A Selective IKK-2 Inhibitor Blocks NF-κB-dependent Gene Expression in Interleukin-1β-stimulated Synovial Fibroblasts*

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NF-κB-induced gene expression contributes significantly to the pathogenesis of inflammatory diseases such as arthritis. IkB kinase (IKK) is the converging point for the activation of NF-κB by a broad spectrum of inflammatory agonists and is thus a novel target for therapeutic intervention. We describe a small molecule, selective inhibitor of IKK-2, SC-514, which does not inhibit other IKK isoforms or other serine-threonine and tyrosine kinases. SC-514 inhibits the native IKK complex or recombinant human IKK-1/IKK-2 heterodimer and IKK-2 homodimer similarly. IKK-2 inhibition by SC-514 is selective, reversible, and competitive with ATP. SC-514 inhibits transcription of NF-κB-dependent genes in IL-1β-induced rheumatoid arthritis-derived synovial fibroblasts in a dose-dependent manner. When the mechanism of NF-κB activation was evaluated in the presence of this inhibitor, several interesting observations were found. First, SC-514 did not inhibit the phosphorylation and activation of the IKK complex. Second, there was a delay but not a complete blockade in IkBα phosphorylation and degradation; likewise there was a slightly slowed, decreased import of p65 into the nucleus and a faster export of p65 from the nucleus. Finally, both IkBα and p65 were comparable substrates for IKK-2, with similar Kₘ and Kₜ values, and SC-514 inhibited the phosphorylation of either substrate similarly. Thus, the effect of SC-514 on cytokine gene expression may be a combination of inhibiting IkBα phosphorylation/degradation, affecting NF-κB nuclear import/export as well as the phosphorylation and transactivation of p65.

NF-κB1 is an inducible transcription factor that regulates the expression of a wide variety of genes including those encoding cytokines, chemokines, adhesion factors, and inducible enzymes such as inducible nitric-oxide synthase and COX-2 (1–3). In addition, NF-κB is required for the activation of several genes involved in the regulation of apoptosis and cell proliferation (4). Thus, modulation of NF-κB activity represents an attractive target for therapeutic intervention of inflammatory diseases such as arthritis and asthma, both of which result from dysregulated immune processes.

The central dogma of NF-κB activation suggests that NF-κB is sequestered in the cytoplasm in resting cells by the inhibitory IkB proteins (5–8). In response to a variety of agonists, IkBα is rapidly phosphorylated, ubiquitinated, and degraded, thus releasing NF-κB for translocation into the nucleus to initiate gene transcription (1, 9–11). A number of recent studies, however, suggest that IkB degradation and nuclear translocation of NF-κB may not be the sole regulatory events in the transcription of NF-κB-dependent genes (12–14). Several investigators (15–18) have shown that upon NF-κB activation, newly synthesized IkBα molecules enter the nucleus, remove NF-κB from DNA, and transport the Rel proteins back to the cytoplasm. In addition, studies using leptomycin B, an inhibitor of nuclear export, have shown that even in unstimulated cells NF-κB-IkBα complexes continuously shuttle in and out of the nucleus (18–21). Finally, the transcriptional activity of NF-κB depends on the post-translational modification of p65 (12–14). For example, several kinases have been shown to phosphorylate p65 (22–27). PKA specifically phosphorylates p65 on serine 276, which enhances p65 binding to DNA and promotes transcriptional activation by recruiting the co-activator, CBP/p300 (22, 23). Likewise IKK-2 phosphorylates p65, albeit on a different serine residue (26). In addition, casein kinase II and PKCα are also thought to phosphorylate p65 (24–27). However, careful enzymatic analyses comparing these enzymes and their specificities against individual serine sites have not been conducted thus far. Although the sites of phosphorylation appear unique for each kinase implying unique functions, the physiologic role for each of these phosphorylation events requires further study.

IkB kinase (IKK) is the convergence point in most signaling pathways activated by many stimuli leading to the inducible phosphorylation and degradation of IkBα. IKK is a multisubunit complex that contains two catalytic subunits, IKK-1 and IKK-2, and the regulatory subunit IKKγ (1, 28–33). Gene knock out studies have clearly demonstrated that IKK-2 and IKKγ subunits of the IKK complex are required for NF-κB activation by all known pro-inflammatory stimuli including lipopolysaccharide (LPS), TNFa, and IL-1β (34, 35). Thus a selective inhibitor of IKK-2 would not only be of great interest as a potential anti-inflammatory agent but also as a valuable
tool to understand the mechanisms regulating NF-κB activation by these inflammatory agonists.

Here we describe a selective IKK-2 inhibitor, SC-514, and we use this inhibitor to study NF-κB activation in rheumatoid arthritis-derived synovial fibroblasts (RASF) cells that have been stimulated with IL-1β. We show that the inhibitor is selective for IKK-2 over 30 other kinases and that it binds specifically at the ATP-binding site of IKK-2. In addition, SC-514 inhibits NF-κB-driven gene expression in a dose-dependent fashion, as measured by a NF-κB-linked reporter gene or endogenous NF-κB-regulated genes. SC-514 is also efficacious in vivo in the rat LPS-induced TNFα model of acute inflammation. Finally, when the mechanism of NF-κB activation was dissected in the presence of this inhibitor, several interesting observations were noted. As expected, the IKK complex is activated normally in the presence of the IKK-2 inhibitor, and the activation of other MAP kinases pathways is likewise unaffected by SC-514. However, the deactivation of IKK-2, presumably via autophosphorylation of the C-terminal serines, may be activated normally in the presence of the IKK-2 inhibitor, and the activation of other MAP kinases pathways is likewise unaffected by SC-514. IKK-2 inhibition by SC-514 demonstrates a slow, decreased level of IκB phosphorylation/degradation and diminished p65 translocation into the nucleus in these cells at maximal kinase inhibition. Interestingly, p65 export out of the nucleus is hastened in the presence of SC-514. The phosphorylation of p65 by rIKK-2 occurs on serine 536 in the transactivation domain with similar catalytic efficiency as that for the IκBs, and SC-514 inhibits both IκB and p65 phosphorylation with comparable IC50 values. Thus, our studies with the selective IKK-2 inhibitor, SC-514, are consistent with the hypothesis that NF-κB activation in IL-1β-stimulated RASF cells is regulated by IKK-2 with novel mechanisms involving nuclear import/export and/or p65 transactivation in addition to the degradation of IκBs.

EXPERIMENTAL PROCEDURES

Materials

Nonidet P-40, BSA, ATP, ADP, LPS, protein A-agarose, anti-FLAG M2-agarose, actin antibody, and dithiothreitol were obtained from Sigma. COX-2 and phospho-HSP-27 antibodies, human and rat TNFα, and PGE2, ELISAs were obtained from Pharmacia Corp. Antibodies specific for IKKγ (FL-419), IKKβ (H-470), IκB-α (C-21), phospho-IκBα (B-9), p65 (F-6), p50(NLS), p53 (C-20), ERK2 (C-14), HSP-27 (C-20), c-Jun (H-79), and phospho-c-Jun (RM-1) were obtained from Santa Cruz Biotechnology. COX-1 antibody was obtained from Oxford. Phospho-p38, phospho-ERK, and phospho-IKK2/1 were from Cell Signaling, and secondary antibodies were supplied by Jackson Immunoresearch. C1AF, DMEM, gentamicin, T4 polynucleotide kinase, nitrocellulose, and secondary antibodies were supplied by Jackson ImmunoResearch. Phospho-p38, phospho-ERK, and phospho-IKK1/2 were from Cell Signaling, and secondary antibodies were supplied by Jackson Immunoresearch. C1AF, DMEM, gentamicin, T4 polynucleotide kinase, nitrocellulose, and secondary antibodies were supplied by Jackson ImmunoResearch.

Cell Culture—Adherent RASF cells were isolated from enucleated eyes of 2-month-old BALB/c mice, and maintained in a humidified atmosphere of 5% CO2 and 95% air at 37°C in DMEM supplemented with 10% fetal bovine serum (FBS), 50 μg/mL gentamicin, 4 mM L-glutamine, and 1 mM sodium pyruvate. For experiments involving IL-1β, RASF cells were incubated with 1 ng/mL IL-1β for 1 h prior to the addition of inhibitors. The cells were then cultured in DMEM containing 1% serum prior to stimulation.

Preparation of Cell Extracts for Western and EMSA Analysis—Confluent RASF cells were pre-treated with SC-514 (1–100 μM) or 0.2% MeSO vehicle and stimulated with IL-1β for 4 h. Total RNA was isolated from RASF cells using an RNeasy Mini Kit according to the manufacturer’s instructions, which included a DNase step. Purified RNA was quantified using a Beckman DU640B spectrophotometer. 200 ng of total RNA was used to determine expression levels of human IL-6, IL-8, and COX-2 by Taqman™ analysis using a 7700 Sequence Detection System (Applied Biosystems). Relative expression levels were normalized to the amount of cyclophilin mRNA. Primer/probe sets specific for human IL-6, IL-8, and COX-2 were designed using Primer Express™ Software (Applied Biosystems) based on published sequences.

Western Analysis of NF-κB Translocation—RASF cells in 96-well plates were pre-treated with SC-514 in 0.2% MeSO or 0.2% MeSO for 1 h prior to stimulation with 1 ng/mL IL-1β for various times. After stimulation, cells were fixed in 3.7% formaldehyde for 10 min, permeabilized in 0.5% Triton X-100 in PBS, and blocked with 5% BSA followed by 5% goat serum for 30 min each. Cells were stained by incubating with an antibody specific primary antibody at 1:1000 for 1 h, washed in 0.1% Triton X-100, followed by incubation with a goat anti-rabbit fluorescein isothiocyanate conjugate at 1:100 and Hoechst stain at 1:2000 for 1 h. Cell images were acquired, analyzed, and quantified for NF-κB translocation using ArrayScan™ HSC software package.

RNA Expression Analysis—Confluent RASF cells were pre-treated with SC-514 (1–100 μM) or 0.2% MeSO vehicle and stimulated with IL-1β for 4 h. Total RNA was isolated from RASF cells using an RNeasy Mini Kit according to the manufacturer’s instructions, which included a DNase step. Purified RNA was quantified using a Beckman DU640B spectrophotometer. 200 ng of total RNA was used to determine expression levels of human IL-6, IL-8, and COX-2 by Taqman™ analysis using a 7700 Sequence Detection System (Applied Biosystems). Relative expression levels were normalized to the amount of cyclophilin mRNA. Primer/probe sets specific for human IL-6, IL-8, and COX-2 were designed using Primer Express™ Software (Applied Biosystems) based on published sequences.

AdNF-κB-SEAP Construct and SEAP Assay—A secreted alkaline phosphatase (SEAP) reporter gene containing an upstream response element with three tandem NF-κB consensus binding sites was cloned into an adenovirus transfer vector, AdBM5. The resulting plasmid was used to co-transform 293 cells with adenovirus DNA to generate recombinant adenovirus particles containing the SEAP reporter construct. Purified virus was produced from three rounds of plaque purification followed by CaPO4 gradient purification. SEAP virus stock was used to transduce adherent RASF cells at a multiplicity of infection of 10 and 24 h prior to stimulation with IL-1β. Production of SEAP was measured in the media after 20 h of IL-1β stimulation.

Preparation of Cell Extracts for Western and EMSA Analysis—Confluent RASF cells were pre-treated for 1 h with vehicle or inhibitor (Z-LLLH, 25 μM; EII-278, 10 μM, and SC-514, 1–100 μM) and then stimulated with 1 ng/mL IL-1β. Whole-cell lysates were prepared for Western analysis at the indicated times by washing once in cold phosphate buffer and incubating the cells on ice for 30 min in whole-cell lysis buffer (10 mM HEPES, 50 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1 mM NaVO3, 10 μM β-glycerophosphate, 1 μM NaF, 1 μM phenylmethylsulfonyl fluoride, 1 μM DTT, and 0.5% Nonidet P-40). Lysates were prepared for IKK-2, IκBα, and p65 Westerns after 5 min of stimulation unless a time course was performed. Lysates were prepared after a 15-min stimulation for the analysis of p38, HSP-27, c-Jun, and ERK. Lysates for Western analysis of COX isoforms were made after 20 h of stimulation. Nuclear lysates for p65 Western analysis and EMSA were prepared using a protocol modified from the method described by Schreiber et al. (38). Briefly, cells were allowed to swell on ice in a low salt buffer (10 mM HEPES, 10 mM KCl, 1 mM EDTA, 1 mM EGTA, 1 mM DTT) for 15 min, after which Nonidet P-40 was added to a final concentration of 0.5%. The cell suspension was pipetted several times to disrupt the cells, and intact nuclei were pelleted by centrifugation. Nuclei were washed once in low salt buffer and resuspended in high salt buffer (20 mM HEPES, 500 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1 mM DTT, and protease inhibitors) for 30 min on ice. Whole-cell and nuclear lysates were cleared of precipitate by centrifugation at 13,000 × g.

Western Analysis—2× Laemmli sample buffer was added to cell lysate and boiled for 2 min. Equal amounts of protein (30–50 μg of lysate or immunoprecipitated IKKks) were separated by SDS-PAGE (8% IKK-2, 10% p65, c-Jun, and COX isoforms, and 12% IκB, HSP-27, and p38). Proteins were transferred to nitrocellulose membranes. The membranes were then blocked in 5% dry milk reconstituted in Tris-buffered saline (100 mM Tris, pH 8.0, 150 mM NaCl) with 0.05% Tween 20 (2% milk) or 5% milk for 30 min at room temperature. Blots were then incubated overnight with primary antibodies (1:300, phospho-IKK1/2; 1:1000, IκBα, phospho-IκBα, IKK-2, p65, HSP-27, phospho-ERK, phospho-c-Jun, c-Jun, phospho-p38, COX-2: 1:5000, p38, ERK, phospho-HSP-27, COX-1) in 1% milk/TBST. The blots were washed 4 times in TBST,
incubated with peroxidase-conjugated goat anti-rabbit or goat antimouse secondary antibodies (1:4000) for 1 h, and washed as above. Enhanced chemiluminescence (ECL plus) was used for detection.

**Electrophoretic Mobility Shift Assay (EMSA)—**Nuclear extracts were prepared as described above and frozen at −80 °C until use. NF-κB probe was generated in a 10 μl reaction containing 20 ng of NF-κB double-stranded oligonucleotide, 2 μl of [γ-32P]-ATP (3000 Ci/mmol), 1 μl of T4 polynucleotide kinase, and nuclease-free H2O for 30 min at 37 °C. Unincorporated [γ-32P]-ATP was removed with a G-50 spin column. For the binding assay, 5 μg of nuclear extract was preincubated in 10 μl Hepes, pH 7.7, 10% glycerol, 50 mM NaCl, 0.5 mM MgCl2, 1 mM DTT, and 2 μg of poly(dI-dC) for 30 min on ice. 1 μl of unlabeled NF-κB double-stranded oligonucleotide (cold competition) or 1 μl (0.2 μg) of anti-p50 or anti-p65 antibodies (supershift) were added to the pre-incubations. Then 1 μl (50,000 CPM) of 32P-oligonucleotide probe was generated in a 10 μl reaction containing 20 ng of poly(dI-dC) for 30 min on ice. The gel was dried, and NF-κB binding was visualized by autoradiography.

**Kinase Assay—**IKK complexes were immunoprecipitated from IL-1β-treated RASF cell lysates (0.5–2 mg) using a NEMO antibody (3–10 μg) followed by the addition of protein A-agarose beads. Antibody complexes were pelleted by centrifugation and washed 3 times with 1 ml of cold whole-cell lysis buffer followed by 2 washes in kinase buffer (25 mM Hepes, pH 7.6, 2 mM MgCl2, 2 mM MnCl2, 10 mM NaF, 5 mM DTT, and 1 mM phenylmethylsulfonyl fluoride). 100–200 μg of immunoprecipitated IKK was analyzed for kinase activity in a reaction containing 10 μM biotinylated IbεBo peptide as substrate and 1 μM [γ-32P]-ATP (2500 Ci/mmol) as described previously (36, 37). After incubation at room temperature for 30 min, 25 μl of the reaction mixture was withdrawn and added to a SAMTM 96 biotin capture plate. After successive wash steps the plate was allowed to air-dry, and 25 μl of scintillation fluid was added to each well. Incorporation of [γ-32P]-ATP was measured using a Top-Count NXT (Packard Instrument Co.). IC50 determinations of rhIKK-2 have been described previously (36, 37). Briefly, various concentrations of [γ-32P]-ATP or peptide substrates were used in the assay at a fixed (3× Kcat) concentration of the substrate and 100 ng of rhIKK-2 in a final volume of 50 μl. Following incubation for 30 min at 25 °C, the reaction was stopped by the addition of 150 μl of AG1X8 resin in 900 mM sodium formate buffer, pH 3 (the resin is in slurry of 1 volume resin to 2 volume of sodium formate buffer). The resin was allowed to settle, and 50 μl of supernatant was transferred to a top count plate followed by the addition of 150 μl of Microscint 40, mixed well, and counted. For p65 FL-GST, 0.043–2.72 μM concentrations were used. Once Ki values were calculated using GrafitTM program, IC50 values were calculated from Vmax values and expressed as micromolar of enzyme/h.

**ATP Binding Assay—**The binding of ATP and inhibitors to IKK-2 was analyzed using FLAG-tagged rhIKK-2 immobilized on anti-FLAG M2-agarose. 30 μg of rhIKK-2 was incubated with anti-FLAG M2-agarose (16 μl of anti-FLAG agarose/μg of rhIKK-2) in 5–3 ml of ELISA buffer (20 mM Tris-HCl, pH 7.2, 150 mM NaCl, 0.1% BSA, and 0.05% Tween 20) for 2 h at 4 °C. The immobilized IKK-2 was pelleted, washed once in ELISA buffer and once in kinase buffer, and resuspended in kinase buffer at a concentration of 12.5 μg/ml. Binding assays were performed in 96-well Millipore MultiScreen plates. The binding reaction consisted of 250 ng of rhIKK-2 and varying concentrations of γ-32P-ATP or inhibitors in 50 μl total volume of kinase buffer. Nonspecific binding was determined by the addition of 1000× unlabeled γ-32P-ATP. The binding reactions were allowed to proceed for 2 h at 4 °C and then washed under vacuum with 200 μl of cold PBS. The filter plates were air-dried, and 30 μl of scintillation fluid was added to each well. The amount of bound γ-32P-ATP was determined using a Top-Count NXT (Packard Instrument Co.).

**Phosphatase Treatment of IKK-2—**rhIKK-2 and native IKK kinase complexes were subjected to phosphatase treatment as described previously (36). Briefly, 4–5 μg of rhIKK-2 or 0.5 mg of native IKK complex were immunoprecipitated as described above, washed, and resuspended in 50 mM Tris-HCl, pH 7.6, 0.1 mM EDTA, and 2 mM MnCl2. The kinase was then incubated for 30 min at room temperature in the presence of calf intestinal alkaline phosphatase or a phosphatase (80 units of CIAP or 1000 units of λ phosphatase per 2 μg of native kinase, 500 units of λ phosphatase per μg of rhIKK-2). Phosphatase was removed from the immobilized kinase by washing three times and resuspending in kinase buffer, and the immunoprecipitated IKK was subjected to kinase or binding assays as described above.

**Rat Model of Acute Inflammation, LPS-induced Serum TNFα—**SC-514 or vehicle (2% Me2SO in saline) was administered either by oral gavage or intraperitoneally to adult male Wistar rats that had been deprived of food overnight. Two hours after compound treatment, 1 mg/kg LPS (Escherichia coli) in saline was administered intraperitoneally 90 min after LPS administration; the animals were bled and serum TNFα levels analyzed by a rat-specific TNFα ELISA.

**RESULTS**

We first characterized the selective inhibitor, SC-514, enzymatically (Fig. 1A). We determined that SC-514 inhibited all forms of recombinant human IKK-2 including rhIKK-2 homo- dimer, rhIKK-1/rhIKK-2 heterodimer, as well as the constitutively active form of rhIKK-2 (Ser177-Glu, Ser186-Glu) with comparable IC50 values in the 3–12 μM range. Furthermore, SC-514 inhibited the activated, native IKK complex immunoprecipitated from IL-1β-stimulated RASF cells with similar potency (Fig. 1, A and B). Note that the other IKK isoforms, rhIKK-1, rhIKK-1, or rhTBK-1 were not inhibited by SC-514. Also note that the native immunoprecipitated kinase can be
Small Molecule IKK-2 Inhibitor

Fig. 2. Inhibition pattern of SC-514 at the ATP and IxB sites. Kinetic rates of IKK-2 were measured as described as described under “Methods” with varying concentrations of SC-514 (0–40 μM) and with either ATP (A) or IxB (B) as the variable substrate, although the fixed substrate was held constant at 5 μM.

Completely inhibited by this selective IKK-2 inhibitor, implying IKK-1 activity within this complex is not enzymatically significant. In addition, SC-514 was >10-fold selective against 28 other kinases, including both tyrosine kinases and other serine-threonine kinases (Fig. 1C).

SC-514 showed competitive inhibition with respect to the ATP site and non-competitive inhibition with respect to the IxB site (Fig. 2). This suggested that SC-514, although not an analog of ATP, occupies the ATP-binding site of IKK-2. To address this, we developed a direct ATP binding assay using the non-hydrolyzable γ-35S-ATP as the ligand (Fig. 3A). The binding of γ-35S-ATP to rhIKK-2 was specific and saturable, with a Kd = 0.05 μM. Binding was reversible, because >80% of γ-35S-ATP was dissociated with the addition of excess unlabeled ligand (Fig. 3B). Binding of γ-35S-ATP also occurred only with the active, phosphorylated form of rhIKK-2. We have shown previously that the rhIKK-2 expressed in a baculovirus system is phosphorylated and active (36, 37). As demonstrated previously, phosphatase-treated rhIKK-2 had no kinase activity, and Western blot analysis confirmed that equal amounts of rhIKK-2 were immunoprecipitated and assayed (Fig. 3C).

When the binding assay was performed on the phosphorylated versus the de-phosphorylated forms of rhIKK-2, only the active, phosphorylated form of rhIKK-2 bound the γ-35S-ATP ligand, because treatment of rhIKK-2 with a phosphatase abolished all specific binding (Fig. 3D). Using this direct binding assay, we next showed that SC-514 competitively inhibited the binding of γ-35S-ATP to rhIKK-2 (Fig. 3E). Note that the Kd value of SC-514 in the direct binding assay was comparable with the Kd value determined by kinetic analysis (Fig. 3F). βγ-Methylene ATP and ADP were also analyzed in each assay and showed that inhibitors with different IC50 values against rhIKK-2 could be clearly delineated in this binding assay. Taken together, these results demonstrate that SC-514 is a selective, competitive inhibitor of the ATP site of activated rhIKK-2.

We next studied the effects of SC-514 on NF-κB-dependent gene transcription in IL-1β-stimulated RASF cells in vitro (Fig. 4). SC-514 demonstrated a dose-dependent inhibition of three representative NF-κB-induced genes, namely IL-6, IL-8, and COX-2 whether measured by RNA or protein. The IC50 values for IL-6 and IL-8 RNA expression were comparable (IC50 = 20 μM), although the inhibition of COX-2 RNA expression was slightly lower (IC50 = 8 μM). Likewise, comparable IC50 values were seen when the gene products were measured for IL-6, IL-8, and PGE2 (Fig. 4B). Note again that PGE2 production was inhibited with slightly greater potency compared with IL-6 and IL-8. Finally, when the RASF cells were transduced with an adenoviral vector containing a specific NF-κB-linked reporter gene (SEAP) and stimulated with IL-1β, SC-514 showed a dose-dependent inhibition of the reporter gene protein expression; the IC50 was similar to those seen for the endogenous genes measured (Fig. 4C). There was minimal cellular toxicity, as measured by the MTT assay, at concentrations of SC-514 that resulted in complete inhibition of NF-κB-driven transcription. Furthermore, the IC50 values for SC-514 observed in the IL-1β-stimulated RASF cells were in agreement with the IC50 value of SC-514 on the rhIKK-2 but well below the IC50 values of the other recombinant kinases such as PRAK and mitogen and stress-activated protein kinase (Fig. 1C). Together, these results show that the selective IKK-2 inhibitor, SC-514, inhibits NF-κB-dependent gene expression in RASF cells induced with IL-1β.

We next evaluated the cellular selectivity of SC-514 on the NF-κB pathway compared with other MAP kinase pathways in IL-1β-stimulated RASF cells (Fig. 5). First, we measured the activity of the endogenous native IKK complex by both kinase activity and Western analysis (Fig. 5A). Note that the IL-1β-stimulated IKK kinase activity was associated with a slowed migration or “bandshift” by Western analysis. Both the kinase activity and bandshift were abrogated to unstimulated levels by treatment with phosphatase (CIAP) (Fig. 5A). Stimulation with LPS, an agonist that does not induce IL-8 or PGE2 expression in RASF cells, failed to activate IKK and did not result in a bandshift. Next, we compared the cellular selectivity of SC-514 and other known nonspecific inhibitors of the NF-κB pathway, E-278 and the proteosome inhibitor, Z-LLLH (Fig. 5B, 39, 40). Treatment with SC-514 did not significantly affect activation of IKK as assessed by kinase activity or Western analysis, suggesting that the phosphorylation of IKK occurred normally in the presence of SC-514 (Fig. 5B). Also, the inhibition of the kinase within the cell is exemplified by the inhibition of both IxBα degradation and p65 translocation, whereas the ex vivo kinase activity is not inhibited because the compund dissociates from the IKK complex during immunoprecipitation. Interestingly, E-278, which has been shown to inhibit NF-κB activation, inhibited IKK activation, demonstrated by the lack of kinase activity and the absence of a bandshift in the Western analysis. As expected, Z-LLLH did not inhibit IKK activation in either assay.

Consistent with the effects on IKK, both E-278 and SC-514 blocked the phosphorylation and degradation of IxBα and also reduced the level of translocation of p65 into the nucleus in IL-1β-treated RASFs (Fig. 5B). As expected, Z-LLLH treatment demonstrated a prominent phospho-IxBα band (doublet in the
Western blot) and also inhibited the translocation of p65 into the nucleus, thus validating the mechanism of action of this inhibitor. Note that, although each inhibitor works via a unique molecular mechanism within the NF-κB pathway, the blockade of NF-κB activation resulted in inhibition of COX-2 protein induction. SC-514 did not inhibit the other MAP kinase pathways that are activated by IL-1β, including p38, MK-2, c-Jun N-terminal kinase, or ERK, because phospho-p38, phospho-Hsp-27, phospho-Jun, and phospho-ERK levels were all unaffected by treatment with SC-514 (Fig. 5B). These cellular data further validate the selectivity of SC-514 for IKK-2 at the concentration used in these experiments.

We further characterized the activation of IKK-2 in the absence and presence of SC-514 in RASF cells. First, we showed that the activation of IKK-2 by upstream kinases in response to IL-1β was unaffected in the presence of SC-514, as determined by the ex vivo immunoprecipitated IKK activity and by Western blot analysis (Fig. 6A). Note that the IL-1β-stimulated IKK activity, both in the presence or absence of SC-514, was phosphatase-sensitive, suggesting that the canonical MAP activation loop was phosphorylated in the presence of an IKK-2 inhibitor.

IKK activation in response to IL-1β was rapid and transient, with peak activity occurring within 5–15 min, followed by a rapid reduction in activity within 40 min (Fig. 6B). In the presence of SC-514, the kinetics of activation and inactiva-
tion were comparable with the IL-1β control. However, the specific activity of the kinase was augmented 3–5-fold when assayed \textit{ex vivo}, presumably after removal of the rapidly reversible SC-514 during immunoprecipitation (Fig. 6B, upper panel). Note that the amount of IKK protein was constant by Western analysis throughout the time course in the absence and presence of SC-514, even though the kinase activity was transient (Fig. 6B, lower panel). In addition, phosphorylation of the IKK complex analyzed by Western analysis with a phospho-specific antibody for IKK was consistent with the transient kinase activity (Fig. 6B, lower panel). These data confirm that SC-514 does not effect the activation of the IKK complex by upstream kinases.

Surprisingly, inhibition of phosphorylation and degradation of IκBα was only observed early in the time course of activation (5 min), and degradation of IκBα still occurred in the compound-treated RASF cells by 15 min (Fig. 6B). Resynthesis of IκBα was detectable by 45 min in control and treated
cells. When we further studied the IκBα phosphorylation, several interesting observations were noted. First, the phosphorylation and degradation of IκBα was dose-dependently inhibited with increasing concentrations of SC-514 from 10 to 300 μM (Fig. 6C, upper panel). Second, to better understand the inhibition of IκBα phosphorylation by SC-514, we treated the cells with the proteosome inhibitor, Z-LLLH, to block the degradation of phosphorylated IκBα, and we then evaluated the kinetics of IκBα phosphorylation in RASF cells by Western analysis for phosphorylated IκBα (Fig. 6C, lower panel). In IL-1β-treated RASF cells, IκBα phosphorylation was seen as early as 3 min, peaked at 5–15 min, and by 45 min had decreased substantially. Again, SC-514 did not completely block the phosphorylation of IκBα but resulted in a significant delay and decrease in phospho-IκBα. Note that at 300 μM of SC-514, complete blockade of IκBα phosphorylation was seen at 5 min, but only partial inhibition was seen at 15 min. Recall from Figs. 1 and 4 that a concentration of 100 μM SC-514 was sufficient to inhibit completely IKK-2 activity in vitro, as well as the NF-κB-driven gene transcription in the RASF cells, respectively. These results suggested that the inhibition of IκBα phosphorylation and degradation is likely...
only one aspect of the IKK-2-dependent regulation of NF-κB-dependent gene transcription. Likewise, because this inhibitor does not block IKK-1 activity, the IκBα may still be phosphorylated by this IKK isoform.

To assess other potential mechanisms by which IKK-2 may regulate NF-κB activation, we evaluated p65 nuclear translocation by three independent assays, EMSA, Western analysis, and nuclear immunolocalization within RASF cells (Fig. 7). Western analysis and DNA binding by EMSA analysis suggested there was a slight delay in p65 translocation by SC-514 at 5 min. Consistent with the minimal effect on IκBα degradation, the amount of p65 in the nucleus was virtually unaffected by SC-514 at 15 and 45 min. However, a dramatic difference was observed in SC-514-treated cells by 60 min post-IL-1β stimulation, namely a reduction in the level of nuclear p65 was noted either by EMSA or Western analysis (Fig. 7). These observations suggest that a more rapid nuclear export of p65 occurred in the presence of IKK-2 inhibition with SC-514, which persisted through 90 min (Fig. 7). This effect of SC-514 could be visualized by immunolocalization of p65; at 45 min both treated and untreated cells demonstrated nuclear staining, while by 90 min the SC-514-treated RASF cells demonstrated little nuclear staining compared with untreated cells. These data suggest that the equilibrium favors export of p65 out of the nucleus in SC-514-treated cells. These observations are consistent with recent findings from several laboratories, which suggest that NF-κB-IκB complexes shuttle rapidly between the nucleus and cytoplasm and imply that post-translational modifications of p65 by IKK-2 may regulate gene transcription by such a mechanism (12–14, 18–21).

Because p65 has been shown to be a substrate for IKK-2, we further characterized the phosphorylation of both truncated peptides as well as the full-length p65 (FL-p65) protein by rhIKKs enzymatically. We directly compared these p65 peptides with IκBα or IκBβ as substrates for rhIKK-2 in terms of both $K_{m}$ and catalytic efficiency (Table I). We also evaluated other isoforms of IKK (TBK-1, IKK-1, and IKK-i) with each substrate to understand the substrate specificity within the IKK family of proteins. First, full-length p65 (FL-p65) was a more efficient substrate for rhIKK-2 compared with FL-IκBα or FL-IκBβ as determined by the 4-fold higher catalytic efficiency ($K_{cat}/K_{m}$, Table I). In contrast to rhIKK-2, p65 was not a very efficient substrate for rhIKK-i, whereas IκBα was a far superior substrate with a catalytic efficiency that was 20-fold higher than that for IκBα or p65. Interestingly, rhTBK-1 utilized p65 as a substrate but rhIKK-1 did not. These data demonstrate the importance and validity of this type of careful enzymatic analysis of substrate selectivity and may help elucidate the unique roles of each IKK isoform within the NF-κB pathway.

We mapped the phosphorylation site(s) by mass spectrometry analysis of phosphopeptides generated by protease digestion of FL-p65 phosphorylated in vitro by rhIKK-2, and we showed that serine 536 in the transactivation domain was the sole site phosphorylated by rhIKK-2 (data not shown).

Second, when p65 peptides encompassing residues 522–540 containing three serines at positions 529, 535, as well as 536 were evaluated, the same specificity was observed as seen with FL-p65 (Table I). Note that substitution of alanine at serine 536 was the only substitution resulting in the loss of substrate competency. These results are consistent with those reported by Sakurai et al. (26) who showed that both rhIKK-2 and rhIKK-1 phosphorylated p65 on serine 536 and extend those results by demonstrating that the catalytic efficiency for rhIKK-2 is much higher than that for rhIKK-1. Of interest, phosphopeptide analysis of p65 phosphorylated in vitro by TBK-1 also showed serine 536 to be the only residue phosphorylated (data not shown). Other investigators have reported direct phosphorylation of p65 by PKA on serine 276 and by casein kinase II on serine 529 (23, 24, 27). We have confirmed these specificities by specific peptide substrate analysis (Table I) and verified that rhIKK-2 does not phosphorylate these peptides. Thus, our results show that p65 is an efficient sub-

**FIG. 7.** SC-514 inhibition of IKK-2 activity decreases the IκBα phosphorylation/degradation, as well as decreases the length of time NF-κB is in the nucleus. EMSA analysis, nuclear p65 Western analysis, and cytosolic IκBα degradation was analyzed from RASF cells pre-treated with 100 μM SC-514 and then stimulated for indicated times with IL-1β. Nuclear extracts were prepared and subjected to EMSA analysis as described under “Methods.” NF-κB binding was visualized by autoradiography. Nuclear translocation was also visualized using a p65 fluorescence assay as described. Each experiment was repeated 3 times with similar results. n.s., not significant.
Small Molecule IKK-2 Inhibitor

**Table I**

| Sequence | IKK-2 | | | | | IKK-1 | | | |
|----------|-------|-------|-------|-------|-------|-------|-------|-------|-------|
|          | $K_m$ | $K_{cat}$ | $K_{cat}$ | $K_m$ | $K_{cat}$ | $K_{cat}$ |
| 0.23 ± 0 | 27 ± 4 | 118 | >40 | 219 ± 6 | 2575 ± 719 | 12 |
| 0.67 ± 0 | 40 ± 6 | 60 | 9 ± 4 | 2057 ± 595 | 223 |
| 0.8 ± 0.2 | 31 ± 0.9 | 38 | 6 ± 0.8 | 2081 ± 176 | 332 |
| 0.5 ± 0.1 | 24 ± 1.6 | 48 | 6 ± 0.8 | 2081 ± 176 | 332 |
| 0.3 ± 0.1 | 34 ± 4 | 110 | >50 | 47 ± 9 | 2181 ± 145 | 46 |
| 0.2 ± 0.6 | 37 ± 4 | 185 | 42 ± 9 | 2233 ± 171 | 53 |
| 0.2 ± 0.6 | 44 ± 8 | 200 | 43 ± 9 | 1898 ± 114 | 44 |
| 0.3 ± 0.1 | 38 ± 2 | 136 | 35 ± 3.5 | 1764 ± 33 | 50 |
| 0.25 ± 0.01 | 41 ± 0.9 | 162 | 60 ± 2 | 595 ± 223 | 12 |

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The inhibition of NF-κB by SC-514, a potent anti-inflammatory drug target in vivo.

**Discussion**

NF-κB regulates the expression of cytokines, chemokines, adhesion factors, and inducible pro-inflammatory receptors. Thus, inhibition of NF-κB activation represents a compelling rationale for the development of a novel anti-inflammatory agent. In fact, several anti-inflammatory agents, namely aspirin, sulfasalazine, and steroids, have been suggested to act at least in part by inhibiting NF-κB activation (41-43). In this report, we have described the characterization of a selective IKK-2 inhibitor, SC-514, and show that it inhibits the activity of the IKK complex and consequently inflammatory gene expression in IL-1β-stimulated RASF cells. We have demonstrated that SC-514 inhibits IKK-2 activity selectively, compared with at least 30 other kinases including those in the MAP kinase inflammatory pathways such as p38, c-Jun N-terminal kinase, and ERK. Furthermore, this selectivity can be maintained in IL-1β-stimulated RASF cells. The demonstrated selectivity of SC-514 validates its use as a pharmacological tool to dissect the effects of IKK-2 inhibition on the activation of the NF-κB pathway in a cell-based system.

We used SC-514 to examine the role of IKK-2 in the activation of NF-κB in IL-1β-stimulated RASF cells, and we made several unexpected observations. First, although IκBα phosphorylation was inhibited in response to SC-514, demonstrating an inhibition of IKK-2 activity, the ex vivo activity of the immunoprecipitated kinase was augmented 3-5-fold in SC-514-treated cells. SC-514 is a reversible inhibitor of IKK-2, and therefore is removed from the IKK complex during immunoprecipitation. One possible explanation is that inactivation of IKK-2 by autophosphorylation of its C terminus cannot occur in the cell when the inhibitor is present in the active site, resulting in a kinase activity that is augmented in comparison to control when the IKK complex is immunoprecipitated. These results are consistent with those reported by Delhase et al. (44) who demonstrated that the overexpression of a mutated IKK-2, in which 10 of the C-terminal serines were replaced by alanine residues, resulted in a more active kinase. Replacing these serines with glutamic acid resulted in decreased IKK-2 activity (44). These data led the authors to hypothesize that the inactivation of IKK activity in the cell is due to a combination of self-inactivation by autophosphorylation of the C-terminal serines in IKK-2, as well as by the recruitment and action of a phosphatase removing the phosphates from the serine residues in the activation MAP kinase kinase loop. Our results support both mechanisms of IKK inactivation. The early ex vivo augmentation of IKK kinase activity is the result of SC-514 inhibi-
tion of IKK-2 autophosphorylation in the C terminus, but the ultimate inactivation of IKK-2 occurring by the action of phosphatase(s) is unaffected by the IKK-2 inhibitor. A direct determination of the phosphorylation of the C-terminal serines of IKK-2 isolated from SC-514-treated cells would be necessary to confirm this hypothesis.

The second unexpected finding in our studies was that SC-514 caused a dose-dependent delay and significant inhibition in IkBα phosphorylation and degradation, but did not result in a complete blockade of IkBα degradation in IL-1β-stimulated RASF cells. The maximal inhibition of IkBα phosphorylation occurred at SC-514 concentrations that completely inhibited NF-κB gene transcription. Likewise, p65 nuclear translocation and DNA binding were also delayed but not completely inhibited. These data are in contrast to results obtained from the use of dominant-negative constructs of either IKK-2 and IkBα, which can completely block IkBα degradation and p65 nuclear translocation (1, 2). However, the amount of overexpression of the dominant-negative proteins is quite high compared with the endogenous protein and thus may have an impact on the integrity of the IKK complexes, impacting IKK isoforms other than IKK-2. In contrast, a small molecule inhibitor such as SC-514 has the advantage in that it does not disrupt the IKK signalosome, allowing for the analysis of potentially unique mechanisms that may be masked by large amounts of overexpressed protein. In the these studies, p65 was found to exit the nucleus more rapidly in the presence of IKK-2 inhibition with SC-514. These results suggest that IkBα phosphorylation and degradation and the subsequent translocation of p65 into the nucleus may be only one point of regulation by IKK-2 in NF-κB activation. In fact, recent reports suggest that NF-κB-IkB complexes shuttle rapidly between the nucleus and cytoplasm and that after cellular activation, DNA binding, transcriptional initiation, as well as the kinetics of nuclear import/export may be additional mechanisms to control NF-κB gene transcription (12–14, 45). Several investigators have shown that p65 transcriptional activity is regulated by inducible phosphorylation. For example, serine 276 in p65 has been shown to be phosphorylated by PKA as well as by mitogen and stress-activated protein kinase, which leads to enhanced DNA binding and recruitment of the transcriptional co-activator, CBP/P300 (23, 24). Other investigators have shown that casein kinase II and even the IKK complex itself specifically phosphorylate p65 on serine 529 and serine 536, respectively (26, 27). Our results, comparing the direct kinetic measurements between these different IKK kinases, demonstrate that p65 is an efficient substrate for IKK-2 and is comparable with respect to $K_m$ and $K_{cat}$ to IkBα as a substrate. With respect to both peptide substrate specificity and phosphopeptide mapping of the in vitro phosphorylation of FL-p65 protein by IKK-2, we have shown that IKK-2 specifically phosphorylates p65 only on serine 536. Furthermore, this phosphorylation is dose-dependently inhibited by SC-514, with a comparable IC$_{50}$ value as that obtained using IkBα as a substrate. SC-514 does not, however, inhibit the phosphorylation of p65 by casein kinase II or PKA. The inhibition of NF-κB-dependent gene expression by SC-514 may thus be a combination of inhibition of IkBα degradation and inhibition of p65 phosphorylation. The importance of specific phosphorylation on different serines of p65 is not currently understood. Whereas phosphorylation of serine 276 has been shown to be important for the recruitment of transcriptional co-activators such as CBP/p300 and for DNA binding (23, 24), the role of serine 536 phosphorylation is not known and may involve other control mechanisms, including nuclear export. For example, p65 phosphorylation has been proposed to play an important role in recruiting modification enzymes such as histone acetyltransferases and histone deacetylases to nuclear NF-κB, resulting in reversible acetylation/deacetylation (46). Chen et al. (47) have suggested that the acylation status of p65 affects its nuclear export/import rate by virtue of altered affinity for binding to IkBα. They propose that when p65 is acetylated in the nucleus, it becomes refractory to inhibition by IkBα, whereas the deacetylated p65 binds to IkBα and is rapidly exported out of the nucleus (47). The treatment of RASF cells with SC-514 did not affect the re-synthesis of IkBα protein seen after stimulation, and hence this mechanism may regulate the rapid export of p65. The delayed import/export rate is consistent with our results, showing a reduction in DNA binding and rapid export of p65 out of the nucleus by 60 min in SC-514-treated cells compared with the untreated, stimulated RASF cells. A combination of delayed import of p65 into and enhanced export out of the nucleus in SC-514-treated cells could contribute to the inhibition of gene transcription. It would be of interest to determine whether SC-514 alters the acetylation/deacetylation state of p65. The phosphorylation of p65 during cellular activation also needs to be further characterized with both specific inhibitors as well as within selective IKK knockout cells, studies ongoing currently.

Although it is not known if serine 536 phosphorylation contributes to the acetylation status of p65, it is intriguing that in our hands, p65 is an efficient substrate for TBK-1 as well. Similar to IKK-2, TBK-1 specifically phosphorylates p65 on serine 536. Interestingly, TBK-1 knock out mice display a similar phenotype to IKK-2 knock out mice, namely embryonic lethality due to massive liver apoptosis. However, TBK-1 knock out fibroblasts show normal IkBα degradation and nuclear translocation of p65 but impaired NF-κB-dependent transcription (48, 49). It is interesting that IKK-2 and TBK-1 both utilize p65 as an efficient substrate, show specificity for serine 536, and have similar knock out phenotypes. However, p65 is not an efficient substrate for the other isoforms, IKK-i or IKK-1, validating that a kinetic approach may provide a means of dissecting unique pathway functions for the various IKK isoforms. Taken together, our results and emerging literature show that post-translational modification of p65 may be another critical point of transcriptional regulation, and highlights the need for further investigation of the physiologic importance of phosphorylation at different sites on p65.

Other structurally unique IKK-2 inhibitors have recently been reported (50) and were analyzed in multiple myeloma cells and in THP-1 monocytic cells (51). We have synthesized one of these inhibitors, PS-1145, and compared it to SC-514 in similar studies as the ones shown here. PS-1145 also demonstrates similar mechanistic results in IL-1β-stimulated RASF cells, further validating these unique mechanisms seen with IKK-2 inhibition using a unique structural inhibitor (data not shown).

In summary, we have described the characterization of a selective IKK inhibitor and show that the inhibition of IKK-2 blocks inflammatory responses in vitro and in vivo. We have dissected the NF-κB pathway in IL-1β-induced RASF cells in the presence of this selective IKK-2 inhibitor and have clearly shown that IKK-2 modulates NF-κB-dependent gene transcription by multiple mechanisms, including both IkBα phosphorylation/degradation and p65 nuclear export, possibly by modulating p65 phosphorylation. Thus, IKK-2 appears to be a novel target for drug development in the treatment of inflammatory diseases like rheumatoid arthritis.

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