Calmodulin-dependent Kinase II Mediates T Cell Receptor/CD3- and Phorbol Ester-induced Activation of IκB Kinase*

Received for publication, July 1, 2001
Published, JBC Papers in Press, July 24, 2001, DOI 10.1074/jbc.M106125200

Kate Hughes†, Sofia Edin, Åsa Antonsson, and Thomas Grundström‡

From the Department of Molecular Biology, Umeå University, 901 87 Umeå, Sweden

Numerous fundamental biological processes involve the NFκB family of transcription factors. The mechanisms by which this family of proteins is regulated are therefore of widespread importance. In most cells, NFκB is bound to inhibitory IκB proteins and sequestered in the cytoplasm. NFκB-inducing signals result in activation of a large multisubunit kinase complex, IKK, which phosphorylates IκB. IκB is subsequently degraded, releasing NFκB, which translocates to the nucleus. We previously reported that inhibitors of the calcium-binding protein calmodulin (CaM) prevent phorbol ester-induced phosphorylation of IκB. Here we show that KN93, an inhibitor of CaM-dependent kinases (CaMKs), also inhibits the phosphorylation of IκB. The effect of both CaM and CaMK inhibitors on IκB phosphorylation is due to the inhibition of the activity of CaMK II because neither drug has any effect when a derivative of CaM II that is insensitive to these inhibitors is expressed. When CaM II is inhibited, phorbol ester is no longer able to activate IKK, placing CaM II in the signaling pathway that leads to IKK activation. CaM and CaMK inhibitors also block T cell receptor/CD3-induced activation but have no effect on the ability of the cytokine tumor necrosis factor α or the phosphatase inhibitor calyculin A to induce degradation of IκB. Finally we show that expression of a constitutively active CaM II results in the activation of NFκB. The results identify CaM II as a mediator of IKK activation specifically in response to T cell receptor/CD3 and phorbol ester stimulation.

NFκB is a family of eukaryotic transcription factors that is expressed in virtually all cell types and implicated in the regulation of numerous genes (for review, see Ref. 1). Fundamental processes such as cell growth, apoptosis, and development are regulated by NFκB, and NFκB is a central mediator of immune, inflammatory, and stress responses (1–5). NFκB is primarily regulated by a family of inhibitory IκB proteins (6, 7). IκB binds to NFκB and masks its nuclear localization sequence, preventing it from being transported to the nucleus. NFκB-activating signals result in the rapid destruction of IκB, exposing the nuclear localization sequence of NFκB, which directs NFκB to the nucleus where it can act on its target genes. The destruction of IκB is initiated by its phosphorylation on specific serine residues, labeling it for degradation through the ubiquitin-proteasome pathway (7). This initiating phosphorylation of IκB is mediated by a large kinase complex termed IKK.IKK is composed of a heterodimer of two kinases, IKKa and IKKβ, an undefined number of the non-kinase protein IKKγ (also denoted NEMO or IKKAP1), and possibly other components (7–9). IKKγ is required for the assembly of the large complex and is indispensable for its activity (10). IKKa and IKKβ can both directly phosphorylate IκB (11), although there is genetic and biochemical evidence that these two kinases have distinct roles. IKKβ mediates NFκB activation in response to proinflammatory cytokines (12), a process that does not require IKKa (13, 14). IKKa, on the other hand, is involved in various aspects of embryonic development (13–15).

IKKα, -β, and -γ are all phosphorylated in response to the cytokine tumor necrosis factor α (TNFα) (16), and treatment of IKK purified from TNFα-stimulated cells with protein phosphatase 2A results in a loss of kinase activity (17). Inducibly phosphorylated sites of IKKa and IKKβ have been mapped to serine residues in the activation loop of their kinase domains and have been shown to be essential for the activity of each kinase (16, 18, 19). With a diversity of signaling pathways leading to the activation of NFκB (1), it is not surprising that a number of proteins have been identified that either directly or indirectly activate IKK (for reviews, see Refs. 8 and 9).

The calmodulin-dependent kinases (CaMKs) are a large family of structurally related proteins that are dependent on the calcium-binding protein calmodulin (CaM) for their activation (20, 21). The catalytic domain of a CaMK is followed by a CaM regulatory domain, which is comprised of an autoinhibitory and a CaM binding domain. The catalytic site is normally sequestered by the autoinhibitory domain through an intramolecular interaction, keeping the kinase in an inactive state. When intracellular calcium (Ca2+) levels rise, Ca2+ binds to and induces a conformational change in CaM that allows it to bind to, among a diversity of other targets, the CaM binding domain of the CaMK. This disrupts the interaction between the autoinhibitory and catalytic domains, activating the kinase. One of the best characterized CaMKs is the multifunctional CaMK II (20–22). CaMK II contains in its C terminus an association domain through which it forms multimers of 8–12 kinase subunits (20, 23). Upon activation by Ca2+/CaM, CaMK II phosphorylates not only exogenous substrates but also a threonine residue (Thr-286) in the autoinhibitory domain of the neighboring subunit of the CaMK II multimer (20–22). This phosphorylation prevents the autoinhibitory domain from reassociating with the kinase domain and results in Ca2+/CaM-independent

* This work was supported by grants from the Swedish Natural Science Research Council, the Swedish Cancer Society, and the Cancer Research Foundation in Umeå. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.
† Present address: Beatson Institute for Cancer Research, Garscube Estate, Switchback Rd., Glasgow G61 1BD, UK.
‡ To whom correspondence should be addressed. Tel.: 46-90-7852531; Fax: 46-90-771420; E-mail: Thomas.Grundstrom@cmb.umu.se.

1 The abbreviations used are: IKK, IκB kinase complex; TNFα, tumor necrosis factor α; CaMK, calmodulin-dependent kinase; CaM, calmodulin; PMA, phorbol 12-myristate 13-acetate; PKC, protein kinase C; TCR, T cell receptor.
CaMK II Mediates TCR and Phorbol Ester Activation of IKK

kinase activity. CaMK II is eventually inactivated by removal of this phosphate by a CaMK-generated phosphatase (24, 25).

We previously reported that activation of transcription by NFκB in response to phorbol ester is blocked by inhibitors of CaM and that this inhibition is due to the prevention of IκB phosphorylation (26). This prompted us to investigate whether a CaMK is involved in the signals leading to IκB phosphorylation. We report here that this is indeed the case and identify CaMK II as a mediator of IκK activation specifically in response to T cell receptor/CD3 and phorbol ester stimulation.

**EXPERIMENTAL PROCEDURES**

**Inducers and Inhibitors**—Phorbol 12-myristate 13-acetate (PMA), W7, and human recombinant TNFα were purchased from Sigma; calyceulin A and GF109203X were purchased from Calbiochem; and KN93 and KN92 were purchased from Seikagaku Corp. Unless otherwise indicated, drugs and antibodies were added to cells at final concentrations of 25 ng/ml PMA, 10 μg/ml W7, 10 ng/ml TNFα, 50 ng/ml calyceulin A, 100 ng/ml OKT3 anti-CD3, and 80 μM KN93 and KN92.

**Plasmids**—The eukaryotic expression plasmids for human wild type and T286D mutated CaMK II β and the parental expression plasmid pSR6.BKS.EBS were purchased from Seikagaku Corp. Unless otherwise indicated, drugs and antibodies were added to cells at final concentrations of 25 ng/ml PMA, 10 μg/ml W7, 10 ng/ml TNFα, 50 ng/ml calyceulin A, 100 ng/ml OKT3 anti-CD3, and 80 μM KN93 and KN92.

**Analysis of IKK—Immunoprecipitation and analysis of IKK activity was based on the protocol of Trushin et al. (30). Cells were resuspended in lysis buffer (40 mM Tris (pH 8.0), 0.3 M NaCl, 0.1% Nonidet P-40, 6 mM EDTA, 6 mM EGTA, 10 mM NaF, 10 mM sodium pyrophosphate, 10 mM β-glycerophosphate, 0.5 mM Na3VO4, 1 mM dithiorthiobitol, and protease inhibitor mixture tablets without EDTA (Roche Molecular Biochemicals)). Lysates were adjusted to 1 mg/ml by protein assay and the samples were boiled at 95 °C. The mixture was incubated with 2 μg of anti-IκKα M-18 antibody (Santa Cruz Biotechnologies, Inc.) for 1 h at 4 °C. The antibody has been used to purify IKK (18) and was more efficient at immunoprecipitating IKK than the anti-IκKα M-280 antibody (data not shown). The mixture was then incubated with protein A-Sepharose for 1 h at 4 °C. The Sepharose was resuspended in 20 mM HEPEs (pH 7.4), 2 mM MgCl2, 2 mM MnCl2, 10 μM ATP, 10 mM NaF, 10 mM sodium pyrophosphate, 10 μM β-glycerophosphate, 0.5 mM Na3VO4, 1 mM dithiorthiobitol, and protease inhibitor mixture tablets without EDTA. 18 μl of γ-[32P]ATP and 5 μg of IκBα were added to each reaction incubated at 30 °C for 30 min. The reaction was stopped by adding sample buffer and boiling the samples at 95 °C. Unincorporated γ-[32P]ATP was removed using Micro Bio-Spin 6 chromatography columns (Bio-Rad). Reactions were separated by SDS-polyacrylamide gel electrophoresis. The bottom part of the gel was Coo- matography columns (Bio-Rad). Reactions were separated by SDS-polyacrylamide gel electrophoresis. The bottom part of the gel was Coo-

**RESULTS**

**Phorbol Ester-induced Phosphorylation of IκB Requires Calmodulin-dependent Kinase II**—We previously reported that phosphorylation and subsequent degradation of IκBα induced by the phorbol ester mitogen PMA was blocked by a number of CaM inhibitors including W7 (26) (Fig. 1A). To investigate whether this was due to the involvement of a CaMK, we analyzed the effect of the CaMK II inhibitor KN93 on the ability of PMA to activate NFκB. Like W7, KN93 prevented the phosphorylation and subsequent degradation of IκBα in Jurkat T cells (Fig. 1B and data not shown). An inactive analogue of KN93, KN92, had no effect (Fig. 1B), indicating that the inhibitory effect of KN93 is due to its interaction with CaMK II. These results suggest that CaM inhibitors block IκBα phosphorylation because CaMK II is involved.

CaMK II is normally present in an inactive conformation due to an intramolecular interaction between its catalytic domain and its autoinhibitory domain (Fig. 2A). Ca2+/CaM activates the kinase by binding near the autoinhibitory domain and releasing the catalytic domain (Fig. 2B). W7 binds directly to CaM and prevents it from interacting with its targets (31) (Fig. 2B, top left). KN93 binds to the CaM binding domain of CaMK II and prevents Ca2+/CaM from binding to and activating the kinase (32, 33) (Fig. 2B, top left). However, once CaMK II is activated it becomes autophosphorylated on threonine 286 and is no longer dependent on CaM for its activity (34, 35) (Fig. 2A) and will therefore not be inhibited by KN93 or W7. A constitutively active kinase resembling the autophosphorylated form is generated by mutating Thr-286 to aspartic acid (T286D) (27) (Fig. 2B, top right). We used this information to address the specificity of KN93 and W7. We argue that if KN93 and W7 are acting on CaMK II, then transient expression of CaMK II T286D would override their ability to inhibit IκBα degradation. A sufficiently high proportion of the cells would have to be transfected to detect any effect on IκBα degradation in the cell extract. Fluorescence-activated cell sorting analyses of Jurkat cells transfected with different green fluorescent protein fusion constructs revealed that indeed most of the cells (>80%) had

---

**Fig. 1.** Mitogen-induced phosphorylation and degradation of IκBα is blocked by inhibitors of CaM and CaMK II. Jurkat cells were stimulated with PMA in the absence or presence of the CaM inhibitor W7 (A and B) and the CaMK II inhibitor KN93 or the inactive KN93 analogue KN92 (B) for the indicated times. IκBα was detected by Western blot analysis. The slower migrating band (P-IκBα) is the induced phosphorylated form of IκBα, *t* minutes.

1:50 in phosphate-buffered saline with 1 mg/ml bovine serum albumin. Cells were rinsed and incubated for 4 h at room temperature with the secondary antibodies fluorescein isothiocyanate-conjugated donkey anti-rabbit IgG and Rhodamine Red X-conjugated goat anti-mouse IgG (both from Jackson Immunoresearch Laboratories) diluted 1:50 in phosphate-buffered saline with 1 mg/ml bovine serum albumin. Cells were rinsed and mounted in a medium containing Citifluor (Chemical Laboratory, The University of Kent, Kent, UK) as an antiafighting agent. Cells were visualized by confocal laser scanning microscopy using a Leica SP2 confocal microscope equipped with an argon and a HeNe laser (Leica Laser Technik). Images were acquired sequentially using the 488 and 546 nm laser lines to excite fluorescein isothiocyanate and Rhodamine Red dyes, respectively, with an ×63 oil immersion PL APO objective. Data presented in the same figure were registered with the same laser and multiplier settings.

---

**RESULTS**

**Phorbol Ester-induced Phosphorylation of IκB Requires Calmodulin-dependent Kinase II**—We previously reported that phosphorylation and subsequent degradation of IκBα induced by the phorbol ester mitogen PMA was blocked by a number of CaM inhibitors including W7 (26) (Fig. 1A). To investigate whether this was due to the involvement of a CaMK, we analyzed the effect of the CaMK II inhibitor KN93 on the ability of PMA to activate NFκB. Like W7, KN93 prevented the phosphorylation and subsequent degradation of IκBα in Jurkat T cells (Fig. 1B and data not shown). An inactive analogue of KN93, KN92, had no effect (Fig. 1B), indicating that the inhibitory effect of KN93 is due to its interaction with CaMK II. These results suggest that CaM inhibitors block IκBα phosphorylation because CaMK II is involved.

CaMK II is normally present in an inactive conformation due to an intramolecular interaction between its catalytic domain and its autoinhibitory domain (Fig. 2A). Ca2+/CaM activates the kinase by binding near the autoinhibitory domain and releasing the catalytic domain (Fig. 2B). W7 binds directly to CaM and prevents it from interacting with its targets (31) (Fig. 2B, top left). KN93 binds to the CaM binding domain of CaMK II and prevents Ca2+/CaM from binding to and activating the kinase (32, 33) (Fig. 2B, top left). However, once CaMK II is activated it becomes autophosphorylated on threonine 286 and is no longer dependent on CaM for its activity (34, 35) (Fig. 2A) and will therefore not be inhibited by KN93 or W7. A constitutively active kinase resembling the autophosphorylated form is generated by mutating Thr-286 to aspartic acid (T286D) (27) (Fig. 2B, top right). We used this information to address the specificity of KN93 and W7. We argue that if KN93 and W7 are acting on CaMK II, then transient expression of CaMK II T286D would override their ability to inhibit IκBα degradation. A sufficiently high proportion of the cells would have to be transfected to detect any effect on IκBα degradation in the cell extract. Fluorescence-activated cell sorting analyses of Jurkat cells transfected with different green fluorescent protein fusion constructs revealed that indeed most of the cells (>80%) had
taken up DNA and expressed the fluorescent proteins, although at varying levels (data not shown). When the Jurkat cells were transiently transfected with CaMK II T286D, we found that KN93 and W7 no longer had any effect on PMA-induced degradation of IκBα, whereas the inhibitors displayed the expected effect in cells transfected with an empty expression vector or wild type CaMK II plasmid (Fig. 2B). We conclude that expression of CaMK II T286D is sufficient to override the effect of the inhibitors and thus that CaMK II is a critical component of the pathway leading to IκBα degradation.

CaM-dependent Kinase II Is Specifically Required by Stimuli That Activate NFκB through Protein Kinase C-dependent Pathways—There are numerous stimuli that activate NFκB, and although most of these result in the activation of IKK and consequent phosphorylation of IκBα, the early events of their signaling pathways are often quite different. To determine the specificity of the requirement of CaMK II, we examined the effect of W7 and KN93 on stimuli that activate NFκB through distinct signaling pathways. Neither drug had any effect on IκBα degradation induced by the cytokine TNFα or the phosphatase inhibitor calcineurin A (Fig. 3). This suggests that CaMK II acts in the phospholipid signaling pathway before a step that is common to different NFκB inducers. W7 and KN93 also blocked the TNFα-induced degradation of IκBα in the early erythroleukemia cell line K562 (data not shown) suggesting that CaMK II is a general, rather than cell-type specific, requirement of phospholipid-ester-induced activation of NFκB.

PMA binds to and activates protein kinase C (PKC), but PMA can also have PKC-independent effects in some systems. To analyze if PMA induction of IκBα degradation in Jurkat cells was dependent on PKC activity, we analyzed the effect of the specific PKC inhibitor GF109203X (36). Fig. 4A shows that PMA-induced degradation of IκBα in Jurkat cells is blocked by the PKC inhibitor. Since inhibition occurs already at 50 nM, a concentration at which GF109203X has been reported not to act on any other kinase (36), we conclude that PMA induction of phosphorylation and degradation of IκBα is dependent on a PKC-initiated pathway in Jurkat cells. GF109203X had no effect on the ability of TNFα or calcineurin A to induce degradation of IκBα, showing that these stimuli activate NFκB independently of PKC (Fig. 4B). Taken together, these data suggest that CaMK II is specifically required for the activation of NFκB in response to mitogenic stimulation and that it acts downstream of PKC.

Stimulation of the T cell receptor (TCR/CD3 complex activates PKC- and Ca2+-dependent pathways that synergistically activate NFκB by inducing the phosphorylation and degradation of IκBα (30, 37–40). We therefore asked if induction of degradation of IκBα by stimulation of the TCR/CD3 complex is blocked by inhibitors of CaM or CaMK II. Jurkat T cells were stimulated by cross-linking the TCR/CD3 complex with antihuman CD3 antibody in the presence or absence of W7 or KN93. Both the CaM and CaMK II inhibitor resulted in a complete block of TCR/CD3-induced degradation of IκBα (Fig. 5A). Induction of degradation of IκBα by cross-linking the TCR/CD3 complex was as sensitive as PMA induction to the PKC inhibitor GF109203X (Fig. 5B).

Phorbol Ester-induced Activation of IKK Requires Calmodulin-dependent Kinase II—Like most analyzed NFκB-activating signals, PMA-induced phosphorylation of IκBα is mediated by IKK (30, 41). The activity of IKK can be measured by immunoprecipitating it from cells and analyzing its ability to phosphorylate exogenous IκBα in an in vitro kinase assay. When immunoprecipitated from cells stimulated with PMA, IKK showed an increased ability to phosphorylate IκBα compared with IKK immunoprecipitated from unstimulated cells (Fig. 6A). However, both W7 and KN93 inhibited this induction of IKK activity (Fig. 6A). None of the drugs affected the efficiency of IKK immunoprecipitation because each sample contained the same amount of IKKα, one of the components of the IKK complex (measured by Western blot analysis, Fig. 6B). CaMK II is therefore part of a PKC-induced PKC-dependent signaling pathway that leads to activation of IKK.

Expression of Constitutively Active CaMK II Results in NFκB Activation—One of the target genes activated by NFκB is IκBα. Newly synthesized IκBα can enter the nucleus, remove NFκB...
CaMK II Mediates TCR and Phorbol Ester Activation of IKK

36011

**Fig. 5.** T cell receptor/CD3-induced degradation of IκBα is blocked by inhibitors of CaM, CaMK II, and PKC. Jurkat T cells were stimulated for 30 min with OKT3 anti-human CD3 antibody (αCD3Ab) in the absence (−) or presence (+) of the CaM inhibitor W7 or the CaMK II inhibitor KN93 (A) or the indicated concentrations of the specific PKC inhibitor GF109203X (B). IκBα was detected by Western blot analysis.

from DNA, and export the complex back to the cytoplasm to restore the original inactive state of NFκB in the cell (42, 43). To further highlight the role of CaMK II in the signaling pathway leading to NFκB activation, the intracellular localization of IκBα in CaMK II-overexpressing cells was assessed by immunohistochemical analysis. Jurkat cells were transiently transfected with different CaMK II expression vectors or empty vector and stained for IκBα (green) and CaMK II (red). In cells transfected with empty expression vector, IκBα was localized mainly to the cytoplasm (Fig. 7A). Stimulation with PMA resulted in a dramatic decrease in cytoplasmic IκBα and the appearance of IκBα in the nucleus (Fig. 7B). This is presumably the result of PMA-induced degradation of IκBα, activation of NFκB, NFκB-induced resynthesis of IκBα, and the subsequent transport of the newly synthesized protein to the nucleus. Overexpression of wild type CaMK II resulted in a more even distribution of IκBα throughout the cell with a slight predominance in the nucleus (Fig. 7C). This could be because overexpression of a wild type CaMK II, although Ca2+/calmodulin-dependent, can enhance the ability of the cell to respond to present amounts of Ca2+ and calmodulin (and perhaps other inducing factors). When expressing the constitutively active T286D mutant of CaMK II, we observed a dramatic increase in nuclear IκBα (Fig. 7D) that was even more pronounced than that observed in PMA-stimulated cells (Fig. 7B). This nuclear redistribution can be blocked by treatment with the proteasome inhibitor lactacystin (data not shown), supporting that the action of CaMK II is through direct activation of NFκB. The effect of CaMK II T286D expression was present in most cells in the transfected culture and not only in the cells that are most heavily expressing the constitutively active CaMK II (Fig. 7D). As mentioned above, most (≥80%) of the cells in the transiently transfected cell cultures were expressing protein from the transfected plasmid, albeit at varying levels, possibly explaining why an effect is seen in most cells. Furthermore, Jurkat cells treated with conditioned medium from CaMK II T286D-expressing cells showed a nuclear distribution of IκBα similar to that seen in Fig. 7D (data not shown), implying that the constitutively active kinase leads to expression and secretion of NFκB-activating products. A likely explanation is the known NFκB induction of a number of genes whose products also are NFκB activators (1). This interpretation is supported by the inhibition of the conditioning of the medium by lactacystin, an inhibitor of IκB degradation (data not shown). We conclude that overexpression of wild type CaMKII and in particular expression of constitutively active CaMKII results in the activation of NFκB.

**DISCUSSION**

It is well established that NFκB is involved in a plethora of biological systems, justifying the need to understand the mechanisms by which this family of proteins is regulated. The phosphorylation and subsequent degradation of the NFκB inhibitor IκBα is a key control point. The IκB kinase IKK has therefore become a subject of intense interest, and attention is now focusing on how IKK is regulated. Initiated by our finding that CaM inhibitors prevent phorbol ester-induced activation of NFκB by blocking phosphorylation of IκBα (26), we here investigated whether this NFκB activation involves a CaMK. We conclude that CaMK II mediates IKK activation specifically in response to TCR/CD3 and phorbol ester stimulation based on the following observations: (i) phosphorylation and degradation of IκBα in response to PMA is blocked by the CaMK II inhibitor KN93 but not by its inactive analogue KN92, (ii) expression of a constitutively active derivative of CaMK II that is insensitive to CaM and CaMK II inhibitors abolishes the effect of these drugs on IκBα degradation, (iii) CaM and CaMK II inhibitors also block TCR/CD3- but not TNFα- and calyculin A-induced degradation of IκBα, (iv) CaM and CaMK II inhibitors prevent the activation of IKK in response to PMA, and (v) expression of

**Fig. 6.** CaMK II is required for mitogen-induced activation of IKK. Jurkat cells were stimulated with PMA for 8 min in the absence (−) or presence (+) of W7 or KN93. IKK was immunoprecipitated and incubated with exogenous IκBα and [γ-32P]ATP, and the resulting phosphorylated IκBα (P-IκBα) was detected by SDS-polyacrylamide gel electrophoresis and autoradiography (A). Immunoprecipitated IKKα was detected by Western blot analysis (B).

**Fig. 7.** Expression of wild type and constitutively active (T286D) CaMK II activates NFκB as measured by nuclear localization of IκB. Jurkat cells were transiently transfected with empty vector (A and B) or with expression vectors encoding CaMK II (C) or CaMK II T286D (D). In panel B, cells were stimulated with PMA for 30 minutes. IκBα (green) and CaMK II (red) were detected by immunohistochemical analysis with a confocal microscope.
constitutively active CaMK II results in NFκB activation.

CaMK II is in itself a large family of proteins. There are four genes encoding CaMK II in vertebrates (α, β, γ, and δ), and each of these is subject to extensive alternative splicing (20, 21). CaMK II isoforms are specifically expressed in the brain, and β isoforms are expressed in the brain and a few other tissues, whereas γ and δ are more broadly expressed. The γ isoform used in our studies was in fact cloned from Jurkat T cells (27) and is so far the only CaMK II known to be expressed in lymphocytes. We cannot, however, exclude the possibility that the PMA-induced IKK activation is mediated by another CaMK II isoform and that we see an effect when expressing the mutant γ isoform because of a functional redundancy between this isoform and a hypothetical other CaMK II isoform.

We have found that PMA-induced activation of NFκB is dependent on CaMK II in the absence of a Ca$^{2+}$ signal. Upon co-stimulation with the Ca$^{2+}$ ionophore ionomycin, NFκB activation is also blocked by calmodulin inhibitors (26) and the CaMK II inhibitor KN93 (data not shown). It is an intriguing question how an enzyme activated by Ca$^{2+}$-loaded CaM can also be required in the absence of a co-stimulus increasing the Ca$^{2+}$ level. The fact that we observe inhibition with CaM inhibitors argues against this being due to the well established mechanism of CaM-independent kinase activity through autoprophosphorylation of Thr-286 (20). The authors showed that both the classical IKK consisting of IKKα, IKKβ, and IKKγ. The authors also showed that both the classical IKK and IKKe are necessary for NFκB activation of NFκB, but how the actions of these kinases are linked is unknown. Since Jurkat cells were also used in their study, CaMK II has to be placed somewhere in this apparently complex signaling network. It has also been shown that both PMA stimulation and overexpression of Raf, which is an effector kinase of Ras, activates IKKβ through the membrane shuttle kinase MEKK1 (55). These authors also used Jurkat T cells in their study. There are therefore numerous alternative pathways from PKC to IKK that could be CaMK II-dependent. Characterizing the influences of these kinases on each other is an obvious topic of future investigations.

It is becoming clear that the signaling pathways that lead to the activation of IKK involve quite a diversity of proteins. Both the nature of the stimuli and the particular type of cell is likely to govern which of these proteins are used. Here we have identified CaMK II as a critical component of mitogenic signaling to IKK in at least some cell types. This knowledge will aid our understanding of not only the regulation of this key kinase complex but also of how the important family of NFκB transcription factors is differentially regulated.

**REFERENCES**

1. Pahl, H. L. (1999) *Oncogene* 18, 6853–6866
2. Ghosh, S., May, M. J., and Kopp, E. B. (1998) *Annu. Rev. Immunol.* 16, 225–260
3. Barkett, M., and Gilmore, T. D. (1999) *Oncogene* 18, 6910–6924
4. Gerondakis, S., Grossmann, M., Nakamura, Y., Pohl, T., and Grummt, R. (1999) *Oncogene* 18, 6888–6895
5. Goridis, S. (1999) *Oncogene* 18, 6875–6887
6. May, M. J., and Ghosh, S. (1997) *Semin. Cancer Biol.* 8, 63–73
7. Karin, M. (1999) *Oncogene* 18, 6867–6874
8. Karin, M., and Delhase, M. (2000) *Semin. Immunol.* 12, 85–98
9. Israel, A. (2000) *Trends Cell Biol.* 10, 129–133
10. Yamaoka, S., Courtois, G., Bessia, C., Whiteside, S. T., Weil, R., Agou, F., Kirk, H. E., Kay, R. J., and Israel, A. (1998) *Cell* 81, 1231–1240
11. Zhou, Y., Baud, V., Delhase, M., Zhang, P., Deerinck, T., Ellisman, M., Johnson, R., and Karin, M. (1999) *Science* 284, 318–322
12. Takeda, K., Takeuchi, O., Tsujimura, T., Itami, S., Adachi, O., Kawai, T., Sanjo, H., Yoshikawa, K., Terada, N., and Akira, S. (1999) *Science* 284, 318–319
21. Means, A. R. (2000) Mol. Endocrinol. 22. Dupont, G., and Goldbeter, A. (1998) Bioessays 20. Lukas, T. J., Mirzoeva, S., and Watterson, D. M. (1998) in 19. Ling, L., Cao, Z., and Goeddel, D. V. (1998) Proc. Natl. Acad. Sci. U. S. A. 22. Mercurio, F., Zhu, H., Murray, B. W., Shevchenko, A., Bennett, B. L., Li, J., Young, D. B., Barbosa, M., Mann, M., Manning, A., and Rao, A. (1997) Science 278, 860–868 17. DiDonato, J. A., Hayakawa, M., Rothwarf, D. M., Zandi, E., and Karin, M. 18. Mercurio, F., Zhu, H., Murray, B. W., Shevchenko, A., Bennett, B. L., Li, J., Young, D. B., Barbosa, M., Mann, M., Manning, A., and Rao, A. (1997) Science 278, 860–868 23. Kanaseki, T., Ikeuchi, Y., Sugiura, H., and Yamauchi, T. (1991) 24. Ishida, A., Kameshita, I., and Fujisawa, H. (1998) J. Biol. Chem. 25. Kitani, T., Ishida, A., Kameshita, I., and Fujisawa, H. (1998) J. Biol. Chem. 26. Hughes, K., Antonsson, K., and Grundstr"om, T. (1998) FEBS Lett. 27. Nghiem, P., Saati, S. M., Martens, C. L., Gardner, P., and Schulman, H. (1993) J. Biol. Chem. 28. Hughes, K., Antonsson, A., and Grundstr"om, T. (1998) FEBS Lett. 29. Onions, J., Hermann, S., and Grundstro"m, T. (1997) J. Biol. Chem. 30. Trushin, S. A., Pennington, K. N., Algeciras Schimnich, A., and Paya, C. V. (1999) J. Biol. Chem. 31. Hidaka, H., and Ishikawa, T. (1992) Cell Calcium 13, 465–472 32. Sumi, M., Kuchi, K., Ishikawa, T., Ishii, A., Hagwara, M., Nagata, T., and Hidaka, H. (1991) Biochem. Biophys. Res. Commun. 181, 968–975 33. Hidaka, H., and Ishikawa, T. (1992) Cell Calcium 13, 465–472 34. Hannon, G. P., and Schultz, H. (1992) Annu. Rev. Biochem. 61, 559–601 35. Schulman, H., Heist, K., and Srinivasan, M. (1995) Prog. Brain Res. 105, 95–104 36. Toullec, D., Pianetti, P., Coste, H., Bellevigue, P., Grand Perret, T., Ajakane, M., Baudet, V., Boissin, P., Bourrier, E., and Lorrolle, F. (1991) J. Biol. Chem. 266, 15771–15781 37. Truneh, A., Albert, F., Golstein, P., and Schmitt Verhulst, A. M. (1985) Nature 313, 318–320 38. Mattila, P. S., Ullman, K. S., Fiering, S., Emmel, E. A., McCutcheon, M., Crabtree, G. R., and Herzenberg, L. A. (1998) EMBO J. 9, 4425–4433 39. Frantz, B., Nordby, E. C., Breen, G., Steffan, N., Paya, C. V., Kincaid, R. L., Tocci, M. J., O’Keefe, S. J., and O’Neill, E. A. (1994) EMBO J. 13, 861–870 40. Tojima, Y., Fujimoto, A., Delhase, M., Chen, Y., Hatakeyama, S., Nakayama, K., Kaneko, Y., Nimura, Y., Motsuyama, N., Ikeda, K., Karin, M., and Nakanishi, M. (2000) Nature 404, 778–782 41. Arenzana-Seisdedos, F., Thompson, J., Rodriguez, M. S., Bachelerie, F., Thomas, D., and Hay, R. T. (1995) Mol. Cell. Biol. 15, 2689–2696 42. Arenzana-Seisdedos, F., Turpin, P., Rodriguez, M., Thomas, D., Hay, R. T., Virelizier, J. L., and Dargemont, C. (1997) J. Cell Sci. 110, 369–378 43. Olwin, B. B., Edelman, A. M., Krebs, E. G., and Storm, D. R. (1984) J. Biol. Chem. 259, 10949–10955 44. Hehner, S. P., Hofmann, T. G., Ushmorov, A., Dienz, O., and Schmitz, M. L. (2000) Mol. Cell. Biol. 20, 2380–2388 45. Haiech, J., Kilhoffer, M. C., Lukas, T. J., Craig, T. A., Roberts, D. M., and Watterson, D. M. (1991) J. Biol. Chem. 19, 4615–4620 46. Schwaninger, M., Tallman, S., Petersen, N., Schreiber, A., Prinz, S., Libermann, T. A., and Spranger, M. (1999) J. Neurochem. 73, 1461–1466 47. Lallena, M. J., Diaz Meco, M. T., Bren, G., Paya, C. V., and Moscat, J. (1999) Mol. Cell. Biol. 19, 2180–2188 48. Waxham, M. N., and Arenowski, J. (1993) Biochemistry 32, 2923–2930 49. Romashkova, J. A., and Makarov, S. S. (1999) Nature 401, 86–90 50. Pomerantz, J. L., and Baltimore, D. (1999) EMBO J. 18, 6694–6704 51. Bonnard, M., Mirtos, C., Suzuki, S., Graham, K., Huang, J., Ng, M., Itie, A., Wakeham, A., Shahinian, A., Henzel, W. J., Elia, A. J., Shillinglaw, W., Mak, T. W., Cao, Z., and Yeh, W. C. (2000) EMBO J. 19, 4976–4985 52. Peters, R. T., Liao, S., and Maniatis, T. (2000) Mol. Cell 5, 513–522 53. Shimada, T., Kawai, T., Takeda, K., Matsumoto, M., Inoue, J., Tatsutomi, Y., Kanamaru, A., and Akira, S. (1999) Int. Immunol. 11, 1357–1362 54. Baumann, B., Weber, C. K., Troppmair, J., Whiteside, S., Israel, A., Rapp, U. R., and Wirth, T. (2000) Proc. Natl. Acad. Sci. U. S. A. 97, 4615–4620