Signal-induced Ubiquitination of IκB Kinase-β*

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Received for publication, September 26, 2003
Published, JBC Papers in Press, September 26, 2003, DOI 10.1074/jbc.M310686200

Initiation of the genetic programs for inflammation and immunity involves nuclear mobilization of transcription factor NF-κB. This signal-dependent process is controlled in part by the β-catalytic subunit of IκB kinase (IKKβ), which marks IκBα and other cytoplasmic inhibitors of NF-κB for proteolytic destruction. The catalytic activity of IKKβ is stimulated by pathologic and physiologic inducers of NF-κB, such as the Tax oncoprotein and proinflammatory cytokines. We now report evidence that these NF-κB-inducers target IKKβ for conjugation to ubiquitin (Ub) in mammalian cells. The apparent molecular size of modified IKKβ is compatible with monoubiquitination rather than attachment of a tandem Ub chain. The modification is contingent upon signal-induced phosphorylation of the activation T loop in IKKβ at Ser-177/Ser-181. The formation of IKKβ-Ub conjugates is disrupted in cells expressing YopJ, a Ub-like protein protease that interferes with the NF-κB signaling pathway. These findings indicate an important mechanistic link between phosphorylation, ubiquitination, and the biologic activity of IKKβ.

The inducible transcription factor NF-κB is biochemically coupled to cell-surface members of the tumor necrosis factor (TNF) receptor, Toll-like receptor, and immunoglobulin superfamily (1). NF-κB is persistently activated in cells expressing the Tax protein of human T-cell leukemia virus type 1, which has potent oncogenic properties (2). This deregulated expression of NF-κB activity also underlies acute and chronic inflammatory diseases (1). Nuclear translocation of NF-κB is controlled in part by the β-catalytic subunit of IκB kinase (IKK), which targets IκBα and other cytoplasmic inhibitors of NF-κB for proteolytic destruction (3). IKK contains two catalytic subunits, designated IKKα and IKKβ, as well as a regulatory subunit called IKKγ (3). IKKβ and IKKγ are essential for proteolytic inactivation of IκBα, whereas IKKα mediates NF-κB subunit processing via an IKKγ-independent mechanism (4).

The catalytic activity of IKKβ is stimulated by signals that trigger its phosphorylation at Ser-177/Ser-181, such as TNF-α (5). These phosphoacceptors lie in a region of the catalytic domain that shares homology with regulatory “T loop” sequences found in mitogen-activated protein kinases and their upstream activators (5). In contrast to its transient pattern of phosphorylation and activation in TNF-stimulated cells, IKKβ is chronically phosphorylated and activated in cells expressing the Tax oncoprotein (6). Tax-induced activation of IKKβ is blocked by YopJ (6), a cysteine protease that removes ubiquitin (Ub)-related modifiers from target proteins in mammalian cells (7). This finding with YopJ raised the possibility that IKKβ might be subject to signal-dependent ubiquitination. In keeping with this possibility, previous studies with partially purified kinase complexes suggested that IKK is conjugated to Ub in vitro (8). However, these pioneering in vitro experiments were conducted prior to molecular cloning of individual IKK subunits (3).

We now demonstrate that IKKβ is conjugated to Ub in vivo following engagement of either the Tax or TNF signaling pathway. Signal-induced ubiquitination of IKKβ is disrupted by wild type but not protease-deficient versions of YopJ. Formation of IKKβ-Ub conjugates is dependent on the presence of IKKγ, which is essential for IKKβ catalytic activity. The apparent molecular size of IKKβ-Ub conjugates is consistent with attachment of a single Ub molecule to IKKβ, excluding efficient recognition by the 26 S proteasome (9). Loss-of-function mutations in IKKβ that block phosphorylation at Ser-177/Ser-181 prevent its ubiquitination. In sharp contrast, the replacement of these sites with phosphomimetic amino acids yields a gain-of-function mutant that is constitutively ubiquitinated in the absence of an NF-κB agonist. We conclude that T loop phosphorylation at Ser-177/Ser-181 generates a conditional Ub-targeting signal in IKKβ. This contingent post-translational mechanism may be applicable to other inducible enzymes under T loop control that lie outside of the NF-κB signaling pathway.

EXPERIMENTAL PROCEDURES

Reagents—Polyclonal anti-IKK (H-470, FL-419) and monoclonal anti-TNF-R1 (TNF-R1) antibodies (H-5) were purchased from Santa Cruz Biotechnology. Rat monoclonal anti-HA antibodies were obtained from Roche Applied Science. Monoclonal antibodies specific for FLAG and T7 epitope tags were purchased from Sigma and Novagen, respectively. Rabbit anti-Tax antibodies were provided by Dr. Bryan Cullen (Duke University). Phospho-specific antibodies that recognize IKKβ following modification at Ser-181 were obtained from Cell Signaling Technology. Expression vectors for Tax, YopJ, Ub, and IKK subunits have been described previously (6, 7, 11). The expression vector for polyclonal (His)6-tagged IKKβ was constructed using pCRU His (Santa Cruz Biotechnology). The expression vector for TNF-R1 was provided by Dr. Brian Seed (Harvard University).

Subcellular Fractionation—Human 293T cells were cultured as described and transfected using the calcium phosphate method (6). Whole cell extracts were prepared in radiomunne precipitation buffer (150 mM NaCl, 10 mM sodium phosphate pH 7.2, 0.1% SDS, 0.5% sodium deoxycholate, 1% Nonidet P-40) containing 5 mM N-ethylmaleimide. Cytoplasmic extracts were prepared by detergent lysis in the presence of phosphatase/protease inhibitors (6) and then equilibrated in ELB buffer (50 mM HEPES, pH 7.4, 250 mM NaCl, 5 mM EDTA, 0.1% Nonidet P-40) prior to immunoprecipitation. Immunocomplexes were washed
cytoplasmic extracts were purified by Ni-NTA in the presence of 3 M GuHCl, fractionated by SDS-PAGE, and immunoblotted with anti-IKK antibodies. IKKβ immunocomplexes were fractionated by SDS-PAGE and immunoblotted for Ub content with monoclonal anti-HA antibodies (top panel). Protein expression levels in cytoplasmic extracts were determined by immunoblotting with the indicated antibodies (lower three panels). B, human 293T cells were transfected with vectors for Tax (0.3 μg), IKKγ (T7-tagged, 40 ng), IKKβ (FLAG-tagged, 50 ng), and Ub (HA-tagged, 2 μg) as indicated. Cytoplasmic extracts were adjusted to 3 M GuHCl and incubated with Ni-NTA resin. Bound proteins were fractionated by SDS-PAGE and immunoblotted with HA-specific antibodies (top panel). Protein expression levels in cytoplasmic extracts were determined by immunoblotting with the indicated antibodies (lower three panels). C, human 293T cells were transfected with vectors for Tax (0.3 μg), IKKγ (T7-tagged, 40 ng), IKKβ (FLAG-tagged, 50 ng), and Ub (HA-tagged, 2 μg) as indicated. His-Ub conjugates were purified from cytoplasmic extracts as described in B and immunoblotted with IKKβ-specific antibodies (top panel). Protein expression levels in cytoplasmic extracts were determined by immunoblotting with the indicated antibodies (lower three panels).

Sequentially with ELB buffer containing 2 M urea and then with radioimmune precipitation buffer. Bound proteins were fractionated by SDS-PAGE, transferred to polyvinylidene difluoride membranes, and detected on immunoblots as described previously (6).

Purification of IKKβ-Ub Conjugates—To perform nickel-chelate affinity chromatography under denaturing conditions (11), cytoplasmic extracts were equilibrated in 3 M guanidine hydrochloride (GuHCl), 150 mM NaCl, and 10 mM imidazole. Alternatively, cells were lysed directly with 6 M GuHCl and incubated in the presence of 3 M GuHCl, fractionated by SDS-PAGE, and immunoblotted with anti-IKKβ antibodies (top panel). Expression levels of IKKβ, IKKγ, and Tax in cytoplasmic extracts were determined by immunoblotting with the indicated antibodies (lower three panels). B, human 293T cells were transfected with vectors for Tax (0.3 μg), IKKγ (T7-tagged, 40 ng), IKKβ (FLAG-tagged, 50 ng), and Ub (HA-tagged, 2 μg). Cells were lysed directly with 6 M GuHCl, and extracts were adsorbed on Ni-NTA beads. Unfractionated cytoplasmic extracts (lanes 1 and 2) and purified Ub conjugates (lanes 3 and 4) were resolved by SDS-PAGE and immunoblotted with IKKβ-specific antibodies. Molecular sizes for protein standards are given in kilodaltons (left margin).

**RESULTS**

**Signal-induced Ubiquitination of IKKβ in Vivo**—The β-catalytic subunit of IKK is required for proteolytic inactivation of IκBα, a principle cytoplasmic inhibitor of NF-κB (3). To determine whether IKKβ is ubiquitinated *in vivo*, we programmed 293T cells with expression vectors for Tax, IKKβ, IKKγ, and a HA-tagged form of human Ub. IKKβ immunocomplexes were prepared from recipient cells and then probed on immunoblots for their HA-Ub content. As shown in Fig. 1A (top), we were unable to detect HA-Ub in IKKβ immunocomplexes derived from control cells lacking either Tax or HA-Ub (lanes 1 and 2). However, Tax induced significant levels of IKKβ ubiquitination.
resultant extracts were subjected to Ni-NTA chromatography. Ub conjugates were fractionated by SDS-PAGE and immunoblotted with IKK activation (10). Ub conjugates were purified from cytoplasmic extracts by nickel-chelate affinity (Ni-NTA) chromatography in the presence of 3 M GuHCl. Affinity-purified Ub conjugates were resolved by SDS-PAGE and immunoblotted with IKK antibodies (11). Alternatively, IKK complexes were immunopurified from cytoplasmic extracts using FLAG-specific antibodies, fractionated by SDS-PAGE, and probed on immunoblots for their IKK content (Fig. 1B, lower panels). These data strongly suggested that IKKβ is subject to Ub conjugation via a signal-dependent mechanism.

To confirm this finding, we used nickel-chelate affinity (Ni-NTA) chromatography to purify IKKβ-Ub conjugates under stringent denaturing conditions (11). For these experiments, 293T cells were transfected with expression vectors for Tax, IKKγ, HA-Ub, and polyhistidine (His)-tagged IKKβ. Histagged IKKβ was purified from recipient cells on Ni-NTA beads in the presence of 3 M GuHCl, eluted, and probed on immunoblots for HA-Ub. We found no evidence for the formation of immunoreactive IKKβ-Ub conjugates in control cells lacking either HA-Ub or Tax (Fig. 1B, top panel, lanes 1–3). However, in keeping with Fig. 1A, Ub-conjugated IKKβ was readily detected in extracts derived from cells coexpressing Tax and HA-Ub (lane 4). Similar results were obtained in complementary experiments with expression vectors for His-Ub rather than His-IKKβ, which enabled us to probe the IKKβ content in affinity-purified Ub conjugates under strong denaturing conditions (Fig. 1C). Based on these results from three independent experimental approaches, we conclude that IKKβ is subject to signal-dependent ubiquitination in transfected mammalian cells. Thus far, we have been unable to detect this modification in untransfected cells, presumably because of limited expression of the relevant IKKβ species at the endogenous protein level.

**Specificity and Extent of IKKβ Ubiquitination—Association of IKKβ with IKKγ is essential for proteolytic inactivation of IkBα and nuclear translocation of NF-κB (3). To determine whether IKKγ is required for signal-induced ubiquitination of IKKβ, we conducted experiments with 293T cells expressing Tax, His-Ub, and IKKβ in the presence or absence of IKKγ. To provide stringent controls for specificity, parallel transfections were performed with point mutants of Tax that are selectively defective for either cAMP-response element-binding protein/activating transcription factor (Tax-M47) or NF-κB (Tax-M22) activation (10). Ub conjugates were purified from cytoplasmic extracts by Ni-NTA chromatography, resolved by SDS-PAGE, and then probed on immunoblots for their IKKβ content. As shown in Fig. 2A (top), wild type Tax failed to induce IKKβ ubiquitination in the absence of ectopic IKKγ (lanes 1 and 2). Provision of IKKγ in trans rescued this modification step in Tax-expressing cells (lane 4). Formation of IKKβ-Ub conjugates was also observed in experiments with Tax-M47 (lane 6) but not with Tax-M22 (lane 5), consistent with their differing capacities to activate IKKβ and NF-κB (6, 10). All of the ectopic proteins were efficiently expressed (Fig. 2A, lower panels). We conclude that IKKγ is required for signal-dependent ubiquitination of IKKβ.

Proteasome-mediated degradation of a substrate requires its
attachment to a multimeric Ub chain containing at least four covalently linked Ub molecules (~8 kDa each) (9). The presence of this degradation marker is typically revealed as a ladder or a smear on SDS-PAGE gels, which we failed to detect in our experiments with Ub-conjugated IKKβ (Fig. 1). In keeping with this observation, Tax has no significant effect on the steady-state level of IKKβ (12). To estimate the molecular size of IKKβ-Ub conjugates under conditions that minimize Ub editing by cellular isopeptidases, proteins were extracted from 293T cells by direct lysis in 6 M GuHCl (11). Ub-conjugated proteins were purified by Ni-NTA chromatography, resolved on an SDS-PAGE gel in conjunction with molecular weight standards, and probed on immunoblots for their IKKβ content (Fig. 2B). An approximate molecular size of 95 kDa was determined for the unmodified form of IKKβ derived from Tax-deficient cells (lane 1), whereas purified IKKβ-Ub conjugates derived from Tax-expressing cells migrated primarily as a single ~105-kDa species (lane 4). The observed electrophoretic mobility shift was compatible with the attachment of a single His-tagged Ub molecule to IKKβ rather than a multimeric Ub chain. We consider this experimental result to be significant, because emerging studies indicate that monoubiquitination regulates the biologic activity of target substrates via a protease-independent mechanism (13).

Role of T Loop Phosphorylation in IKKβ Ubiquitination—Tax-induced activation of IKKβ is contingent upon its phosphorylation at Ser-177 and Ser-181 (6). To explore the role of Ser-177/Ser-181 in IKKβ ubiquitination, 293T cells were transfected with vectors for a mutant of IKKβ containing alanine replacements at these two sites (IKKβ-SA), IKKγ, Tax, and His-Ub. We then isolated His-Ub-conjugates from recipient cells by chromatography on Ni-NTA beads, fractionated them by SDS-PAGE, and probed the resolved proteins on immunoblots for the presence of IKKβ. As shown in Fig. 3A (top), mutations that disrupt phosphorylation of Ser-177/Ser-181 in IKKβ blocked its ubiquitination in the presence of Tax (lanes 3 and 4). Similar results were obtained with a kinase-dead mutant of IKKβ that is defective for ATP-binding (IKKβ.KM, lanes 5 and 6). IKKβ was constitutively ubiquitinated in Tax-deficient cells when Ser-177 and Ser-181 were both replaced with the phosphomimetic glutamic acid (IKKβ. SE, lane 7), which generates a constitutively active IκB kinase (8). We conclude that Tax-induced phosphorylation and ubiquitination of IKKβ are biochemically linked.

Phosphorylation of IKKβ at Ser-177/Ser-181 is also required for its activation by proinflammatory mediators such as TNF (5). To explore whether proinflammatory signaling pathways for NF-κB activation lead to IKKβ ubiquitination, we programmed 293T cells with expression vectors for His-Ub, IKKβ, IKKγ, and TNF-R1 rather than Tax. As shown in Fig. 3B (top), TNF-R1 induced significant levels of IKKβ ubiquitination relative to control cells lacking the ectopic receptor (lanes 1 and 2). However, this signal-dependent response was disrupted in cells coexpressing TNF-R1 and IKKβ-SA (lanes 3 and 4). Immunoblotting experiments confirmed that enforced expression of TNF-R1 stimulated IKKβ phosphorylation and that IKKβ-SA was efficiently phosphorylated (Fig. 3B, lower panels). We conclude that both Tax and TNF-R1 induce IKKβ ubiquitination via a mechanism involving T loop phosphorylation of this catalytic subunit at Ser-177/Ser-181.

Attachment of Ub to IKKβ Is Disrupted by YopJ—Tax-induced activation of endogenous IKKβ is blocked in cells expressing the Yersinia virulence factor YopJ (6). Recent studies indicate that YopJ removes Ub-related modifiers from target proteins in mammalian cells (7). To determine whether YopJ interferes with stable formation of IKKβ-Ub complexes, 293T cells were transfected with expression vectors for Tax, HA-Ub, IKKβ, IKKγ, and YopJ. IKK complexes were immunopurified from recipient cells, resolved by SDS-PAGE, and then probed for the presence of HA-Ub on immunoblots. As expected, Tax stimulated ubiquitination of IKKβ in YopJ-deficient cells (Fig. 4, top panel, lanes 1 and 2). Coexpression with YopJ completely blocked this signal-dependent modification, which was accompanied by diminished T loop phosphorylation of IKKβ (lane 3, top two panels). In contrast, a protease-deficient mutant of YopJ containing a Cys → Ala substitution in its catalytic triad (7) had no significant effect on signal-induced ubiquitination of IKKβ (top panel, lane 4). All of the ectopic proteins were efficiently expressed, including the protease-deficient mutant of YopJ (Fig. 4, lower panels). Thus, the capacity of YopJ to disrupt signal-induced ubiquitination of IKKβ is dependent on its protease function (7). It remains unclear how this protease function also impinges on the phosphorylation status of IKKβ.

In summary, we have found that IKKβ is ubiquitinated in response to engagement of either the Tax or TNF signaling pathway. The apparent molecular size of Ub-modified IKKβ is most compatible with the attachment of a single Ub tag (Fig. 2B), precluding efficient recognition of the conjugate by the 26 S proteasome (9, 13). In keeping with prior in vitro studies (8), several lines in vivo evidence indicate a role for Ub in the regulation of IKKβ catalytic activity. First, ubiquitination of IKKβ is contingent upon the presence of IKKγ (Fig. 2A), a regulatory subunit that is essential for proper functional control of IKKβ (3, 4). Second, the modification is disrupted by YopJ (Fig. 4), which interferes with signal-dependent activation of endogenous IKKβ (6). Third, the formation of IKKβ-Ub conjugates is triggered by phosphorylation of IKKβ at Ser-177/Ser-181 (Fig. 3), a prerequisite for its activation by either Tax or TNF (5, 6). We propose that phosphorylation at Ser-177/Ser-181 generates a substrate recognition motif, permitting the Ub-conjugating machinery to dock with and monoubiquitinate IKKβ (14). In turn, this modification regulates the biologic action of IKKβ via a proteasome-independent mechanism, perhaps involving the intervention of an IKKβ-specific Ub receptor (15). Resolution of this model awaits assignment of the Ub acceptor sites and functional studies with ubiquitination-defective mutants of IKKβ.

Acknowledgments—We gratefully acknowledge Dr. Kim Orth (University of Texas Southwestern) for the expression vector encoding protease-deficient YopJ and Dr. Eugene Oltz (Vanderbilt University) for helpful discussions.

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J. Biol. Chem. 2003, 278:48903-48906.
doi: 10.1074/jbc.M310686200 originally published online September 26, 2003

Access the most updated version of this article at doi: 10.1074/jbc.M310686200

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