Cell, Vol. 89, 413–424, May 2, 1997, Copyright ©1997 by Cell Press

The Transcriptional Activity of NF-κB Is Regulated by the IκB-Associated PKAc Subunit through a Cyclic AMP–Independent Mechanism

Haihong Zhong,* Helena SuYang,*‡
Hediye Erdjument-Bromage,† Paul Tempst,†
and Sankar Ghosh*
*Section of Immunobiology
and Department of Molecular Biophysics
and Biochemistry
Howard Hughes Medical Institute
Yale University School of Medicine
New Haven, Connecticut 06520
†Molecular Biology Program
Memorial Sloan-Kettering Cancer Center
New York, New York 10021

Summary

Stimulation of cells with inducers of NF-κB such as LPS and IL-1 leads to the degradation of IκB-α and IκB-β proteins and translocation of NF-κB to the nucleus. We now demonstrate that, besides the physical partitioning of inactive NF-κB to the cytosol, the transcriptional activity of NF-κB is regulated through phosphorylation of NF-κB p65 by protein kinase A (PKA). The catalytic subunit of PKA (PKAc) is maintained in an inactive state through association with IκB-α or IκB-β in an NF-κB–IκB–PKAc complex. Signals that cause the degradation of IκB result in activation of PKAc in a cAMP-independent manner and the subsequent phosphorylation of p65. Therefore, this pathway represents a novel mechanism for the cAMP-independent activation of PKA and the regulation of NF-κB activity.

Introduction

The transcription factor NF-κB consists of homodimers or heterodimers of proteins that belong to the Rel family (Verma et al., 1995; Baldwin, 1996). Members of this family share a 300 aa (amino acid) region known as the Rel homology domain (Verma et al., 1995; Baldwin, 1996). In mammalian cells, this family includes the oncogene v-*rel*, its cellular counterpart c-Rel, p105 (p50), p100 (p52), p65, and RelB. In Drosophila, two Rel proteins have been described: Dorsal, a morphogen important for dorso-ventral patterning; and Dif, an inducible transcription factor that is critical for immune responses (Kopp and Ghosh, 1995; Baldwin, 1996). NF-κB/Rel transcription factors are present in most cell and tissue types as inactive cytosolic proteins. The retention of NF-κB in the cytosol is due to its interaction with inhibitory proteins called IκBs. NF-κB activity can be induced by a wide spectrum of signals, including mitogens, cytokines, bacterial lipopolysaccharide, viruses, viral proteins, double-stranded RNA, and ultraviolet light (Kopp and Ghosh, 1995). Despite the differences among these signals, one of their common targets is the cytoplasmic NF-κB–IκB complex. Treatment of cells with inducers of NF-κB activity leads to phosphorylation, ubiquitination, and degradation of the IκB proteins, thus releasing NF-κB and allowing it to translocate into the nucleus to activate transcription (Verma et al., 1995; Baldwin, 1996). Although attention has primarily been focused on the kinases involved in phosphorylation of IκBs, some NF-κB/Rel proteins, in particular p65, are also inducibly phosphorylated during the activation of NF-κB (Naumann and Scheidereit, 1994; Neumann et al., 1995). However, the nature of this phosphorylation and its effect on NF-κB activity are not yet understood.

Most Rel proteins have a consensus recognition site (RRXS) for cyclic AMP–dependent protein kinase (PKA) located approximately 25 amino acids N-terminal to the nuclear localizing signal (NLS) in the Rel homology domain (Mosialos and Gilmore, 1992). It has been suggested that PKA-mediated phosphorylation of NF-κB is involved in the inducible and constitutive activation of NF-κB (Blank et al., 1992; Verma et al., 1995). Naumann and Scheidereit have demonstrated that, unlike p50 and c-Rel, p65 is constitutively phosphorylated in mature B cells and inducibly phosphorylated in Hela cells (Naumann and Scheidereit, 1994). Dephosphorylation of nuclear extracts from induced cells reduces the DNA-binding activity of NF-κB in vitro. In addition, PKA can phosphorylate the recombinant p65–p65 homodimer and p65–p50 heterodimer, leading to an enhancement of their DNA-binding activity. Further support for a role of PKA in the activation of NF-κB comes from studies in Drosophila (Norris and Manley, 1992). In Drosophila Schneider cells, the nuclear translocation and transactivating ability of transfected Dorsal can be enhanced by cotransfection of PKA, which phosphorylates Dorsal at the PKA consensus site. It is also known that Dorsal becomes phosphorylated concomitant with its transport to the nucleus in the embryo (Whalen and Steward, 1993; Gillespie and Wasserman, 1994). Although transfection studies suggest that PKA increases the activity of NF-κB, some reports have indicated that elevation of cAMP reduces NF-κB activity, possibly by stabilizing IκB-α and impairing the nuclear transport of p65 (Chen and Rothenberg, 1994; Neumann et al., 1995). However, the mechanism by which active PKA might stabilize IκB-α has not yet been explained.

Although these individual observations suggest that PKA plays a role in regulating NF-κB activity, the mechanism of PKA activation during induction of NF-κB remains unknown. It is generally accepted that the only source of PKA is cytosolic, tetrameric complexes of the PKA regulatory and catalytic subunits (R_2C_2) (Taylor et al., 1990; Scott, 1991; Francis and Corbin, 1994; Walsh and Van Patten, 1994). Elevation of cAMP levels causes binding of cAMP to the regulatory subunits, and the consequent allosteric change leads to release of the catalytic subunits. This active PKA affects both the regulation of key metabolic enzymes and the expression of numerous target genes. However, the inability of inducers of NF-κB, such as LPS, IL-1, or PMA/PHA, to elevate cAMP levels has stymied efforts to formulate a

‡Present address: Tularik Inc., 2 Corporate Drive, San Francisco, California 04080.

mechanism that could explain how PKA might be activated in cells treated with these inducers.

We now demonstrate that the PKA catalytic subunit (PKAc), but not the PKA regulatory subunit (PKAr), binds IκB proteins and is associated with the NF-κB–IκB complex. PKAc interacts with IκB-α and IκB-β through sequences from the N terminus of the protein, and this interaction inhibits the catalytic activity of PKAc. Stimulation of cells with inducers of NF-κB activity, such as LPS, that do not alter the levels of intracellular cAMP, leads to degradation of IκB proteins and the consequent activation of IκB-bound PKAc. Thus, this pathway represents a novel mechanism by which a portion of PKA in cells is regulated in a cAMP-independent manner. The active PKAc then phosphorylates NF-κB p65 at the PKA consensus site in the Rel domain and leads to a dramatic increase in the transcriptional activity of NF-κB. Specific inhibition of PKA activity in cells stimulated with inducers of NF-κB potently inhibits transcriptional activation by NF-κB, but not its nuclear translocation or binding to DNA. Therefore, our studies reveal novel mechanisms for regulating the transcriptional activity of NF-κB and the activity of a portion of PKA in a cAMP-independent manner.

Results

The PKA Catalytic Subunit Copurifies with IκB-β

During the purification of IκB-β protein from rabbit lung cytosol (Figure 1A), we noticed a 42 kDa protein that copurified with IκB-β (Figure 1B) (Thompson et al., 1995). Sequencing of this 42 kDa polypeptide showed that it was the α isoform of the catalytic subunit of cyclic AMP-dependent protein kinase (PKAc α) (Figure 1C) (Scott, 1991; Francis and Corbin, 1994). Because the pIs of IκB-β and PKAc are markedly different (4.5 versus 8.6), the coelution of these proteins on ion exchange columns strongly suggested that they were associated with each other. However, to our surprise, we did not observe any polypeptide between ~52 and 55 kDa, corresponding to the regulatory subunit of PKA (Figure 1B) (Scott, 1991; Francis and Corbin, 1994). The absence of PKAr in these preparations was also verified by immunoblotting (data not shown). Since the activity of PKAc is normally inhibited through its association with PKAr (Scott, 1991; Francis and Corbin, 1994), we wanted to determine if the IκB-β-associated PKAc was catalytically active. We therefore tested the purified IκB-β fractions containing PKAc for kinase activity, using the phosphorylation of a fluorescent kempeptide as an assay (see Experimental Procedures), but we could not detect any PKA activity in these fractions, even in the presence of cAMP (data not shown). This suggested that association of PKAc with IκB-β caused inhibition of PKA catalytic activity.

To determine whether PKAc was also present in earlier fractions from the purification of NF-κB–IκB, we chromatographed fractions from Sephacryl S-200 (step 4) (Figure 1A), that were enriched for NF-κB–IκB complexes, on a Mono Q column. Assaying the fractions both for PKA activity in the presence of cAMP and for NF-κB activity after deoxycholate (DOC) treatment indicated that NF-κB–IκB complexes and the PKA holoenzyme (R_2C_2) could be separated from one another (Figure 1D). Immunoblot analysis of the same fractions with antibodies to PKAr and PKAc showed that while PKAr was present only in the fractions where PKA could be activated by cAMP (fractions 19–24), PKAc was present both in fractions containing PKAr (fractions 19–24) and with NF-κB–IκB complexes (fractions 13–18) (Figure 1D). As previously seen with PKAc that copurified with IκB-β (Figure 1B), the PKAc present in the NF-κB–IκB fractions could not be activated by cAMP. To prove that the PKAc detected in the NF-κB–IκB fractions was not intrinsically defective, we carried out a denaturation-renaturation assay for PKA from a purified NF-κB–IκB–PKAc–containing fraction (fraction 16) that contained PKAc but did not exhibit PKA activity (Figures 1D and 1EI) (Hager and Burgess, 1980). Protein kinase activity was detected from the slice corresponding to ~42–45 kDa, which is around the expected molecular weight of PKAc (Figure 1EII), demonstrating that association with NF-κB–IκB-α/β complexes inactives PKAc.

PKAc Associates with IκB-α and IκB-β in NF-κB–IκB Complexes

To further examine the association of IκB proteins with PKAc and PKAr, we generated constructs by fusing IκB-α and IκB-β to glutathione *S*-transferase and expressed the recombinant fusion proteins in bacteria (Figure 2AI). We then mixed the purified GST–IκB proteins with ^{35}S-labeled PKAc, PKAr, and NF-κB p65, made by in vitro translation in rabbit reticulocyte lysates (Figure 2AII). After a short incubation, the GST proteins were precipitated by binding to glutathione–agarose beads and were washed extensively, and the bound proteins were analyzed by SDS–PAGE, followed by fluorography (Figure 2AIII). As expected, only p65 and PKAc, but not PKAr, were bound to the IκB proteins.

We then wanted to determine whether the interaction between PKAc and IκB-α/IκB-β could be observed when the various proteins were coexpressed in cells. We therefore cotransfected into COS-1 cells cDNAs encoding NF-κB p65 and IκB-α or IκB-β, along with a cDNA encoding PKAc, tagged with an epitope from influenza virus. Following transfection, the cells were lysed and immunoprecipitations were performed with antibodies against IκB-α, IκB-β, and p65 (Figure 2B). The immunoprecipitates were fractionated by SDS–PAGE and immunoblotted with an antibody against the flu epitope. In all cases, the flu-tagged PKAc could be precipitated, indicating that it was bound to both NF-κB–IκB-α and NF-κB–IκB-β complexes (Figure 2B).

The results presented above indicated that both in vitro and in transfected cells, PKAc could associate with IκB-α and IκB-β. To determine whether PKAc was a component of both NF-κB–IκB-α and NF-κB–IκB-β complexes in vivo, we examined the composition of NF-κB–IκB complexes in cells, using immunoprecipitation and immunoblotting techniques. We began by immunoprecipitating NF-κB–IκB complexes from 70Z/3 and Jurkat cell extracts, using antibodies against NF-κB p65. The immunoprecipitates were fractionated by SDS–PAGE and immunoblotted with an antibody directed against the catalytic subunit of PKA. PKAc was very efficiently coimmunoprecipitated with p65, suggesting

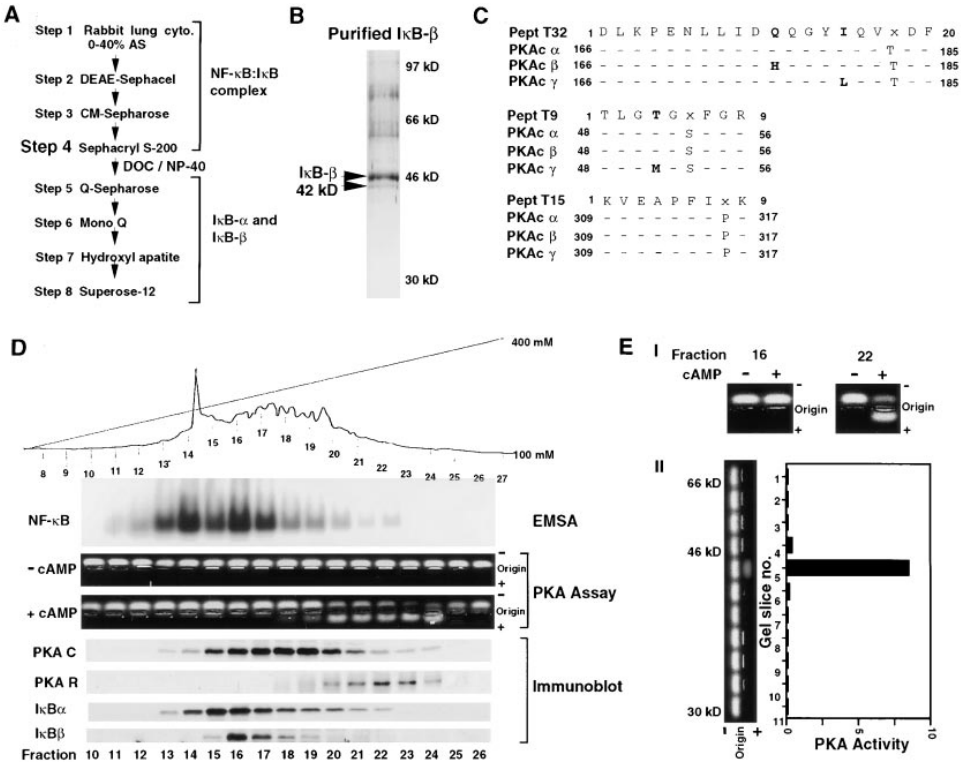

Figure 1. PKAc Copurifies with IκB-β from Rabbit Lung Cytosol

(A) The purification scheme used for purifying IκB-β from rabbit lung cytosol is shown (Thompson et al., 1995).

(B) A silver-stained gel of the peak fraction of purified IκB-β from the Superose 12 column (see Experimental Procedures).

(C) The protein sequence of the 42 kDa copurifying band reveals that it is the α isoform of the catalytic subunit of PKA. The three isoforms of PKAc, although highly homologous, have some key differences (shown in bold) that allow them to be distinguished from one another. (X) indicates amino acids that could not be unequivocally assigned by protein sequencing.

(D) The Sephacryl-S200 fraction (step 4 in Figure 1A) that contains partially purified NF-κB–IκB complex was chromatographed on a FPLC MonoQ column, and the bound proteins were eluted with a gradient from 75 mM to 500 mM NaCl (the region of the gradient from 100 mM to 400 mM NaCl is shown). The fractions containing protein (fractions 10–26) were assayed for NF-κB DNA-binding activity after DOC/NP-40 treatment, PKA activity with and without cAMP, and immunoblot analyses with antibodies to the catalytic subunit of PKA, the regulatory subunit of PKA, IκB-α, and IκB-β. The cAMP-induced PKA activity was seen in fractions 19–24, fractions that also contained the PKAc and PKAr subunits. The NF-κB–IκB complex containing fractions 12–18 does not contain PKAr but does have PKAc.

(E) (I): Fraction 16 contains DOC-induced NF-κB activity but no cAMP-induced PKA activity, in contrast to fraction 22, which does not contain NF-κB activity but has cAMP-induced PKA activity. (II): To determine whether the PKAc protein seen on immunoblots of fraction 16 was active, a denaturation-renaturation assay for PKA activity was performed on 300 μl of this fraction (Hager and Burgess, 1980). PKA activity was only detected from the slice around 42–45 kDa (slice 5). The photograph of the agarose gel used for the fluorescent kempeptide assay is on the left, while the quantitation of the assay is on the right.

that it was associated with NF-κB–IκB complexes (Figure 2C, lanes 1 and 3). Boiling the extracts before immunoprecipitation resulted in the loss of coimmunoprecipitating PKAc, suggesting that it was bound to NF-κB–IκB complexes by noncovalent interactions (Figure 2C, lanes 2 and 4). We then wanted to determine whether IκB-α, IκB-β, or both IκBs could be coprecipitated with PKAc. Therefore, we carried out immunoprecipitations with antibodies against PKAc and PKAr and analyzed the precipitated proteins by immunoblotting with antibodies against IκB-α and IκB-β (Figure 2D). The PKAc immunoprecipitates contained both IκB-α (Figure 2D, lane 1) and IκB-β (Figure 2D, lane 4), while the PKAr immunoprecipitates contained neither of the two IκB isoforms (Figure 2D, lanes 2 and 5). Incubating parallel blots with antibodies to PKAc indicated the presence of PKAc in both immunoprecipitates (Figure 2D, lanes 7 and 8). Therefore, this result demonstrated that PKAc is present in both NF-κB–IκB-α and NF-κB–IκB-β complexes. Finally, we carried out immunoprecipitations with antibodies against IκB-α or IκB-β and used immunoblotting to examine whether PKAc was present in both immunoprecipitates (Figure 2E). Again, the results indicated that PKAc could be immunoprecipitated with both NF-κB–IκB-α and NF-κB–IκB-β complexes (Figure 2E, upper panel, lanes 3 and 4). If a parallel blot was probed with the PKAr antibody, the results indicated that PKAr was only coprecipitated with PKAc (Figure 2E, lower panel, lane 5). Hence, these results clearly establish the presence of two pools of PKAc in cells, one bound to PKAr and another bound to NF-κB–IκB complexes.

The ATP-Binding Domain of the PKA Catalytic Subunit Interacts with IκB

The lack of PKA activity in the purified IκB/PKAc fractions (Figure 1A and data not shown) and in purified NF-κB–IκB–PKAc complexes (Figure 1D) suggested that the binding of IκB to PKAc inhibits the enzymatic activity

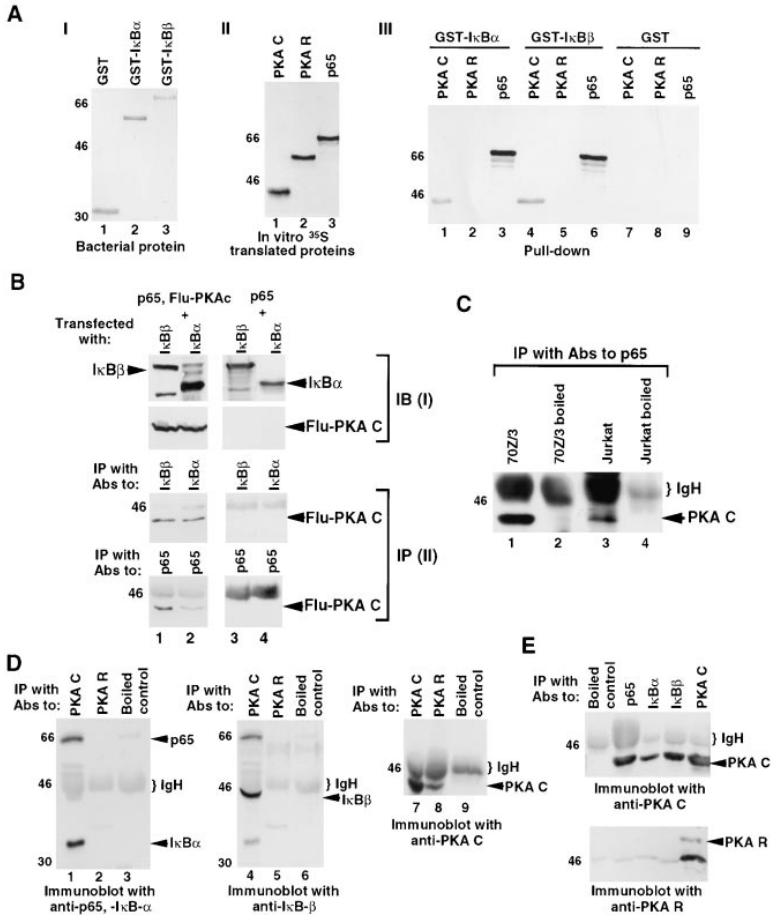

Figure 2. PKAc, but Not PKAr, Directly Associates with IκB-α and IκB-β

(A) The PKAc, PKAr, and NF-κB p65-encoding cDNAs were translated in vitro using the TNT system from Promega. The purified GST proteins (Thompson et al., 1995) (~0.5 μg) were mixed with 1 μl of the in vitro translated proteins and incubated on ice for 10 min, and then glutathione–agarose beads were used to pull down proteins associated with the GST proteins. The beads were washed, and the precipitated proteins were fractionated on SDS–PAGE and analyzed by fluorography.

(B) Expression vectors (pCDNA3) containing cDNAs for NF-κB p65 (lanes 1–4) and HA (flu)-tagged PKAc (lanes 1 and 2) with either IκB-β (lanes 1 and 3) or IκB-α (lanes 2 and 4) were used for transfection into COS-1 cells. (I): Twenty-four hours after transfection, extracts were prepared and immunoblots were carried out with anti-IκB-β (upper panel, lanes 1 and 3), anti-IκB-α (upper panel, lanes 2 and 4), and anti-flu antibody (lower panel) to determine the expression of the transfected constructs. (II): Immunoprecipitations were then carried out on the extracts using the indicated antibodies. After extensive washing of the PAS immunoprecipitates, the bound proteins were eluted by boiling in SDS sample buffer and fractionated on SDS–PAGE, followed by immunoblotting with anti-HA antibodies.

(C) Immunoprecipitations were carried out using antibodies against NF-κB p65, from cytosolic extracts of 70Z/3 and Jurkat cells (lanes 1 and 3). Identical immunoprecipitations were carried out on samples that had been boiled with SDS and neutralized with Triton X-100 before adding the immunoprecipitating antibody (lanes 2 and 4). The immunoprecipitates were fractionated on SDS–PAGE and then immunoblotted with an antibody against PKAc. The absence of PKAc in the boiled samples (lanes 2 and 4) indicated that PKAc was associated with NF-κB–IκB complexes by noncovalent interactions.

(D) Extracts from 70Z/3 cells were immunoprecipitated with antibodies against PKAc and PKAr. The immunoprecipitates were fractionated on 10% SDS–PAGE and analyzed by immunoblotting with antibodies against IκB-α (lanes 1–3), IκB-β (lanes 4–6), and PKAc (lanes 7–9). The boiled controls were precipitated with the PKAc antibody.

(E) Immunoprecipitations on 70Z/3 cells were carried out as in Figure 2D, with antibodies against p65, IκB-α, IκB-β, and PKAc. The immunoprecipitates were analyzed by immunoblotting after SDS–PAGE, using antibodies against PKAc (upper panel) or PKAr (lower panel). The boiled control was precipitated with IκB-β antibody.

of PKA. To demonstrate that IκB proteins can directly inhibit PKA activity, we performed an assay in which increasing amounts of GST–IκB-α, GST–IκB-β, GST, and PKAr were added to a fixed amount of purified PKAc (Figure 3A). The results showed that both IκB-α and IκB-β were able to inhibit the catalytic activity of the purified PKAc preparation (Figure 3A).

The catalytic activity of PKAc is inhibited by its regulatory subunit PKAr through a direct masking of the substrate binding domain (Francis and Corbin, 1994; Walsh and Van Patten, 1994). To determine whether IκBs were also inhibiting the catalytic activity of PKAc through a similar mechanism, we set up an experiment to map the region in PKAc used for interaction with IκB. We tested the ability of GST–IκB-α and GST–IκB-β proteins to interact with different ^{35}S-labeled deletions of PKAc (Figures 3BI and 3BII). The results of the pull-down experiment indicated that sequences toward the N terminus of PKAc (minimally, from amino acids 46–76) were critical for interaction with IκBs (Figure 3BIII). The three-dimensional structure of PKAc has revealed that the protein has a bilobate structure, a smaller lobe made up primarily of sequences from the N terminus (amino acids 40–125) and a larger lobe consisting primarily of sequences from the C terminus (amino acids 140–280) (Knighton et al., 1991a, 1991b). The catalytic site of the kinase is localized in a deep cleft between the two lobes. The smaller N-terminal lobe is primarily used for binding to ATP, while substrate binding is carried out with sequences from the larger, C-terminal lobe. Therefore, the region of PKAc that appears to be critical for interaction with IκBs is different from the substrate binding region of PKAc that is masked by the regulatory subunit (Francis and Corbin, 1994). Hence, the lack of PKA activity in IκB-PKAc complexes (Figure 1A) is possibly due to the masking of the ATP-binding domain of PKAc.

If the interaction of IκB with PKAc is confined to the N-terminal ATP-binding domain, it might leave the C-ter-

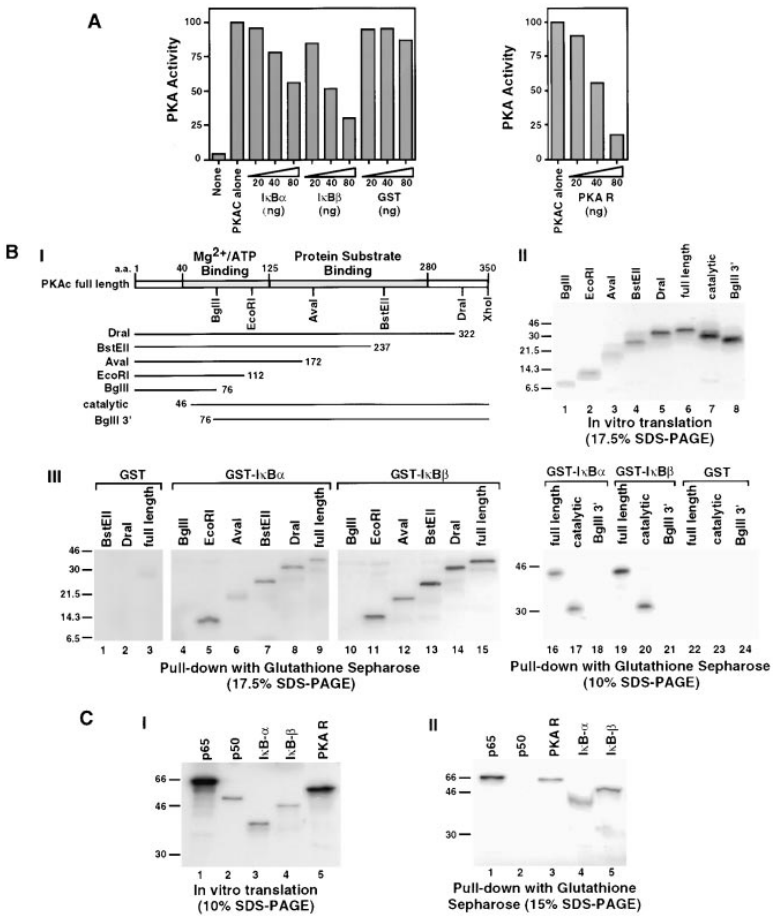

Figure 3. Inhibition of PKA Activity by IκB Is Due to Masking of the Catalytic Domain

(A) Activity of PKA was measured by using the fluorescent kempeptide assay system (see Experimental Procedures). PKA activity was determined from the ratio of phosphorylated peptide to total peptide, by quantitating the amount of each peptide after scanning photographs of the agarose gels. The PKAc and the PKAr used were purified proteins from bovine brain. The purified GST–IκB proteins were the same as described earlier (Figure 2A). In each case, 10 ng of PKAc was used, while the amount of GST–IκB-α, GST–IκB-β, GST, and PKAr varied from 20 ng to 80 ng.

(B) (I): Deletions from the C terminus of PKAc were made by truncating the plasmid containing the cDNA at various sites and in vitro translating these templates. (II): The proteins truncated from the N terminus of PKAc were generated by subcloning these smaller proteins into pCDNA3, followed by their expression in vitro in reticulocyte lysates. The amount of lysates added to each assay contained approximately equal amounts of the truncated PKAc proteins, as determined by analyzing the proteins on a 17.5% SDS–PAGE. (III): The proteins associating with the GST–IκB proteins were precipitated by using glutathione–agarose and analyzed by SDS–PAGE and fluorography.

(C) The ^{35}S-labeled Rel proteins translated in vitro, mixed with GST-PKAc, and precipitated by binding to glutathione–agarose beads. The precipitated proteins were analyzed by SDS–PAGE and fluorography. (I): Each in vitro translated protein (4 μl) was analyzed by SDS–PAGE and fluorography. (II): To compensate for the more efficient translation of p65 and PKAr, 0.5 μl of p65, 1 μl of PKAr, and 5 μl of the other proteins (p50, IκB-α, and IκB-β) were used for the pull-down experiment.

minal substrate binding domain of PKAc partially unmasked. Since we knew that PKAr is not a component of the NF-κB–IκB complexes, it appeared likely that this region of PKAc interacts with either p50 or p65, since they both contain consensus PKA phosphorylation sites and therefore can act as substrates (Mosialos and Gilmore, 1992). To test whether PKAc displays any preference between p50 and p65, we produced a GST–PKAc protein and mixed it with ^{35}S-labeled, in vitro translated p50, p65, and PKAr proteins (Figure 3C). Surprisingly, precipitation of complexed proteins with glutathione–agarose indicated that GST–PKAc forms a stable complex with only p65, and not p50. To further localize the sequences on PKAc that interact with p65, we used a truncated PKAc that contained sequences from the C terminus of the protein (amino acids 159–350) for the pull-down experiment and confirmed that the C-terminal substrate binding domain of PKAc binds to p65 (data not shown). These interaction experiments therefore suggest a model in which the N-terminal ATP-binding domain of PKAc binds to IκB, while the C-terminal region establishes interactions with the substrate p65 protein. Dual sites of interaction might thus allow PKAc to bind stably and avidly to the NF-κB–IκB complex.

Stimulation with NF-κB Inducers Activates PKA without Altering cAMP Levels

Because binding of PKA to IκB inhibits kinase activity, we assumed that the removal of IκB should activate the bound PKA. Stimulation of cells with inducers of NF-κB activity, such as LPS, results in the phosphorylation and degradation of IκB-α and IκB-β proteins (Thompson et al., 1995; Baldwin, 1996). Therefore, we wanted to test whether the degradation of IκBs correlates with the activation of PKA in LPS-stimulated cells. We stimulated 70Z/3 pre-B cells with LPS for up to 2 hr, a period in which the majority of IκB-α and IκB-β is degraded (Figure 4A) (Thompson et al., 1995; SuYang et al., 1996). Assaying the extracts for PKA activity revealed that PKA is activated in these cells in a manner that correlates with the degradation of IκBs (Figure 4B). As a control, we used forskolin, which does not affect IκB (Figure 4A) but activates PKA by dissociating the PKA R_2C_2 complexes through elevation of cAMP levels (Figure 4B) (Francis and Corbin, 1994; Walsh and Van Patten, 1994). To rule out the possibility that activation of PKA in LPS-treated cells is due to an increase in cellular levels of cAMP, we measured the level of cAMP in cell extracts using a Biotrak enzyme immunoassay kit (Amersham)

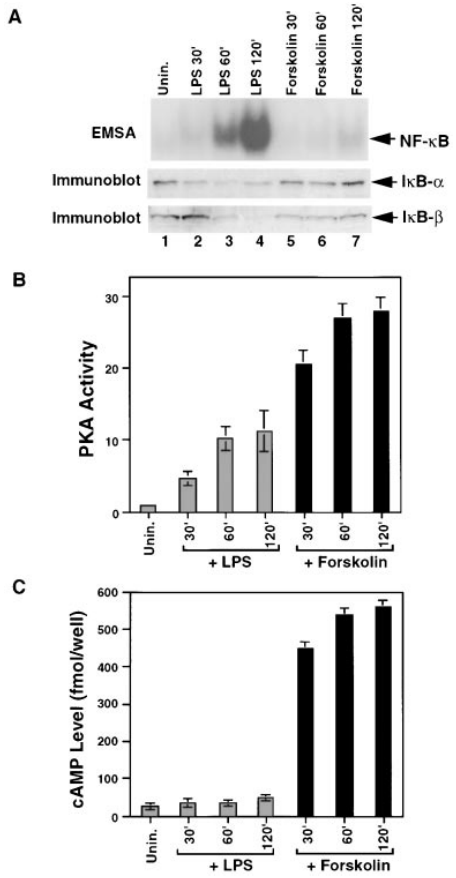

Figure 4. Stimulation with LPS Activates PKA but Does Not Increase cAMP Levels

(A) 70Z/3 pre-B cells were treated with 10 μg/ml LPS or 20 μM forskolin for the indicated periods. Cytosolic and nuclear extracts were prepared, and the nuclear extracts were used to examine NF-κB DNA binding in EMSA, whereas the cytosolic extract was used to perform immunoblots with antibodies to IκB-α and IκB-β.

(B) Total cell extracts from 70Z/3 cells, stimulated as in (A) and made in parallel, were used to determine the activity of PKA using the fluorescent kempeptide assay. The mean values and standard deviations are shown; at least three independent experiments were done for each condition. The activity of PKA is presented as fold activity compared to uninduced cells.

(C) The same samples as in (B) were used to determine the amount of cAMP, using a Biotrak EIA assay kit (Amersham).

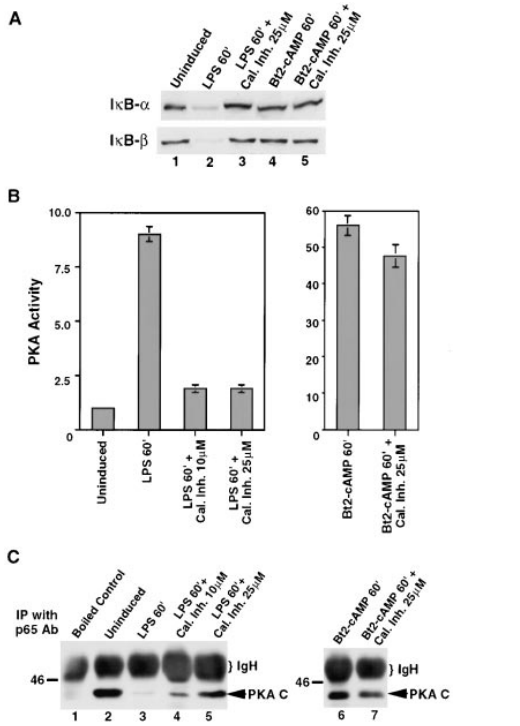

Figure 5. Proteasome Inhibitors Affect LPS-Dependent, but Not cAMP-Dependent, Activation of PKA

(A) 70Z/3 cells were treated with 10 μM, 25 μM, or no calapain inhibitor I, before stimulating with 10 μg/ml LPS for 60 min. A parallel experiment was performed in which 70Z/3 cells were stimulated with 50 μM dibutyryl cAMP with or without pretreatment with calpain inhibitor I. Extracts were made from the cells and used for immunoblot analysis.

(B) PKA activity was measured from the same samples as (A), using the fluorescent kempeptide assay.

(C) Immunoprecipitations were carried out with antibodies against NF-κB p65, from extracts of cells treated with LPS or dibutyryl cAMP, with or without calapain inhibitor I, as shown in (A). The immunoprecipitates were analyzed on SDS–PAGE and immunoblotted with an antibody against PKAc.

(Figure 4C). As expected, cells stimulated with forskolin showed an ~30-fold increase in cellular cAMP levels. In contrast, cells stimulated with LPS demonstrated no significant change in cAMP levels. Therefore, the increase in PKA activity in LPS-treated cells was not dependent on changes in cAMP levels.

Proteasome Inhibitors Block LPS-Dependent, but Not cAMP-Dependent, Activation of PKA

To prove that activation of PKA in LPS-treated cells is the result of degradation of IκB-α and IκB-β, we used calpain inhibitor I to block signal-induced IκB degradation. It has been demonstrated previously that treatment of cells with inducers of NF-κB activity (e.g., LPS) leads to the phosphorylation and degradation of IκB-α and IκB-β (Verma et al., 1995; Baldwin, 1996). Pretreatment of cells with peptide aldehyde inhibitors such as calpain inhibitor I, MG132, or PSI efficiently blocks the degradation of phosphorylated IκBs in stimulated cells (Verma et al., 1995; Baldwin, 1996). We treated 70Z/3 cells with LPS or dibutyryl cAMP, with or without pretreatment with calpain inhibitor I. The cellular extracts were then immunoblotted to determine the levels of IκB-α and IκB-β proteins (Figure 5A) and assayed for PKA activity (Figure 5B). We also carried out immunoprecipitations with the p65 antibody, followed by immunoblotting with the PKAc antibody to determine the amount of PKA that remained associated with NF-κB–IκB complexes in these stimulated cells (Figure 5C). The results showed that treatment of cells with both LPS and dibutyryl cAMP, an analog of cAMP, activated PKA (Figure 5B); however, only LPS treatment caused the degradation of IκB-α and IκB-β (Figure 5A). Pretreatment of cells with calpain inhibitor I blocked both IκB degradation (Figure 5A, lane 3) and PKA activation (Figure 5B) in LPS-stimulated cells, but did not affect activation of

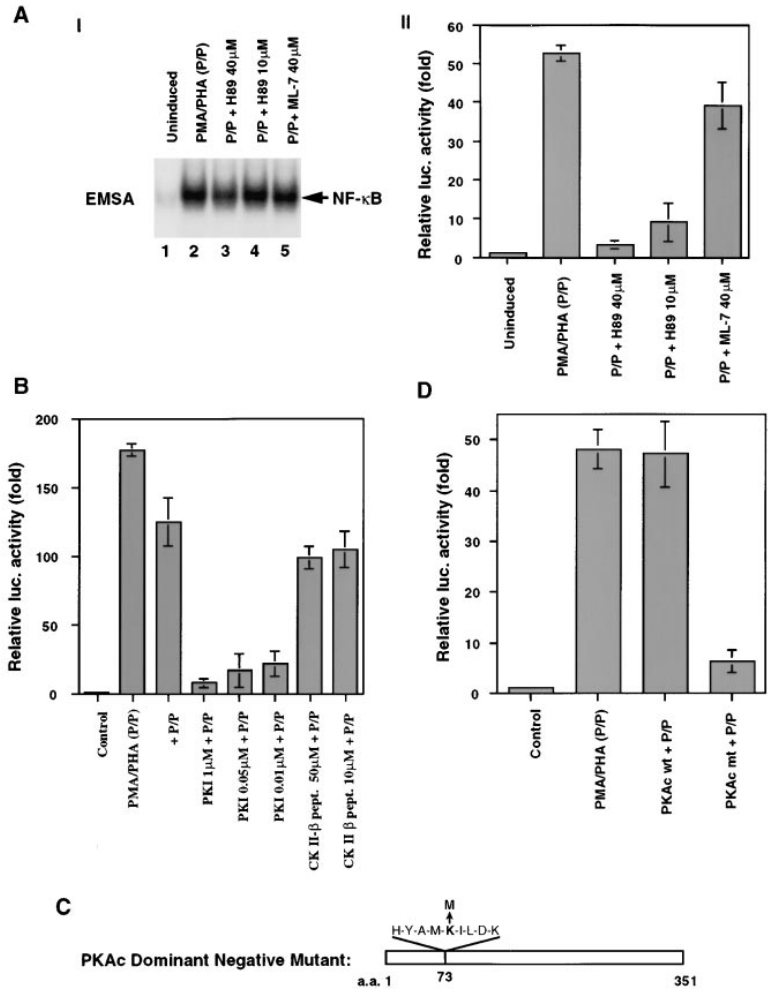

Figure 6. Inhibition of NF-κB Activation by Interfering with PKA

(A) Jurkat cells were transfected with the pBIIx luc, an NF-κB-dependent reporter construct, using lipofectamine reagent (GIBCO-BRL). Twenty-four hours after transfection the cells were treated with PMA/PHA, with or without H-89 or ML-7, at the indicated concentrations. The cells were then lysed, and the total cell extracts were used both to assay for κB-specific DNA binding and for luciferase activity.

(B) Hela cells transfected with pBIIx luc, were used for permeabilization. Following permeabilization with the TransPort reagent (GIBCO-BRL), the inhibitory PKI peptide and the control peptide (which inhibits casein kinase II autophosphorylation) were added to the cells. After allowing the peptides to enter, cells were treated with PMA/PHA for 4 hr, after which they were harvested and lysed and the activity of luciferase was determined.

(C) A schematic diagram of the PKAc protein with the mutation at lysine 73 into methionine is shown.

(D) Jurkat cells were transfected with pBIIx luc, alone or with PKAc, wild-type or the mutant. Twenty-four hours after transfection, the cells were stimulated with PMA/PHA, and the expression of the luciferase reporter was quantitated.

PKA in cells treated with dibutyryl cAMP (Figures 5A and 5B). In all cases, the activation of PKA by LPS correlated with the loss of PKAc in p65 immunoprecipitates (Figure 5C, lane 3). However, in LPS-stimulated cells pretreated with calpain inhibitor I, PKAc could still be coimmunoprecipitated with p65 (Figure 5C, lanes 4 and 5). Therefore, the degradation of IκB proteins in LPS-stimulated cells leads to the release and activation of the associated PKAc.

Inhibition of NF-κB Activation upon Interfering with PKA

To elucidate the possible role of PKA-mediated phosphorylation on NF-κB function, we used three different approaches to specifically inhibit PKA activity in cells. We first used the isoquinoline-sulfonamide derivative, H-89, which is one of the most potent inhibitors of PKA (K_i [inhibition constant] of ~0.048 mM). As a control, we used ML-7, another inhibitor that is specific for myosin light chain kinase. We tested the effect of the two inhibitors on LPS-induced activation of NF-κB and found that both inhibitors had little effect on the NF-κB DNA-binding complex (Figure 6AI, lanes 3–5). We then tested the effect of these inhibitors on NF-κB-dependent transcription, using the expression of luciferase from a reporter construct, pBIIx luc, as an assay. We observed dramatic inhibition of PMA/PHA-induced, NF-κB-dependent transcription in the presence of 10 μM H-89 (Figure 6AII), a concentration that does not affect DNA binding by NF-κB (Figure 6AI, lane 4). In contrast, ML-7, when used at up to 40 μM, had little effect on NF-κB-dependent transcription (Figure 6AII). This experiment provides strong evidence for the involvement of PKA in modulating transcriptional activation by NF-κB without affecting DNA binding by NF-κB.

We next used the specific PKA inhibitory peptide, PKI (amino acids 6–22) amide, to examine the role of PKA in NF-κB-dependent transcription (Figure 6B). PKI is a 16 aa peptide that contains a PKA pseudosubstrate sequence and specifically inhibits PKAc by binding to the substrate binding site (K_i of ~1.7 nM). To allow the peptide to enter the cell, we permeabilized the cells with the TransPort cell permeabilization kit (GIBCO-BRL), which does not lead to a significant loss of cytosolic components. Hela cells transfected with the NF-κB-dependent luciferase reporter construct were permeabilized 24 hr after transfection and were treated with either the PKI inhibitory peptide or a control peptide, which competitively inhibits casein kinase II autophosphorylation. After allowing the peptides to enter, the

cells were stimulated with PMA/PHA for 4 hr and luciferase activity was assessed. Although permeabilization itself caused a slight decrease in the level of reporter gene expression, the inclusion of the PKI peptide, but not the control peptide, caused nearly complete inhibition (>90%) of reporter gene expression (Figure 6B).

Finally, we generated a mutant, catalytically inactive form of PKAc by altering lysine 73 in PKAc to a methionine (K73M) (Figure 6C). A similar change to a conserved lysine has been demonstrated to inactivate other kinases (Taylor et al., 1990; Scott, 1991). We verified that this mutation results in a catalytically inactive form of PKA by expressing it in COS cells and assaying PKA activity (data not shown). We then tested the ability of this mutated kinase to interfere with NF-κB function by cotransfecting it with the κB-dependent luciferase reporter construct. We expected the transfected mutant PKAc to interfere with PKAc-dependent activation of NF-κB, and, as shown in Figure 6D, introduction of this mutant form into cells caused a dramatic decrease in the amount of transcription from the reporter construct when cells were stimulated with PMA/PHA. In contrast, transfection of wild-type PKAc along with the reporter construct exhibited marginal effects on PMA/PHA-induced transcription (Figure 6C). This result further strengthens the hypothesis that phosphorylation by PKA enhances transcriptional activation mediated by NF-κB. Since neither activation of PKA alone (Figure 4A) nor inhibition of PKA by H-89 (Figure 6A) affects the nuclear translocation and DNA binding by NF-κB, the role of PKA appears to be a modulator of transcriptional activity of NF-κB entering the nucleus.

Transcriptional Activity of NF-κB Is Stimulated by Phosphorylation of p65 by PKA

A possible mechanism to explain the effect of PKA on transcription by NF-κB might be the direct phosphorylation of NF-κB subunits by PKAc at the consensus site for PKA phosphorylation. Although almost all Rel proteins have a conserved PKA phosphorylation site (Mosialos and Gilmore, 1992), previous studies have shown that only inducibly phosphorylated p65 and p105 can be detected in stimulated Hela and Jurkat cells (Naumann and Scheidereit, 1994; Neumann et al., 1995). Since PKAc is directly associated with IκB-α and IκB-β, which in turn interacts only with p65, it is possible that degradation of IκB proteins allows PKA to specifically phosphorylate p65. We have also found that PKAc interacts with only p65, and not p50, through its substrate binding region (Figure 3C and our unpublished data), and therefore, degradation of IκBs should lead to a preferential phosphorylation of the bound p65 protein.

To directly demonstrate that PKAc can phosphorylate p65, we used a truncated form of p65 (amino acids 1–313), which retains the Rel homology domain and the PKA phosphorylation site, as a substrate for PKA phosphorylation. This purified, bacterially expressed p65 protein was efficiently phosphorylated by purified PKAc (Figure 7A, lane 1), and the phosphorylation was blocked by the inclusion of the PKA inhibitory peptide, PKI (Figure 7A, lane 2). We then wanted to determine whether p65 was inducibly phosphorylated by PKA when cells were stimulated with LPS. We therefore metabolically labeled unstimulated and LPS-stimulated 70Z/3 cells with ^{32}Pi and immunoprecipitated labeled proteins using the p65 antibody. A number of ^{32}P-labeled proteins were seen, including a protein around 65 kDa (Figure 7B, lane 2), and we confirmed that this 65 kDa protein was p65 by reimmunoprecipitating with the p65 antibody (Figure 7B, lane 4). We then used the dual immunoprecipitation technique to examine the effect of inhibiting IκB degradation on the inducible phosphorylation of p65. We treated ^{32}P-labeled cells with either PDTC (an antioxidant that prevents signal-induced phosphorylation of IκB), calpain inhibitor I (which blocks the degradation of inducibly phosphorylated IκB), or H-89 (which inhibits PKA). The results of the labeling experiments indicate that both PDTC and calpain inhibitor I completely block the inducible phosphorylation of p65 (Figure 7B, lanes 5 and 6), whereas H-89 inhibits such phosphorylation (Figure 7B, lane 7). Therefore, the inducible phosphorylation of p65 upon LPS treatment appears to be dependent on the degradation of IκB and the activity of PKA. To further localize the site of phosphorylation on p65 in LPS-stimulated cells, we isolated the inducibly phosphorylated p65 protein and digested it with either trypsin, chymotrypsin, LysC, or CNBr (Figure 7C). The phosphopeptides were then separated on a high-resolution Tris-tricine gel system (Schagger and von Jagow, 1987). The sizes of the phosphopeptides obtained (∼9 kDa for LysC, ∼3.4 kDa for chymotrypsin, ∼1.5 kDa for CNBr, and ∼0.5 kDa for trypsin), when compared to predicted peptides based on the sequence, indicated that they most likely include Ser 276 of the p65 protein (Figure 7C). These results therefore suggest that the inducible phosphorylation of p65 in LPS-stimulated cells occurs on Ser 276 in the consensus PKA phosphorylation site.

We then generated a site-directed mutant of p65 in which the serine 276 was altered to a nonphosphorylatable alanine residue (S276A) (Figure 7D). We tested the ability of this protein to transactivate the κB-dependent reporter construct upon transfection into COS cells. Immunoblots of the transfected cells indicated that both the wild-type and mutant p65 proteins were expressed in equivalent amounts (data not shown). However, there was a marked difference in the ability of the two p65 forms to transactivate, as measured by the expression of luciferase from a reporter construct (Figure 7D). To examine the effect of PKA on transactivation by NF-κB, we cotransfected Jurkat cells with wild-type or K73H mutant PKAc and wild-type or S276A mutant p65, and measured κB-dependent luciferase activity (Figure 7E). Transfection of wild-type or mutant PKAc alone had no effect on NF-κB. Transfection of wild-type and mutant p65 alone resulted in low levels of expression of luciferase, as seen earlier (Figure 7D). Cotransfection of PKAc with p65 led to a dramatic increase in the expression of the reporter gene, and this increase was not seen if the K73M mutant of PKAc was used (Figure 7E). The reciprocal experiment, in which wild-type PKAc was transfected with wild-type and S276A mutant p65, revealed the same effect on NF-κB-dependent transcription (Figure 7E). These results provide compelling evidence that phosphorylation of p65 by PKA at serine 276 causes a significant stimulation of transcriptional activity of NF-κB.

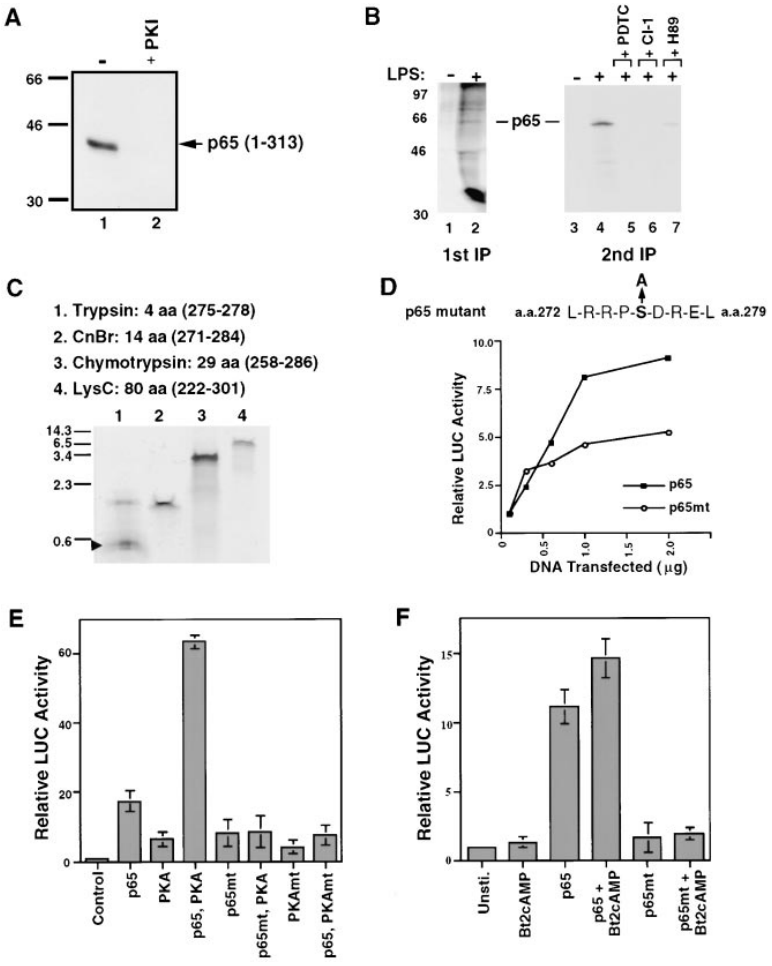

Figure 7. Transcriptional Activity of NF-κB Is Affected by Phosphorylation of p65 by PKA

(A) A truncated version of p65 from residues 1–313 was expressed in bacteria and purified to near homogeneity. The purified protein (30 ng) was used as a substrate for phosphorylation by 20 U of purified PKAc. Assays were carried out at 30°C for 30 min before analysis on SDS–PAGE, followed by autoradiography. Approximately 10 ng of PKI was used in lane 2 to inhibit PKAc.

(B) 70Z/3 cells were labeled with ^{32}Pi for 2 hr before stimulation with LPS for 15 min. The cells were lysed, p65 was immunoprecipitated and separated by SDS–PAGE, and the dried gel was exposed for autoradiography for 3 hr. Lane 1 contained unstimulated cells; lane 2 contained LPS-stimulated cells. After reimmunoprecipitation with the p65 antibody (Experimental Procedures), the secondary immunoprecipitates were separated on SDS–PAGE and detected by autoradiography for 40 hr (lane 3 contained unstimulated cells; lane 4 contained LPS-stimulated cells). To examine the effect of IκB degradation on p65 phosphorylation, ^{32}P-labeled 70Z/3 cells were pretreated with PDTC (300 μM) (lane 5), calpain inhibitor I (50 μM) (lane 6), and H-89 (40 μM) (lane 7) for 1 hr before stimulation with LPS. The cells were lysed and subjected to primary and secondary p65 immunoprecipitations.

(C) The site for LPS-induced phosphorylation on p65 was mapped using phosphopeptide mapping. The ^{32}P-labeled proteins from LPS-stimulated cells were transferred to nitrocellulose, and after autoradiography, the labeled p65 band was excised and digested with the indicated enzymes and CNBr. The upper band in the trypsin lane probably results from incomplete digestion.

(D) The sequence of the PKA phosphorylation site in p65 and the serine residue that is changed to alanine in the mutant are indicated. The mutant protein is not phosphorylated by PKAc in vitro (data not shown). The wild-type and mutant p65 proteins were expressed in Jurkat cells along with the pBIIx luc construct. Twenty-four hours after transfection, the transfected cells were harvested and the expressed luciferase was quantitated. A concentration of 0.5 μg p65 was used for transfection in the subsequent assays.

(E) Jurkat cells were transfected with 0.5 μg of p65 (wild-type or mutant) and 1 μg of PKAc (wild-type or mutant), along with the pBIIx luc reporter construct. Cells were harvested for luciferase assay 24 hr after transfection.

(F) Jurkat cells were transfected with p65 wild-type and mutant, along with the pBIIx luc, similar to that in (E). Twenty-four hours after transfection, the indicated cells were treated with 50 μM dibutyryl cAMP for 2 hr, before harvesting all the samples to assay luciferase activity.

To test whether this effect of PKA on NF-κB activity could be seen by costimulating cells with PKA activators such as dibutyryl cAMP, we transfected p65 into Jurkat cells using the κB-dependent luciferase reporter and stimulated them with dibutyryl cAMP (Figure 7F). However, costimulation with dibutyryl cAMP resulted in only a modest stimulation of the transcriptional activity of NF-κB (Figure 7F). This suggests that efficient phosphorylation of p65 occurs only when PKAc is a component of the NF-κB–IκB complex and, hence, is in close vicinity to the substrate. This also suggests that cAMP-induced PKA is unlikely to significantly affect the activity of NF-κB.

Discussion

The results presented in this paper describe novel mechanisms by which the activity of both PKA and NF-κB is regulated. The most surprising finding in this work is that IκB-associated PKAc is regulated in a cAMP-independent manner. Essentially, this is accomplished by replacing the regulatory subunit of PKA by IκB to form a pool of cytosolic NF-κB–IκB–PKAc complex, in which the association between PKAc and NF-κB–IκB blocks the catalytic activity of PKA. This pool of PKA is insensitive to changes in levels of intracellular cAMP; instead, treatment of cells with inducers of NF-κB activity that result in the degradation of IκB causes the activation of this PKAc. We have also demonstrated that transcriptional activity of NF-κB can be influenced through phosphorylation by PKA, a finding that brings NF-κB into the larger group of transcription factors whose activity can be regulated directly by phosphorylation. Well characterized examples include AP-1 and CREB, whose direct phosphorylation by Jun kinases or PKA, respectively, helps to determine whether the transcription factor is able to activate transcription (Karin, 1995; Sassone-Corsi, 1995). Therefore, in the case of NF-κB, it appears

that its activity is regulated at two levels: (1) its release from cytosolic complexes with IκB, and (2) its phosphorylation at a specific site by PKA. Such a layering of regulatory mechanisms might help cells to more finely tune the expression of genes regulated by NF-κB.

We also show that inhibition of PKA activity in cells treated with inducers of NF-κB activity does not significantly affect the dissociation of NF-κB–IκB complexes, the translocation of NF-κB to the nucleus, or the binding of NF-κB to DNA. Instead, what is dramatically affected is the transcriptional activity of NF-κB, as determined by its ability to drive transcription of a κB-dependent reporter construct. This effect of PKA is mediated through the direct phosphorylation of NF-κB p65 (Figures 7B and 7C), since a site-directed mutation at the consensus PKA phosphorylation site in p65 abolishes the dependence of NF-κB on PKA for transcriptional activity (Figure 7F). We are also struck by the novelty of the IκB–PKA interaction, since it appears to involve N-terminal sequences of PKAc that comprise the ATP-binding domain (Knighton et al., 1991b; Francis and Corbin, 1994; Walsh and Van Patten, 1994). Since no other protein that interacts specifically with this region of PKAc has been characterized, the structural features used for this interaction remain to be defined (Francis and Corbin, 1994; Walsh and Van Patten, 1994). Although theoretically the interaction of IκB proteins with PKAc might allow the binding of the regulatory subunit to the C terminus of PKAc, we have not observed any PKAr in NF-κB–IκB–PKAc complexes. The lack of PKAr in the NF-κB–IκB–PKAc complex might be due to steric factors, or due to the interaction of the C-terminal substrate binding region of PKAc with p65. Therefore, in our proposed model, the degradation of IκB leads to the activation of PKAc by unmasking the ATP-binding domain, and the active PKAc immediately phosphorylates p65, the substrate to which it is already bound. Such a coordinated phosphorylation might be necessary, since in cells where the level of cAMP is unaltered, free, active PKAr in the cytosol might rapidly inactivate any released PKAc before it can phosphorylate its intended substrate, p65, which is taken up into the nuclear import pathway.

The involvement of PKA in the regulation of Rel transcription factors has been most intensively studied in Drosophila, where activation of PKA leads to the stimulation of transcription by Dorsal (Norris and Manley, 1992). In addition, cotransfection of Dorsal with PKA leads to enhanced transcription, and the enhancement is blocked if a catalytic mutant of PKA is used (Norris and Manley, 1992). It has also been reported that Dorsal is multiply phosphorylated concomitant to its entry into the nucleus in the embryo (Whalen and Steward, 1993; Gillespie and Wasserman, 1994). Despite these results, a critical role for PKA in the activation of Dorsal has not been widely accepted because there was no explanation for how PKA might be activated during the time when Dorsal was activated. Also, the inability to generate flies completely lacking PKA has prevented the direct analysis of the role of PKA in dorso-ventral patterning (Lane and Kalderon, 1993). Our results provide an explanation for how PKA may modulate the activity of Dorsal in a cAMP-independent manner during the time when the Drosophila IκB homolog, Cactus, is degraded (Whalen and Steward, 1993; Gillespie and Wasserman, 1994). The knowledge that PKA might be activated during dorso-ventral patterning also raises questions about the role that active PKA might play in regulating the activity of other developmental regulators such as the hedgehog protein.

It remains to be determined whether all NF-κB–IκB complexes contain PKAc, or whether a fraction of the cytosolic NF-κB–IκB complexes can exist without PKAc. The lack of associated PKAc in a fraction of the complexes could have a significant regulatory impact if these complexes were targeted differently from those containing PKAc, since unphosphorylated NF-κB released from such PKAc-deficient NF-κB–IκB complexes would be less effective transcriptionally. In such a scenario, the activity of unphosphorylated NF-κB might then be regulated by costimulation with inducers that activate PKAc through the elevation of cAMP levels, although we have found that cAMP-induced PKA does not significantly affect NF-κB activity (Figure 7F). However, such a dependence of NF-κB activity on costimulation might allow the cell to use NF-κB activity in two ways: (1) unphosphorylated NF-κB could bind to κB sites and drive low level transcription, or (2) NF-κB phosphorylated by PKA could drive transcription at a high level.

In summary, the results presented in this report provide a novel insight into the regulation of two very important regulatory molecules in the cell, the transcription factor NF-κB and the protein kinase PKA. In particular, the discovery of a novel mechanism for regulating PKA, probably the most well studied protein kinase, was highly surprising. We believe that the interaction between PKAc and ankyrin repeats might not be confined to IκB proteins and might include other regulatory proteins. Identifying other such anchors for PKAc will be an important area of investigation in the future.

Experimental Procedures

Purification of NF-κB–IκB and IκB from Rabbit Lung Cytosol and Sequencing of the 42 kDa Copurifying Polypeptide

The purification of IκB proteins has been described previously (Figure 1A) (Thompson et al., 1995). The 42 kDa band from the peak fraction of the Superose 12 column (step 8) was used for internal sequence analysis (Figure 1A), and three tryptic peptides were sequenced. The yields of the three PKAc peptides were: T9, 1.5 pmol; T15, 1.2 pmol; and T 32, 1 pmol. The 42 kDa protein appears to stain less efficiently with silver, since the yield of peptides from the sequencing reactions indicated that it was present in equimolar amounts compared to peptides from IκB-β (T15-β, 1.4 pmol; T27-β, 1.6 pmol; T41-β, 1.5 pmol) (Thompson et al., 1995). The mean absorbance of peptide T32 from PKAc (24 amino acids, no Trp) at 214 nm was 3.5 mAU, compared to peptide T27-β from IκB-β (21 amino acids, no Trp), which was 3.0 mAU. Because these peptides are of comparable length and devoid of Trp, they are likely to have similar molar extinction coefficients. Therefore, their equal absorption at 214 nm strongly suggests that the proteins from which they were derived were present in roughly equimolar amounts.

Antibodies and Reagents

The sources of the different antibodies used were as follows: anti-p65 was from Biomol; anti-IκB-α (C-21) and anti-PKAr were from Santa Cruz Biotechnology; anti-IκB-β was made in this laboratory (Thompson et al., 1995); and anti-PKAc was a gift from Dr. Susan Taylor (UCSD). The H-89 and ML-7 inihibitors were from Calbiochem.

The PKI (amino acids 6–22) amide peptide (TYADFIASGRTGRRNAI-NH_2), casein kinase II autophosphorylation inhibitory peptide (QLQLQAASNFKSPVKTIR), forskolin, and dibutyryl cAMP were from Biomol. The fluorescent and biotinylated kempeptide assay kits for PKA were from Promega, the TransPort cell permeabilization kit was from GIBCO-BRL, and the Biotrak enzyme immunoassay kit for measuring cAMP levels was from Amersham.

Immunoprecipitation and immunoblots were carried out using protocols described previously (Thompson et al., 1995).

Site-Directed Mutations of NF-κB p65 and PKAc

Site directed mutations of NF-κB p65 and PKAc were generated using PCR mutagenesis techniques. The sequences of the sense mutagenic oligos used were: p65, GCG ATC AG**C** AGG CCG CCG TAG CTG; and PKAc, TAA GAT C**A**T CAT GGC GTA GTG G (the sites of mutations are indicated in bold). The 5′ and 3′ mutated PCR products were then run on agarose gels, purified, and mixed. PCR reactions were then performed on this mixed template, using primers from the 5′ and 3′ ends of the respective cDNAs. The long PCR product, corresponding to the entire cDNA, was then subcloned into the pCDNA3 expression vector. The mutations were verified by sequencing.

PKA Assay

In the phosphorylation of fluorescent kempeptide assay, addition of phosphate to the peptide was assessed by its altered mobility on an agarose gel run at neutral pH, where the phosphorylated peptide migrates toward the anode (Figures 1C and 1D). The extent of phosphorylation was determined by quantitating the amount of negatively charged peptide after scanning images of the fluorescent gels. In all cases, the PKA activity is reported as folds over the PKA activity in uninduced or untreated cells.

In Vivo Labeling and Phosphopeptide Mapping

Cells were metabolically labeled with ^{32}Pi, using previously published protocols (Naumann and Scheidereit, 1994; DiDonato et al., 1996). To identify the labeled p65 protein, primary immunoprecipitates, using anti-p65, were boiled in 1% SDS buffer, diluted in TNT, and then reimmunoprecipitated with anti-p65 (Naumann and Scheidereit, 1994). For carrying out phosphopeptide mapping, primary anti-p65 immunoprecipitates were fractionated by SDS–PAGE and transferred to nitrocellulose. The phosphorylated p65 was identified by autoradiography, excised, and digested with trypsin, chymotrypsin, LysC, and CNBr, following established protocols (DiDonato et al., 1996). The resulting peptides were analyzed by Tris-tricine gel electrophoresis (Schagger and von Jagow, 1987), and the phosphorylated peptides were detected by autoradiography. The molecular weight markers were used to generate a calibration curve, and the Rf values of the phosphopeptides were used to determine their molecular weights.

Acknowledgments

Correspondence regarding this paper should be addressed to S. G. The authors would like to acknowledge the invaluable technical help of Iris Douglas, Lynne Lacomis, and Mary Lui. We would like to thank Dr. Susan Taylor for the PKAc antibody and Dr. I. S. Singh for helpful discussions. We would also like to thank Drs. J. A. Steitz, A. Horwich, M. Solomon, D. G. Schatz, M. Schlissel, D. Sengupta, and members of the laboratory for suggestions and for carefully reading the manuscript. This work was supported by funding from the Howard Hughes Medical Institute and the National Institutes of Health (grant RO1 AI 33443) to S. G., and by a grant from the National Science Foundation (grant DBI-9420123) to P. T.

Received January 2, 1997; revised March 21, 1997.

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