THE JOURNAL OF BIOLOGICAL CHEMISTRY
Vol. 271, No. 23, Issue of June 7, pp. 13868–13874, 1996

Printed in U.S.A.

Characterization of IκB Kinases

IκB-α IS NOT PHOSPHORYLATED BY Raf-1 OR PROTEIN KINASE C ISOZYMES, BUT IS A CASEIN KINASE II SUBSTRATE*

(Received for publication, October 3, 1995, and in revised form, March 6, 1996)

Petra Janosch‡, Miriam Schellerer‡, Thomas Seitz‡, Peter Reim‡, Manfred Eulitz‡, Markus Brielmeier‡, Walter Kölch‡, John M. Sedivy§, and Harald Mischak‡¶

From the ‡Institut für Klinische Molekularbiologie und Tumorgenetik, Forschungszentrum für Umwelt und Gesundheit, Marchioninistrasse 25, D-81377 München, Federal Republic of Germany and the §Department of Molecular Biology, Cell Biology, and Biochemistry, Brown University, Providence, Rhode Island 02912

The NF-κB transcription factor is activated by a wide variety of stimuli, including phorbol esters such as 12-*O*-tetradecanoylphorbol-13-acetate. In its inactive state, NF-κB is sequestered in the cytoplasm tethered to an inhibitor protein, IκB. Activation comprises the rapid phosphorylation of IκB-α at N-terminal sites, which presumably marks IκB-α for proteolytic degradation and leads to release of NF-κB into the nucleus. In addition, IκB-α is constitutively phosphorylated at the C terminus, which may be a prerequisite for proper IκB function. Protein kinase C (PKC) is activated by 12-*O*-tetradecanoylphorbol-13-acetate and has been previously reported to phosphorylate IκB-α *in vitro*. As PKC has turned out to constitute a multigene family encoding isozymes with different biological functions, we have reinvestigated IκB-α phosphorylation by PKC using recombinant PKC isozymes expressed in insect cells. While crude PKC preparations were efficient IκB-α kinases, highly purified PKC isozymes completely failed to phosphorylate IκB-α. Biochemical separation of porcine spleen yielded at least two fractions with IκB-α kinase activity, both of which were devoid of detectable PKC isozymes. One peak contained both Raf-1 and casein kinase II (CKII). Purified Raf-1 does not phosphorylate IκB-α directly, but associates with CKII, which efficiently phosphorylates the C terminus of IκB-α. Two-dimensional phosphopeptide mapping and high pressure liquid chromatography-mass spectroscopy analysis showed that all IκB-α kinases induced phosphorylation at the same prominent sites in the C terminus. Our results clearly indicate that PKC isozymes α, β, γ, δ, ϵ, η, and ζ as well as Raf-1 are not IκB-α kinases. They furthermore demonstrate that IκB-α is targeted by several kinases, one of which appears to be CKII.

NF-κB is composed of a dimer of related proteins belonging to the Rel superfamily (reviewed in Refs. 1 and 2). The induction of NF-κB activity is an immediate-early event, when cells are exposed to inflammatory cytokines, such as tumor necrosis factor-α or interleukin-1, phorbol esters, *e.g.* TPA,[1] UV radiation, or hydrogen peroxide (reviewed in Ref. 3). The activation of NF-κB is a unique paradigm for the regulation of a transcription factor by subcellular compartmentalization. In the inactive state, NF-κB is complexed with cytosolic proteins collectively designated as inhibitors of NF-κB, IκB, which retain NF-κB in the cytoplasm. The prototypic and best studied IκB is IκB-α. Activation induces the phosphorylation of IκB-α, ubiquitination, and its subsequent proteolytic degradation (4–6). As a consequence, NF-κB can translocate to the nucleus and activate the transcription of target genes. The role of IκB-α phosphorylation is not entirely defined, but recent work suggests that IκB-α is phosphorylated on multiple sites located at the C and N termini (4, 6–8). Phosphorylation of the C-terminal sites is constitutive (7, 8), while phosphorylation of the N terminus has been suggested to be the target of inducible phosphorylation (4, 6). Phosphorylation of these sites does not suffice to release NF-κB, but rather seems to mark IκB-α for degradation (5).

The central role of phosphorylation in the NF-κB activation pathway has evoked an intense interest in the identification of kinases that phosphorylate IκB-α. A prime candidate for such kinases has been PKC. Phorbol esters, which activate PKC, are efficient inducers of NF-κB, and PKC has been reported to phosphorylate IκB *in vitro* (9). These studies were carried out at a time, however, when PKC was characterized mainly as a biochemical entity that could be activated by phorbol esters, phospholipids, and calcium. Molecular cloning of PKC cDNAs has shown that PKC is a multigene family comprising at least 10 genes, whose protein products differ in structure and biological effects (reviewed in Ref. 10). The classical PKC isozymes α, βI, βII, and γ feature all the properties of the initial biochemical description. The novel PKCs (PKC-δ, -ϵ, -η, -θ, and -μ) lack the calcium-binding domain and hence are calcium-independent. The atypical PKCs (PKC-ζ and PKC-λ) do not bind and respond to phorbol ester or calcium, but instead, at least PKC-ζ may be activated by ceramide (11). In the cell, ceramide is generated in response to tumor necrosis factor-α and can mediate NF-κB induction (12). Moreover, overexpression of individual PKC isozymes in NIH 3T3 fibroblasts and 32D promyelocytes has revealed very diverse biological effects. PKC-ϵ and PKC-η can transform NIH cells, whereas PKC-δ inhibits proliferation (13).[2] In 32D cells, PKC-α and PKC-δ, but not

* This work was supported in part by a grant from the Deutsche Krebshilfe Mildred Scheel Stiftung (to H. M.), by National Institutes of Health Grant GM-R01-41690 and Presidential Young Investigator Award DMB-9057715 from the National Science Foundation (to J. M. S.), and by a grant from the Deutsche Forschungsgemeinschaft (to W. K.) 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.

¶ To whom correspondence should be addressed: Tel.: 89-7099-224; Fax: 89-7099-500.

[1] The abbreviations used are: TPA, 12-*O*-tetradecanoylphorbol-13-acetate; PKC, protein kinase C; CKII, casein kinase II; PAGE, polyacrylamide gel electrophoresis; GST, glutathione *S*-transferase; HPLC, high pressure liquid chromatography; MBP, myelin basic protein.

[2] P Janosch, M. Schellerer, T. Seitz, P. Reim, M. Eulitz, M. Brielmeier, W. Kölch, J. M. Sedivy, and H. Mischak, unpublished observation.

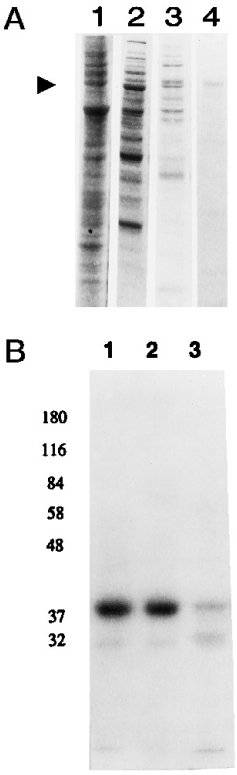

FIG. 1. **IκB-α kinase activity can be separated from PKC-α by further purification.** *A*, Coomassie Blue stain of a 10% SDS-polyacrylamide gel showing the purification of PKC-α expressed in insect cells. Lysates of Sf9 cells infected with a PKC-α baculovirus were prepared and purified by fast protein liquid chromatography as described under "Materials and Methods." PKC-α was detected by examining the fractions for phospholipid-dependent histone kinase activity. Active fractions were pooled and used for further purification. *Lane 1*, crude cellular extract; *lane 2*, pool of active fractions from Q-Sepharose fast flow column; *lane 3*, pool of active fractions from hydroxylapatite column; *lane 4*, pool of active fractions from phenyl-Superose column. The *arrowhead* indicates the immunoreactive PKC-α band as detected on parallel Western blots with a PKC-α-specific antiserum (Transduction Laboratories). *B*, *in vitro* kinase assays of the PKC-containing fractions with IκB-α as substrate. *Lane 1*, pool of active fractions from Q-Sepharose fast flow column; *lane 2*, pool of active fractions from hydroxylapatite column; *lane 3*, pool of active fractions from phenyl-Superose column.

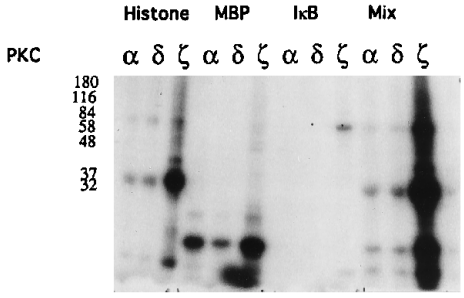

FIG. 2. **Kinase assay of purified PKC-α, -δ, and -ζ using histone, MBP, or IκB-α as substrate.** The PKCs and the substrates used are indicated. *Mix* refers to a mixture of histone, IκB-α, and MBP. The sizes (in kilodaltons) and positions of the molecular mass markers are indicated on the left.

other isozymes, make these cells susceptible to differentiation into macrophages upon TPA treatment (14).

These observations prompted us to reinvestigate the phosphorylation of IκB-α using recombinant PKC isozymes produced in the baculovirus/Sf9 cell expression system. Unexpectedly, highly purified PKC isozymes failed to phosphorylate IκB-α, while crude PKC preparations were active as IκB-α kinases. The IκB-α kinase activity could be separated from

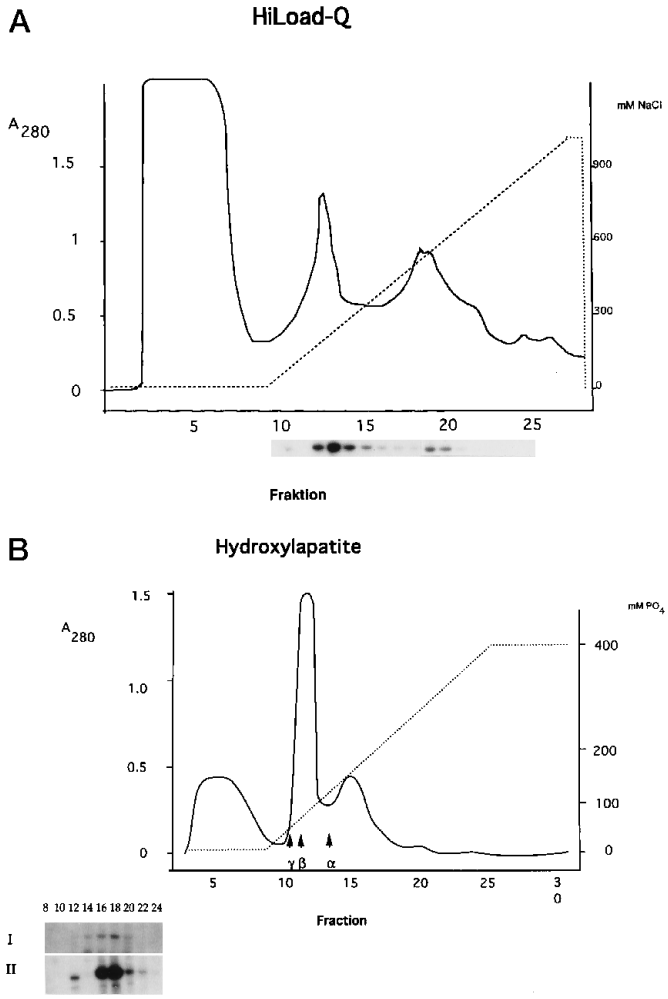

FIG. 3. **Isolation of IκB-α kinases from porcine spleen.** *A*, spleen lysate was loaded onto a HiLoad-Q fast protein liquid chromatography column and eluted with a salt gradient. The absorbance profile (——) as well as the salt gradient (· · · ·) are indicated. 2 μl of each fraction were tested in kinase assays with IκB-α as substrate. They are shown below the chromatogram. *B*, active fractions were pooled and loaded onto a hydroxylapatite column. Since both pools from the HiLoad-Q column gave essentially identical UV profiles, the profile of only one (pool I) is shown here. The absorbance profile (——) as well as the salt gradient (· · · ·) are indicated. The IκB-α kinase activities of 2 μl of each fraction from both pool I and pool II purification are shown below the chromatogram. The *arrows* indicate the elution positions of PKC-α, -β, and -γ determined in parallel experiments.

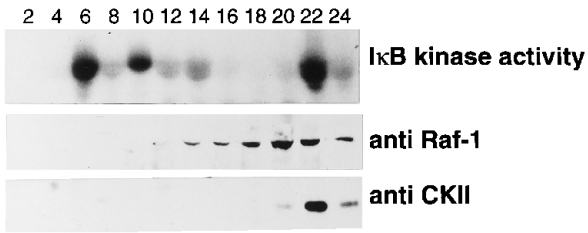

FIG. 4. **Western blot analysis of IκB-α kinase purification.** Fractions from a Mono-Q column (indicated at the top) were used in IκB kinase assays and for Western blot analysis. Samples were resolved by SDS-PAGE and transferred to nitrocellulose membranes. The blots were stained with an antiserum recognizing Raf-1 (crafVI) or CKII.

PKC by further purification. In concordance with these results, we could not detect PKC isozymes in IκB-α kinase preparations from chromatographically fractionated porcine spleen extracts. These separations yielded two different peaks with IκB-α kinase activity, one of which copurified with Raf-1 and CKII, which both have previously been shown to function as IκB-α

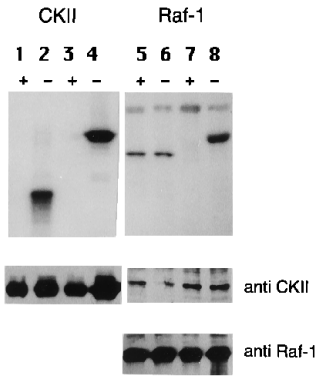

FIG. 5. **Kinase assays using CKII and Raf-1.** Kinase assays were performed as described under "Materials and Methods" using IκB-α (*lanes 3*, *4*, *7*, and *8*), casein (*lanes 1* and *2*), or Mek (*lanes 5* and *6*) as substrate. The kinases used are indicated at the top, and the presence or absence of heparin is indicated (+ and −).

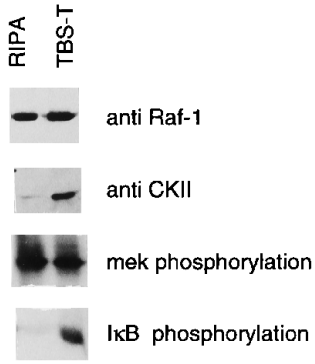

FIG. 6. **Kinase activity and Western blot analysis of Raf-1 preparations washed with either Tris-buffered saline/Tween 20 (1%) (*TBS-T*) or radioimmune precipitation assay buffer (*RIPA*).** The substrates or antibodies used are indicated.

kinases (7, 8). Purified CKII as well as the other IκB-α kinase fractions phosphorylated the same sites in IκB-α located at the C terminus.

MATERIALS AND METHODS

Expression and Purification of PKC Isozymes—PKC isozymes were purified from Sf9 cells infected with baculoviruses expressing the different PKCs (15). Purification of PKC isozymes was carried out essentially as described (16). In short, cell pellets of 3×10^9 baculovirus-infected Sf9 cells were lysed in 100 ml of Tris buffer (pH 7.5) containing 1 mM EDTA, 2 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride, and 1% Triton X-100. The homogenate was centrifuged at 100,000 × *g* for 60 min, and the clear supernatant was applied to a 20 × 100-mm Q-Sepharose fast flow column. The column was eluted with a 500-ml linear gradient of 0–500 mM NaCl. Fractions containing high PKC activity (as measured by histone phosphorylation) were pooled and applied to a 10 × 100-mm hydroxylapatite column. The column was eluted with a 200-ml linear gradient of 0–400 mM KH_2PO_4 (pH 7.5) containing 2 mM dithiothreitol and 1 mM EDTA. Active fractions were pooled again, adjusted to 1 M KCl, and loaded onto a 5 × 50-mm phenyl-Superose column. The column was eluted with a 50-ml linear gradient of 1 to 0 M NaCl, and the purity of the active fractions was assayed by SDS-PAGE. Fractions containing PKC as the major protein were pooled; dialyzed overnight against 20 mM Tris buffer (pH 7.5) containing 1 mM EDTA, 1 mM dithiothreitol, and 50% glycerol; and subsequently stored at −80 °C.

Preparation of Raf-1 and IκB-α—Raf-1 was expressed in Sf9 cells as a GST fusion protein and purified as described (17). GST-IκB-α was a kind gift from Dr. Ulli Siebenlist. GST-IκB-α was prepared as described (17), cleaved with thrombin to remove the GST portion, and stored at −70 °C.

Kinase Assays—PKC activity was determined in 20 mM Tris-HCl (pH 7.5), 1 mM $CaCl_2$, 10 mM $MgAc_2$, 1 μM TPA, 80 μg/ml phosphatidylethanolamine, 20 μg/ml phosphatidylserine, and 10 μM ATP/[γ-^{32}P]ATP (specific activity of 2 Ci/mmol). Raf-1, CKII, and IκB-α kinase assays

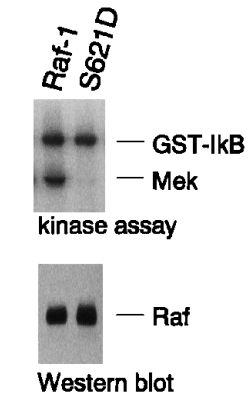

FIG. 7. **Kinase activity of wild-type Raf-1 or a kinase-negative mutant (S621D) using Mek and IκB-α as substrates.**

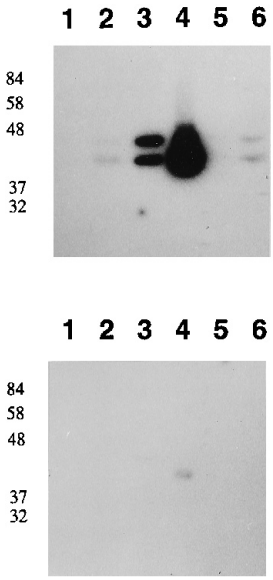

FIG. 8. **In-gel kinase assay of protein preparations that contain IκB-α kinase activity.** *Lane 1*, pool I of the IκB-α kinase purification; *lane 2*, pools I and II; *lane 3*, pool II; *lane 4*, purified CKII; *lane 5*, immunoprecipitated Raf-1; *lane 6*, immunoprecipitated Raf-1 after washing with radioimmune precipitation assay buffer. The sizes (in kilodaltons) and positions of the molecular mass markers are indicated. The *upper panel* shows the autoradiograph of a gel copolymerized with IκB-α, and the *lower panel* shows an autoradiograph of the same samples run on a gel without IκB-α.

were performed in 20 mM Tris-HCl (pH 7.5), 1 mM $CaCl_2$, 10 mM $MgAc_2$, and 10 μM ATP/[γ-^{32}P]ATP (specific activity of 2 Ci/mmol). The reactions were incubated at 30 °C for 10 min. Subsequently, the kinase reactions were stopped by boiling the sample in SDS-PAGE buffer. The reactions were resolved on 10% SDS gels, and the gels were exposed to Kodak XAR-2 film.

Purification of IκB-α Kinases from Spleen—5 g of frozen porcine spleen were homogenized in a mortar under liquid nitrogen. Purification of the IκB-α kinases was carried out following a procedure similar to that described above for PKC, using a Mono-Q or Q-Sepharose fast flow column followed by a hydroxylapatite column.

Two-dimensional Peptide Maps—1 μg of IκB-α was phosphorylated as described above, and bands of phosphorylated IκB-α were cut out from an SDS gel and processed for two-dimensional tryptic peptide mapping as described using pH 8.9 buffer for the first dimension and 1-butanol/pyridine/acetic acid/water (15:10:3:12) for the second dimension (17, 18).

HPLC-Mass Spectroscopy Analysis—Analysis was performed on an ABI Model 172 micropurification system connected to a Perkin-Elmer API 100 quadrupol mass spectrometer and a Berthold radioactivity detector. IκB-α was phosphorylated and digested as described above. Tryptic peptides from 5 μg of IκB-α were loaded onto a Pharmacia Biotech reversed-phase column. A gradient of 0–35% acetonitrile in 100 min and subsequently of 35–70% in 10 min was run at 40 μl/min.

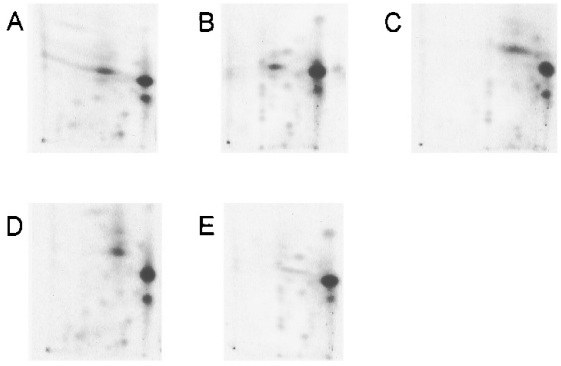

FIG. 9. **Phosphopeptide maps of IκB-α phosphorylated with different kinases *in vitro*.** IκB-α was phosphorylated with a crude PKC-α preparation, purified Raf-1, CKII, or IκB-α kinases purified on a hydroxylapatite column. Phosphorylated IκB-α was isolated by SDS-PAGE and processed for two-dimensional phosphopeptide mapping as described under "Materials and Methods." Sequencing-grade proteases were purchased from Boehringer Mannheim and used according to the instructions provided by the manufacturer. Electrophoresis at 1000 V/20 min in pH 8.9 buffer was in the horizontal direction. Thin-layer chromatography in phosphochromatography buffer was in the vertical direction. The origin is in the lower-left corner. *A*, trypsin and Asp-N digest of IκB-α phosphorylated with crude PKC-α; *B*, trypsin and Asp-N digest of IκB-α phosphorylated with Raf-1; *C*, trypsin and Asp-N digest of IκB-α phosphorylated with CKII; *D*, trypsin and Asp-N digest of IκB-α phosphorylated with IκB-α kinase pool I; *E*, trypsin and Asp-N digest of IκB-α phosphorylated with IκB-α kinase pool II.

Masses were determined in 0.1-atomic mass unit steps over an m/z range from 500 to 3000 with an orifice voltage of 20 V.

In-gel Kinase Assays—Protein was resolved on a 10% SDS-polyacrylamide gel containing 0.2 mg/ml IκB-α. After electrophoresis, the gel was washed twice for 10 min with 250 ml of 50 mM HEPES (pH 7.4), 5 mM 2-mercaptoethanol, and 20% isopropyl alcohol. After equilibration for 1 h at room temperature with 250 ml of 50 mM HEPES (pH 7.4) and 5 mM 2-mercaptoethanol, the gel was denatured twice for 30 min at room temperature with 6 M guanidinium Cl in 50 mM HEPES (pH 7.4) and 5 mM 2-mercaptoethanol. The gel was subsequently renatured overnight at 4 °C in 500 ml of 50 mM HEPES (pH 7.4) and 5 mM 2-mercaptoethanol containing 0.04% Tween. The gel was equilibrated for 30 min at 30 °C in kinase buffer (25 mM HEPES (pH 7.4), 5 mM 2-mercaptoethanol, 10 mM $MgCl_2$, and 90 μM Na_3VO_4) and incubated in kinase buffer containing 250 μCi of [γ-^{32}P]ATP (specific activity of 3000 Ci/mmol) for 1 h at 30 °C. The gel was washed extensively four times for 4 h in 500 ml of 5% trichloroacetic acid and 10 mM sodium pyrophosphate and subsequently dried and exposed to x-ray film.

Western Blot Analysis—Proteins were resolved on a 10% SDS-polyacrylamide gel and subsequently electroblotted onto nitrocellulose filters. Nonspecific binding to nitrocellulose was blocked with 5% nonfat dry milk for 30 min, and the blots were subsequently incubated with primary antibody and, after extensive washing with Tris-buffered saline and 1% Tween 20, with secondary antibody for 1 h each. The primary antibodies used were either a monoclonal antibody against PKC-α (Upstate Biotechnology, Inc.) or antisera against classical PKCs (201, directed against the pseudosubstrate domain of the PKCs), PKC-δ (19), c-Raf (17), or CKII (Upstate Biotechnology, Inc.). Secondary antibodies coupled to horseradish peroxidase were purchased from Dianova. Immunoreactive bands were visualized using the ECL system (Amersham Corp.).

RESULTS

Different PKC isozymes were produced in the baculovirus/Sf9 cell system and purified by successive chromatography on Q-Sepharose fast flow, hydroxylapatite, and phenyl-Superose columns. Column fractions were monitored for PKC activity employing histone as substrate. Active fractions were pooled and further purified. The presence of PKC was confirmed by Western blotting with appropriate PKC isozyme-specific antibodies. In parallel, the purity was assessed by staining duplicate gels with Coomassie Blue. A routine purification of PKC-α is shown in Fig. 1. PKC-α preparations of <10% purity (Fig. 1*A*) efficiently phosphorylated recombinant IκB-α purified from *Escherichia coli* (Fig. 1*B*). The IκB-α kinase activity, however, could be separated from PKC-α by further purification, suggesting that it copurified with kinases that were distinct from PKC-α. Additional evidence was obtained with the specific PKC inhibitor GF109203X, which did not affect the IκB-α kinase activity, but reduced the histone kinase activity by >95% (data not shown).

Similar results were obtained with PKC-βII, -γ, -δ, -ϵ, -η, and -ζ. The IκB-α kinase activity of these PKC preparations could be removed by enrichment of PKC above 50% (by phenyl-Superose chromatography, step 3 in the purification protocol). A representative assay is shown in Fig. 2. Purified preparations of PKC-α, -δ, and -ζ efficiently phosphorylated histone or myelin basic protein (MBP), but completely failed to phosphorylate 38-kDa IκB-α. To exclude the possibility that the IκB-α preparations contained an unspecific PKC inhibitor, a mixture of histone, MBP, and IκB-α was used as substrate. IκB-α was not phosphorylated and did not reduce the phosphorylation of histone or MBP by PKC.

To corroborate these findings, we attempted to purify IκB-α kinases from porcine spleen. Spleen lysates were separated by chromatography on a HiLoad-Q-Sepharose column (Fig. 3*A*). Individual fractions were assayed for kinase activity using IκB-α as substrate. Two peaks of IκB-α kinase activity were recovered, pooled, and further fractionated on a hydroxylapatite column (Fig. 3*B*). Their elution profiles differed from those of PKC-α, -β, and -γ, which were determined in parallel experiments.

The peaks containing IκB-α kinases were examined for the presence of PKC by kinase assays using histone as substrate and by Western blotting with anti-PKC antibodies (data not shown). Both methods failed to detect appreciable amounts of PKC in the IκB-α kinase fractions, confirming the results obtained in the course of purification of PKC isozymes expressed in insect cells. As Raf-1 and CKII have been reported to phosphorylate IκB-α, the Western blots were also tested for the presence of these two kinases. As shown in Fig. 4, pool II contained readily detectable amounts of Raf-1 and CKII. While Raf-1 was also present in fractions that were devoid of appreciable IκB-α kinase activity, CKII was present only in the fractions of the second peak containing IκB-α kinase activity.

To assess the question of which of these two enzymes does in fact phosphorylate IκB-α, we used purified CKII (Upstate Biotechnology, Inc.) and purified Raf-1 (17). As shown in Fig. 5, both enzyme preparations contained IκB-α kinase activity. The addition of heparin, a CKII inhibitor (20), completely abolished the IκB-α kinase activity of both the Raf-1 and CKII preparations. As shown in Fig. 5, the Raf-1 kinase activity against Mek was not inhibited at all with heparin, while CKII activity using casein as substrate was abolished in the presence of heparin. Furthermore, the Raf-1 preparations, although pure as judged by Coomassie Blue staining (Ref. 17 and data not shown), did contain detectable amounts of CKII, as can be seen in the Western blot analysis shown in Fig. 5. These results suggest that CKII associates with Raf-1 under the conditions used and that the associating CKII, but not Raf-1, phosphorylates IκB-α.

To eliminate association of CKII with Raf-1, the immobilized GST-Raf fusion protein was washed with radioimmune precipitation assay buffer (Tris-buffered saline containing 1% Triton X-100, 0.5% deoxycholate, and 0.1% SDS). As shown in Fig. 6, these additional washes resulted in the essentially complete absence of both CKII as well as IκB-α kinase activity, while the Raf-1 kinase activity (using Mek as substrate) was unchanged. To further substantiate the finding that the CKII associating with Raf-1, and not Raf-1 itself, phosphorylates IκB-α, we assayed the Mek and IκB-α kinase activity of both wild-type

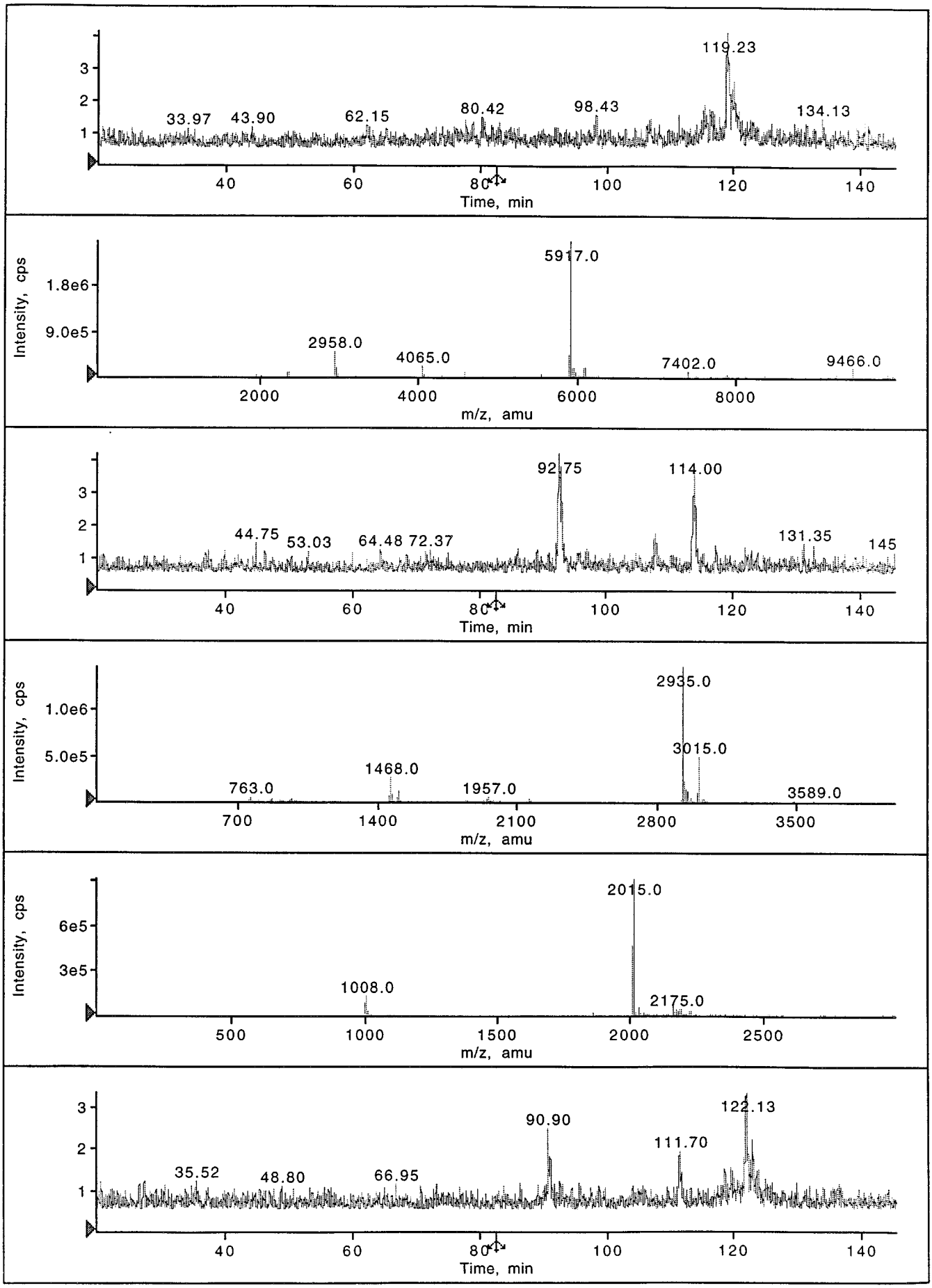

FIG. 10.

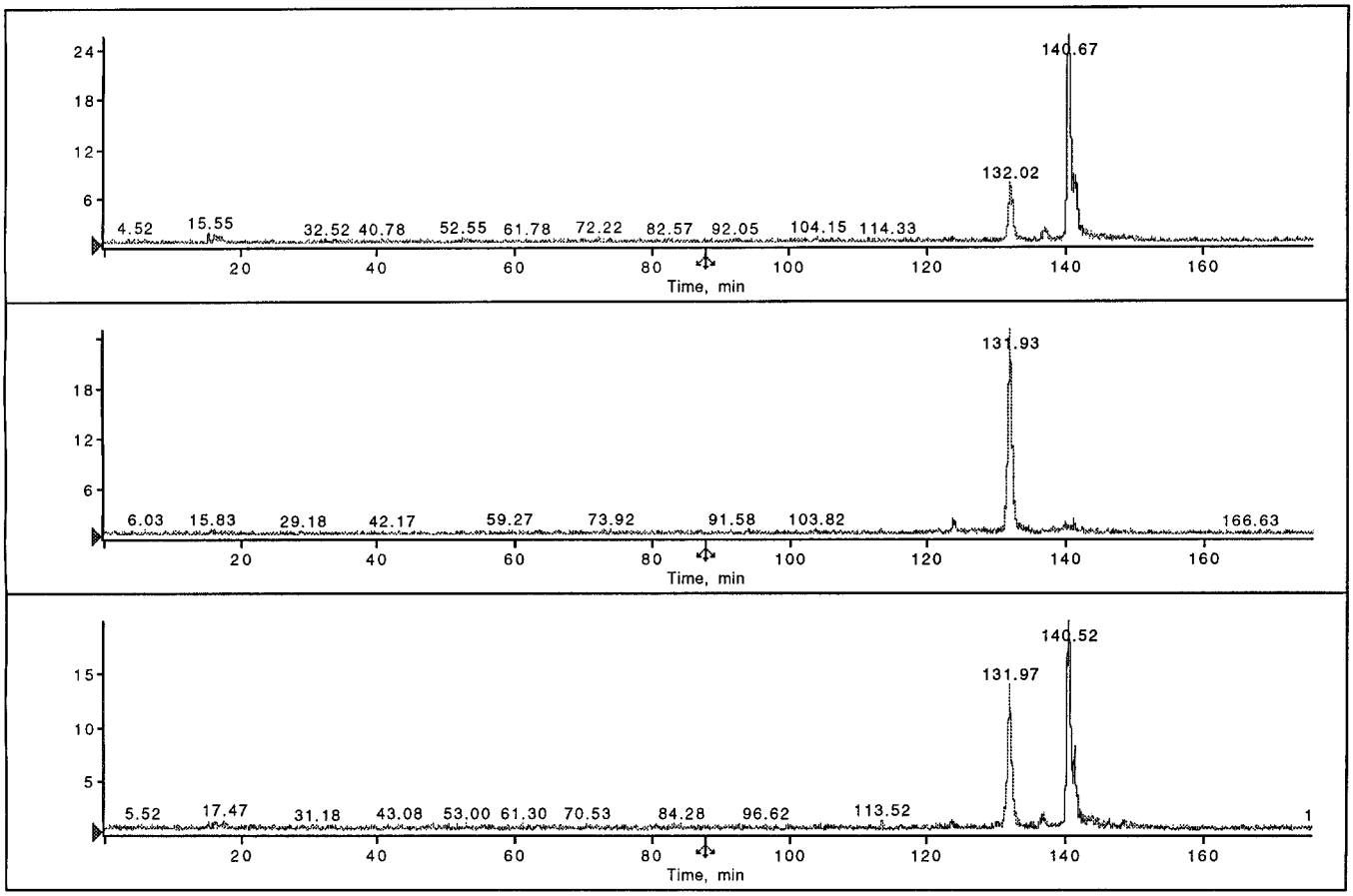

FIG. 11. **Comigration of the CKII-phosphorylated synthetic IκB-α peptide (amino acids 265–289) with the radiolabeled peptide resulting from the trypsin and Asp-N digest of CKII-phosphorylated IκB-α on HPLC.** The *upper panel* shows the radioactivity trace of digested IκB-α; the *middle panel* shows the radioactivity trace of the synthetic peptide, and the *lower panel* shows a mixture of the two samples.

and kinase-negative Raf-1. Both wild-type and kinase-negative preparations of Raf-1 contained roughly equal amounts of IκB-α kinase activity, while the Mek kinase activity was present only in the wild-type Raf-1 preparation, but was completely absent in the kinase-negative mutant (Fig. 7).

As several protein kinases can at least be partially renatured after SDS electrophoresis and their presence detected in in-gel kinase assays, we attempted to visualize IκB-α kinase activity in in-gel kinase assays using SDS gels that were polymerized in the presence or absence of recombinant IκB-α. The results of these experiments are shown in Fig. 8. While the presence of CKII was readily detectable using this approach in fractions of pool II of our IκB-α kinase preparations as well as in Raf-1 immunoprecipitates, no activity could be detected in pool I. These experiments further supported that CKII efficiently phosphorylates IκB-α, but unfortunately, they failed to help identify the IκB-α kinase present in pool I.

These results demonstrate that IκB-α serves as substrate for at least two different kinases. Therefore, the pattern of phosphorylation sites induced by these kinases was compared by two-dimensional phosphopeptide mapping (Fig. 9). IκB-α was phosphorylated by crude PKC-α preparations, purified Raf-1, CKII, or the IκB-α kinases purified from spleen; isolated by SDS-PAGE; and digested with proteases. Phosphopeptides were resolved on thin-layer cellulose plates by electrophoresis at pH 8.9 in the first dimension followed by ascending chromatography in the second dimension and autoradiographed. The standard digestion protocol with trypsin proved unsatisfactory, resulting in smeary maps (data not shown), probably due to the large size of the tryptic phosphopeptide. Therefore, a combination of trypsin and Asp-N endoproteases was used for subsequent experiments, which allowed the resolution of two major phosphopeptides. All IκB-α kinases (Fig. 9) yielded identical phosphopeptide maps, indicating that a common set of phosphorylation sites is targeted by different kinases.

To identify the phosphorylation sites in IκB-α, we performed HPLC-mass spectroscopy analysis of the tryptic digests. Digestion with trypsin yielded one radioactive peak that eluted at ~35% acetonitrile (Fig. 10) and comigrated with a peptide with a mass of 5917 Da. This corresponds to the mass of the C-terminal tryptic peptide IQQQ . . . (amino acids 265–314, 5906 Da). Digestion with trypsin and Asp-N yielded two radioactive peaks (Fig. 10). The major mass detected in the first peak was 2935 Da, with a second peak at 3015 Da, which was absent in the unphosphorylated preparation. This corresponds exactly to the peptide IQQQLGQLTLENLQMLPESEDEESY (amino acids 265–289) and its singly phosphorylated form, respectively. The masses found in the second peak, however (2015 and 2175

FIG. 10. **HPLC-mass spectroscopy analysis of IκB-α phosphorylation sites.** Analysis was performed as described under "Materials and Methods." The *top panel* shows the radioactivity in the HPLC run of a tryptic digest of IκB. The *second panel* is the reconstructed mass spectrum of the radioactive peak. The *third panel* shows the radioactivity in the HPLC run of a trypsin and Asp-N digest of IκB-α. The *fourth* and *fifth panels* are the reconstructed mass spectra of the first and second radioactive peaks, respectively. The *bottom panel* shows the radioactivity in the HPLC run of a mixture of the tryptic with the trypsin and Asp-N digest of phosphorylated IκB-α. *cps*, counts per second (masses detected per second); *amu*, atomic mass units.

Da, the latter only present in phosphorylated IκB-α), could not be correlated with any of the predicted masses from the IκB-α sequence. Since both peptides resulting from the additional Asp-N digest must be derived from the only radiolabeled peak in the trypsin digest, they must lie within the large tryptic peptide at amino acids 265–314. To investigate this assumption, we generated a GST-IκB-α mutant protein with a stop codon at amino acid 236. This protein in fact was not phosphorylated by any of the IκB-α kinases tested here (data not shown). Hence, the phosphorylation sites of all IκB-α kinases described here must lie within amino acids 242–307, and no phosphorylation of Ser-32 or Ser-36 could be detected.

To prove the identity of the phosphorylated peptide (amino acids 265–288), we synthesized the identical peptide. This synthetic peptide was readily phosphorylated by CKII and comigrated exactly with the first peptide resulting from the trypsin and Asp-N digest of IκB-α (Fig. 11).

DISCUSSION

PKC isozymes expressed in insect cells were purified and assayed as IκB-α kinases. Enriched PKC preparations (~10% pure) efficiently phosphorylated IκB-α, whereas highly purified PKCs, including PKC-ζ, failed. This result was not due to a loss of PKC activity or to the presence of a PKC inhibitor in the IκB-α preparations used. Furthermore, in crude preparations of PKC that still contained IκB-α kinase activity, we could inhibit the histone and MBP kinase activity with a highly specific PKC inhibitor, while the activity of the IκB-α kinase remained unchanged. In a complementary experiment, we partially purified IκB-α kinases from porcine spleen. Fractions active as IκB-α kinases were devoid of PKC, further proving that PKC is not an IκB-α kinase. The latter experiments demonstrated that at least two IκB-α kinase activities could be separated. One of the IκB-α kinase peaks contained both Raf-1 and CKII; the latter was in fact identified as IκB-α kinase. A Raf-1 mutant, BXB, rendered constitutively activated by deletion of the regulatory domain, has been shown to phosphorylate IκB-α *in vitro* and to activate NF-κB transcription in cells (17, 21). While the *in vitro* phosphorylation of IκB-α is certainly due to the presence of contaminating CKII, it still remains unclear how Raf-1 can initiate the down-regulation of IκB and hence NF-kB activation if it does not phosphorylate IκB.

From our studies, it is clear that IκB-α can be targeted by at least two distinct kinases. This finding is not unexpected, given the pleiotropic modes of NF-κB induction. These different signaling pathways seem to converge on the level of IκB-α phosphorylation, as distinct kinases phosphorylate the same set of sites resolved on two-dimensional phosphopeptide maps of trypsin/Asp-N-digested IκB-α. Unfortunately, the kinase activity present in pool I could not be renatured after SDS electrophoresis under various different conditions. Hence, we are unable to even speculate on the nature of this second IκB-α kinase.

We could not detect any phosphorylation of IκB-α serines 32 and 36, which have been indirectly implicated as IκB-α phosphorylation sites (4, 6). Since kinases that phosphorylate these sites might only be active in, for example, TPA-stimulated cells, we also purified IκB-α kinases from TPA-stimulated WEHI 3Z cells (data not shown). We again only obtained the two pools of IκB-α kinases that were described above, but no activity that phosphorylated IκB-α on other sites, including serines 32 and 36, could be detected. Although IκB-α point mutants at serines 32 and 36 have been reported to be resistant to tumor necrosis factor-α-induced degradation, direct phosphorylation of these sites has not yet been shown.

Recently, two laboratories reported that IκB-α is constitutively phosphorylated by CKII (6, 8), which is in good agreement with our data. The phosphorylation sites described for CKII match the sites we identified here. Although McElhinny *et al.* (8) show phosphorylation of IκB-α by PKC, they also state that the efficiency is ~1000-fold less than with CKII, suggesting that the phosphorylation actually results from impurities in the PKC preparation. The authors were, as we were, unable to detect phosphorylation of IκB-α at the N terminus, including serines 32 and 36. It is tempting to speculate that the phosphorylation of IκB-α at the C terminus is required for an additional phosphorylation at serines 32 and 36. Since the IκB-α protein used to detect IκB-α kinases was unphosphorylated, the "inducible" phosphorylation at the N terminus would not be detected. In conclusion, our data indicate that PKC isozymes and Raf-1 are not IκB-α kinases; however, Raf-1 associates with an IκB-α kinase, CKII.

Acknowledgments—We thank A. Kieser for thoughtfully and critically reading the manuscript and Dr. Ulli Siebenlist for the GST-IκB-α plasmid.

REFERENCES

1. Thanos, D., and Maniatis, T. (1995) *Cell* **80,** 529–532
2. Siebenlist, U., Brown, K., and Franzoso, G. (1995) in *Inducible Gene Expression* (Baeuerle, P. A., ed) Vol. 1, pp. 93–141, Birkhaeuser Boston, Inc., Cambridge
3. Baeuerle, P. A., and Henkel, T. (1994) *Annu. Rev. Immunol.* **12,** 141–179
4. Traeckner, E. B.-M., Pahl, H. L., Henkel, T., Schmidt, K. N., Wilk, S., and Baeuerle, P. A. (1994) *EMBO J.* **14,** 2876–2883
5. Chen, Z., Hagler, J., Palombella, V. J., Melandri, F., Scherer, D., Ballard, D., and Maniatis, T. (1995) *Genes Dev.* **9,** 1586–1597
6. Brown, K., Gerstberger, S., Carlson, L., Franzoso, G., and Siebenlist, U. (1994) *Science* **267,** 1485–1488
7. Barroga, C. F., Stevenson, J. K., Schwarz, E. M., and Verma, I. M. (1995) *Proc. Natl. Acad. Sci. U. S. A.* **92**, 7637–7641
8. McElhinny, J. A., Trushin, S. A., Bren, G. D., Chester, N., and Paya, C. V. (1996) *Mol. Cell. Biol.* **16,** 899–906
9. Gosh, S., and Baltimore, D. (1990) *Nature* **344,** 678–682
10. Goodnight, J., Mischak, H., and Mushinski, J. F. (1994) *Adv. Cancer Res.* **63,** 159–209
11. Lozano, J., Berra, E., Municio, M. M., Diaz-Meco, M.-T., Dominguez, I., Sanz, L., and Moscat, J. (1994) *J. Biol. Chem.* **269,** 19200–19202
12. Wiegmann, K., Schütze, S., Machleidt, T., Witte, D., and Krönke, M. (1994) *Cell* **78,** 1005–1015
13. Mischak, H., Goodnight, J., Kolch, W., Martiny-Baron, G., Schæchtle, C., Kazanietz, M. G., Blumberg, P. M., Pierce, J. H., and Mushinski, J. F. (1993) *J. Biol. Chem.* **268,** 6090–6096
14. Mischak, H., Pierce, J. H., Goodnight, J., Kazanietz, M. G., Blumberg, P. M., and Mushinski, J. F. (1993) *J. Biol. Chem.* **268,** 20110–20115
15. Kazanietz, M. G., Areces, L. B., Bahador, A., Mischak, H., Goodnight, J., Mushinski, J. F., and Blumberg, P. M. (1993) *Mol. Pharmacol.* **44,** 298–307
16. Huang, K.-P., and Huang, F. L. (1991) *Methods Enzymol.* **200,** 241–252
17. Häfner, S., Adler, H., Mischak, H., Janosch, P., Heidecker, G., Wolfman, A., Pippig, S., Lohse, M., Üffing, M., and Kölch, W. (1994) *Mol. Cell. Biol.* **14,** 6696–6703
18. Kölch, W., Heidecker, G., Kochs, G., Vahidi, H., Mischak, H., Finkenzeller, G., Hummel, R., Marmee, D., and Rapp, U. R. (1993) *Nature* **364,** 249–252
19. Mischak, H., Bodenteich, A., Kolch, W., Goodnight, J., Hofer, F., and Mushinski, J. F. (1991) *Biochemistry* **30,** 7925–7931
20. Hathaway, G. M., Lubben, T. H., and Traugh, J. A. (1980) *J. Biol. Chem.* **255,** 8038–8041
21. Li, S., and Sedivy, J. M. (1993) *Proc. Natl. Acad. Sci. U. S. A.* **90,** 9247–9251

Characterization of IκB Kinases: IκB-α IS NOT PHOSPHORYLATED BY Raf-1 OR PROTEIN KINASE C ISOZYMES, BUT IS A CASEIN KINASE II SUBSTRATE

Petra Janosch, Miriam Schellerer, Thomas Seitz, Peter Reim, Manfred Eulitz, Markus Brielmeier, Walter Kölch, John M. Sedivy and Harald Mischak

J. Biol. Chem. 1996, 271:13868-13874.