Description of an IL-1-Responsive Kinase That Phosphorylates the K Protein. Enhancement of Phosphorylation by Selective DNA and RNA Motifs†

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ABSTRACT: The K protein was first identified in the heterogeneous ribonucleoprotein particle (hnRNP). Subsequently, K protein was shown to bind sequence-specific single- and double-stranded DNA, stimulate transcription, and bind Src, Fyn, Lyn, and Vav via SH3 interactions. The K protein also binds to the κB enhancer motif which stimulates its phosphorylation *in vitro* by an associated serine/threonine kinase. To gain more insight into this unique nucleic acid-dependent phosphorylation process, we set out to examine the regulation of this kinase. We demonstrate that the K protein exists in a complex with an IL-1-responsive kinase and that phosphorylation of the K protein by this kinase is augmented by either cognate DNA or RNA sequences. The IL-1-responsive kinase activity associated with the K protein is reduced by phosphatase treatment, suggesting that the K protein kinase activity is regulated by phosphorylation. The observation that phosphorylation of the K protein is DNA- or RNA-dependent and IL-1-responsive suggests that the function of the K protein is tightly regulated.

Interleukin-1 (IL-1) is a potent immunoregulatory hormone that acts by inducing expression of a myriad of genes whose products are key elements of the immune response (Dinarello, 1991). As with other inducers, activation of gene expression by IL-1 is the result of a tightly coordinated chain of events that begins with the activation of transcription factors, followed by transcription, processing, and transport of messenger RNA. Transcriptional activation of gene expression by IL-1 is a well-recognized process that is mediated by inducible transcriptional factors including NF-κB (Osborn et al., 1989; Iwasaki et al., 1993) and AP-1 (Chedid et al., 1991). Whether IL-1 can also regulate gene expression beyond the transcriptional step has not been well explored.

The κB enhancer element (GGGGACTTTCC) regulates expression of many genes involved in the immune response, and the expression of human immunodeficiency virus (HIV) genes (Grimm & Baeuerle, 1993). The κB motif is recognized by the NF-κB family of transcription factors (Sen & Baltimore, 1986; Ghosh et al., 1990; Kieran et al., 1990; Nolan et al., 1991; Sparacio et al., 1992) as well as other structurally unrelated proteins (Rustgi et al., 1990; Adams et al., 1991). It is not known whether the κB motif-binding proteins, that are not related to NF-κB, regulate transcription from this element. We originally identified a 65 kDa κB motif-binding protein that was phosphorylated *in vivo* and *in vitro* by a serine/threonine kinase(s) (Ostrowski et al., 1991). Unexpectedly, cloning of the cDNA encoding this κB motif-binding protein identified it as the heterogeneous ribonucleoprotein (hnRNP) K protein, a protein structurally unrelated to NF-κB. Expression of the cloned cDNA in *Escherichia coli* showed that the K protein is the genuine κB motif-binding phosphoprotein (Ostrowski et al., 1994).

The K protein is 1 of at least 20 major proteins that exist in the hnRNP complex (Dreyfuss et al., 1993). The proteins contained within the complex are thought to play a role in processing pre-mRNA (Dreyfuss et al., 1993). The K protein is unusual among the hnRNP proteins; its primary structure does not contain the consensus RNA-binding domains present in other hnRNP proteins (Matunis et al., 1992, 1994), it binds to both single- and double-stranded DNA in a sequence-specific way (Takimoto et al., 1993; Ostrowski et al., 1994), and is transcriptionally active (Takimoto et al., 1993; Gaillard et al., 1994). Collectively, these observations provide evidence that the K protein is involved in regulation of gene expression. K protein has also been shown to bind SH3 domains of Src, Fyn, Lyn, and Vav (Hobert et al., 1994; Taylor & Shalloway, 1994; Weng et al., 1994).

In order to elucidate the ways by which the function of K protein might be regulated, we set out to characterize the K protein-associated kinase and study the mechanism by which K protein is phosphorylated. We demonstrate that the K protein copurifies and is phosphorylated by an IL-1-responsive kinase. This kinase cannot efficiently phosphorylate the K protein unless the complex is engaged by sequence-selective DNA or RNA.

MATERIALS AND METHODS

Cell Lines. The murine thymoma EL-4 6.1 C10 (Lowenthal & MacDonald, 1986) cells were grown in suspension. Cells were grown at 37 °C in complete RPMI 1640 medium supplemented with 5% FCS or Fetal Clone, 2 mM glutamine, 50 μM β-mercaptoethanol, penicillin (100 units/mL), and streptomycin (0.01%) and humidified with a 7/93% CO_2/air gas mixture.

Reagents. Streptavidin−agarose was purchased from GIBCO/BRL (Gaithersburg, MD). Bovine myelin basic

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protein (MBP) was obtained from Sigma (St. Louis, MO). Purified casein kinase II (CKII), mitogen-activated protein kinase (MAPK), glycogen synthase kinase 3 (GSK) (Boyle et al., 1991), and antibodies to phosphotyrosine, CK II, Raf-1, pp90^{RSK}, MAPK, MAPKK (MEK), and cdc2 kinases (Draetta, 1990; Davis, 1993) were kindly provided by Drs. R. Seger and E. Krebs (University of Washington, Seattle, WA). Recombinant *src*-homology 3 (SH3) domain-containing proline-rich kinase, SPRK, and anti-SPRK antibody (Gallo et al., 1994) was a gift from Dr. Godowski (Genentech Corp., South San Francisco, CA).

The following synthetic oligonucleotides were used to make a DNA-affinity matrix by coupling to streptavidin–agarose beads as described previously (Ostrowski et al., 1991; Ostrowski & Bomsztyk, 1993):

ds-κB^{w}

5′-CAGA**GGGGACTTTCC**GAGAGGAAGCT-3′ (sense)

3′-GTCT**CCCCTGAAAGG**CTCTCCTTCGA-5′ (antisense)

ds-κB^{m}

5′-CAGA**GCTCACTTTCC**GAGAGGAAGCT-3′ (sense)

3′-GTCT**CGAGTGAAAGG**CTCTCCTTCGA-5′ (antisense)

The κB motif (Grimm & Baeuerle, 1993) is shown in boldface letters, and the mutated bases are underlined. To couple the synthetic oligonucleotides to streptavidin–agarose beads, the antisense strand was synthesized with biotin at the 5′ end (DMT-biotin-C6-PA; Cambridge Research Biochemicals, Wilmington, DE) (Ostrowski & Bomsztyk, 1993).

Cytoplasmic and Nuclear Protein Extraction. Nuclear and cytoplasmic extracts were prepared by a modified method of Dignam et al. (1983) as described previously (Ostrowski et al., 1991). In addition to dithiothreitol (0.5 mM), phenylmethanesulfonyl fluoride (0.5 mM), and leupeptin (10 μg/mL), both lysis, extraction, and dilution buffers contained the following phosphatase inhibitors (all from Sigma): 30 mM *p*-nitrophenyl phosphate, 10 mM NaF, 0.1 mM Na_3VO_4, 0.1 mM Na_2MoO_4, and 10 mM β-glycerophosphate.

K Protein Purification. The K protein was purified as described previously (Ostrowski & Bomsztyk, 1993). Briefly, nuclear or cytoplasmic extracts were chromatographed on a DEAE-Sephacel column, and after a wash with binding buffer containing 100 mM NaCl, proteins were eluted with 30 mL of 200 mM NaCl elution buffer (20 mM HEPES–NaOH, pH 7.5, 2 mM EGTA, and 2 mM EDTA). The DEAE-Sephacel eluate was diluted to 70 mL with binding buffer (5 mM Tris-HCl, pH 7.5 , 25 mM NaCl, 1.0 mM EDTA, and 0.05% NP-40) and further purified on the tandem DNA-affinity column. The top three modules contained streptavidin beads bound to the double-stranded mutant κB oligonucleotide, and the bottom module contained wild-type κB motif. After the modules were washed with binding buffer containing 175 mM NaCl, the K protein was eluted from the bottom module with elution buffer containing 400 mM NaCl. The eluate was then dialyzed, and concentrated (Micro-ProDicon; Spectrum, Los Angeles, CA).

Production of Antibodies and Immunoprecipitation. Anti-K protein antibody 54 was raised against a synthetic peptide, (GCG)QNSVKQYADVEGF, which represents C-terminal amino acid residues 452–464 of the murine K protein (Ostrowski et al., 1994); the GCG amino acid residues were added to allow coupling and increase solubility. The peptide was coupled to keyhole limpet hemocyanin (KLH) using *m*-maleimidobenzoyl-*N*-hydroxysuccinimide ester (MBS) (Imject Kit; Pierce, Rockford, IL) and used to immunize rabbits at 3 week intervals (Harlow & Lane, 1988). The same antibody 54 was used for both immunoprecipitation and immunoblotting studies.

Beads for immunoprecipitation were made by mixing protein A–Sepharose 4 Fast Flow (Pharmacia, Uppsala, Sweden) beads with preimmune or immune serum in binding buffer for 1 h (1 μL of serum/10 μL beads) at 4 °C and were stored in the same buffer at 4 °C for later use. Immunoprecipitation was carried out by mixing the preimmune or immune beads with the sample containing the K protein for 1–2 h in binding buffer (4 °C). After centrifugation and multiple washes, the beads loaded with the immunoprecipitated proteins were used for further analysis.

Phosphorylation Reaction. For the phosphorylation reaction in solution, 50 μL of the concentrated eluate from the DNA-affinity module was diluted to 270 μL with binding buffer to reduce the NaCl concentration below 100 mM; higher concentrations inhibit phosphorylation. Samples were incubated with or without 1μg/mL DNA or RNA for 1 h at 4 °C. The phosphorylation reaction was carried out for 30 min at 30 °C after addition of 30 μL of 10X kinase buffer (final concentration; 20 mM HEPES, 10 mM $MgCl_2$, and 5.0 mM DTT) containing 5–10 μCi (1.66–3.33 pmol) of [γ-^{32}P]ATP (3000 Ci/mmol; NEN Research, Boston, MA). The phosphorylation reaction was terminated by adding 1.2 mL of ice-cold acetone. Samples were vigorously vortexed, kept overnight at −70 °C, and microcentrifuged at 14 000 rpm at 4 °C for 30 min, and after the supernatant was discarded, the protein pellet was dissolved with 1X loading buffer (60 mM Tris-HCl, pH 6.8, 2% SDS, 10% glycerol, and 5% β-mercaptoethanol)(Laemmli, 1970) and boiled for 5 min.

The following protocol was used to phosphorylate immunoprecipitated K protein. Protein A beads bearing preimmune IgGs (20 μL) were first mixed with nuclear extracts or purified K protein preparation diluted with binding buffer containing the full complement of phosphatase inhibitors (30 mM *p*-nitrophenyl phosphate, 10 mM NaF, 0.1 mM Na_3VO_4, 0.1 mM Na_2MoO_4, and 10 mM β-glycerophosphate) for 2 h at 4 °C. The preimmune beads were pelleted, and the supernatant was then mixed for 2 h at 4 °C with protein A beads (20 μL) containing antiserum 54 to the K protein. After immunoprecipitation, beads were washed once with 1.0 mL of binding buffer containing 175 mM NaCl plus the full complement of phosphatase inhibitors, once with 1.0 mL of binding buffer without phosphatase inhibitors and finally once with 1.0 mL of kinase buffer (20 mM Hepes, pH 7.5, 10 mM $MgCl_2$, and 5 mM DTT). The phosphorylation reaction was carried out for 30 min at 30 °C, after resuspending the washed beads in 90 μL of kinase buffer containing 5 μCi of [γ-^{32}P]ATP. The reaction was stopped by the addition of 30 μL of 4X loading buffer and boiling the beads. Eluted proteins were analyzed by SDS–PAGE and autoradiography.

RESULTS AND DISCUSSION

We have previously shown that the K protein is phosphorylated *in vivo* and *in vitro* by a serine/threonine kinase

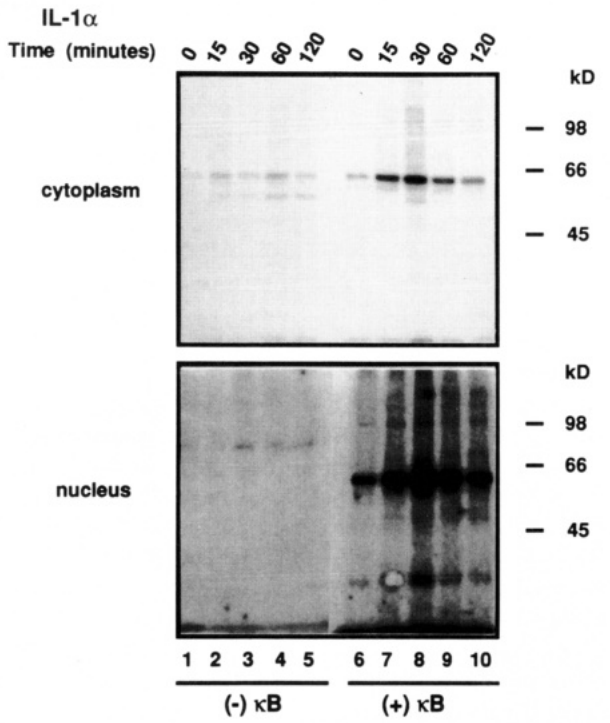

FIGURE 1: Effects of κB enhancer element on the level of phosphorylation of K protein purified by tandem DNA-affinity chromatography from IL-1-treated cells. EL-4 murine thymoma cells at 10^6 cells/mL RPMI 1640 medium were treated with 10^{-9}M IL-1α (Ostrowski et al., 1991). At given time points, 5×10^8 cells were harvested, nuclear and cytoplasmic extracts were prepared, and K protein was purified. 50 μL aliquots of purified K protein were diluted to 270 μL with binding buffer containing [γ-^{32}P]ATP (5.3 nM) and incubated with (1 μg/mL), *(+)* κB (lanes *6*−10), or without, (−) κB, (lanes 1−5), double-stranded synthetic oligonucleotide containing the κB enhancer element (1 h at 4 °C) and phosphorylated for 30 min at 30 °C. Precipitated protein pellets dissolved in loading buffer were then separated on 10% SDS−PAGE and silver-stained. The dried gels were autoradiographed; 4 h for cytoplasmic extracts (cytoplasm) and 20 h for nuclear extracts (nucleus).

(Ostrowski et al., 1991, 1994). *In vitro*, the level of phosphorylation of the K protein by the copurifying kinase was enhanced in the presence of synthetic oligonucleotides containing the κB motif (Ostrowski et al., 1994). To further characterize the K protein-associated kinase, EL-4 cells were treated with 10^{-9}M IL-1α, and at given time points, equal aliquots of cells were harvested. Both cytoplasmic and nuclear proteins were purified from IL-1-treated cells using tandem DNA-affinity chromatography (Ostrowski & Bomsztyk, 1993). Purified samples were used in phosphorylation reactions with or without preincubation with a synthetic oligonucleotide containing the κB motif. Without preincubation with DNA, the level of phosphorylation of the cytoplasmic and the nuclear K protein was the same in samples isolated from 0 to 120 min after IL-1 treatment (Figure 1, lanes 1−5). However, in nuclear and cytoplasmic samples preincubated with an oligonucleotide containing the κB enhancer element (lanes 6−10), the level of phosphorylation of the K protein by the associated kinase was greatly enhanced. In both cytosolic and nuclear extracts from cells treated with IL-1, the copurified kinase activity peaked at 30 min; the activity returned to the base line after 120 min (Figure 1, lanes 6−10). These results demonstrate that phosphorylation of the K protein by the copurifying kinase is an IL-1-responsive process.

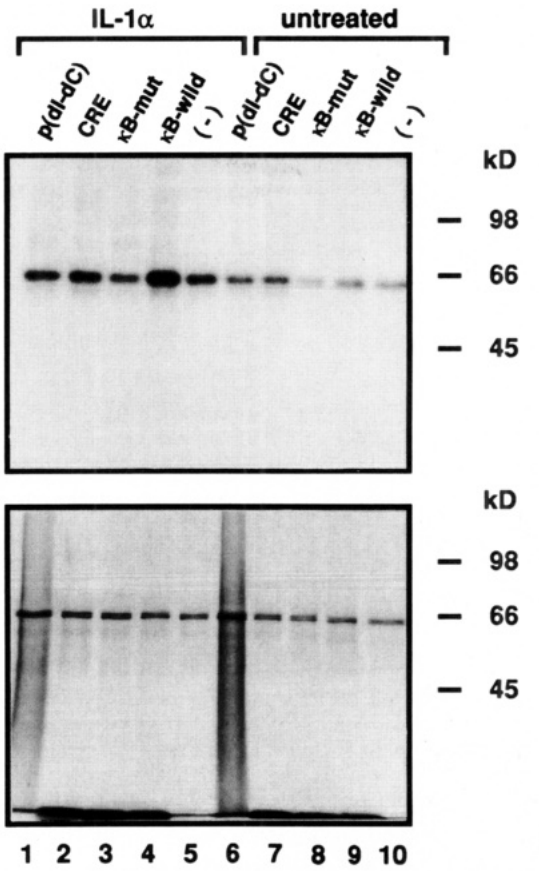

FIGURE 2: Comparison of the effect of different DNA sequences on the level of phosphorylation of K protein purified by the κB tandem DNA-affinity column. Nuclear extracts prepared from untreated (lanes 6−10) and IL-1-treated (10^{-9} M for 30 min) (lanes 1−5) EL-4 cells were purified by sequential anion-exchange and tandem DNA-affinity chromatography as before (Ostrowski & Bomsztyk, 1993). Equal aliquots of purified samples were incubated with no DNA (lanes 5 and 10) or 1 with μg/mL of one of the double-stranded synthetic DNAs, poly(dI-dC) (lanes 1 and 6), oligonucleotide containing the CRE (5′-ATACTGACCTTGGCTGACGTCAGAGAGA-3′) (Gonzales et al., 1989) (lanes 2 and 7), mutated κB (lanes 3 and 8), or wild-type κB (lanes 4 and 9) motif. Phosphorylation was carried out as before; acetone-precipitated proteins were separated by SDS−PAGE, silver-stained (lower panel), and autoradiographed (upper panel).

DNA containing different nucleotide sequences was used to evaluate the sequence-specificity of the DNA-enhanced phosphorylation of the K protein by the associated IL-1-responsive kinase. Equal aliquots of samples purified from IL-1- treated or untreated EL-4 cells were phosphorylated as before and separated on SDS−PAGE. The autoradiogram is shown in Figure 2 (upper panel) and, Figure 2 (lower panel) shows the silver-stained gel. Addition of poly(dI-dC) (lane 1), double-stranded synthetic oligonucleotides containing the cAMP-responsive element (CRE)(Gonzales et al., 1989) (lane 2), or the mutated κB motif (lanes 3) had little or no effect on the level of phosphorylation of the κB-binding protein purified from the IL-1-treated EL-4 cells compared to the wild-type κB motif where the level of phosphorylation increased (lane 4). This experiment indicates that the DNA-stimulated phosphorylation in extracts purified from IL-1-treated cells is DNA sequence-selective. Because phosphorylation of the K protein purified from IL-1-treated cells was stimulated most by the DNA that contains the κB motif, these results suggest that the potentiation is actually the result of binding of DNA to the K protein. The

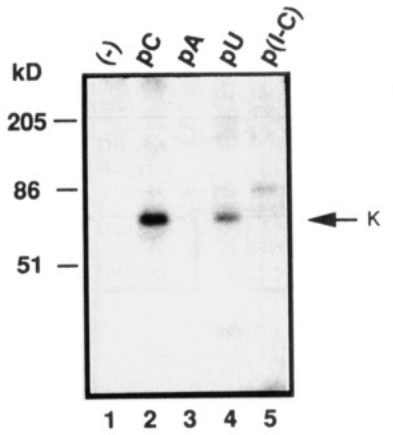

FIGURE 3: Comparison of the effect of different RNA sequences on the level of phosphorylation of K protein purified by the κB tandem DNA-affinity column. Nuclear extracts purified from IL-1-treated (10^{-9}M for 30 min) EL-4 cells were purified by DNA-affinity chromatography as in Figure 2. Equal aliquots of purified samples were incubated with no RNA (−) (lane 1) or 1 μg/mL RNA, poly(C) (lane 2), poly(A) (lane 3), poly(U) (lane 4), and poly-(I-C) (lane 5). Phosphorylation was carried in solution, and proteins were precipitated as before. Phosphorylated proteins were analyzed by SDS−PAGE and autoradiography.

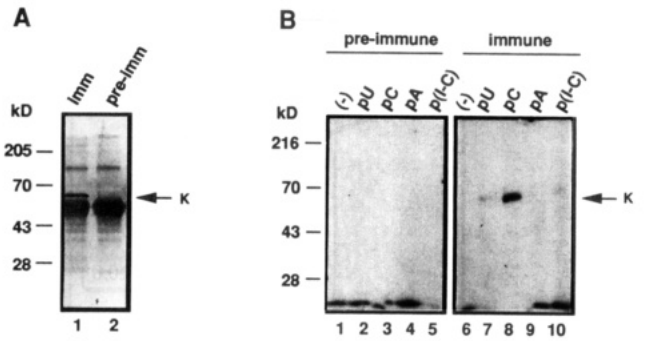

FIGURE 4: Phosphorylation of immunoprecipitated K protein by the associated kinase in the presence of single- and double-stranded RNA. Panel A: 150 μL of K protein preparation from the tandem κB-affinity column was mixed with 10 μL of preimmune protein A−IgG beads (lane 2) in 250 μL of binding buffer. After 1 h (at 4 °C), beads were centrifuged, and the supernatant was then mixed with 10 μL of immune protein A−IgG beads (lane 1) (1 h at 4 °C). Proteins eluted from beads were analyzed by SDS−PAGE followed by immunoblotting with the same antipeptide antibody (antibody 54 at 1:10 000 dilution). Panel B: 50 μL of purified K protein preparation was diluted with binding buffer containing no RNA (lanes 1 and 6) or 1 μg/mL poly(U) (lanes 2 and 7), poly(C) (lanes 3 and 8), poly(A) (lanes 4 and 9), and poly(I-C) (lanes 5 and 10). The mixture was incubated with preimmune protein A−IgG beads (lanes 1−5) for 1 h at 4 °C. Beads were centrifuged, and the supernatant was then mixed with immune protein A−IgG beads (lanes 6−10) as described above. Beads were then submitted to phosphorylation (18 nM [γ-^{32}P]ATP) with or without 1μg/mL of RNA as denoted. ^{32}P-Labeled proteins eluted from the beads were then analyzed by SDS−PAGE and autoradiography.

significance of the different levels of phosphorylation of K protein purified from untreated cells in the presence of the different DNA is not clear, but the overall phosphorylation levels of the K protein purified from untreated cells (lanes 6−10) were far below the levels of K protein phosphorylation in extracts purified from IL-1-treated cells (lanes 1−5). Given that these phosphorylations were done in solution, the phosphorylation of K protein purified from untreated cells may represent the activity of another kinase(s) that is copurified on the DNA-affinity column.

In addition to binding specific double-stranded DNA motifs (Takimoto et al., 1993; Ostrowski et al., 1994), the K protein also binds tenaciously to single-stranded oligo-(dC) (Matunis et al., 1992). In contrast to the κB motif, binding of oligo(dC) decreases phosphorylation of the K protein by the copurified or associated kinase (Ostrowski et al., 1994). Because the K protein is also known to bind poly-(C) RNA (Matunis et al., 1992, 1994), we used conditions described above (Figure 2) to test the effect of different RNA sequences on the level of phosphorylation in solution of the K protein purified as above by DNA-affinity chromatography. Results of this experiment are illustrated in Figure 3. This experiment revealed that poly(C) RNA (compare lanes 1 and 2) and to a lesser degree poly(U) RNA (lane 4) stimulated phosphorylation of a 60−65 kDa band that corresponds to the K protein. Poly(A) (lane 3) and the double-stranded poly(I-C) RNA (lane 5) did not detectably induce phosphorylation of the K protein.

To ensure that the poly(C) RNA-stimulated phosphorylation was that of the K and not another copurifying protein, we carried out phosphorylation of proteins immunoprecipitated with antiserum to the C-terminus of the murine K protein (serum 54). K protein was immunoprecipitated from the DNA-affinity-purified preparation by sequential mixing first with protein A beads containing preimmune and then immune IgGs. An immunoblot developed with the same anti-K antiserum (Figure 4A) illustrates that the immune (lane 1) but not the preimmune (lane 2) protein A beads specifically immunoprecipitated K protein. The same immunoprecipitation procedure was then used to test the effect of different RNAs on the level of K protein phosphorylation. Preimmune and immune protein A−IgG beads were sequentially loaded with proteins from the DNA-affinity-purified K protein preparation with or without 1μg/mL RNAs with different sequences. After being washed, beads were resuspended in kinase buffer containing no or 1 μg/mL of the same RNA, and phosphorylation was carried out on the beads. Figure 4B illustrates an autoradiograph of ^{32}P-labeled proteins eluted from the beads containing either preimmune or immune IgGs. Regardless of the type of RNA used, no ^{32}P-labeled 60−65 kDa band was eluted from the preimmune beads (lanes 1−5). When proteins were phosphorylated on the beads bearing anti-K antibodies, the strongest ^{32}P-labeled band was eluted from beads incubated with poly(C) RNA (lane 8). In conclusion, these results demonstrate (Figures 1−4) that the K protein phosphorylation by the associated kinase can be enhanced by both DNA and RNA in a sequence-selective manner. The nucleic acid sequence-specificity of K protein phosphorylation does not appear to be exclusive to poly(C) RNA, since poly(U), to a lower extent, can also enhance the level of phosphorylation of the K protein by the copurifying kinase(s) when phosphorylation is done in solution (Figure 3, compare lanes 1 to lanes 2 and 4). The significance of these differential effects induced by the different nucleic acid sequences remains to be defined, but it may reflect different binding affinities of K protein to these nucleic acid sequences. It is interesting to note that the double-stranded poly(I-C) RNA did not increase the level of K protein phosphorylation (Figures 3 and 4B), suggesting that the K protein-associated kinase is not the inducible dsRNA-activated kinase (Katze, 1992).

In the above series of experiments, we used a K protein preparation purified with the DNA-affinity column. It is

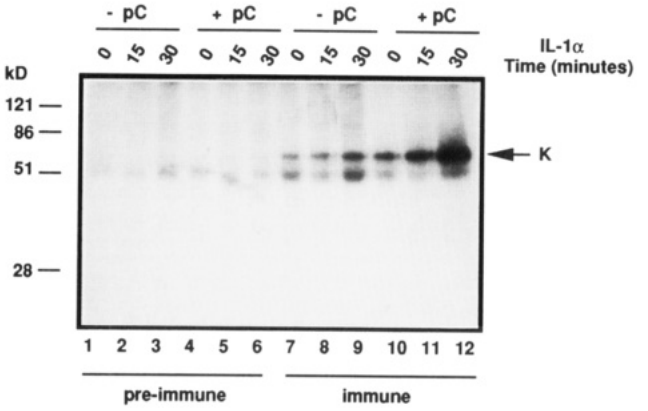

FIGURE 5: Effects of poly(C) RNA on *in vitro* phosphorylation of K protein immunoprecipitated from crude nuclear proteins extracted from IL-1-treated EL-4 cells. EL-4 cells were treated for the indicated time points (0, 15, and 30 min) with 2×10^{-10} M IL-1α, and nuclear protein extracts were prepared as before. 20 μL of protein A beads bearing preimmune rabbit serum was mixed for 2 h (4 °C) with 100 μg of nuclear extracts diluted with 1.0 mL of binding buffer containing the full complement of phosphatase inhibitors (preimmune, lanes 1−6). After preclearing with the preimmune serum, the beads were pelleted, and the supernatant was mixed with 20 μL of protein A beads bearing antipeptide serum 54 (2 h, 4 °C) (immune, lanes 7−12). Preimmune and immune beads were washed 3 times as before, and phosphorylation (18 nM [γ-^{32}P]ATP) was carried out with (+pC, lanes 4−6 and 10−12) or without (−pC, lanes 1−3 and 7−9) 1 μg/mL poly(C) RNA. Proteins were eluted from the beads by boiling in loading buffer and were analyzed by SDS−PAGE and autoradiography.

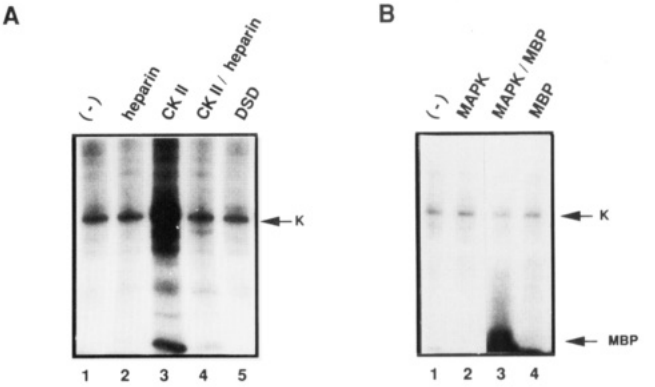

FIGURE 6: Phosphorylation of the K protein by CK II but not by MAPK. Crude nuclear extracts from EL-4 cells were mixed for 1 h (4 °C) with mutant κB DNA−agarose beads (20 μL) (Ostrowski et al., 1991). The bead suspension was centrifuged, and the supernatant was then mixed with wild-type κB DNA−agarose beads (20 μL). Phosphorylation was carried out directly on the beads as above. Panel A: Phosphorylation without any exogenous reagent added (lane 1) and in the presence of heparin (0.2 μg/mL), (lane 2), casein kinase II (1 μL) (lane 3), CK II and heparin (lane 4), and the CK II peptide substrate DSD (RRRDDDSDDD at 0.1 mM) (lane 5). Panel B: Phosphorylation in the absence of added exogenous reagent (lane 1) and in the presence of purified MAPK (1μL) (lane 2), MAPK and MBP (2 μg/mL) (lane 3), and MBP alone (lane 4).

therefore possible that the K protein copurifies with a kinase that binds to the DNA column independently of K, rather than a kinase that exists in a complex with the K protein. The experiment illustrated in Figure 4 provides evidence that the K protein and the IL-1-responsive kinase do indeed exist in a complex. If so, then the K protein and the associated IL-1-responsive kinase ought to coimmunoprecipitate from crude nuclear extracts. To test this, nuclear extracts from IL-1-treated EL-4 cells were first mixed with protein A beads bearing preimmune serum, and then the supernatant was incubated with beads containing the anti-K protein IgG. After being washed, the preimmune and immune beads were resuspended in kinase buffer and phosphorylation was carried out with or without poly(C) RNA (Figure 5). The results showed a 60−65 kDa band that was phosphorylated on the immune (lanes 7−12) but not on the preimmune beads (lanes 1−6), and thus representing the K protein. The intensity of this phosphorylated band was by far stronger in nuclear extracts from IL-1-treated cells (30 min) when the phosphorylation was carried out in the presence of poly(C) RNA (Figure 5, compare lane 7 to lanes 9 and 12). This experiment provides further evidence that the K protein does exist in a complex with an IL-1-responsive kinase(s) and that the phosphorylation reaction mediated by this kinase is facilitated by either cognate RNA or DNA motifs. Until the K protein-associated inducible kinase is identified, it is not certain whether the RNA- and DNA- facilitated phosphorylation reaction of K protein by the IL-1-responsive kinase(s) is mediated by the same or different K protein-associated enzymes.

The *in vitro* phosphorylation of the K protein by the associated IL-1-responsive kinase requires only magnesium as cofactor (data not shown), suggesting that it may not be one of the second messenger-dependent kinases such as protein kinase A or C which have been reported to respond to treatment of cells with IL-1 (Ostrowski et al., 1988; Shirakawa et al., 1989; Munoz et al., 1991). Because the K protein forms a complex with an IL-1-responsive kinase, it provided us with a convenient way to study this enzyme and to test whether it is a known cytokine-inducible protein kinase. We have previously shown that the K protein can be phosphorylated by CK II (Ostrowski et al., 1994). The following set of experiments was designed to test whether CK II is the K protein-associated kinase. Nuclear extracts from EL-4 cells precleared with mutated κB DNA−agarose beads were mixed with κB wild-type DNA−agarose beads and phosphorylated on the beads (Ostrowski et al., 1991). Heparin, which inhibits the CK II-mediated phosphorylation (Hathaway et al., 1980), did not alter the level of phosphorylation of the K protein by the associated K protein kinase (Figure 6A, compare lanes 1 and 2). In contrast, phosphorylation mediated by exogenously added CK II was effectively blocked by heparin (Figure 6A, compare lanes 3 and 4). These results suggest that the kinase that is associated with the K protein is not CK II. This is further supported by the observation that the addition of CK II peptide substrate DSD (Sommercorn & Krebs, 1987) in excess into the phosphorylation reaction did not compete away the phosphorylation of the K protein (compare lanes 1 and 5).

To test the ability of MAP kinase to phosphorylate K protein, nuclear extracts precleared with mutated κB DNA beads were mixed with wild-type κB beads and phosphorylated as described above. Addition of exogenous MAP kinase to the phosphorylation buffer did not increase the level of phosphorylation of the K protein (Figure 6B, compare lanes 1 and 2). Failure of MAP kinase to phosphorylate the K protein was not due to the presence of inhibitors, since myelin basic protein, MBP, a MAP kinase substrate (Seger et al., 1992), was very efficiently phosphorylated when added to the reaction (lane 3). Finally, addition of an excess of MBP did not compete away the phosphorylation of the K

protein bound to κB beads (lane 4). This series of experiments demonstrates that the K protein-associated kinase is not MAPK. Moreover, antibodies to MAPK, MAPKK (MEK), Raf-1 (Li et al., 1991), pp90^{RSK} (Chen et al., 1991), cdc2, and SPRK (Gallo et al., 1994; Ing et al., 1994) did not recognize the K protein kinase. JNK1 (SAPK) is a novel member of the MAPK group of kinases that binds and phosphorylates c-Jun (Derijard et al., 1994; Kyriakis et al., 1994). Like MAPK, JKN1 (SAPK) is activated by dual phosphorylation at threonine and tyrosine, but immunoblots of the K protein-associated kinase using antiphosphotyrosine antibodies were negative (data not shown). These results suggest that the K protein kinase-associated kinase may not be a member of the MAPK group of kinases, nor is it one of the other known kinases that we considered above.

Cytokines and other growth factors activate kinase cascades whereby activation of a growth factor-responsive kinase is mediated through phosphorylation by an upstream kinase (Davis, 1993). If the IL-1-responsive K protein-associated kinase activity is regulated by phosphorylation, then dephosphorylation of the kinase by phosphatase treatment ought to diminish or prevent phosphorylation of K protein by this kinase. Two different types of experiments illustrated in Figure 7 were carried out to test this hypothesis. The crude nuclear extracts are rich in phosphatases (Walter & Mumby, 1993), and we routinely use several protein phosphatase inhibitors to preserve the integrity of the phosphoprotein extracted from the nucleus. If the IL-1-responsive K protein-associated kinase is regulated by phosphorylation, it should be regulated and serve as a substrate for one or more of the nuclear phosphatases. To test whether nuclear phosphatases could inactivate the IL-1-responsive K protein-associated kinase, nuclear extracts were diluted 20-fold with binding buffer containing either no (Figure 7A, lanes 1−2) or the full complement (lanes 3−4) of phosphatase inhibitors that we routinely add to buffers used to prepare nuclear extracts. After incubation for 4 h at 25 °C, the K protein was immunoprecipitated and phosphorylated on beads as before. Prior to mixing with the protein A beads, the full complement of phosphatase inhibitors was restored in the nuclear extract suspension that was diluted without these inhibitors. The autoradiograph from this experiment demonstrates that phosphorylation of the K protein immunoprecipitated from nuclear extracts that were incubated in a buffer containing diluted levels of phosphatase inhibitors diminished the IL-1-responsive component of K protein phosphorylation (compare lanes 2 and 4 vs lanes 1 and 3). This result is consistent with the contention that the IL-1-responsive K protein-associated kinase activity is regulated by phosphorylation. This is supported by another type of an experiment where we used exogenous phosphatase to inactivate the K protein kinase (Figure 7B). The K protein−K protein kinase complex immunoprecipitated from crude nuclear extracts with protein A beads bearing anti-K protein serum was treated with or without 0.1 unit of alkaline phosphatase for 15 min at 30 °C. After extensive wash, beads were resuspended in kinase buffer, and phosphorylation reaction was carried out as before. The autoradiograph shows that phosphatase treatment of immunoprecipitates prevented the subsequent phosphorylation of the K protein by the associated kinase (compare lanes 1−2 with lanes 3−4).

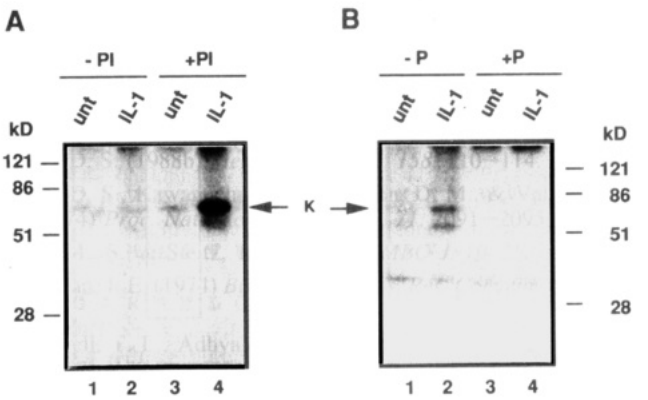

FIGURE 7: Effects of phosphatases on *in vitro* phosphorylation of K protein by the associated kinase immunoprecipitated from crude nuclear extracts. Panel A: Equal amounts of nuclear protein extracts (100 μg) from untreated (unt) and IL-1α-treated EL-4 cells (2×10^{-10} M for 30 min) (IL-1) containing the full complement of phosphatase inhibitors (30 mM *p*-nitrophenyl phosphate, 10 mM NaF, 0.1 mM Na_3VO_4, 0.1 mM Na_2MoO_4, and 10 mM β-glycerophosphate) were diluted 20-fold with binding buffer (50 mM Tris-HCl, pH 7.5, 25 mM NaCl, 1.0 mM EDTA, and 0.05% NP-40) containing either none (−PI, lanes 1−2) or the full complement (+PI, lanes 3−4) of phosphatase inhibitors. After incubation for 4 h at 25 °C, the full complement of phosphatase inhibitors was restored in the nuclear extract suspensions that were diluted without phosphatase inhibitors. Each sample was precleared with 20 μL of beads bearing preimmune serum, and K and associated proteins were immunoprecipitated with beads containing anti-K antibody (54). After three rounds of washing, phosphorylation was carried out in the presence of poly(C) RNA as before (Figure 5). Panel B: The same nuclear extracts used in Panel A were precleared with preimmune beads, and K protein was immunoprecipitated with beads bearing anti-K serum. After being washed, the beads were resuspended for 15 min at 30 °C in 100 μL of phosphatase buffer (50 mM Tris-HCl, pH 7.5, 10 mM $MgCl_2$) containing no (−P, lanes 1−2) or 1.0 unit/mL (+P, lanes 3−4) of bovine intestinal mucosa alkaline phosphatase (Sigma). Beads were washed once with 1.0 mL of binding buffer containing 175 mM NaCl, once with 1.0 mL of binding buffer, and once with 1.0 mL of phosphorylation buffer, and phosphorylation was carried out in the presence of 1 μg/mL poly(C) RNA. Panels A and B represent autoradiographs of ^{32}P-labeled proteins eluted from the beads by boiling and resolved by SDS−PAGE and autoradiography.

The intrinsic molecular mechanism(s) responsible for the observed IL-1-responsive phosphorylation remain(s) to be explored, but at least two possibilities exist. (i) Treatment of cells with IL-1 activates a kinase that is already bound to the K protein. For example, another kinase binds to the K protein in response to IL-1 and then phosphorylates/activates the kinase that is already bound to the K protein. (ii) IL-1 both activates and promotes binding of the kinase to the K protein. The fact that protein tyrosine kinases bind to the K protein via SH3 domains (Hobert et al., 1994; Taylor & Shalloway, 1994; Weng et al., 1994), while the serine/threonine IL-1-responsive K protein kinase binds to another domain of K protein independently of SH3 interactions (Van Seuningen et al., 1994), is consistent with the first scenario. Regardless of the mechanism(s) responsible for the IL-1-mediated effects, phosphorylation of the K protein by the associated kinase cannot fully proceed unless specific nucleic acid motifs are added to the phosphorylation reaction [Figures 1−3 (Ostrowski et al., 1994)]. If this *in vitro* observation recapitulates an *in vivo* situation, then these experiments would suggest that following treatment with IL-1, the K protein kinase is primed, but not able to phosphorylate unless the K protein is engaged by cognate nucleic acid motifs.

Once phosphorylated, the K protein is presumably allowed to interact with a set of molecular partners responsible for conveying its effects. Although there are examples of binding between a kinase and a transcription factor, the type of DNA- or RNA-facilitated phosphorylation of the K protein by a bound kinase has not previously been described. For example, JNK1 (SAPK) binds c-Jun, but unlike the K protein-associated kinase, JNK1 phosphorylates c-Jun in a DNA-independent manner (Derijard et al., 1994).

In summary, we have demonstrated that the K protein is a substrate and forms a complex with an IL-1-responsive kinase. Phosphorylation of the K protein by this kinase is augmented by selective DNA or RNA motifs. Purification of the K protein-associated kinase is now in progress to obtain partial amino acid sequences and establish its identity.

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