Activation of Nuclear Transcription Factor NF-κB by Interleukin-1 Is Accompanied by Casein Kinase II-mediated Phosphorylation of the p65 Subunit*

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In fibroblasts and hepatoma cells, interleukin-1 (IL-1) treatment results in the rapid nuclear accumulation of the transcription factor NF-κB, present largely as p65 (RelA)/p50 heterodimers. It is well established that this process is dependent in large part upon the phosphorylation and subsequent degradation of the cytosolic inhibitor IκB. We looked for other IL-1-induced modifications of NF-κB components and found that, in both cell types, IL-1 stimulation led, within minutes, to phosphorylation of both NF-κB p65 and p50. Phosphorylation of p65 was sustained for at least 30 min after addition of the cytokine and occurred principally upon serine residues. Immunoprecipitates of NF-κB complexes contained an associated protein kinase, the biochemical characteristics of which were indistinguishable from casein kinase II (CKII). Purified CKII efficiently phosphorylated p65 in vitro, apparently on the same major sites that became phosphorylated in intact IL-1-treated cells. Although IL-1 treatment caused little apparent stimulation of total cellular CKII activity, the fraction that was specifically associated with NF-κB complexes was markedly elevated by the cytokine. The association of CKII with NF-κB occurred in the cytoplasm, suggesting that this phosphorylation might be involved either in control of translocation of the activated complex or in modulation of its DNA binding properties.

Interleukin-1 (IL-1)† receptors are widely distributed on cells of many lineages (1). Thus IL-1 can exert effects on connective tissues, for example inducing mitogenesis through secretion of PDGF; effects on lymphoid cells, such as inducing up-regulation of surface immunoglobulin and secretion of cytokines; and effects on hepatocytes, for example inducing secretion of acute phase proteins such as C-reactive protein. All of these responses are triggered through a single type of receptor (IL-1R1) (2), which is the archetype for a diverse family of factors/DNA binding activities that recognize a consensus motif, 5′-GGGRNNYYCC-3′. All of these factors are dimers composed of subunits that share a common domain prototypically found in c-Rel (6). The transactivating activity of the NF-κB factors is regulated by a distinct set of proteins termed IκBs, which are characterized by a series of ankyrin repeats. IκBs function by sequestering NF-κB dimers in the cytosol. A variety of stimuli can induce activation of NF-κB (8), including viruses, lipopolysaccharides, and pro-inflammatory cytokines such as IL-1 and tumor necrosis factor (TNF) a. Activation of NF-κB involves its translocation from the cytoplasm to the nucleus, following dissociation from one or more IκBs. Cytokines bring about this dissociation by activating a newly identified protein kinase complex (9) that phosphorylates IκB at two critical residues located at the amino terminus (residues Ser-32 and Ser-36 in the case of IκBo, 10–11). The phosphorylated form of IκB, although still bound to p65/p50 heterodimers (10, 12–13), is then subject to rapid proteolysis through the ubiquitin-proteasome pathway (14). Removal of IκB exposes a nuclear localization sequence present in NF-κB, allowing the factor to translocate to the nucleus. In addition, several reports have shown that p50/p50 homodimers, which appear to bind to NF-κB sites but not transactivate, are regulated by a mechanism not involving cytoplasmic/nuclear translocation but rather are resident in the nucleus and regulated by a nuclear protein Bcl-3, which when complexed prevents association of this form of NF-κB with its recognition site (15). Recently, it was reported that other subunits of NF-κB, including p65 and p50 are phosphoproteins (16). Furthermore, activation of NF-κB in HeLa cells treated with H2O2, phorbol 12-myristate 13-acetate, or TNFa (17) or in endothelial cells treated with TNFa (18) is accompanied by an increased level of p65 phosphorylation. In this report, we show that treatment of fibroblasts or hepatoma cells with IL-1 also results in phosphorylation of p65 on serine residues, and we identify the kinase responsible as casein kinase II, raising the possibility that this is an additional level at which its activity is regulated.

EXPERIMENTAL PROCEDURES

HepG2 hepatoma cells (ATCC HB-8065) and human diploid gingival fibroblasts (19) were maintained as described previously (20). Carrier-free [32P]orthophosphate was obtained from NEN Life Science Products while (γ-32P)ATP (3000 Ci/mmol) was from Amersham Corp. Purified polyclonal rabbit anti-pest antibodies specific for NF-κB p65, NF-κB p50, and IκBo/MAD-3 together with corresponding immunizing peptides were obtained from Santa Cruz Biotechnology, Inc. Rabbit polyclonal antisera raised against synthetic peptides corresponding to

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§ The abbreviations used are: IL-1, interleukin-1; EMSA, electrophoretic mobility shift assay; HGF, human gingival fibroblast, HIV, human immunodeficiency virus; PMSF, phenylmethylsulfonyl fluoride; PAGE, polyacrylamide gel electrophoresis; TNF, tumor necrosis factor; CKII, casein kinase II; NF-κB, nuclear factor κB.
amino acids 198–215 of the β-subunit and amino acids 376–391 of the α-subunit of human casein kinase II have been described previously (21) and were the kind gift of Minoo Afdahi, Department of Biochemistry, Immunex Corporation. Recombinant human IL-1α was expressed and purified as described (22). Recombinant human NF-κB p65 was a gift from Dr. Guido Franzoso (Laboratory of Immunoregulation, National Institute of Allergy and Infectious Diseases, Bethesda, MD). Casein kinase II, purified to greater than 95% from Pisaster ochraceus, was purchased from Upstate Biotechnology, Inc.

**Electrophoretic Mobility Shift Assay (EMSA)**—Approximately 5 × 10^5 cells were washed with ice-cold PBS and resuspended in 400 μl of buffer A: 10 mM HEPES, pH 7.9, 10 mM KCl, 0.1 mM EDTA, 1 mM EGTA, 1 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride, 1 mM leupeptin. After 15 min at 4 °C, 25 μl of 10% Nonidet P-40 was added. Cells were vortexed briefly, nuclei were pelleted by microcentrifugation, and supernatants were removed (cytoplasmic extracts). Pellets was resuspended in 200 μl of buffer B (20 mM HEPES, pH 7.9, 0.4 M NaCl, 1 mM EDTA, 1 mM EGTA, 1 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride, and 1 mM leupeptin). After 30 min at 4 °C, lysates were centrifuged and supernatant was removed (nuclear extract). Protein concentration of extracts was measured using the BCA assay (Pierce).

Nuclear extracts (10 μg) were mixed with 0.02 units of poly(dI-dC) (Pharmacia Biotech Inc.) and end-labeled NF-κB oligonucleotide probe (23) (1 × 10^−2.5 × 10^−5 pmol per reaction) in binding buffer (2.5 mM HEPES, pH 7.5, 1 mM EDTA, 1.5 mM dithiothreitol, 10% glycerol) and were incubated for 20 min at room temperature. For supershift assays, 0.2 μg of anti-NF-κB subunit antibodies were included in the reaction mixture. Samples were separated on native 6% polyacrylamide gels (Novex) in low ionic strength buffer (0.25 × Tris-borate-EDTA). Dried gels were exposed to a storage phosphor screen (Kodak) overnight and quantitated using a PhosphorImager (Molecular Dynamics).

**Cell Labeling and Immunoprecipitation**—10-cm dishes of HepG2 cells (approximately 80% confluent) or post-confluent fibroblasts were pre-incubated for 30 min in serum-free RPMI 1640 medium lacking phosphate and containing 20 mM HEPES, pH 7.4. This medium was then replaced with 3 ml/dish of the same medium supplemented with 0.4–1.0 mCi/ml [32P]orthophosphoric acid and incubation continued for 30 min with 20 μM NF-κB B oligonucleotide probe (IP buffer). Lysates were passed 10 times through a 25 gauge hypodermic needle, then twice with 50 mM Tris-HCl, pH 7.5, 20 mM NaCl, 2 mM EGTA, and supernatants were removed (cytoplasmic extracts). Pellets was resuspended in 200 μl of buffer B (20 mM HEPES, pH 7.9, 0.4 M NaCl, 1 mM EDTA, 1 mM EGTA, 1 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride, and 1 mM leupeptin). After 30 min at 4 °C, lysates were centrifuged and supernatant was removed (nuclear extract). Protein concentration of extracts was measured using the BCA assay (Pierce).

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**RESULTS**

The aim of this study was to determine if stimulation of HepG2 hepatoma cells or human gingival fibroblasts (HGF) with IL-1 leads to phosphorylation of other components of NF-κB besides the IkBα inhibitor family. In both of these cell types, as shown by EMSA supershift analysis (Fig. 1), all of the IL-1-inducible nuclear NF-κB complexes contain p65, and a majority contain p50. After treatment with IL-1 for various times, lysates from cells metabolically labeled with [32P]orthophosphate were prepared and immunoprecipitated with anti-p65 antibody. To control for the presence of nonspecifically recognized proteins, we carried out duplicate immunoprecipitations, one of which contained an aliquot of the peptide against which the anti-p65 antibody was raised. As reported by
that this phosphoprotein corresponded to IκB in blot analysis with a specific antibody (not shown), and we found and (less prominently) from HGF, a phosphoprotein of 40 kDa as indicated in Fig. 2. From lysates of untreated HepG2 cells and HGF, a phosphoprotein of 40 kDa was specifically immunoprecipitated. We performed Western blot analysis with a specific antibody (not shown), and we found that this phosphoprotein corresponded to IκB. After 5 min of IL-1 treatment, the 40-kDa species disappeared and was replaced by a slightly slower migrating species (shown as IκB(P) in Fig. 2) which also reacted with anti-IκB. At subsequent times, IκB was undetectable as either a labeled phosphoprotein (Fig. 2) or by Western blotting (not shown). These data are entirely consistent with previous reports (27–28) in which activators of NF-κB complexes was carried out by inclusion of specific antibodies directed against p65 or p50, as indicated.

NF-κB/Rel family members and the IκB are physically associated phosphoproteins and are readily co-precipitated as shown in Fig. 2. From lysates of untreated HepG2 cells and (less prominently) from HGF, a phosphoprotein of 40 kDa was specifically immunoprecipitated. We performed Western blot analysis with a specific antibody (not shown), and we found that this phosphoprotein corresponded to IκB. After 5 min of IL-1 treatment, the 40-kDa species disappeared and was replaced by a slightly slower migrating species (shown as IκB(P) in Fig. 2) which also reacted with anti-IκB. At subsequent times, IκB was undetectable as either a labeled phosphoprotein (Fig. 2) or by Western blotting (not shown). These data are entirely consistent with previous reports (27–28) in which activators of NF-κB were shown to increase the phosphorylation of IκB and thereby decrease its electrophoretic mobility. A protein that we tentatively identify as NF-κB p50 was transiently phosphorylated in both cell types following IL-1 treatment. Interestingly, Li et al. (16) have reported that p50 phosphorylation was increased by treatment of Jurkat cells with phorbol 12-myristate 13-acetate and phytohemagglutinin and that phosphorylated p50 exhibited more stable DNA binding than the unphosphorylated form. Our most striking observation was an IL-1-mediated increase in the level of p65 phosphorylation. Although barely detectable in unstimulated HepG2 cells or fibroblasts, p65 was the most prominent phosphorylated species in immunoprecipitates made from cells treated with IL-1 for 5 min. Phosphorylation of P65 was maximal after 15 min and still evident for as long as 60 min after cytokine addition (not shown). IL-1-stimulated phosphorylation of an unidentified co-precipitated protein of about 130 kDa was consistently observed in HepG2 cells but was not investigated further. We isolated p65 from IL-1-stimulated HepG2 cells for phosphoamino acid analysis and found that phosphorylation occurred exclusively upon serine residues (Fig. 3).

IL-1 is known to activate a number of cytosolic serine/threonine kinases, including members of the mitogen-activated protein kinase family (20), stress-activated protein kinase/p38/JNK family (21, 29–31), and a novel β-casein kinase (32–33). One of the preferred phosphorylation sites of the β-casein kinase purified from lungs of IL-1-injected rabbits is serine 57 of β-casein (34).5 The primary sequence surrounding this residue bears some resemblance to a sequence in human p65 immediately COOH-terminal to the Rel-homology domain, surrounding a serine at position 340. Specifically, both sequences have prolines at the −6, +4, +6, +8, and +10 positions relative to the serine residue and hydrophobic residues at the −8 and +1 positions. p65 also contains two potential serine phosphorylation sites (35) for casein kinase II (minimal recognition sequence: Ser/Thr-X-X-Glu [Asp]) located at amino acid positions 276 and 539. We reasoned that the IL-1-stimulated kinase present in NF-κB immunoprecipitates might be one of these activities and that if it remained complexed with NF-κB during immunoprecipitation, it should be possible to detect it based on its ability to phosphorylate exogenously added casein. Fig. 4a shows that extensively washed p65 immunoprecipitates from untreated HepG2 cells contain a kinase activity capable of phosphorylating both α- and β-caseins and that the level of this activity is markedly stimulated if the cells are first treated with IL-1. The association of kinase activity with NF-κB in the immunoprecipitates is specific since it is prevented by inclusion of excess immunizing peptide and visualized by phosphoimaging. Migration positions of 14C-labeled molecular weight standards (Amersham Corp.) are indicated on the right.

2 G. D. Virca and T. A. Bird, unpublished observations.

3 T. A. Bird and G. D. Virca, unpublished data.
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jected to chymotryptic digestion and two-dimensional phosphopeptide mapping. These maps were compared with maps of casein phosphorylated by authentic casein kinase II (Fig. 4b). Although some of the digestion products (particularly in the case of α-casein) remained at the origin, the resolvable phosphopeptides were found to be essentially identical. Both kinases phosphorylated α-casein predominantly on serine and β-casein on threonine residues, with less phosphorylation on serine (data not shown).

Taken together, these data confirm the identity of the NF-κB-associated kinase as casein kinase II. Because the anti-p65 antibody co-precipitates p50, and possibly other, associated proteins, it is not possible to determine from our data if p65 directly associates with the kinase. It is perhaps notable that the primary sequence of human p50 (37) also contains a potential casein kinase II phosphorylation site (SDLE at position 338–341) that is conserved in both the rabbit and mouse p50 genes (38).

It was important to determine if the kinase associated with NF-κB in cell extracts was the same as that responsible for its phosphorylation in intact cells. Accordingly, we compared the phosphopeptide patterns of p65 phosphorylated in IL-1-stimulated HepG2 cells with recombinant p65 phosphorylated in vitro by purified casein kinase II. Fig. 4c shows that casein kinase II efficiently phosphorylated recombinant p65 (samples of IL-1-induced rabbit β-casein kinase were also able to phosphorylate the recombinant protein, but at less than one-tenth the rate of casein kinase II, data not shown). To generate conveniently small fragments for mapping, phosphorylated p65 was digested sequentially with chymotrypsin and trypsin. In both samples, two major phosphopeptides (labeled x and y in Fig. 4d) exhibited no net charge, but migrated to a similar extent in the chromatographic dimension. A minor spot (z) was present in the in vitro labeled sample, but was barely visible in the in vitro phosphorylated material and may result from additional phosphorylation of p65 by a different kinase. In both samples, variable amounts of radioactive material remained at the origin, but the difficulty of preparing sufficient labeled p65 from cell cultures precluded further detailed analysis. It is not clear if casein kinase II phosphorylates p65 at either or both of the potential consensus phosphorylation sites described above, or indeed at a different site. Casein kinase II did not phosphorylate, in vitro, a recombinant p65 truncated to contain only the Rel homology domain,4 and so it is likely that the phosphorylation site(s) lie in the COOH-terminal portion of the molecule.

It is now appreciated that casein kinase II activity is acutely regulatable by components of signal transduction pathways. Thus, modest but rapid stimulation of casein kinase II activity can occur in response to growth factors including EGF (39),

\[ \text{Phosphorylation of NF-κB p65 occurs on serine residues.} \]

\[ { }^{32} \text{P-labeled p65 from untreated HepG2 cells (top panels) or cells treated for 15 min with IL-1 (bottom panels) was carefully excised from SDS-PAGE gels, extracted, and hydrolyzed. Samples were subjected to two-dimensional phosphoamino acid analysis and visualized using a PhosphorImager (left panels). Internal phosphoamino acid standards were detected using ninhydrin and are shown in the panels on the right (PS, phosphoserine; PT, phosphothreonine; PY, phosphotyrosine).} \]

\[ \text{Fig. 4. IL-1-treatment causes the association of casein kinase II with NF-κB complexes.} \]

\[ a, \text{NF-κB was immunoprecipitated from unstimulated or IL-1-treated (20 ng/ml, 15 min) HepG2 cells in the absence (–) or presence (+) of immunizing peptide as indicated. Equal amounts of the washed immunoprecipitates were assayed for associated kinase activity using α- and β-caseins as substrates. Reaction products were analyzed by SDS-PAGE. The assay was carried out in the absence (left panel) or presence (right panel) of 20 μg/ml heparin.} \]

\[ b, \text{two-dimensional chymotryptic phosphopeptide maps were prepared from caseins phosphorylated by the NF-κB co-precipitated kinase (right panels) or casein kinase II (left panels). Sample application points are indicated (ori.); electrophoresis was in the horizontal dimension (anode at left) and chromatography in the vertical dimension.} \]

\[ c, \text{phosphorylation of recombinant NF-κB p65 (150 ng) by purified casein kinase II (15 ng) in vitro.} \]

\[ d, \text{comparison of phosphopeptides generated by trypsin/chymotrypsin digestion of recombinant p65 phosphorylated in vitro by purified casein kinase II (left panel) and endogenous p65 immunoprecipitated from orthophosphate-labeled HepG2 cells stimulated for 15 min with IL-1 (right panel).} \]

4 T. A. Bird, K. Schooley, S. K. Dower, H. Hagen and G. D. Virca, unpublished results.
FIG. 5. IL-1 has a minimal effect on total cellular casein kinase II activity. a, detergent lysates were prepared from HepG2 after treatment for the indicated times with IL-1. Casein kinase II was immunoprecipitated with a mixture of polyclonal antibodies that recognize the α- and β-subunits and assayed using β-casein as a substrate. The inset shows the PhosphorImager output from a typical experiment. Mean results (± S.E.) from three independent experiments are plotted. b, after treatment of HGF cells with (bottom panel) or without (top panel) IL-1 for 15 min, cytosolic extracts were prepared and applied to a Mono-Q anion exchange column. The column was eluted with a linear NaCl gradient, and fractions were collected as described under “Experimental Procedures.” Each of the fractions was assayed for α- and β-casein kinase activities. The positions of IL-1-stimulated β-casein kinase and casein kinase II are indicated. Similar results were obtained with HepG2 cells.

insulin, and insulin-like growth factor 1 (40). Although the underlying mechanism is not completely understood, it may involve the phosphorylation of either casein kinase II itself or accessory factors (39). We therefore wished to test whether increased phosphorylation of p65 was attributable to an IL-1-stimulated increase in casein kinase II activity. After stimulation of HepG2 cells for various times with IL-1, whole-cell detergent lysates were prepared and immunoprecipitated with antibodies to casein kinase II. The washed immunoprecipitates were assayed for their ability to phosphorylate casein in an immune-complex kinase assay. The assay specifically measures casein kinase II since no activity could be detected in the presence of 20 μg/ml heparin or if non-immune serum was used (data not shown). IL-1 caused a somewhat variable but small increase (to 150% of control) in immunoprecipitable casein kinase II activity, evident after 5 min of stimulation (Fig. 5a). TNF-α was recently reported to increase casein kinase II activity measured in crude cytosolic extracts of Swiss 3T3 and L929 cells (41), the kinetics and magnitude of the reported effects were similar to our findings with IL-1. We also directly measured casein kinase II activity in cytosolic extracts of HGF after separation on an anion exchange column (Fig. 5b). Casein kinase II is detected as a sharp peak of predominantly α-casein phosphorylating activity that elutes at about 380 mM NaCl (fractions 21–24) and that is inhibited by low concentrations of heparin or unlabeled GTP (data not shown). In contrast to the immunoprecipitation assay data described above, and in agreement with data reported by others (33), a 15-min pretreatment with IL-1 did not increase this peak of casein kinase II activity. Similar results were consistently obtained with HepG2 and a variety of other cell lines (data not shown). The peak of β-casein-specific, IL-1-stimulated kinase which elutes in fractions 7–12 (Fig. 5b) corresponds to the activity described by Guesdon et al. (32–33). Taken together, the above experiments suggest that the small IL-1-induced increase in total casein kinase II activity is restricted to that fraction of the enzyme (presumably associated with nuclei, membranes, or other organelles) which is only extractable by detergent; although, we cannot rule out the possibility that IL-1 induces an alteration in a subset of the kinase molecules such that they are more reactive with our antisera. In either case, it seems unlikely that such a small effect on casein kinase II activity is alone sufficient to account for the dramatic increase in p65 phosphorylation.

The subcellular location of casein kinase II has been reported to vary between the cytoplasm and nucleus at specific phases of the cell cycle (42) or to be constitutively nuclear (43). In HepG2 cells and fibroblasts, NF-κB p65-containing complexes migrate into the nucleus upon IL-1 stimulation (Fig. 1). It is, therefore, possible that in resting cells, cytosolic NF-κB would be in a different subcellular location from casein kinase II and that their association occurs simply as a consequence of nuclear translocation of the transcription factor. Accordingly, cytosolic and nuclear extracts were prepared from HepG2 cells after various periods of IL-1 stimulation and immunoprecipitated with anti-p65 for determination of associated casein kinase II activity (Fig. 6, a and c). A casein kinase II-associated pool of NF-κB is established first in the cytoplasm and is detectable between 2 and 5 min after addition of IL-1. Accumulation of this form of NF-κB in the nucleus is delayed until the 15 min time point is reached, and peak levels of casein kinase II activity associated with both nuclear and cytoplasmic NF-κB are reached after 30 min of IL-1 treatment. The level of casein kinase II detectable in the nuclear fraction correlates well with the appearance of immunoreactive p65 in the nucleus, as shown by Western blotting (Fig. 6b). These data are consistent with a model in which casein kinase II associates with (and phosphorylates) cytoplasmic NF-κB which then translocates to the nucleus. An obvious mechanism, although difficult to directly test at the present time, would be one in which dissociation of IκB from p50/p65 heterodimers exposes a phosphorylation site on one or both subunits for constitutively active, cytoplasmic casein kinase II.

**DISCUSSION**

The results of the experiments described above suggest that NF-κB p65 is one of a number of transcriptional regulators that are physically associated with and phosphorylated by casein
isolated NF-κB analog. This is close to the size expected for the catalytic kinase II. Indeed, a 43-kDa moiety was affinity labeled using possible that the activity identified by this group was casein cAMP-dependent protein kinase. In light of our data, it is binding activity. These authors did not identify the kinase but assay and that phosphorylation correlated with increased DNA directly phosphorylate p65 and p50 during an human T cells was associated with a kinase activity that could transcriptional activity of NF-κB. Another important component of the regulation of p65 is cytosolic compartment.

Another important component of the regulation of p65 is interaction with cytosolic IκB, which both inhibits the DNA binding activity of the complexes and causes retention in the cytoplasm. It is therefore possible, since we have shown that phosphorylation of p65 by casein kinase II in intact cells takes place in the cytosol, that this phosphorylation might facilitate IκB dissociation and/or translocation of NF-κB into the nucleus. Alternatively, phosphorylation of p65 may inhibit the re-binding of IκB or other inhibitors. Finally, it is of interest that casein kinase II has recently been proposed by several groups as the kinase most likely responsible for constitutive phosphorylation of the COOH-terminal domain of IκB (51–53). This phosphorylation takes place on serines 283, 289, and 293 and on threonine 291. A requirement for these phosphorylations in both normal turnover and HIV-induced degradation of IκB was suggested by the observation (53) that immunodepletion of casein kinase II abrogated both processes in in vitro IκB degradation assays. Thus, it is possible that CKII may exert functionally opposite effects on NF-κB and its inhibitor.

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