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Functional Role of c-Src in IL-1–Induced NF-κB Activation: c-Src Is a Component of the IKK Complex

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Interleukin-1 (IL-1) mediates numerous host responses through the rapid activation of nuclear factor-κB (NF-κB), but the signal pathways leading to NF-κB activation are regulated at multiple stages. Here, we propose a novel regulatory system for IL-1–induced NF-κB activation by a tyrosine kinase, c-Src. The kinase activity of c-Src increases in an IL-1–dependent manner and the ectopic expression of c-Src augments IL-1–induced NF-κB activation, suggesting the involvement of c-Src in IL-1 signaling. However, a Src family inhibitor, PP2 failed to inhibit IL-1–induced NF-κB activation, and the expression of a c-Src mutant lacking kinase activity (c-Src KD) augmented IL-1–induced NF-κB activation as well as wild type c-Src, indicating that the tyrosine kinase activity is not required for IL-1–induced NF-κB activation. Furthermore, a physiological interaction between c-Src and IκB kinase γ (IKKγ) was observed, implying the involvement of c-Src in the IKK-complex. While c-Src augmented IL-1–induced IKK activation independent of its kinase activity, the region comprising amino acids 361–440 in the c-Src kinase domain are required for NF-κB activation. The same region of c-Src is also required for IL-1–induced IKK activation and the association with IKKγ. Taken together, our results suggest that c-Src plays a critical role in IL-1–induced NF-κB activation through the IKK complex.

Key words: c-Src, c-Src KD, IκB-Complex, IKKγ, IL-1–induced NF-κB activation.

The transcription factor NF-κB is one of the key regulators of genes involved in the immune and inflammatory response (*1*, *2*). In mammalian cells, NF-κB is composed of a homo- or heterodimer of various DNA-binding subunits including RelA, RelB, c-Rel, p105/NF-κB 1, and p100/NF-κB 2. In most cell types, NF-κB dimers are kept in the cytoplasm through their association with IκB proteins, which mask their nuclear localization sequence (*3*, *4*). In response to diverse extracellular stimuli, including viral infection, bacterial lipopolysaccharides, phorbol ester, and inflammatory cytokines such as IL-1 and tumor necrosis factor-α (TNF-α), IκBα is rapidly phosphorylated at two serines within the amino-terminal regulatory domain by the IκB kinase (IKK) complex. The IKK complex contains two catalytic subunits, IKKα and IKKβ, and a regulatory subunit, IKKγ / NEMO (*5–10*). Phosphorylation at these sites by IKKs triggers polyubiquitination of the IκBα and targets them for rapid degradation by the 26S proteasome (*11*, *12*). The degradation of IκBα results in the release of NF-κB and allows its translocation into the nucleus and the subsequent activation of various target genes.

Signaling pathways leading to NF-κB activation by IL-1 have been intensively studied in recent years. After IL-1 binds to IL-1 receptor type I (IL-1RI), a complex is formed between the IL-1RI and IL-1R accessory protein (IL-1R AcP) (*13*, *14*). The cytosolic proteins MyD88 (*15*) and Tollip (*16*) are recruited to this complex, where they function as adaptors to recruit IL-1 receptor–associated kinase (IRAK) in turn (*17*, *18*). Then, IRAK, a serine/threonine kinase, interacts with TNF receptor–associated factor-6 (TRAF6) (*19*). Moreover, it has been reported that TRAF6 activates IKKs through TAK1, NIK, and MEKK1, suggesting essential roles of TRAF6 in IL-1–mediated NF-κB activation (*20*, *21*). The signaling cascade described above is based mostly on results obtained through the isolation of specific protein complexes, analysis of protein–protein interaction through yeast two-hybrid screens, and data from gene knockout studies. However, the detailed mechanisms of IL-1–dependent signaling pathways remain to be elucidated.

c-Src is a member of a family of nonreceptor tyrosine kinases defined by a common structure that includes the Src homology (SH) protein interaction domains: the SH3 domain interacts with proline-rich regions, and the SH2 domain interacts with phosphorylated tyrosine residues (*22*, *23*). The tyrosine kinase catalytic domain comprises the second half of the molecule and is followed by a short carboxyl-terminal tail containing a negative regulatory tyrosine (Y527), which is normally phosphorylated by C-terminal Src kinase (Csk) (*24*).

We have previously demonstrated that c-Src kinase is activated by IL-1 stimulation in a human glioblastoma cell line, T98G (*25*). To understand better the function of c-Src and to elucidate the possible involvement of c-Src in IL-1–mediated NF-κB activation, we constructed various deletion mutants to test their ability to modify IL-1–dependent NF-κB activation. An interesting finding is

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that c-Src augments NF-κB activation, but its kinase activity is not required for IL-1–induced NF-κB activation. Furthermore, we found that the partial kinase domain (amino acids 361–440) of c-Src is important for IL-1–induced NF-κB activation through binding to IKKγ / NEMO.

MATERIALS AND METHODS

Antibodies and Reagents—Mouse monoclonal antibodies against FLAG-peptides (M2), Myc-peptides (9E10) and HA-peptides were purchased from Sigma (St. Louis, MO), Santa Cruz Biotechnology (Santa Cruz, CA), and Roche (Roche Diagnostics, Tokyo), respectively. Rabbit polyclonal antibodies against c-Src and JNK1 and goat polyclonal antibody against IKKγ were purchased from Santa Cruz Biotechnology, Inc. Peroxidase-conjugated swine anti-rabbit IgG and peroxidase-conjugated goat anti–mouse and anti–rabbit IgG antibodies were obtained from Dako (Dako-Japan, Tokyo). Human recombinant IL-1α (IL-1) was kindly provided by Dainippon Pharmaceutical Co. (Suitashi, Osaka). The Src family inhibitor PP2 and Dual luciferase assay kit were purchased from Calbiochem-Novabiochem Co. (Merck Japan, Tokyo) and Promega, respectively.

Cell Culture—A human glioblastoma cell line, T98G, and human embryonic kidney cells, HEK293T (JCRB, Kamiyoga, Tokyo), were maintained in Dulbecco's modified Eagle's medium (Nissui Seiyaku, Tokyo) supplemented with 10% heat-inactivated fetal bovine serum (FBS, Nippon Bio-Supply Center, Tokyo), 4 mM glutamine, 100 units/ml penicillin G, and 100 μg/ml streptomycin.

Plasmids—The cDNA encoding the full length c-Src was amplified from total RNA from a murine thymoma cell line, EL4, by RT-PCR. The PCR products were purified and inserted into the *Eco*RI and *Bam*HI sites of pCMV5 (ATCC, Rockville, MD, USA). The kinase dead mutant of c-Src cDNA (c-Src KD) contains one point mutation, a substitution of arginine for lysine[296]. c-Src deletion fragments were generated by PCR with c-Src as a template. The PCR products were cloned into the *Eco*RI and *Bam*HI sites of pCMV5. The cDNA encoding IκBα (amino acids 1–60) and IKKγ was amplified from 293 cells, and inserted into *Bam*HI and *Eco*RI sites of pGEX-2T. Reporter gene vectors, pNF-κB luciferase and pRL-TK, were purchased from Stratagene and Promega, respectively.

Transient Transfection and Luciferase Assay—Plasmid DNAs were transfected into T98G cells by LIPOFECTAMINE2000 (Invitrogen). The final amount of transfected DNA in each well of a 24 well plate was adjusted to 1 μg with empty vector, pCMV5. 0.5 μg of pCMV5-c-Src or pCMV5-c-Src KD was cotransfected with 0.01 μg of pRL-TK (Promega) and 0.1 μg of pNF-κB-Luc (Invitrogen) or pIL-8-Luc (–133 to +46) (*26*, *27*). After 48 h of transfection, the cells were harvested and the luciferase activities were measured by Lumat LB9501 (Bertold Japan, Tokyo). The efficiency of transfection was normalized to that of sea pansy luciferase activities.

Immunoprecipitation and Immunoblotting—Cells were harvested and lysed in lysis buffer [10 mM Tris-HCl (pH 7.4), 158 mM NaCl, 1% Triton X-100, 1% sodium deoxycholate, 1 mM EGTA, 1 mM Na_3VO_4 2 μg/ml Aprotinin, 2 μg/ml Leupeptin] on ice and the cellular debris was removed by centrifugation at 15,000 rpm for 15 min to obtain whole cell extracts. Aliquots (250 μg) of cell lysate were mixed with Protein G-Sepharose (Pharmacia-LKB Biotechnologies, Uppsala, Sweden) and various antibodies overnight at 4°C. The immune complexes were precipitated by centrifugation at 10,000 rpm, washed three times with lysis buffer, and boiled in Leammli's sample buffer. The boiled samples were subjected to SDS–polyacrylamide gel electrophoresis, and the proteins were transferred to nitrocellulose membranes. Immunoblotting was performed with various antibodies and anti–rabbit or mouse IgG antibody conjugated horseradish peroxidase, and visualized by the enhanced chemiluminescence Western blotting detection system (Amersham).

In Vitro Kinase Assay—The immunoprecipitates were washed twice with lysis buffer and three times with kinase buffer (30 mM HEPES pH7.4, 20 mM $MgCl_2$, 1 mM dithiothreitol). The kinase reaction [20 μl of kinase buffer, 10 μM ATP and 0.5 μg GST-SAM68 (1–180) for c-Src, 1 μg GST-IκBα (amino acid 1–60) for IKK or GST-c-Jun for JNK] was carried out with [γ^{32}-P]ATP for 20 min at 30°C as described elsewhere (*28*). The samples were resolved by 15% polyacrylamide gel electrophoresis, and phosphorylated GST-SAM68, GST-IκBα and GST-c-Jun were visualized by autoradiography.

RT-PCR—Total RNA separation and RT-PCR analysis were performed according to the manufacturer's protocols (Takara Shuzo, Shiga, Japan) using an oligo(dT) 20-primer and 1 μg total RNA for first strand cDNA synthesis. PCR was performed at an annealing temperature of 57°C for 20 amplification cycles. The PCR products were resolved and electrophoresed in a 1% agarose gel in Tris/borate/EDTA. The primers used were as follows: human IL-8, 5′-GAGCCAGGAAGAAACCACCGGA-3′ (upstream) and 5′-GTCAGTGGTGGACCTGACCT-3′ (downstream); human GAPDH, 5′-GTCAGTGGTGGACCTGACCT-3′ (upstream) and 5′-TGAGGAGGGGAGATTCAGTG-3′ (downstream).

RESULTS

C-Src Augments IL-1–Induced NF-κB Activation but Its Kinase Activity Is Not Required—While the IL-1/IL-1R–mediated signaling pathway has been explored extensively, and many signaling molecules have been identified so far (12–21), cross-talk among the signaling molecules is complicated and less defined. To explore the possible involvement of tyrosine kinase, c-Src, in IL-1 signaling, we examined whether IL-1 induces the activation of c-Src. As shown in Fig. 1A, c-Src was immediately activated in response to IL-1 in a human glioblastoma cell line, T98G, by *in vitro* kinase assay using with GST-SAM68 as a substrate. PP2, a potent inhibitor of Src-type tyrosine kinase, almost completely abrogated the IL-1–induced c-Src activation. Since IL-1 is a potent activator of NF-κB (*6*), the effect of c-Src on IL-1–induced NF-κB activation was also tested by the luciferase reporter gene assay. While PP2 significantly inhibited IL-1–induced c-Src activation, it failed to suppress IL-1–induced NF-κB activation (Fig. 1B). When T98G cells were transfected with wild type c-Src, they exhibited a moderate increase

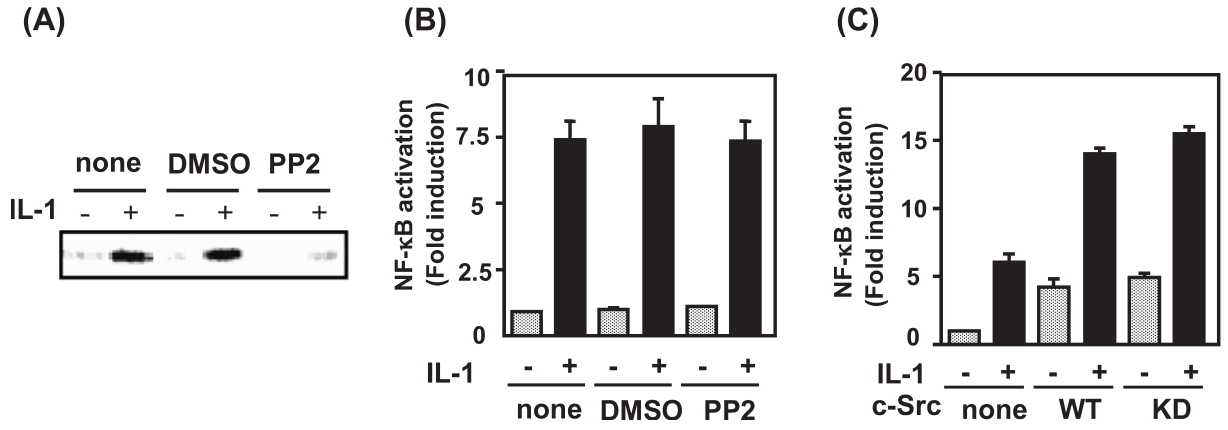

Fig. 1. **c-Src augments IL-1–induced NF-κB activation, but its tyrosine kinase activity is not required.** (A) T98G cells were incubated with the Src family inhibitor PP2 (10 μM) or DMSO for 1 h prior to stimulation with IL-1 (10 ng/ml) for 15 min. Cell extracts were immunoprecipitated with anti–c-Src antibody and c-Src activity was measured by an in vitro kinase assay using GST-SAM68 as a substrate. (B) T98G cells were transiently transfected with an NF-κB–responsive reporter (pNF-κB-Luc) and analyzed 48 h later. Where indicated, the cells were incubated with PP2 (10 μM) for 1 h prior to stimulation with IL-1 (10 ng/ml) for 12 h. (C) T98G cells were transiently cotransfected with NF-κB–responsive reporter (pNF-κB-Luc), and either the empty vector or plasmids bearing genes encoding wild type (WT) or a kinase dead mutant (KD) of c-Src, and analyzed 48 h later. Where indicated, the cells were treated with IL-1 (10 ng/ml) for 12 h. Luciferase activity was normalized for transfection efficiency. Data are presented as multiples of the level of activation obtained for the vector control group, which was set as 1. Results are expressed as mean ± SD of three independent experiments.

(~4 to 5-fold increase) in NF-κB activation *per se*, and IL-1–induced NF-κB activation was enhanced significantly. Interestingly, a kinase dead mutant of c-Src (c-Src KD) also induced activation as strongly as the wild type both in the presence and absence of IL-1 stimulation (Fig. 1C). These data suggest that c-Src is involved in IL-1–mediated NF-κB activation, but that its tyrosine kinase activity is not required for this activity.

C-Src Kinase Activity Is Not Required for the IL-1–Induced IKK Activation—Since IKK activation is a key step in IL-1–induced NF-κB activation (*5–10*), we then examined the effect of c-Src on IL-1–induced IKK activation by performing an *in vitro* kinase assay using GST-IκBα [1–60] as a substrate. As shown in Fig. 2A, pretreatment with PP2 had little effect on IL-1–induced IKK activation, indicating that c-Src kinase activity is not involved in IL-1–induced IKK activation. Furthermore, IL-1–induced IKK activation was markedly augmented by the transfection of wild type c-Src as well as by c-Src KD (Fig. 2B). Thus, c-Src induces IKK activation independent of its tyrosine kinase activity.

C-Src Kinase Activity Is Required for the IL-1–Induced JNK Activation—Since IL-1 activates not only NF-κB but also JNK, we next examined the effect of c-Src on IL-1–induced JNK activation. Cell lysates of IL-1–stimulated T98G cells were analyzed for JNK activity in vitro using GST-c-jun as a JNK substrate. As shown in Fig. 3A, JNK activity was induced markedly by IL-1 treatment, and this induction was completely inhibited by pretreatment with PP2. While the expression of wild type c-Src augmented IL-1–induced JNK activation, the kinase dead mutant, c-Src KD, abrogated IL-1–induced JNK activation almost completely (Fig. 3B). Thus, these results suggest that the c-Src tyrosine kinase activity is involved more prominently in JNK activation than in NF-κB activation in IL-1 signaling.

Amino Acids 361–440 of C-Src Are Required for IL-1–Induced NF-κB and IKK Activation—c-Src is a multidomain protein containing an unique N-terminal domain:

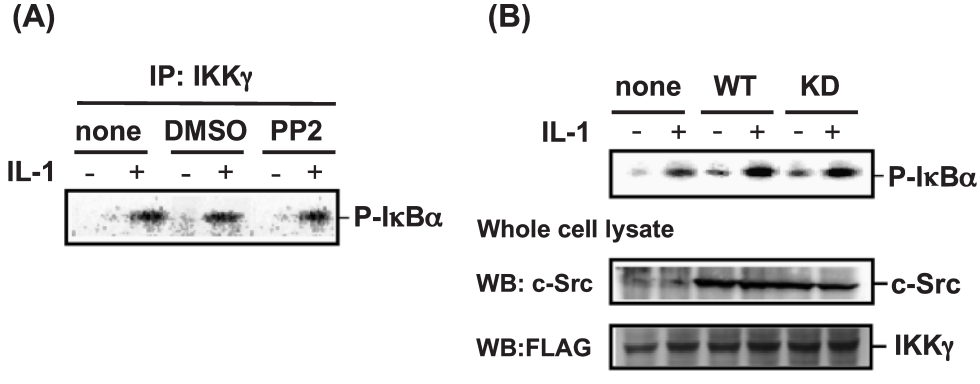

Fig. 2. **The tyrosine kinase activity of c-Src is not required for IKK activation.** (A) T98G cells were incubated with Src family inhibitor PP2 (10 μM) or DMSO for 1 h prior to stimulation with IL-1 (10 ng/ml) for 20 min. (B) T98G cells were transiently cotransfected with pCMV5-FLAG–tagged IKKγ and either the empty vector or plasmids bearing genes encoding WT or KD c-Src. Cells were treated with IL-1 (10 ng/ml) for 20 min and cell lysates were prepared. After normalization for protein content, cell extracts were immunoprecipitated with anti-IKKγ antibody (A) or anti-FLAG antibody (B) and a kinase assay was performed using GST-IκBα (amino acids 1–60) as a substrate. (Blots) Blots were probed with a FLAG specific antibody for FLAG-tagged IKKγ and with a c-Src specific antibody for c-Src.

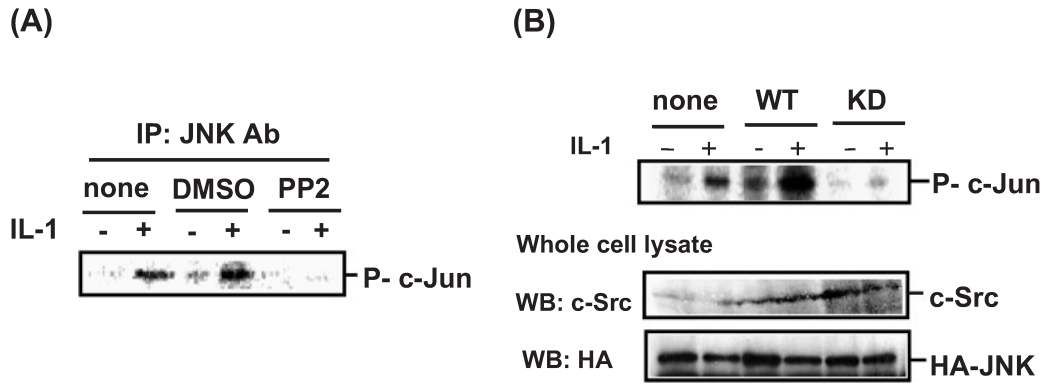

Fig. 3. **The tyrosine kinase activity of c-Src is required for JNK activation.** (A) T98G cells were incubated with Src family inhibitor PP2 (10 μM) or DMSO for 1 h prior to stimulation with IL-1 (10 ng/ml) for 20 min. (B) T98G cells were transiently cotransfected with pCMV5-HA-tagged JNK1 and either the empty vector or plasmids bearing genes encoding WT or KD c-Src. Cells were treated with IL-1 (10 ng/ml) for 20 min and cell lysates were prepared. After normalization for protein content, cell extracts were immunoprecipitated with anti-JNK1 antibody (A) or anti-HA antibody (B) and a kinase assay was performed using GST-c-Jun as substrate. (Blots) Blots were probed with a HA specific antibody for HA-tagged JNK1 and with a c-Src specific antibody for c-Src.

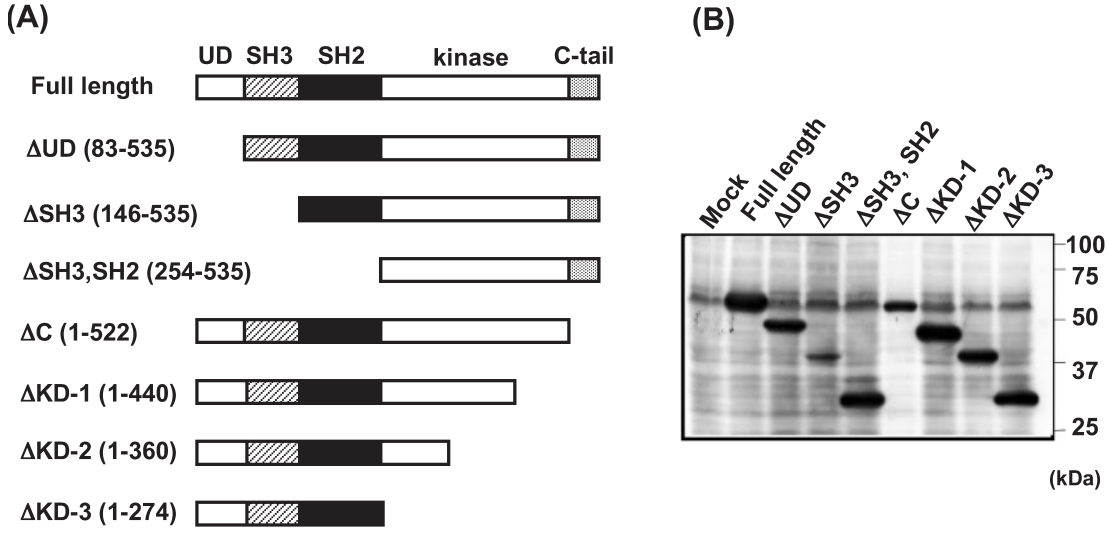

Fig. 4. **Deletion mutants of c-Src.** (A) Scheme showing deletion mutants of c-Src. (B) HEK293 cells were transiently transfected with expression vectors of the indicated c-Src deletion mutants. After 48 h, whole cell extracts were subjected to immunoblot analysis with anti-Myc antibody.

UD (residues 1–80), SH3 (residues 83–144), SH2 protein interaction domains (150–247), a catalytic region (269–522), and a negative-regulatory tyrosine located near the carboxyl terminus (residue 523) (*21–23*). To identify the functional domain of c-Src in IL-1–induced NF-κB activation, we generated a series of deletion constructs and analyzed the expression of these constructs by immunoblot assay (Fig. 4, A and B). Using these constructs, we determined the ability of c-Src mutants to activate NF-κB in response to IL-1 by a luciferase assay. The c-Src mutant lacking the UD region augmented NF-κB activation both in the presence and absence of IL-1 stimulation (ΔUD in Fig. 5A) to the same extent as wild type c-Src, indicating that the UD region of c-Src is not required for IL-1–induced NF-κB activation. Similarly, the deletion of SH3, or SH3 and SH2 still resulted in IL-1–induced NF-κB activation (ΔSH2 or ΔSH 2/SH3 in Fig. 5A). Thus, the N-terminal regions of c-Src, including UD, SH2 and SH3, are not required for NF-κB activation. Furthermore, ΔC, which does not have a negative-regulatory tyrosine in the C-terminal region, still augmented IL-1–induced NF-κB activation. While the deletion of amino acid residues 441–535 (ΔKD-1) also allowed marked IL-1–induced NF-κB activation, the deletion of amino acid residues 361–535 (ΔKD-2) and the whole kinase region (ΔKD-3) abrogated IL-1–induced NF-κB activation dramatically. These results clearly indicate that the region including amino acids 361–440 of c-Src, which includes the kinase domain, is required for IL-1–induced NF-κB activation.

Concomitantly, we examined the effects of the above c-Src deletion mutants on IL-1–induced IKK activation by an in vitro kinase assay. Consistent with the results of the luciferase assay for NF-κB, deletion mutants lacking parts of the N-terminal region, such as ΔUD, ΔSH2, ΔSH2/SH3, were capable of activating IKK in response to IL-1 at levels almost the same as those observed with

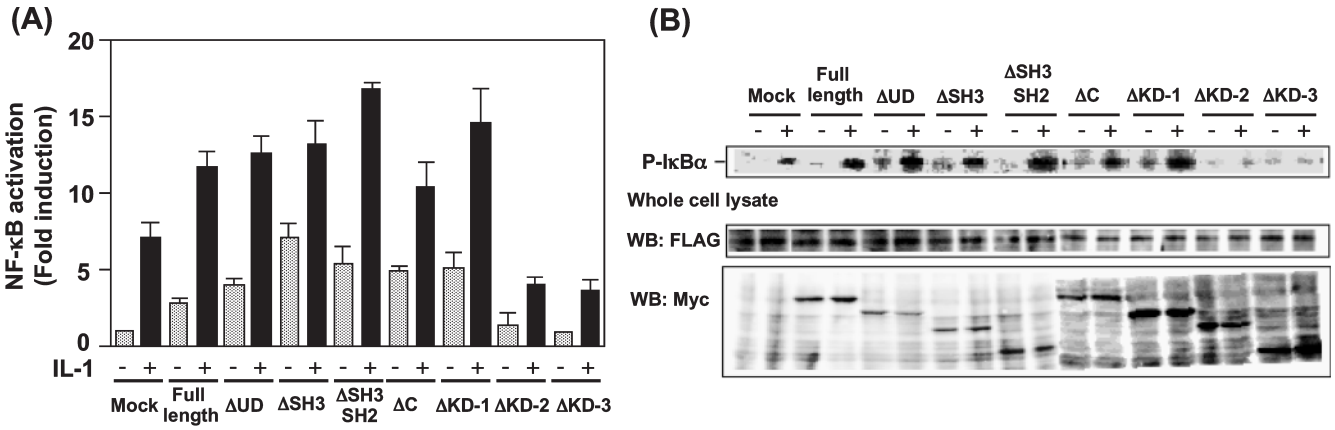

Fig. 5. **Amino acids 361–440 of c-Src are required for IL-1–induced NF-κB and IKK activation.** (A) T98G cells were transiently cotransfected with an NF-κB–responsive reporter (pNF-κB-Luc) and either the empty vector, plasmids bearing genes encoding deletion mutants of c-Src, or Flag-tagged TRAF6 and analyzed 48 h later. Where indicated, the cells were treated with IL-1 (10 ng/ml) for 12 h. Luciferase activity was normalized for transfection efficiency. Data are presented as multiples of the level of activation obtained for the vector control group, which was set as 1. Results are expressed as mean ± SD of three independent experiments. (B) T98G cells were transiently cotransfected with expression plasmids bearing the encoding deletion mutants of c-Src and FLAG-IKKγ. Cells were treated with IL-1 (10 ng/ml) for 20 min and cell lysates were prepared. After normalization for protein content, cell extracts were immunoprecipitated with anti-FLAG antibody and a kinase assay was performed using GST-IκBα (amino acids 1–60) as a substrate. (Blots) Blots were probed with a FLAG specific antibody for FLAG-tagged IKKγ and with a Myc specific antibody for Myc-tagged c-Src deletion mutants.

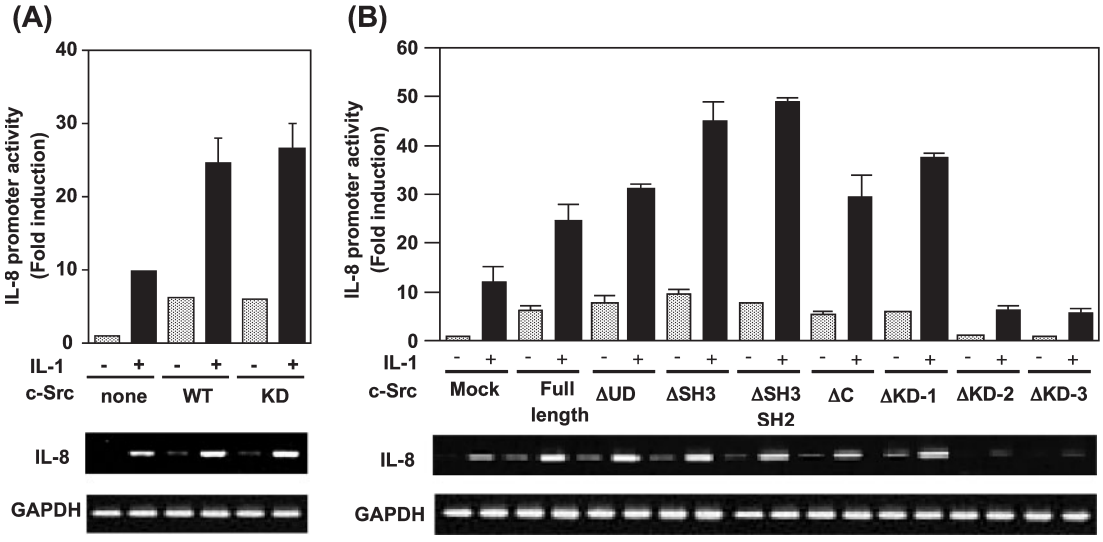

Fig. 6. **Amino acids 361–440 of c-Src are required for IL-1–induced IL-8 expression.** (A, B) Constructs encoding KD and deletion mutants of c-Src were transiently cotransfected with IL-8 promoter-luciferase vector (pIL-8-Luc) and analyzed 48 h later. Where indicated, the cells were treated with IL-1 (10 ng/ml) for 12 h. Luciferase activity was normalized for transfection efficiency. Data are presented as multiples of the level of activation obtained for the vector control group, which was set as 1. Results are expressed as mean ± SD of three independent experiments. (Lower panels) Constructs encoding the kinase dead and deletion mutants of c-Src were transiently transfected and the cells were treated with IL-1 (10 ng/ml) for 6 h. Total RNA was prepared and analyzed by RT-PCR. RT-PCR products were electrophoresed in 1% agarose gels and visualized by ethidium bromide staining.

wild type c-Src, or even much higher levels (Fig. 5B). Furthermore, ΔC or ΔKD-1, which lack either the C-tail domain or amino acid residues 441–535 also augmented IL-1–induced IKK activation. However, ΔKD-2 and ΔKD-3 failed to induce significant IKK activation in response to IL-1. These results also support the notion that amino acids 361–440 of the kinase domain play a critical role in the activation of NF-κB.

Amino Acids 361–440 of C-Src Are Required for IL-1–Induced IL-8 Expression—Since it has been well established that NF-κB activation is required for the activation of the IL-8 gene (*25–27*), we explored how c-Src mutants modulate IL-1–induced IL-8 gene expression using an IL-8 promoter assay. Consistent with the ability to activate NF-κB, as shown in Fig. 2, not only wild type c-Src, but also the kinase dead c-Src KD, induced a significant activation of basal as well as IL-1–induced IL-8 promoter activity (Fig. 6A). Furthermore, ΔUD, ΔSH3, ΔSH3/SH2, ΔC and ΔKD-1, which induced NF-κB activation, induced IL-8 promoter activity markedly similar to

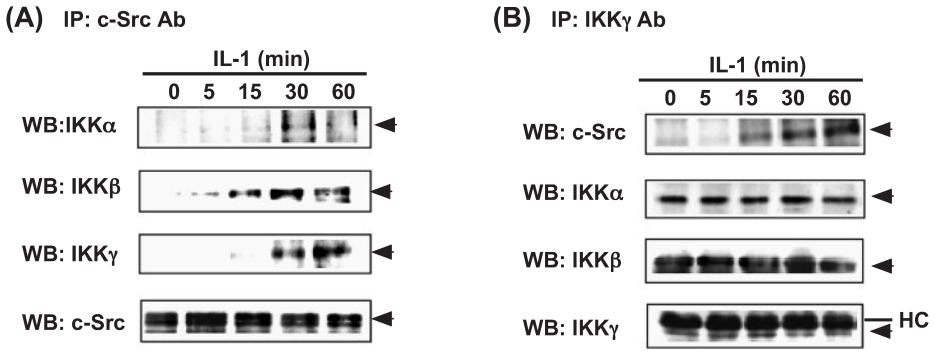

Fig. 7. **c-Src is a component of the IKK complex.** T98G cells were stimulated with IL-1 (10 ng/ml) for the indicated periods. Cell extracts were immunoprecipitated with anti-IKKγ antibody and immunoblotted with anti–c-Src antibody, anti-IKKα antibody, anti-IKKβ antibody, or anti-IKKγ antibody. HC indicates the immunoglobulin heavy chain.

wild type c-Src. Interestingly, these deletion mutants, particularly the ΔSH3, ΔSH3/SH2 constructs, augmented IL-1–induced IL-8 promoter activity more strongly than wild type c-Src. However, the ΔKD-2 or ΔKD-3 constructs had no enhancing effects on IL-8 promoter activity and failed to augment IL-1–induced IL-8 promoter activity (Fig. 6B), a result that is largely consistent with the results obtained for NF-κB activation as shown in Fig. 5. IL-8 mRNA expression by RT-PCR (shown in the bottom panel) confirmed the above results. Thus, the region comprising amino acids 361–440 of the kinase domain, which is required for NF-κB activation, is also necessary for IL-8 gene expression.

C-Src Interacts with IKKγ in an IL-1–Dependent Manner—Since c-Src augments IL-1–induced IKK activation as shown in Fig. 2B, we assumed that c-Src acts upstream of IKK. To determine whether c-Src is physically associated with the IKK complex, cell lysates were immunoprecipitated with anti c-Src or anti-IKKγ antibodies, separated by SDS-PAGE, and blotted with anti-IKKγ or anti–c-Src antibodies, respectively. As shown in Fig. 7A, the anti–c-Src antibody coimmunoprecipitated IKKγ as well as IKKα and IKKβ in an IL-1–dependent manner. Similarly, c-Src coimmunoprecipitated with IKKα and IKKβ by the anti-IKKγ antibody (Fig. 7B). Of note is that c-Src is associated with the IKK complex in an IL-1–dependent manner. These results suggest that c-Src is associated with or included in the IKK complex.

C-Src Interacts with IKKγ through the Region Comprising Amino Acids 361–440 of the Kinase Domain—The above experiments clearly indicate that c-Src interacts with IKKγ in a stimulation- dependent manner. We then determined whether the kinase activity of c-Src is required for the interaction with IKKγ. FLAG-tagged IKKγ was coexpressed with wild type or c-Src KD, and their association was tested by detecting c-Src in the anti-FLAG immunoprecipitates. An association between wild type c-Src and IKKγ was observed even without IL-1 stimulation. Furthermore, c-Src KD was shown to be associated with IKKγ**,** suggesting that that the tyrosine kinase activity of c-Src is not required for the interaction with IKKγ (Fig. 8A). To confirm further the function of c-

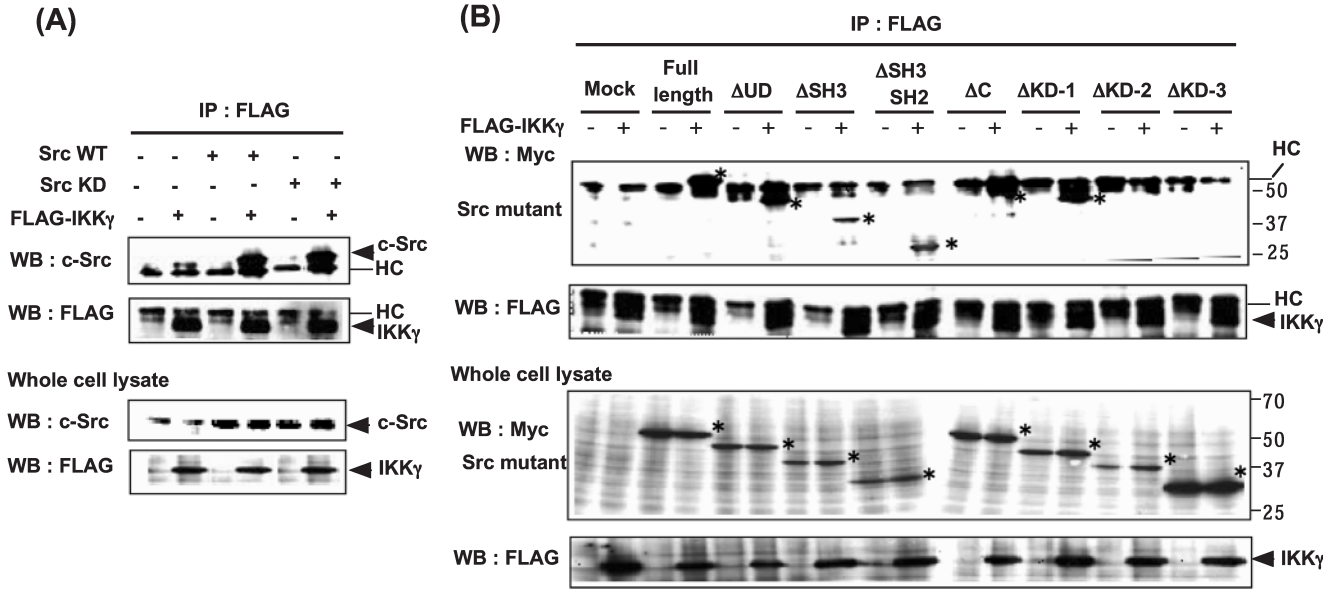

Fig. 8. **Amino acids 361–440 of c-Src are required for the association with IKKγ.** HEK293 cells were transiently cotransfected with expression plasmids bearing the WT, KD or deletion mutants of c-Src and FLAG-IKKγ. After 48 h, cell extracts were immunoprecipitated with anti-FLAG antibody and immunoblotted with anti–c-Src antibody, anti-Myc antibody and anti-FLAG antibody. * indicates the band of the c-Src deletion mutant. (Blots) (A) Blots were probed with anti–c-Src antibody, FLAG specific antibody for FLAG-tagged IKKγ and (B) with a Myc specific antibody for Myc-tagged c-Src deletion mutants and a FLAG specific antibody for FLAG-tagged IKKγ.

Src in the association with IKKγ, several deletion mutants of c-Src were co-transfected with FLAG-tagged IKKγ to examine their ability to interact with IKKγ. As shown in Fig. 8B, ΔUD, ΔSH3, ΔSH3/ SH2, ΔC and ΔKD-1, which have the ability to induce NF-κB activation, associated with IKKγ. In contrast, ΔKD-2 and ΔKD-3, which lack either amino acids 361–535 or 275–535, failed to interact with IKKγ. Thus, the region comprising amino acids 361–440 of the kinase domain, which is required for NF-κB activation, is also essential for the association with IKKγ.

DISCUSSION

IL-1 is a major inflammatory cytokine that plays an important role in immunological and inflammatory reactions by rapidly activating transcription factors, NF-κB and AP-1, through the induction of various inflammatory genes (*1*, *2*, *26*, *27*, *29–31*). Since a number of molecules involved in the IL-1 signaling pathway have been identified in recent years (*12–20*), the IL-1/IL-1R–mediated signal cascade is well-understood. However, previous studies have suggested the possibility that some tyrosine kinases participate in IL-1 signaling. Munoz *et al.* have reported that IL-1 induces the activation of tyrosine kinases in a T helper type 2 cell line, D10A, leading to the tyrosine phosphorylation of several proteins, indicating the involvement of some tyrosine kinase in the IL-1 signaling pathway (*29*), a hypothesis that has also been supported by al-Ramadi *et al.* (*31*). Furthermore, it has been shown that a tyrosine residue of the IL-1 receptor is essential for NF-κB activation by IL-1 stimulation through PI-3 kinase, suggesting that some tyrosine kinase phosphorylates the tyrosine residue of the IL-1 receptor, and relays the signal to the PI3-kinase/NF-κB pathway (*32*, *33*).

Previously, we found that the IL-1–mediated activation of a tyrosine kinase, c-Src, is required for AP-1 activation in a human glioblastoma cell line, T98G cells (*25*). We have now studied the function of c-Src more extensively for its ability to mediate the IL-1–dependent signaling pathway. We first investigated how c-Src influences IL-1–induced NF-κB activation. Obviously, the ectopic expression of wild type c-Src induces NF-κB activation, and augments IL-1–induced NF-κB activation significantly. Our interesting finding is that not only wild type c-Src, but also a c-Src mutant that lacks kinase activity (c-Src KD), activates NF-κB in both the presence and absence of IL-1 treatment. In addition, the inhibition of c-Src by PP2 treatment has no effect on IL-1–induced NF-κB activation. Thus, although c-Src appears to have the ability to activate NF-κB, we could not confirm that IL-1–induced c-Src kinase activity *per se* is required for the IL-1–induced NF-κB activation. The activation of NF-κB is controlled by a family of IκB repressor proteins (IκBα, IκBβ, and IκBγ) that sequester NF-κB in the cytoplasm (*3*, *4*). The phosphorylation-dependent inactivation of IκB proteins leads to the mobilization of NF-κB to the nucleus where it can act as a transcription factor. These phosphorylation pathways have been studied extensively for IκBα and include two distinct mechanisms involving serine or tyrosine IκBα phosphorylation. The most comprehensively studied pathway regulating IκBα includes the phosphorylation of two serine residues (*32* and *36*) by the IκB kinase complex (IKK). Phosphorylation at these sites leads to the ubiquitination of IκBα at nearby lysine residues, and degradation by proteasome (*3–12*). An alternative, less well-characterized pathway of NF-κB activation involves the tyrosine phosphorylation of IκBα at residue 42 (*34–36*), which is capable of activating NF-κB in the absence of ubiquitin-dependent degradation of IκBα. The phosphorylation of IκBα at tyrosine 42 can only be observed during the NF-κB activation following hypoxia/reoxygenation or pervanadate treatment, and differs from the canonical proinflammatory pathway, which mediates NF-κB activation through the serine phosphorylation of IκBα by the IKK complex (*35*, *36*). In this paper, c-Src inhibition by PP2 treatment or a c-Src–deficient cell line from c-Src (–/–) knockout mice showed a significant reduction in IκBα tyrosine phosphorylation and NF-κB activation (*36*). While the observation by al-Ramadi *et al.* (*31*) that the Src protein tyrosine kinase Lck is required for IL-1–mediated costimulatory signaling in Th2 cells confirms our notion that c-Src acts upstream of the IKK complex in IL-1 signaling as shown in this study, the observation that a kinase-dead mutant of c-Src can also augment IL-1–induced IKK activation is rather conflicting. We assume that c-Src induces NF-κB activation through the physical involvement of IKK activation, not due to the tyrosine kinase activity in the IL-1 signaling pathway, since IKK activity was detected in the precipitates of c-Src in an IL-1 stimulation–dependent manner (data not shown). Alternatively, the involvement of c-Src in NF-κB activation may be regulated differently by cell type– and signal type–specific machineries.

Further analysis of a series of c-Src deletion mutants for IL-1–mediated NF-κB activation revealed that amino acids 361–440 of c-Src contribute to NF-κB activation. The same regions of c-Src was also shown to be required for IL-1–induced IKK activation. These findings suggest that c-Src activates IKK through the comprising amino acids 361–440, resulting in NF-κB activation. Next, we examined whether c-Src is involved in the IKK complex by immunoprecipitation and kination analysis. In c-Src precipitates, IKK activity was detected in an IL-1 stimulation–dependent manner, suggesting the involvement of c-Src in the IKK-complex. Interestingly, we observed that c-Src and IKKγ formed a stable complex. In addition, we detected the same interaction between the kinase dead mutant of c-Src (c-Src KD) and IKKγ, suggesting that this interaction is not dependent on its tyrosine kinase activity. Namely, the region of c-Src comprising amino acids 361–440, is required for the association with IKKγ. Thus, the data reveal that the interaction of c-Src with IKKγ correlates well with its ability to activate IKK and NF-κB. An interaction between c-Src and IKK has also been reported by Huang *et al.* (*37*), who showed that Tyr188 and Tyr199 of IKK are phosphorylated by c-Src, which is important for NF-κB activation, and leads to the expression of ICAM-1. In our opinion, the interaction between IKKγ and c-Src seems to depend on IL-1 stimulation. So far, it has been reported that the overexpression of signaling components in the IL-1 signaling pathway, including IRAK and TRAF6, induces AP-1 and NF-κB activations without IL-1 (*38*, *39*). In our study, the overexpression of c-Src induced a moderate activation of

NF-κB in the absence of IL-1 stimulation, although the activation level was a lower than with IL-1 stimulation. Thus, the overexpression of c-Src partially mimics the IL-1 signaling pathway. In HEK293 cells, the CMV promoter system works much better than in T98G cells, as shown in Fig. 8. Moreover, because it is still very difficult to detect the interactions in the absence of IL-1, we transfected FLAG-IKKγ (Fig. 8) to gain more sensitivity. These factors may have allowed us to detect interactions between IKKγ and c-Src without IL-1 stimulation.

On the other hand, c-Src tyrosine kinase activity is required for JNK activation. These results were confirmed by the data in which the inhibition of c-Src by PP2 treatment inhibited IL-1–induced JNK activation completely. Furthermore, we showed previously that activation of AP-1, which is known to be the target of JNK, is dependent on c-Src tyrosine kinase activity (*25*). Thus, we surmise that c-Src induces NF-κB and JNK through different mechanisms. In our study, neither kinase activity nor the tyrosine phosphorylation of c-Src is apparently required for IL-1–induced NF-κB activation, because the kinase dead mutant and the tyrosine kinase inhibitor PP2 were not effective in NF-κB activation. In the cases of several protein kinases, it has been shown that kinase activity is not required for their entire functions. For example, the kinase activity of c-Src is not required for osteoclast differentiation (*40*), and IKKα can be involved in keratinocyte differentiation without its kinase activity (*41*). We propose the possibility that c-Src may be involved in IL-1–induced NF-κB activation as a scaffold protein. We have already reported that c-Src interacts with TRAF6 in an IL-1 stimulation–dependent manner (*25*). c-Src might act as bridge between TRAF6 and IKKγ. In our previous research, we have found the kinase activity of c-Src to be essential for AP-1 activation (*25*), and have shown that the kinase activity of c-Src seems to be involved in JNK activation, suggesting that there might be two kinds of complexes involving c-Src in the IL-1 signaling pathway. Right now we have no explanation for the difference in these two c-Src–containing protein complexes, one for NF-κB activation and the other for the JNK-AP-1 pathway. Future experiments are needed to clarify this discrepancy.

Previously, it has been demonstrated by Wooten *et al.* (*42*) that c-Src is required for NGF-induced NF-κB activation and cell survival. They showed that IKK exists as a complex with atypical protein kinase C (aPKC), Src and IκB in PC12 cells. Furthermore, they found that the absence of c-Src impairs the recruitment of aPKC into the IKK complex and markedly impairs NGF-induced NF-κB activation. These data suggest that c-Src may play a role in the coupling of aPKC with the NF-κB pathway *via* the interaction of tyrosine-phosphorylated PKC with IKK in NGF-signaling. It has been reported that aPKCs may also be involved in the activation of IKKβ in IL-1–stimulated cells (*43*). Although we could not detect the an association of two members of the aPKC subfamily of isozymes, ζPKC and ζ/ι PKC with c-Src or IKKγ even with IL-1 stimulation in T98G cells (data not shown), it can be considered that c-Src may play a role as an adapter protein that recruits critical intermediators other than aPKC to the IKK complex in the IL-1 signaling pathway.

In Figs. 5A and 6B, the activities of the reporters NF-κB and IL-8 in ΔKD-2 and ΔKD-3 are seen to be lower than in the case of the empty vector. Previously, it was reported that the IL-8 promoter is regulated by NF-κB and AP-1 (*26*, *27*). As described above, there could be two protein complexes that include c-Src in the IL-1 signaling pathway. ΔKD-2 and ΔKD-3 might exhibit different behaviors in NF-κB and AP-1 activation. This could cause the differences in the effects of these mutants in NF-κB and IL-8 promoter activation. Although we have no evidence, ΔKD-2 and ΔKD-3 might lack motifs that interact with TRAF6 and IKKγ and act as dominant negative mutants. This notion is supported by the fact that phosphorylation of IκBα was completely abolished in presence of ΔKD-2 and ΔKD-3 as shown in Fig. 5. To explain this mechanism, an analysis of the interaction motif of c-Src needs to be performed.

In conclusion, we have found a novel regulatory mechanism involving c-Src in the IL-1–dependent signaling pathway. We showed that c-Src acts as a positive factor in IL-1–induced NF-κB activation without its tyrosine kinase activity. Further experiments are needed to determine how the interaction of c-Src with IKKγ leads to the induction of NF-κB activation.

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