; Sigma); they were visualized by autoradiography at -70°C.

Two-dimensional tryptic peptide analysis

Phosphorylated proteins were either immunoprecipitated (pp40 and I κ B β using pp40 antiserum) or fractionated directly on a 10% SDS-PAGE. Proteins were transferred to Immobilon without fixation, and radiolabeled proteins were visualized by autoradiography of the wet filter. Protein bands of interest were excised and analyzed as described previously (Luo et al. 1990). After exhaustive digestion with trypsin and oxidation with performic acid, samples were analyzed by two-dimensional mapping as described previously (Luo et al. 1990); electrophoresis on cellulose thin-layer chromatography plates was carried out at 1.0 kV for 27 min at pH 8.9. Electrophoresis was from left to right with the origin in the center (crosses in Fig. 3) and the negative electrode on the right. Ascending chromatography was performed for 9 hr in a buffer composed of butanol, pyridine, acetic acid, and water [97 : 75 : 15 : 60 (vol/vol), pH 5.3]. Plates were dried after chromatography and exposed to film at -70°C for the given times.

In vitro kinase phosphorylations

For cAMP-dependent protein kinase reactions, samples were assayed (in 10 μ l) in the presence of 10 mM MgCl₂ and either 10 mM ATP or [γ -³²P]ATP (NEN-Dupont, >3000 Ci/mole). Reactions were initiated by the addition of 1 μ l of purified catalytic subunit of cAMP-dependent protein kinase from beef heart

(0.14 mg/ml) prior to incubation at 30°C for 10 min. Where indicated, 3 μ M HA [HA 1004, N-(2-guanidinoethyl)-5 isoquinoline sulfonamide hydrochloride; Seikagaku America, Inc., St. Petersburg, FL] was included to inhibit the cAMP-dependent protein kinase reaction (Hidaka et al. 1984). Reactions were terminated by the addition of 3 μ M HA to prevent further phosphorylation by PKA.

PKC

Samples were phosphorylated in the presence of 20 mM Tris-HCl (pH 7.5), 5 mM Mg-acetate, 0.1 mM CaCl_2 , 8 μ g/ml of phosphatidylserine, 0.8 μ g/ml of diolein, and either 10 μ M ATP or [γ - 32 P]ATP. Reactions were initiated by the addition of 0.5 μ l of rat brain protein kinase C (0.32 mg/ml) (sp. act. 460 nmole/min per mg of protein) prior to incubation at 30°C for 10 min. Reactions were terminated by the addition of 6 μ M H-7. Appropriate controls included reactions in which ATP, phosphatidylserine, or CaCl_2 were not added to confirm that specific phosphorylation by PKC was observed.

CKII

Samples were phosphorylated in the presence of 20 mM Tris-HCl (pH 7.5), 10 mM MgCl_2 , 150 mM KCl, and either 20 μ M ATP or [γ - 32 P]ATP. Reactions were initiated by the addition of 3 μ l of rat testes CKII (0.1 μ g) prior to incubation of the samples at 30°C for 20 min. Reactions were terminated by the addition of 10 μ M heparin. Appropriate controls included the absence of ATP or the inclusion of EDTA in the reaction mixture.

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