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Herbimycin A Abrogates Nuclear Factor-κB Activation by Interacting Preferentially with the IκB Kinase β Subunit

Shinichi Ogino, Kazuhiro Tsuruma, Takashi Uehara, and Yasuyuki Nomura

Department of Pharmacology, Graduate School of Pharmaceutical Sciences, Hokkaido University, Sapporo, Japan

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ABSTRACT

NF (nuclear factor)-κB is known to be a critical transcription factor in inflammatory responses. We have reported that herbimycin A, a potent Src tyrosine kinase inhibitor, attenuates the NF-κB activation triggered by cytokines, bacterial endotoxin, and hydrogen peroxide. Accompanying the suppression by this agent, NF-κB–dependent gene expressions, such as cytokine, chemokine, and inducible-type nitric oxide, are specifically inhibited in glial cells. In the present study, we attempted to elucidate the possible target protein for herbimycin A on this pathway. We demonstrate here that herbimycin A preferentially inhibits IKK (IκB kinase)β. Furthermore, substituting alanine for the cysteine at 59 (Cys59) in IKKβ resulted in the insensitivity to herbimycin A, suggesting that this compound may interact with the Cys59 residue located near the catalytic ATP binding site. Taken together, these results indicate that herbimycin A can be considered a novel candidate for an anti-inflammatory drug agent through its specific inhibition of IKKβ, which results in prevention of the expression of NF-κB–dependent genes implicated in the pathogenesis of inflammatory responses.

Transcription factor NF-κB plays several essential roles in the inducible gene expression that contribute to a diverse range of biological processes (e.g., development, immune, and inflammatory responses) (Baeuerle and Baltimore, 1996). These responses occur not only in the immune system but also in the brain (Nomura and Kitamura, 1993; Uehara et al., 1998). In resting cells, the majority of NF-κB is sequestered in the cytoplasm as an inactive form because of its association with IκBα protein. After a variety of extracellular stimuli, such as tumor necrosis factor (TNF)α and interleukin (IL)-1β, IκBα is phosphorylated specifically (Ser32 and Ser36) by IκB kinases (IKKs) (DiDonato et al., 1997; Mercurio et al., 1997; Regnier et al., 1997; Woronicz et al., 1997; Zandi et al., 1997) and then degraded through a ubiquitin-proteasome–dependent mechanism. Degradation of IκBα allows NF-κB to translocate to the nucleus, where it activates the transcription of specific target genes (Perkins, 2000; Ghosh and Karin, 2002). The IKKs have been characterized as ~700 to 900-kDa protein complexes consisting of at least two catalytic subunits, IKKα and IKKβ, and a regulatory subunit, NF-κB essential modulator (also known as IKKγ/IKKAP1) (Rothwarf et al., 1998; Yamaoka et al., 1998). IKKα and IKKβ are two highly homologous kinases, both containing a conserved N-terminal kinase domain and a C-terminal region with a leucine zipper (LZ) and a helix-loop-helix motif (Karin, 1999a). Although the LZ motif is responsible for dimerization of IKKα and IKKβ, both the LZ and helix-loop-helix motifs are important for modulating the kinase activities of IKKα/β (Karin, 1999b). These IKKα/β dimers are phosphorylated at Ser176 and Ser180 in IKKα or Ser177 and Ser181 in IKKβ by NF-κB–inducing kinase (NIK) (Malinin et al., 1997; Stancovski and Baltimore, 1997; Ling et al., 1998) or mitogen-activated protein kinase kinase kinase 1 (Lee et al., 1997). It has been proposed that NIK is a downstream regulator of the TNFα signaling pathway that may be activated by cytoplasmic adaptor proteins, such as ribosome inactivating proteins or TRAF2 (Malinin et al., 1997; Stancovski and Baltimore, 1997). These proteins facilitate the interaction of NIK-IKKα/β with receptor signalsomes, such as TNFR, IL-1R, and TLR4. Overexpression of wild-type NIK potently activates NF-κB, whereas a catalytically inactive NIK mutant dominantly interferes with TNFα- and IL-1β–induced NF-κB activation (Malinin et al., 1997; Ling et al., 1998).

We have reported previously that herbimycin A, a potent Src tyrosine kinase inhibitor, suppresses the NF-κB activation and subsequent induction of inducible nitric oxide synthase and chemokines by treatment with several stimuli in C6 glioma cells (Uehara et al., 1989, 1998, 1999; Nishiya et

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S.O. and K.T. contributed equally to this work.

ABBREVIATIONS: NF, nuclear factor; IL, interleukin; GST, glutathione *S*-transferase; IKK, IκB kinase; LZ, leucine zipper; NIK, NF-κB-inducing kinase; TNF, tumor necrosis factor; DTT, dithiothreitol; TRAF, TNF receptor-associated factor; PCR, polymerase chain reaction; HEK, human embryonic kidney; pRL-TK, *Renilla reniformis* luciferase reporter plasmid; PAGE, polyacrylamide gel electrophoresis; NAC, *N*-acetyl cysteine.

al., 1995, 2000). However, the target molecule of herbimycin A in NF-κB–activating pathways remains to be identified. In this study, we attempted to elucidate the target protein for herbimycin A. Our results show that herbimycin A selectively inhibits IKKβ through the possible interaction with cysteine 59, which is located near the catalytic site in the kinase domain in IKKβ. We concluded that herbimycin A is a novel potent inhibitor of IKKβ in NF-κB signaling.

Materials and Methods

Materials. Recombinant human IL-1 and anti-Flag monoclonal antibody M2 were purchased from Sigma-Aldrich (St. Louis, MO). Anti-IKKα (M-280), anti-IKKβ (H-4), anti-IκBα (C-21), and anti–NF-κB (p65) antibodies were obtained from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Anti-phospho IκBα (Ser32) and phospho IKKα (Ser180)/β (Ser181) antibodies were purchased from New England Biolabs (Beverly, MA). Herbimycin A was obtained from Wako Pure Chemicals (Osaka, Japan).

Expression Vectors and Site-Directed Mutagenesis. FLAG epitope-tagged NIK, NIK (K429A/K430A) mutant, IKKα (K44A) mutant, and IKKβ (K44A) mutant were generous gifts from Dr. David V. Goeddel (Tularik Inc., South San Francisco, CA). NIK and IKKs were subcloned into pRK vector. Several mutants of NIK, IKKα, and IKKβ were generated by the overlapping PCR method. To clarify the possible modification site with herbimycin A in IKK, variants of IKKα and IKKβ were prepared by PCR. In addition, seven cysteine residues in the kinase domain of IKK were displaced with an alanine residue by the overlapping PCR method.

Transfection, Immunoprecipitation, and Immunoblotting Analyses. HEK 293 T cells were transiently transfected with expression plasmids (8 μg each) by the calcium phosphate method. After 36 h, cells were washed twice with cold PBS and lysed in lysis buffer [50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1 mM EGTA, 1 mM EDTA, 1 mM Na_3VO_4, 1 mM NaF, 1% NP40, and the Complete protease inhibitors (Roche Molecular Biochemicals, Indianapolis, IN)]. Immunoprecipitation and immunoblotting analyses were performed as described previously (Ko et al., 2002). In brief, the total cell lysate was centrifuged at 15,000 rpm for 30 min at 4°C. The supernatant was incubated with protein G-Sepharose for 1 h at 4°C and then centrifuged for 10 min. The resultant supernatant was incubated for 16 h with the first antibody, which had been precoupled with protein G-Sepharose. The immunoprecipitates were washed twice with lysis buffer and three times with washing buffer (50 mM Tris/HCl, pH 7.5, 137 mM NaCl, 1 mM EGTA, 1 mM vanadate, and 0.1% Triton X-100). Otherwise, cells were washed twice with ice-cold PBS and added to the SDS sample buffer. Protein (20 μg) from the lysate was fractionated by electrophoresis in 10% polyacrylamide gel and transferred onto a nitrocellulose membrane. Horseradish peroxidase-conjugated anti-mouse or -rabbit IgG (Amersham Biosciences Inc., Piscataway, NJ) was used as the secondary antibody. The antibody-reactive bands were revealed by chemiluminescent detection (enhanced chemiluminescence Western detection kit).

Reporter Assays. For the reporter assays, cells were transfected with 0.25 μg pNF-κB Luc vector (BD Biosciences Clontech, Palo Alto, CA) and 1.25 ng of pRL-TK (internal control plasmid). Reporter gene activity was determined with the luciferase assay system (Promega, Madison, WI). In particular, to confirm the possible target site for herbimycin A, Hs683 glial cells were transfected with 5 μg of C59A-mutated IKKβ together with pNF-κB Luc and pRL-TK vectors. The cells were treated with or without herbimycin A and then stimulated with 5.0 ng/ml IL-1β for 6 h.

Immunohistochemistry. To analyze the localization of NF-κB (p65 subunit), we used human Hs683 glioma cells transiently transfected with several genes. Cells were fixed in 4% paraformaldehyde, permeabilized in 0.5% Triton X-100, and incubated with anti–NF-κB (1:100 dilution) and anti-phospho IKK (1:100 dilution) antibodies for 1 h at 37°C. After reaction with the first antibody, cells were incubated with anti-mouse IgG conjugated with Alexa-488, anti-goat IgG conjugated with Alexa-488, and anti-rabbit IgG conjugated with Alexa-594 (Molecular Probes, Eugene, OR) for 1 h at 37°C. All images were taken on a laser-scanning confocal microscope (LSM510; Carl Zeiss Inc., Thornwood, NY) (Tanaka et al., 2000; Furuta et al., 2003).

In vitro Kinase Assay. HEK 293 cells were transfected with wild-type IKKβ and then incubated for 36 h. The cells were washed rapidly with PBS and lysed with ice-cold lysis buffer, after which IKKβ was immunoprecipitated. The immunoprecipitated IKKβ with protein G-Sepharose beads was collected by centrifugation, then washed three times with lysis buffer and once with kinase assay buffer without ATP. The immunoprecipitates were then suspended in 20 μl of kinase assay buffer (20 mM Tris-HCl, pH 7.6, 10 μM $MgCl_2$, 100 μM ATP, and 20 μM β-glycerophosphate) containing 5 μCi [γ-^{32}P]ATP and 1 μg of IκBα as substrate with or without several concentrations of herbimycin A for 20 min at 25°C. The reaction was stopped by the addition of Laemmli buffer, the proteins were separated by electrophoresis on 10% SDS-PAGE, and phosphorylated IκBα was visualized by autoradiography or on a Fuji BAS2000 apparatus (Tokyo, Japan). Quantitative data were obtained from Fuji BAStation software.

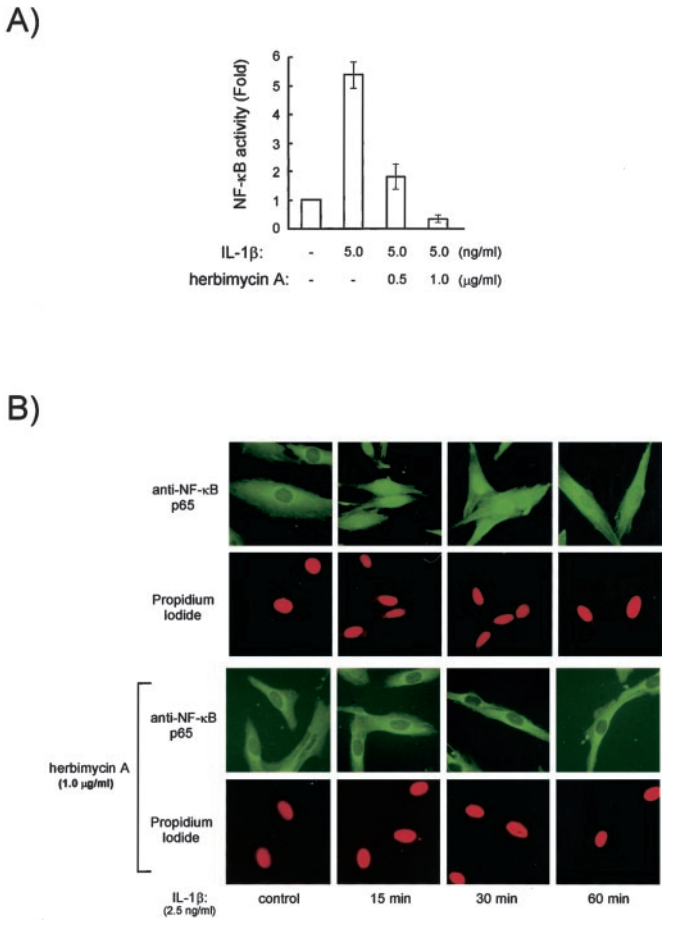

Fig. 1. Herbimycin A suppressed the nuclear translocation of NF-κB in glial cells. Human Hs683 glioma cells were transiently transfected with pNF-κB and together with pRL-TK by Effectene transfection reagent and then treated with or without herbimycin A (A). After 12 h, the transfected cells were stimulated with IL-1β for 6 h. The activity was assayed as described under *Materials and Methods*. Human glioma cells were incubated with 1 μg/ml herbimycin A for 12 h before treatment with 2.5 ng/ml IL-1β (B). The cells were fixed and subjected to indirect immunofluorescence staining with anti–NF-κB pAb. Propidium iodide was used to stain the nuclei.

Results

Herbimycin A Inhibited NF-κB Activation. We initially examined the inhibitory effect of herbimycin A on IL-1β–stimulated reporter gene expression and translocation of the NF-κB p65 subunit. Herbimycin A alone (1 μg/ml) did not affect the NF-κB activity in this system (data not shown). As shown in Fig. 1A, treatment of human Hs683 glioma cells with herbimycin A resulted in a dose-dependent decrease in the NF-κB activity detected by luciferase assay. Immunohistochemical analysis revealed that the NF-κB p65 subunit was localized mostly in the cytosol in the quiescent state and translocated to the nucleus at 15 to 30 min after IL-1β treatment. After 60 min, p65 returned to the cytosol (Fig. 1B). Herbimycin A inhibited completely the translocation of p65 induced by IL-1β treatment.

Effects of Herbimycin A on Phosphorylation of IKK and IκBα. To identify the target molecule for herbimycin A, we investigated the phosphorylation states of IκBα by overexpressing several genes, such as wild types and kinase-negative mutants of NIK, IKKα, and IKKβ with or without herbimycin A. Transfection of the wild types of NIK and IKKβ, but not IKKα, resulted in significant phosphorylation of IκBα in a herbimycin A-sensitive manner (Fig. 2A). These results suggest strongly that both NIK and IKKβ, but not

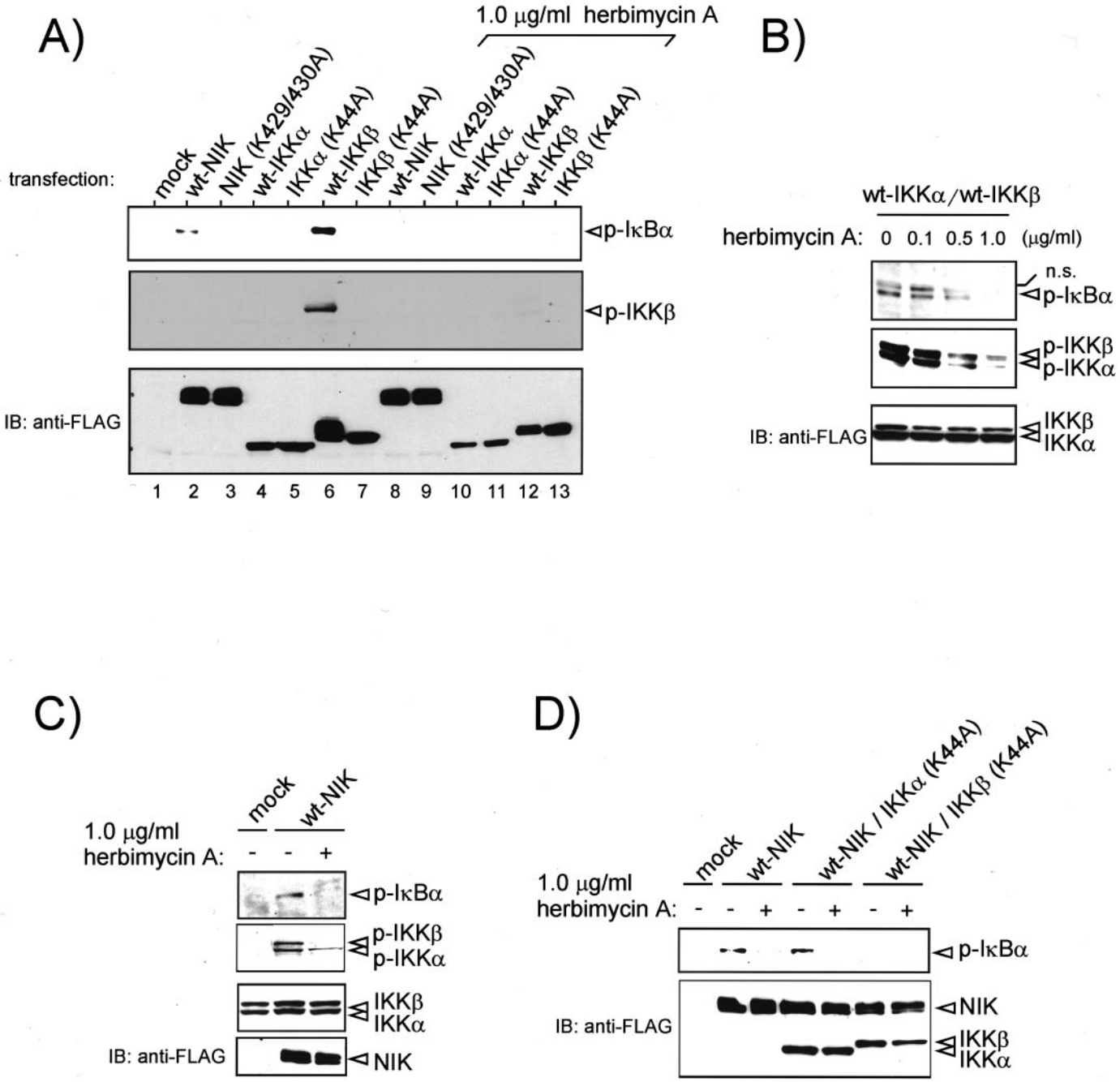

Fig. 2. Herbimycin A inhibited NIK- and IKKβ-induced phosphorylation of IκBα. HEK 293 T cells were transfected with several genes for 24 h and then treated with or without the indicated concentration of herbimycin A for an additional 12 h. The total cell lysates were prepared and then subjected to Western blot analysis using anti-phospho IκBα (Ser329), anti-phospho-IKKs, anti-IKKs, and anti-FLAG antibodies.

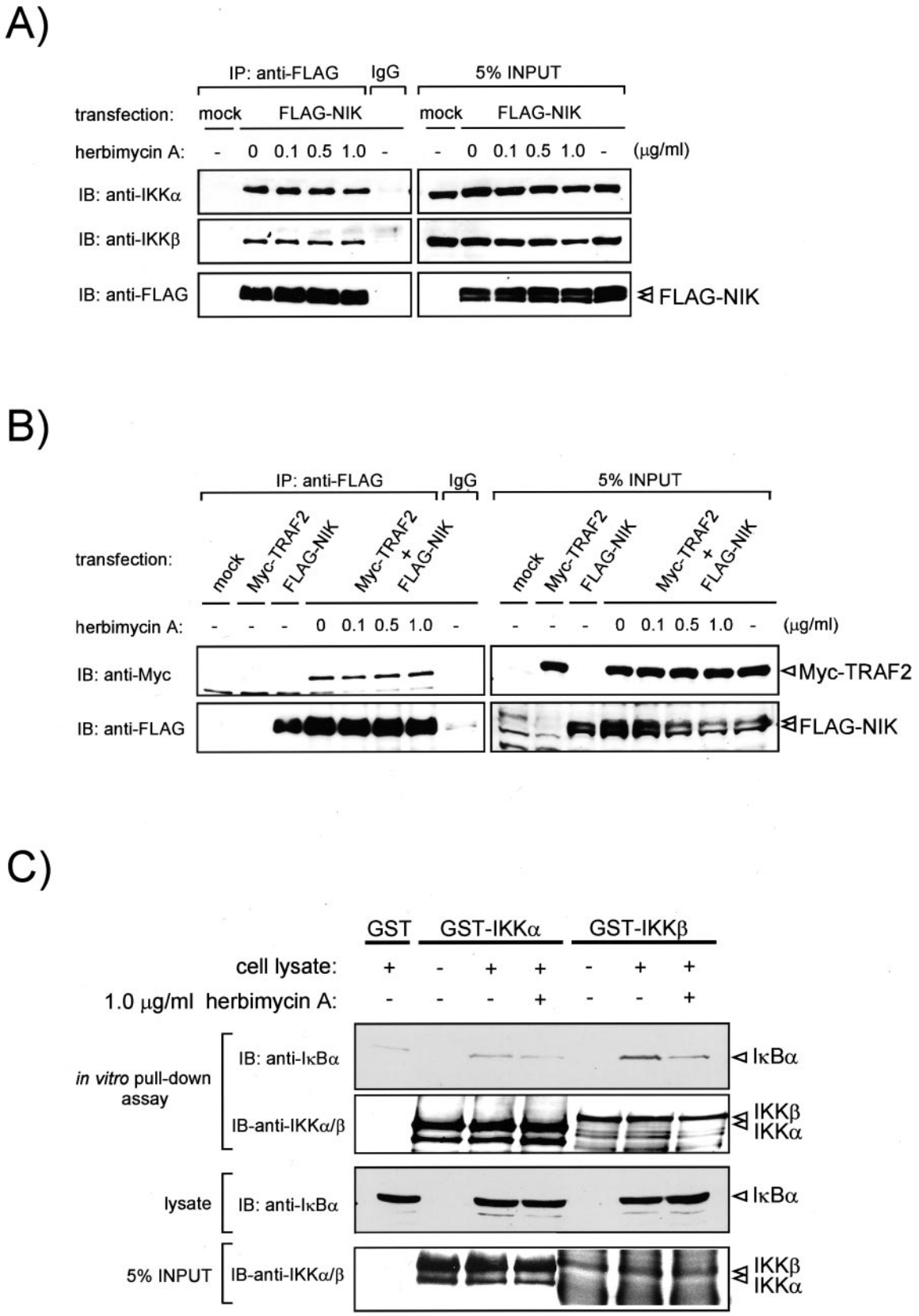

Fig. 3. Effects of herbimycin A on protein-protein interaction. HEK 293 cells were transfected with FLAG-NIK and/or Myc-TRAF2 and then treated with or without herbimycin A (A and B). The lysates were prepared and immunoprecipitated with anti-FLAG antibody. The immunoprecipitates were resolved by SDS-PAGE and then subjected to Western blot analysis with anti-IKKα, anti-IKKβ, anti-FLAG, and anti-Myc antibodies. C, in vitro pulldown assay. GST-fused IKKα or IKKβ precoupled with glutathione-Sepharose 4B was incubated with cell lysates prepared from glial cells for 12 h at 4°C with or without herbimycin A. The precipitated proteins were then resolved by SDS-PAGE and subjected to Western blot analysis with anti-IKKs and anti-IκBα antibodies.

IKKα, play a crucial role in NF-κB activation. It has been reported previously that transfection of IKKα or IKKβ alone into cells results in the formation of a homodimer (Karin, 1999a,b). However, IKKα and IKKβ exist as heterodimers in cells. Therefore, we examined the effect of herbimycin A on simultaneous cotransfection of IKKα and IKKβ. Although no phosphorylation of IKKα and IκBα was observed by transfection of IKKα alone, cotransfection of IKKα plus IKKβ resulted in a marked phosphorylation of IKKα (Fig. 2B). Under this condition, the phosphorylation of IKK and IκBα was attenuated by herbimycin A in a concentration-dependent manner (Fig. 2B). NIK alone also induced the phosphorylation of IKKα, IKKβ, and IκBα (Fig. 2C). It was interesting that IKKα phosphorylation by NIK transfection was insensitive to herbimycin A. Specific phosphorylation of IκBα by overexpression of NIK was abrogated completely by kinase-negative IKKβ but not by kinase-negative IKKα (Fig. 2D).

Effects of Herbimycin A on the Protein-Protein Interactions in NF-κB Signaling. It is possible that the inhibitory effects described above were a result of the incomplete interaction between signal molecules because of herbimycin A. We therefore investigated whether this compound interferes with the interaction between NIK and IKK or TRAF2. However, neither of these interactions was abrogated by treatment with herbimycin A (Fig. 3, A and B). We further examined the effect of herbimycin A on the binding of recombinant GST-fused IKKs to IκBα. The significant interactions between GST-IKKα or GST-IKKβ and IκBα were observed in a herbimycin A-sensitive manner (Fig. 3C).

Herbimycin A Blocked the Kinase Activity of IKKβ. Because overexpression of IKKβ, but not IKKα, resulted in the phosphorylation of IκBα (Fig. 2A), we studied the inhibitory effects of herbimycin A on IKKβ kinase activity in vitro. We performed an in vitro kinase assay using the immunoprecipitates with an anti-FLAG antibody from FLAG-IKKβ–expressing cell lysates and recombinant IκBα as a substrate.

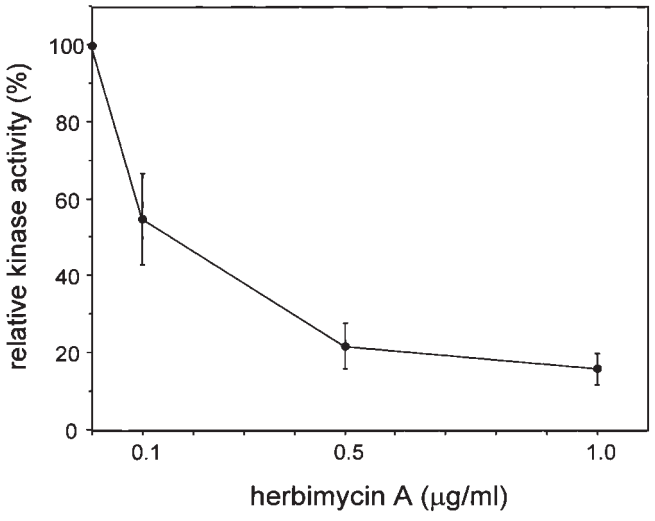

Fig. 4. IKKβ activation was sensitive to herbimycin A. HEK 293 T cells were transiently transfected with the FLAG-epitope tagged IKKβ. Cell extracts were prepared and immunoprecipitated with anti-FLAG antibody. Then, the immunoprecipitates were incubated with recombinant GST-fused IκBα as a substrate in vitro. The expression levels of IKKβ in total cell extracts were determined by immunoblotting with anti-FLAG antibody. Each intensity was quantified using the Fuji BAStation software program. Values are mean ± S.E. of four separate experiments.

Herbimycin A dose-dependently impaired the phosphorylation of IκBα (Fig. 4).

Treatment with DTT Disrupted the Inhibitory Effects of Herbimycin A. Because the reducing agents, such as dithiothreitol (DTT), inactivate the reaction of herbimycin A with substrate protein (Senga et al., 2000), we investigated the effects of simultaneous treatment with herbimycin A and DTT on IL-1β–induced phosphorylation of IκBα. Treatment with DTT alone did not affect the state of IκBα phosphorylation compared with that of nontreatment cells. On the other hand, the protein levels of IκBα detected with anti-IκBα antibody were decreased in samples in which the phosphorylation of IκBα was evident. Although herbimycin A completely disrupted the IκBα phosphorylation stimulated by IL-1β, treatment with DTT recovered the inhibitory effect (Fig. 5).

In addition, we examined the effects of *N*-acetyl cysteine (NAC) as an another thiol compound. NAC is known to be a potent inhibitor on the NF-κB pathway (Schreck et al., 1991; Hayakawa et al., 2003). High concentrations of NAC (more than 10 mM) are usually required to inhibit this pathway, but this condition was cytotoxic to cells in our system (data not shown). Hence, we could not detect the significant inhibition of NF-κB activation or the phosphorylation of IKKs and IκBα caused by challenge with IL-1 or overexpression of several genes.

Herbimycin A Selectively Affected IKKβ Cys59 Near the Catalytic Site. It is possible that herbimycin A interacts with or modifies IKKβ and thereby inhibits the NF-κB pathway. In this study, we constructed variants of IKKβ to clarify the possible modification site(s). Each of the seven cysteine residues in the kinase domain of IKKβ was displaced with an alanine residue (Fig. 6A). Each mutant was transfected transiently, and we then investigated the change in sensitivity of herbimycin A on IKK and IκBα phosphorylation. None of the variants altered the intrinsic kinase activities (Fig. 6B). However, herbimycin A effectively inhibited the phosphorylation in all mutant-transfected cells, with the exception of the C59A mutant of IKKβ, which showed insensitivity for herbimycin A. Although IKKα has a structure similar to that of IKKβ (Fig. 6A), significant phosphorylation of IKKα and IκBα was not observed in IKKα-transfected cells (Fig. 2A).

The C59A Mutant of IKKβ Was Insensitive to Herbimycin A. Next, we attempted to characterize the herbimycin A-insensitive C59A IKKβ mutant. Immunohisto-

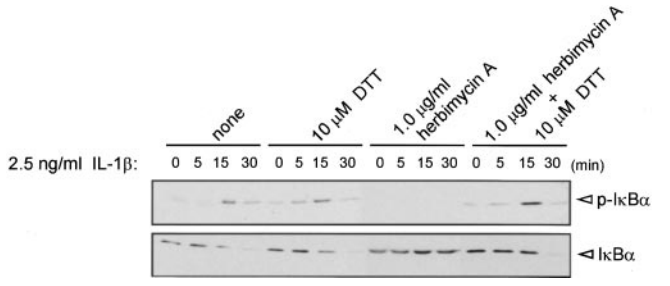

Fig. 5. The inhibitory effect of herbimycin A on NF-κB signaling was reversed by DTT treatment. Hs683 cells were pretreated with 10 μM DTT and/or herbimycin A for 12 h and then stimulated with or without 2.5 ng/ml IL-1β for the indicated periods. Cell extracts were prepared and subjected to Western blot analysis with anti-phospho IκBα (Ser32) and anti-IκBα antibody.

chemical analysis revealed that the wild type or C59A IKKβ induced their own phosphorylation in a herbimycin A-sensitive or -insensitive manner, respectively (Fig. 7A). It has been well established that activation of IKK is dependent on phosphorylation of the IKKβ subunit and that IKKα is not implicated in IKK activation (Delhase et al., 1999; Karin, 1999b; O'Mahony et al., 2000). However, endogenous IKK complexes are composed of IKKα·IKKβ heterodimers. Therefore, we attempted to characterize the effect of the C59A mutant in heterodimers against herbimycin A. As shown in Fig. 2B, coexpression of IKKα and IKKβ induced the phosphorylation of IKKα, IKKβ, and IκBα in a herbimycin A-sensitive manner. Next, we checked the effect of herbimycin A on IKKα and IKKβ phosphorylation in cells transfected by both IKKα and C59A-mutated IKKβ. Treatment with herbimycin A disrupted the phosphorylation in wild-type IKKα/IKKβ-transfected cells but did not alter the phosphorylation in wild-type IKKα- and mutated C59A IKKβ-transfected cells (Fig. 7C). Last, we examined whether the IKKβ C59A mutant abrogates NF-κB activation challenged with IL-1β in glioma cells. As shown in Fig. 7D, treatment with herbimycin A resulted in a decrease in NF-κB activity as detected by the luciferase assay in mock-transfected cells. It is noteworthy that the cells overexpressed by the C59A mutant of IKKβ showed significant insensitivity to herbimycin A.

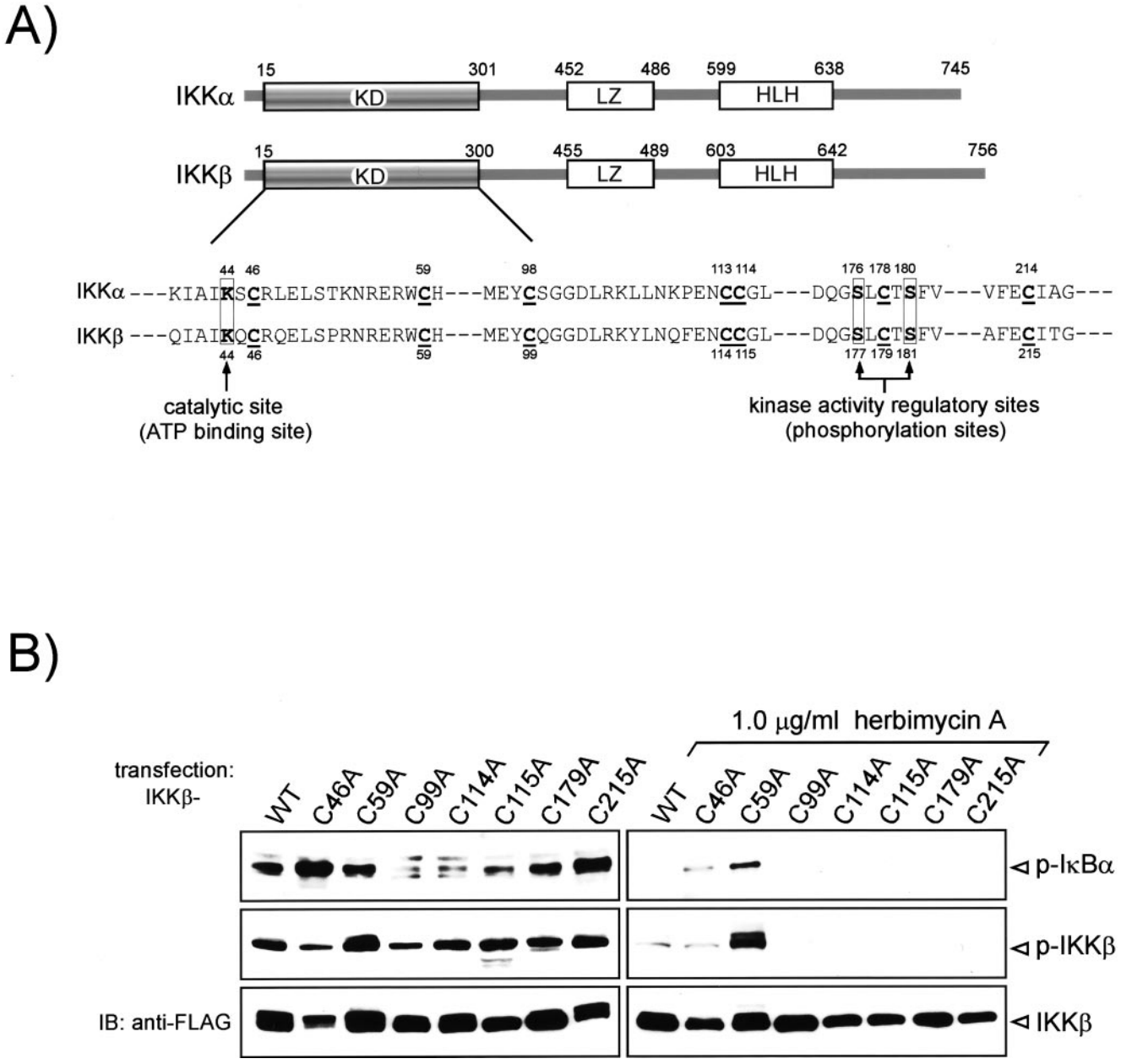

Fig. 6. Determination of the target site of herbimycin A in IKK β. A, Schematic representation of Cys residues at the kinase domain (KD) of IKKα and IKKβ. B, HEK 293 T cells were transiently transfected with the indicated mutant of IKKβ (B) and then incubated with or without herbimycin A. Cell extracts were prepared and subjected to Western blot analysis with anti-phospho IKK and anti-phospho IκBα antibodies. The expression levels of IKKβ in total cell extracts were determined by immunoblotting with anti-FLAG antibody.

Discussion

We have demonstrated previously that treatment with herbimycin A selectively attenuates the gene induction regulated by NF-κB in glial cells (Nishiya et al., 1995, 2000; Uehara et al., 1998, 1999). In this report, we attempted to elucidate the possible target for herbimycin A. We focused on NIK and IKK, which regulate NF-κB signaling upstream of IκBα. The IκBα phosphorylation by transfection of wild-type NIK or IKKβ, but not of wild-type IKKα, was sensitive to herbimycin A (Fig. 2A). Moreover, we indicate here that 1) NIK alone stimulates phosphorylation of IKKs and subsequently, IκBα, but phosphorylation of IKKβ and IKKα is inhibited completely and partially, respectively, by herbimycin A treatment (Fig. 2C); 2) transfection of IKKα together with IKKβ stimulates phosphorylation of IκBα and IKKs themselves in a herbimycin A-sensitive manner (Fig. 2B); and 3) NIK-induced phosphorylation of IκBα occurs via IKKβ but not IKKα (Fig. 2D). From these observations, we conclude that 1) NIK induces both IKKα and IKKβ phosphorylation and the subsequent IκBα phosphorylation and 2) IKKβ triggers the phosphorylation of itself and IKKα and subsequently, IκBα. Therefore, we assert that IKKα phosphorylation by NIK is inhibited partially by herbimycin A, probably caused by blockage of the pathway from IKKβ to IKKα but not from NIK to IKKα (Fig. 2C). Thus, we speculate that herbimycin A may attenuate IKKβ activity at least and thereby impair such subsequent reactions as IκBα phosphorylation and NF-κB activation, because NIK is known to activate the IKK upstream of the IKK complex (Woronicz et al., 1997). However, some problems remain in determining the effects of herbimycin A on the IKKα molecule. It is difficult at present to determine the kinase activity of IKKα in vivo. Because IKKα could not stimulate the phosphorylation of IκBα (NF-κB activation) in vivo, it is hard to characterize the inhibitory effects of herbimycin A on IKKα in our system. Thus, IKKα may also be another target for herbimycin A, although we could not detect the NF-κB activation by overexpression of IKKα. On the other hand, the inhibitory effect of herbimycin A on NF-κB signaling may be based on suppression of the interaction between NIK and the IKK complex. We therefore attempted to confirm the effect of herbimycin A on the interaction between IKK and NIK. However, we could not observe a significant abrogation of these interactions (Fig. 3A). On the other hand, IκBα interacted with IKKβ more tightly than did IKKα in vitro, and the interaction between IκBα and IKKβ was attenuated slightly by treatment with herbimycin A (Fig. 3C). These results suggested that herbimycin A achieved its effect through inhibition of both IKKβ kinase activity and the interaction between IKK and IκBα.

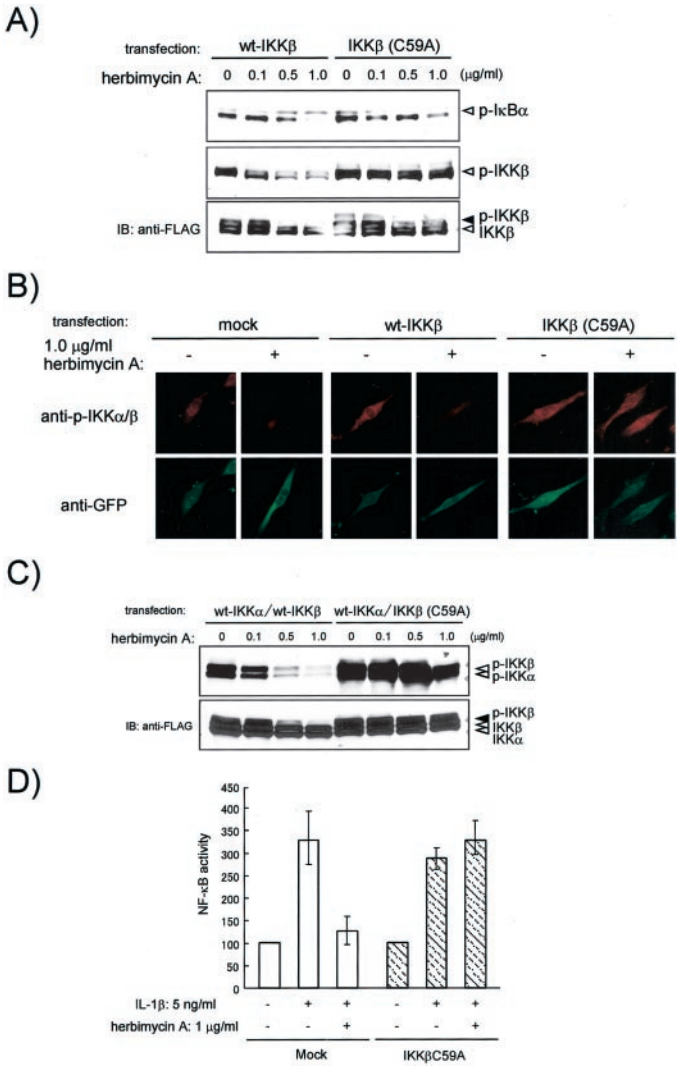

Fig. 7. The C59A IKKβ mutant was insensitive to herbimycin A. HEK 293 T cells were transfected with FLAG-tagged wild type (wt) or FLAG-tagged C59A-mutated IKKβ and then treated with several concentrations of herbimycin A (A). Cell lysates were prepared and subjected to Western blot analysis with anti-phospho IκBα, anti-phospho IKK, and anti-FLAG antibodies. Hs683 cells were transiently transfected with wild type or C59A-mutated IKKβ together with enhanced green fluorescent protein (GFP) and treated with herbimycin A (B). The cells were fixed and subjected to immunofluorescent analysis with anti-phospho IKKs and anti-GFP antibodies. HEK 293 T cells were cotransfected with FLAG-tagged IKKα and FLAG-tagged wt or C59A-mutated IKKβ and then treated with the indicated dose of herbimycin A (C). Cell extracts were prepared and subjected to Western blot analysis with anti-phospho IKKs. The expression levels of IKKα and IKKβ in total cell extracts were determined by immunoblotting with anti-FLAG antibody. IKKβ C59A mutant abrogates NF-κB activation challenged with IL-1β in glioma cells (D). To confirm the possible target site for herbimycin A, Hs683 glial cells were transfected with mock or C59A-mutated IKK β together with pNF-κB Luc and pRL-TK vectors. The cells were treated with or without herbimycin A and then stimulated with 5.0 ng/ml IL-1β for 6 h. The activity was assayed as described under *Materials and Methods*.

Herbimycin A has been isolated as a potent selective inhibitor of v-Src tyrosine kinase (Uehara et al., 1989). This inhibition occurs via the irreversible binding of herbimycin A to a particular Cys residue in the C-terminal of v-Src (Uehara et al., 1989; Fukazawa et al., 1994; Senga et al., 2000). We also observed that herbimycin A blocked IKKβ activity dose dependently both in vivo and in vitro, and this effect disappeared with simultaneous addition of DTT (Figs. 2 and 4). Based on the above findings, it seemed possible that IKKβ could be covalently modified by herbimycin A at Cys residue(s) localized in or near its kinase domain. We then constructed several variant IKKs with cysteine-to-alanine mutations in their kinase domains. It was surprising that only the C59A mutant showed remarkable resistance against herbimycin A, and the C46A mutant was also less sensitive to this inhibitor than other mutants (Fig. 6B). On the other hand, none of the mutants of IKKα resulted in any change compared with the wild-type IKKα (data not shown). These results suggested strongly that herbimycin A selectively in-

teracted with or modified Cys59 and partly Cys46 in IKKβ. These results may have been related to the three-dimensional structure of IKKβ or to a disturbance of IKKβ by interacting proteins in the IKK complex, such as IKKβ or NF-κB essential modulator. In addition, the inhibitory effect of herbimycin A on the IKKα·IKKβ heterodimer also disappeared in the cells overexpressing the C59A mutant of IKKβ (Fig. 6C). The kinase activity of heterodimerized IKKs was dependent on that of IKKβ (Karin, 1999a,b). From these observations, it seems possible that herbimycin A binds specifically to IKKβ (Cys59 or Cys46) in the IKK complex and thereby inhibits IKK activity. It has been reported previously that prostaglandin A1 and arsenite inhibit the IKK activity caused by binding at Cys178 of IKKα and Cys179 of IKKβ, sites that are both located near the kinase activity-regulatory (phosphorylation) sites (Kapahi et al., 2000; Rossi et al., 2000). In addition, aspirin, which is used widely as an anti-inflammatory agent, has the ability to inhibit selectively only IKKβ activity by interfering competitively with ATP binding (Yin et al., 1998). Although we cannot currently explain the detailed interactive mechanism of herbimycin A or other compounds on the IKKβ molecule, the differences between modification/interaction sites in IKKβ by these chemicals may be dependent on their own structure or size. To finish, we attempted to determine whether the IKKβ C59A mutant prevents herbimycin A-dependent inhibition of NF-κB activation by IL-1β. As shown in Fig. 7D, the effects of herbimycin A on NF-κB activation triggered by IL-1β disappeared in the C59A mutant-overexpressed cells. From these results, we conclude that Cys59 in IKKβ is a target for herbimycin A and consequently, abrogates the NF-κB pathway.

In summary, our results indicate that herbimycin A may interact mainly with the Cys59 of IKKβ located in or near the catalytic (ATP binding) site. Therefore, this modification may lead to the inhibition of ATP binding or an interaction with substrate proteins, such as IκBα. In fact, herbimycin A also attenuated the interaction between IKKβ and IκBα in vitro (Fig. 3C). Hence, we consider herbimycin A is a potent inhibitor that induces modification specifically at the Cys59 (or Cys46) of IKKβ. Based on these findings, herbimycin A may be a useful tool for analysis of NF-κB signaling and could be a new candidate anti-inflammatory drug with a novel function.

Acknowledgments

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Address correspondence to: Prof. Yasuyuki Nomura, Department of Pharmacology, Graduate School of Pharmaceutical Sciences, Hokkaido University, Sapporo 060-0812, Japan. E-mail: nomura@pharm.hokudai.ac.jp