Site-specific Monoubiquitination of IkB Kinase IKKβ Regulates Its Phosphorylation and Persistent Activation

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Transcription factor NF-κB governs the expression of multiple genes involved in cell growth, immunity, and inflammation. Nuclear translocation of NF-κB is regulated from the cytoplasm by IkB kinase-β (IKKβ), which emarks inhibitors of NF-κB for polyubiquitination and proteosome-mediated degradation. Activation of IKKβ is contingent upon signal-induced phosphorylation of its T loop at Ser-177/Ser-181. T loop phosphorylation also renders IKKβ a substrate for monoubiquitination in cells exposed to chronic activating cues, such as the Tax oncoprotein or sustained signaling through proinflammatory cytokine receptors. Here we provide evidence that the T loop-proximal residue Lys-163 in IKKβ serves as a major site for signal-induced monoubiquitination with significant regulatory potential. Conserved replacement of Lys-163 with Arg yielded a monoubiquitination-defective mutant of IKKβ that retains kinase activity in Tax-expressing cells but is impaired for activation mediated by chronic signaling from the type 1 receptor for tumor necrosis factor-α. Phosphopeptide mapping experiments revealed that the Lys-163 → Arg mutation also interferes with proper in vivo but not in vitro phosphorylation of cytokine-responsive serine residues located in the distal C-terminal region of IKKβ. Taken together, these data indicate that chronic phosphorylation of IKKβ at Ser-177/Ser-181 leads to monoubiquitin attachment at nearby Lys-163, which in turn modulates the phosphorylation status of IKKβ at select C-terminal serines. This mechanism for post-translational cross-talk may play an important role in the control of IKKβ signaling during chronic inflammation.

Initiation of the genetic programs for inflammation and immunity is mediated in part by the inducible transcription factor NF-κB (1). In quiescent cells, NF-κB is sequestered in the cytoplasmic compartment by virtue of its association with the labile inhibitor IκBα or other structurally related proteins (2). Biological inducers of NF-κB include proinflammatory cytokines such as tumor necrosis factor-α (TNF), the lipopolysaccharide component of Gram-negative bacteria, and the Tax oncoprotein of human T-cell leukemia virus type 1 (3–5). Cellular stimulation with these agents leads to activation of a multicomponent kinase called IKK, which phosphorylates IκBα at Ser-32/Ser-36 (6). In turn, the phosphorylated Ser-32/Ser-36 motif docks with a specific ubiquitin (Ub)-protein ligase complex, resulting in Ub conjugation at nearby Lys-21/Lys-22 (7). Polyyubiquitinated IκBα is then degraded by the 26 S proteasome, enabling NF-κB to translocate to its nuclear site of action (6, 7).

The capacity of IKK to integrate a wide spectrum of activating cues has evoked significant interest in its structure, function, and signal-dependent regulation (8). The IKK holoenzyme is composed of α and β catalytic subunits, both of which contain kinase, leucine zipper, and helix-loop-helix domains (9). IKKβ is primarily responsible for phosphorylation of IκBα in vivo, whereas IKKα targets distinct cellular substrates in an alternative pathway for NF-κB induction (10). Activation of IKKβ by TNF, Tax, and other inducers of NF-κB is triggered by phosphorylation of this kinase at Ser-177/Ser-181 (11, 12). These phosphoacceptors lie in a region of the catalytic domain that shares homology with the regulatory T loop sequences found in mitogen-activated protein kinases and their upstream activators (11). The C-terminal region of IKKβ (amino acids 670–705) harbors a second set of TNF-inducible phosphoacceptors whose precise function remains unclear relative to Ser-177/Ser-181 (11). Prior studies suggest that modification of the T loop and C-terminal phosphoacceptors is mediated by IKKβ itself (11, 13). Signal-induced phosphorylation and activation of IKKβ are critically dependent upon its association with a noncatalytic subunit termed IKKγ/Nemo (9). Activated IKKβ also phosphorylates IKKγ, which may serve to amplify IKK signaling (12, 14, 15).

More recent studies indicate that IKKβ and IKKγ are substrates not only for phosphorylation but also for ubiquitination, although to different extents. Specifically, IKKγ is polyubiquitinated during the cellular response to microbial products, proinflammatory cytokines, antigen receptor agonists, and genotoxic stress (16). This modification is dependent on the C-terminal zinc finger domain of IKKγ, a frequent target for mutations that can impair NF-κB signaling and cause human immunodeficiency disease (17, 18). In contrast to IKKγ, IKKβ is conjugated to a single Ub unit under conditions of chronic stimulation via the Tax oncoprotein or the type 1 receptor for TNF (TNF-R1) (19, 20). Monoubiquitin attachment is contingent upon T loop phosphorylation at Ser-177/Ser-181 and disrupted in cells expressing YopJ, a Ub-like protein protease that interferes with IKK/NF-κB signaling (19). However, the underlying targeting mechanism and regulatory potential of IKKβ monoubiquitination remain unknown.

In this study, we provide genetic and biochemical evidence that chronic phosphorylation of IKKβ at Ser-177/Ser-181 leads to ligation of monoubiquitin at the T loop-proximal residue Lys-163. Replacement of Lys-163 with Arg yields a ubiquitination-defective mutant that is resistant to chronic activation mediated by TNF-R1. This functional defect is not apparent in cells expressing the Tax oncoprotein, suggesting an agonist-specific requirement for monoubiquitination. We also provide evidence that monoubiquitin attachment at Lys-163 regulates the in
vivo phosphorylation status of IKKβ at select TNF-responsive serines located in its C-terminal region (amino acids 670–705) (11). Comparative studies of in vivo versus in vitro phosphorylated IKKβ suggest that the Ub-dependent serines are targeted by a kinase other than IKKβ itself. Taken together, our findings indicate that IKKβ is regulated by a novel mechanism involving post-translational cross-talk between monoubiquitination and phosphorylation. This mechanism may play an important role in the acquisition of a deregulated IKKβ phenotype during chronic inflammation (21).

EXPERIMENTAL PROCEDURES

Antibodies—Polyclonal antibodies specific for IKK subunits (H-470, FL-419) and IκBα (C-21) were purchased from Santa Cruz Biotechnology. Monoclonal antibodies directed against RIP, IKKα, and IKKβ were obtained from BD Biosciences. Monoclonal antibodies specific for the HA (Roche Applied Science), FLAG (Sigma), and T7 (Novagen) epitope tags were obtained from the indicated commercial sources. Monoclonal anti-TNF-R1 antibodies (H-5) were purchased from Santa Cruz Biotechnology. Phospho-specific antibodies that recognize modified Ser-181 in IKKβ, Ser-376 in IKKγ, and Ser-32/Ser-36 in IκBα were obtained from Cell Signaling Technology. Polyclonal anti-IKKα antibodies were provided by Nancy Rice (NCI, National Institutes of Health, Bethesda). Polyclonal anti-Tax antibodies were provided by Dr. Bryan Cullen (Duke University).

Plasmids—Expression vectors for Tax, TNF-R1, Ub, and IKK subunits have been described (19). The epitope-tagged derivative of Tax was constructed by PCR-assisted amplification with 5′ primers that fused sequences encoding the FLAG epitope (MDYKDDDDK) in-frame with the N-terminal coding sequences of Tax. The RIP expression vector was provided by Dr. Adrian Ting (Mount Sinai School of Medicine). The full-length cDNA for IKKβ harboring Ser→Ala substitutions at positions 670, 672, 675, 679, 682, 689, 692, 695, 697, and 705 was provided by Dr. Michael Karin (University of California, San Diego) (11), modified by the addition of FLAG coding sequences, and inserted into pCMV4. Site-directed mutations were introduced into the full-length cDNA for IKKβ using the QuikChange (Stratagene) as specified by the manufacturer and confirmed by DNA sequencing.

Transfections and Subcellular Fractionation—Human 293T cells were cultured as described and transfected using the calcium phosphate method (12). Whole cell extracts were prepared in RIPA buffer (150 mM NaCl, 10 mM sodium phosphate, pH 7.2, 0.1% SDS, 0.5% sodium deoxycholate, 1% Nonidet P-40). Where indicated, N-ethylmaleimide (5 mM) was included to inhibit cellular isopeptidases and to disrupt IKKβ-IKKγ complexes (19). Cytoplasmic extracts were prepared by detergent lysis in the presence of phosphatase/protease inhibitors (12) and then purified by 1-chloro-3-tosylamido-7-aminobutyric acid (TLCK)-treated chymotrypsin, and subjected to two-dimensional phosphopeptide mapping as described (15).

RESULTS

Differential Ubiquitination of IKKβ and IKKγ—In prior transfection studies, we found that Tax-induced monoubiquitination of IKKβ is contingent upon the presence of the regulatory subunit IKKγ (19). These data suggested that the relevant Ub-conjugating machinery can recognize and modify IKKβ within the context of a higher order complex containing IKKγ. To test this model, we determined whether Ub-conjugated forms of IKKβ are associated with IKKγ. For these studies, epitope-tagged versions of the two IKK subunits were coexpressed with Tax in mammalian cells and then immunoprecipitated with epitope-specific antibodies. Resultant complexes were dissociated with 6 M guanidine hydrochloride and fractionated by nickel-chelate affinity (Ni-NTA) chromatography to capture His-tagged IKKβ (19). Ni-NTA eluates were probed on immunoblots (Fig. 1A) for their content of ubiquitinated and phosphorylated IKKβ (top and middle panels, respectively). Both of these species were readily detected in Ni-NTA eluates derived from IKKβ, IKKγ, or Tax immunoprecipitates (Fig. 1A, lanes 2, 4, and 6). Parallel fractionation studies with extracts from Tax-deficient cells confirmed the signal-dependent nature of each modification (Fig. 1A, lanes 1, 3, and 5). We conclude that IKKγ-bound forms of IKKβ are targeted for monoubiquitination.

Emerging studies indicate that IKKγ is polyubiquitinated during the cellular response to TNF, antigen receptor signaling, or genotoxic stress (16). Given our findings that IKKγ-bound forms of IKKβ acquire a different Ub signature (Fig. 1A), we next investigated whether IKKγ is conjugated to polyubiquitin or monoubiquitin in Tax-expressing cells. For these studies, extracts from transfected populations were treated with N-ethylmaleimide to disrupt IKKβ-IKKγ complexes, and then each subunit was isolated by immunoprecipitation (19). Resultant immunocomplexes were fractionated by SDS-PAGE and probed for Ub content on immunoblots. As shown in Fig. 1B, Tax stimulated monoubiquitination of IKKβ (lanes 1 and 2), whereas IKKγ was polyubiquitinated in the same cellular background (lanes 3 and 4). In keeping with prior studies of TNF-treated cells (17), removal of the C-terminal zinc finger motif in IKKγ blocked Tax-induced polyubiquitination (data not shown). Thus, ectopically expressed IKKβ and IKKγ are differentially modified with Ub in the presence of Tax, underscoring the subunit-specific nature of the monoubiquitination pathway under investigation.

To determine whether these distinct patterns of subunit modification are recapitulated at physiologic substrate levels, we next monitored Tax-induced ubiquitination of endogenous rather than ectopic IKK. As detected on immunoblots with antibodies to the Ub tag (Fig. 1C, top panels), the endogenous pool of IKKβ complexes contained a major immunoreactive species of ~105 kDa, consistent with the predicted...
molecular size of monoubiquitinated IKKβ (lane 4). Parallel studies with control cells lacking either tagged Ub or Tax confirmed the signal-dependent nature of this modification (Fig. 1C, lanes 1–3). In keeping with the results shown in Fig. 1B, endogenous IKKγ was polyubiquitinated in Tax-expressing cells (Fig. 1C, lane 8). Changes in the ubiquitination pattern could not be attributed to Tax-mediated stabilization or...
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**FIGURE 2.** Effect of Lys → Arg mutations in IKKβ on monoubiquitination in vivo. A, schematic diagram highlighting the kinase domain, leucine zipper (LZ), and helix-loop-helix (HLH) regions of human IKKβ. Relative positions of the T loop phosphoacceptors (Ser-177/Ser-181), C-terminal serine cluster (11), and 43 Lys residues (lower tick marks) in IKKβ are indicated. All of the Lys residues within each of the bracketed regions were simultaneously mutated to Arg, yielding mutants of IKKβ designated R1–R5. B, human 293T cells were transfected with expression vectors for IKKγ (T7-tagged, 15 ng), Ub (HA-tagged, 100 ng), and FLAG-tagged versions of either wild type (WT) IKKβ or the indicated Lys → Arg mutants (25 ng each). Whole cell extracts were prepared by detergent lysis in the presence of 10 mM N-ethylmaleimide. IKKβ complexes were immunoprecipitated with anti-FLAG antibodies, fractionated by SDS-PAGE, and immunoblotted with monoclonal anti-HA antibodies ([top panel] or anti-IKKβ antibodies ([bottom panel]). C, cells were transfected and analyzed for monoubiquitination ([top panel]) and total ([middle panel]) IKKβ as described in B, except that an expression vector for Tax (125 ng) was included in the transfection mixture. Relative expression levels of Tax in recipient cell extracts were determined by immunoblotting ([lower panel], D, cells were transfected with expression vectors for Tax (300 ng), IKKγ (T7-tagged, 30 ng), Ub (HA-tagged, 150 ng), and FLAG-tagged versions of IKKβ mutants R1–R5 (see A, 50 ng each). Transfectants were monitored for Tax-induced monoubiquitination ([top panel]) as described in B. Relative expression levels of IKKβ (T loop phosphorylated and total; [middle panel]) and Tax ([lower panel]) in recipient cell extracts are shown. E, cells were transfected with expression vectors for IKKγ (T7-tagged, 30 ng) and either IKKβ.SA (Ser-177/Ser-181 → Ala), IKKβ.SE (Ser-177/Ser-181 → Glu, lane 2), or variants of IKKβ.SE containing the indicated Lys → Arg mutations (FLAG-tagged, 50 ng). IKK complexes were immunopurified from cytoplasmic extracts with anti-T7 antibodies and assayed for Ikβ kinase activity in the presence of γ-32P(γ-ATP). Reaction mixtures were resolved by SDS-PAGE and analyzed by sequential autoradiography ([top panel]) and immunoblotting with IKK subunit-specific antibodies ([lower panels]).

degradation of either IKK subunit (Fig. 1C, lower panels). These data provide direct biochemical evidence for differential ubiquitination of IKKβ versus IKKγ at the endogenous protein level.

Lys-163 Is the Primary Ub Acceptor Site in IKKβ—Protein ubiquitination is mediated by the sequential action of at least three enzymes (termed E1, E2, and E3) that together catalyze the activation and ligation of Ub to susceptible lysine residues in a substrate (22). A prerequisite for understanding the mechanism and functional consequences of IKKβ monoubiquitination is to identify the relevant Tax-responsive acceptor site(s). In this regard, recent structural studies indicate that the spacing between a substrate’s inducible phosphorylation motif and its Ub acceptor site is a key determinant of ubiquitination efficiency (23). In what may be a related observation, we have found that Tax-induced phosphorylation of T loop residues Ser-177/Ser-181 in IKKβ is a prerequisite for its monoubiquitination (19).

These mechanistic results raised the possibility that IKKβ is monoubiquitinated at a Lys residue positioned relatively close to the T loop phosphoacceptors. To identify candidate Ub acceptors, site-directed mutagenesis was used to engineer Arg replacements at each of the 11 Lys residues in the N-terminal kinase domain of IKKβ (Fig. 2A). We then programmed Tax-deficient cells with these IKKβ mutants and monitored their in vivo ubiquitination status by immunoblotting. As shown in Fig. 2B, all of the kinase constructs were comparably expressed (lower panel). With the notable exception of mutant K171R (Lys-171 → Arg), which was constitutively monoubiquitinated, none of the Lys → Arg mutants of IKKβ were appreciably modified with Ub in Tax-deficient cells (Fig. 2B, top panel). The K171R mutant was also constitutively phosphorylated at T loop residues Ser-177/Ser-181 (data not shown), consistent with our prior studies indicating that the two modifications are linked (19).

Similar experiments were conducted with Tax-expressing cells. As shown in Fig. 2C, Tax induced monoubiquitination of wild type IKKβ ([top panel, lane 1], whereas mutants of IKKβ containing Lys → Arg replacements at positions 44, 147, 163, or 238 were significantly
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Impaired for Ub attachment (top panel, lanes 3, 5, 6, and 10). The observed defects in monoubiquitination could not be attributed to Tax-induced changes in IKKβ protein expression (Fig. 2C, middle panel) or varying levels of Tax expression (lower panel). Moreover, mutations that simultaneously disrupted all of the Lys residues located within the leucine zipper, the helix-loop-helix, or the intervening regions of IKKβ coexpression with Tax led to persistent monoubiquitination of wild-type IKKβ (lanes 3, 4, and 6). In sharp contrast, IKKβ kinase activity was fully retained following replacement of Lys-163 with Arg (lane 5). Taken together with Fig. 2C, we conclude that the K44R, K147R, and K238R mutations inhibit ubiquitination by perturbing the structural and/or functional integrity of the IKKβ kinase domain, whereas the K163R mutation selectively removes the Ub attachment site. Consistent with this interpretation, subsequent studies confirmed that removal of Lys-163 from IKKβ blocks Ub attachment in vivo (see Fig. 5A).

Tax and Proinflammatory Receptors Converge on the Same Ub Acceptors—Similar to the Tax oncoprotein, chronic cellular stimulation through TNF-R1 leads to monoubiquitination of IKKβ (19). We have shown previously that Tax activates the IKK complex via a direct binding mechanism (24). In contrast, TNF-R1 acts indirectly on IKK via intermediate adaptors, such as RIP, which recruit the kinase to the cytoplasmic tail of TNF-R1 (2, 5). Given these divergent mechanisms of action, we reasoned that Tax and TNF-R1 might target distinct Ub acceptors in IKKβ. To explore this possibility, we transfected 293T cells with expression vectors for IKKβ, IKKγ, and either TNF-R1 or Tax. We then monitored in vivo ubiquitination of IKKβ under each stimulatory condition.

As expected, neither wild type IKKβ nor the K163R mutant was monoubiquitinated in the absence of chronic cellular stimulation (Fig. 3, top panel, lanes 1 and 2). In keeping with results shown in Fig. 2C, coexpression with Tax led to persistent monoubiquitination of wild type IKKβ, whereas the K163R mutant was defective for this modification (Fig. 3, lanes 3 and 4). Despite the distinct mechanism by which TNF-R1 activates IKK signaling, the pattern of IKKβ subunit ubiquitination induced by TNF-R1 was indistinguishable from that obtained with Tax-expressing cells (Fig. 3, lanes 5 and 6). Immunoblotting experiments confirmed that Tax, TNF-R1, and IKK substrate targets were efficiently expressed (Fig. 3, lower panels). These biochemical data suggest that monoubiquitination at Lys-163 is a common post-translational modification step in the mechanism of Tax and TNF-R1 action on IKKβ.

Functional Consequences of IKKβ Monoubiquitination—Both Tax and TNF-R1 stimulate the catalytic activity of IKKβ via a mechanism involving phosphorylation of its T loop at Ser-177/Ser-181 (11, 12). This modification appears to be mediated by IKKβ itself (13). In turn, activated IKKβ phosphorylates a set of distinct protein substrates, including the associated regulatory subunit IKKγ and cytoplasmic inhibitors of NF-κB such as IκBα (6, 12, 14, 15). To determine whether these biochemical steps are dependent on monoubiquitination, we analyzed the in vivo phosphorylation status of K163R, IKKγ, and IκBα in Tax-expressing cells.

For these studies, cytoplasmic extracts from recipient cells were fractionated by SDS-PAGE and probed on immunoblots for Ub content with monoclonal anti-HA antibodies (top panel). Relative expression levels of IKKβ, Tax, and TNF-R1 were determined in parallel immunoblotting studies (lower panels).

![FIGURE 3. Tax and TNF-R1 target the same Ub acceptor site in IKKβ.](image-url)

Human 293T cells were programmed with expression vectors for His-tagged IKKβ (wild type versus K163R, 100 ng each), IKKγ (T7-tagged, 30 ng), Ub (HA-tagged, 200 ng), and either Tax (250 ng) or TNF-R1 (125 ng) as indicated. Cytoplasmic extracts were adjusted to 3 M guanidine hydrochloride and incubated with Ni-NTA resin. Bound proteins were fractionated by SDS-PAGE and probed on immunoblots for Ub content with monoclonal anti-HA antibodies (top panel). Relative expression levels of IKKβ, Tax, and TNF-R1 were determined in parallel immunoblotting studies (lower panels).
of RIP (Fig. 4C). Thus, Lys-163 is required for sustained IKKβ signaling from cell-surface TNF-R1, whereas RIP and Tax can circumvent this requirement from their sites of action in the cytoplasmic compartment. These results suggest an important mechanistic role for monoubiquitination in stable targeting of IKK in TNF-treated cells (11). To determine whether Ub attachment is dispensable for the induction of IKK activity in TNF-treated cells (11). To determine whether Ub attachment regulates phosphoryl group transfer to this serine cluster, we performed a series of experiments with IKKβ mutants that disrupt the Ub acceptor (mutant K163R) or the C-terminal phosphoacceptors (mutant M10; Ser→Ala replace-
ments were resolved on two-dimensional phosphopeptide maps to inves-
tigate the modification status of serines positioned in the C-terminal
region of IKKβ. As shown in Fig. 5C, unmodified IKKβ,SE contained at least six major chymotryptic phosphopeptides (designated PP-1 through PP-6). Disrup-
tion of the C-terminal serine cluster in IKKβ,SE eliminated PP-2 through PP-6 (Fig. 5D). In contrast, disruption of the Ub acceptor in IKKβ completely eliminated PP-3 and significantly altered the content of PP-2 and PP-6 (Fig. 5E). The observed in vivo effect of the K163R mutation on PP-2, PP-3, and PP-6 was highly specific, because replacement of T loop-proximal residue Lys-198 with Arg yielded the same phosphorylation pattern as IKKβ,SE (Fig. 5F). We conclude that Lys-163 is required for proper in vivo phosphorylation of the C-terminal serine cluster in IKKβ.

To investigate whether autophosphorylation generates PP-2, PP-3, and PP-6, we next assessed the in vitro modification pattern of purified IKKβ,SE proteins. For these studies, 293T cells were programmed with IKKβ,SE containing Lys versus Arg at position 163 and grown in media lacking [32P]orthophosphate. Ectopic proteins were immunopurified under highly stringent washing conditions (2 mM urea) in order to remove kinases that might loosely associate with IKKβ,SE and phosphorylate its C-terminal serine cluster. Purified IKKβ,SE was then incubated with [γ-32P]ATP and analyzed by two-dimensional phosphopeptide mapping. In keeping with the in vivo fingerprints, PP-4 and PP-5 were clearly

FIGURE 4. Agonist-specific effects of Lys-163 → Arg mutation in IKKβ on T loop phosphorylation and kinase activity. Human 293T cells were transfected with expression vectors for FLAG-tagged forms of IKKβ (wild type versus K163R, 50 ng each), IKKγ (T7-tagged, 30 ng), and either Tax (250 ng) (A), TNF-R1 (125 ng) (B), or RIP (125 ng) (C) as indicated. Cytoplasmic extracts were fractionated by SDS-PAGE. Resolved polypeptides were probed on immunoblots with antibodies for phosphorylated and unmodified forms of IKKβ (top panels), IKKγ (middle panels), or IκBα (lower panels). Blots were stripped and probed with antibodies specific for Tax, TNF-R1, or RIP as indicated (bottom panels).

Role of Monoubiquitin in IKKβ Phosphorylation at C-terminal Sites—Delhase et al. (11) reported that IKKβ autophosphorylates at a cluster of C-terminal serines following transient cellular stimulation with TNF. Unlike T loop phosphorylation of IKKβ at Ser-177/Ser-181, which greatly stimulates kinase activity, modification of these C-terminal sites is dispensable for the induction of IKKβ activity in TNF-treated cells (11). To determine whether Ub attachment regulates phosphoryl group transfer to this serine cluster, we performed a series of experiments with IKKβ substrates containing mutations that disrupt the Ub acceptor (mutant K163R) or the C-terminal phosphoacceptors (mutant M10; Ser→Ala replace-
ments at positions 670, 672, 675, 679, 682, 689, 692, 695, 697, and 705) (11). To exclude the potential for any subtle effects on the stoichiometry of T loop phosphorylation, both of the mutations were introduced into a constitutively active form of IKKβ harboring Glu residues in place of Ser-177/Ser-181 (IKKβ,SE). These Glu residues function efficiently as T loop phosphomimetics (see Fig. 2E) and facilitate chronic phosphorylation of the C-terminal serines in IKKβ (11).

We first monitored the resultant IKKβ constructs for Ub attachment following their enforced expression in 293T transfectants. All of the IKKβ mutants were expressed at comparable levels as demonstrated by immunoblotting (Fig. 5A, middle panel). As shown in the top panel of Fig. 5A, IKKβ,SE was chronically ubiquitinated in the absence of overt cellular stimulation (lane 2). In keeping with the essential role of T loop phosphorylation in Ub conjugation, ubiquitination was blocked when the T loop phosphomimetics were replaced with Ala (mutant IKKβ,SA) (Fig. 5A, lane 1). Likewise, replacement of Lys-163 with Arg in IKKβ,SE impaired Ub attachment (Fig. 5A, lane 3). In contrast, disruption of the C-terminal serine cluster in IKKβ was without effect (Fig. 5A, lane 4). Thus, C-terminal phosphorylation of IKKβ is not a prerequisite for monoubiquitination.

To determine whether monoubiquitination is required for C-terminal phosphorylation, transfectants expressing the same set of IKKβ mutants were metabolically radiolabeled with [32P]orthophosphate. Kinases complexes were isolated from recipient cells by immunoprecipitation, fractionated by SDS-PAGE, and analyzed by sequential autoradiography and immunoblotting. As shown in Fig. 5B (top panel), all of the IKKβ,SE constructs were efficiently phosphorylated relative to IKKβ,SA, a kinase-dead mutant (see Fig. 2E). As such, the radiolabeled kinases were digested in situ with chymotrypsin, and the resultant peptides were resolved on two-dimensional phosphopeptide maps to inves-
tigate the modification status of serines positioned in the C-terminal
region of IKKβ.
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FIGURE 5. Effect of Lys-163→Arg mutation in IKKβ on phosphorylation of its C-terminal serine cluster. A, human 293T cells were transfected with vectors for IKKγ (T7-tagged, 30 ng), Ub (HA-tagged, 250 ng), and either IKKβ.SA, IKKβ.SE, or variants of IKKβ.SE containing mutations that inhibit monoubiquitination (K163R) or C-terminal phosphorylation (M10) (FLAG tagged, 100 ng each). IKKβ complexes were purified from whole cell extracts with anti-FLAG antibodies, fractionated by SDS-PAGE, and immunoblotted for either Ub (top panel) or IKKβ (middle panel) content. Expression levels of IKKγ in recipient cell extracts are shown (lower panel). B, cells were transfected as described in A (except that the Ub expression vector was omitted) and radiolabeled with \(^{32}\)Porthophosphate for 4 h. IKK complexes were immunopurified with anti-FLAG antibodies and fractionated by SDS-PAGE. Resolved proteins were analyzed by sequential autoradiography (top panel) and immunoblotting with IKK subunit-specific antibodies (lower panels). C–H, cells were transfected and metabolically radiolabeled as described in B. Alternatively, \(^{32}\)Porthophosphate was omitted from the growth media (G and H). Radiolabeled IKKβ was immunopurified, resolved by SDS-PAGE, transferred to a PVDF membrane, and digested in situ with chymotrypsin. Peptides were separated by sequential electrophoresis and chromatography on TLC plates. The same protocol was used to process extracts from unlabeled cells, except that immunopurified IKKβ was subject to in vivo phosphorylation in the presence of \(^{32}\)P-ATP prior to SDS-PAGE. Radiolabeled chymotryptic fragments derived from IKKβ following either in vivo phosphorylation (PP-1 through PP-6, top four panels; spots 1–6) or in vitro phosphorylation (PP-4 and PP-5, lower two panels; spots 4–5) are indicated. Mixing experiments confirmed complete overlap of PP-4 and PP-5 derived from in vitro versus in vivo phosphopeptide fingerprints for IKKβ.SE (data not shown).

DISCUSSION

Current knowledge about the substrate targets and functional range of monoubiquitin in mammalian cell biology is still rudimentary as compared with polyubiquitin, which earmarks many proteins for proteasome-mediated degradation (20, 26). In prior studies, we found that chronic signaling mediated by the Tax oncoprotein or the proinflammatory receptor TNF-R1 leads to IKKβ monoubiquitination, albeit under conditions of IKK subunit overexpression (19). In the present report, we provide several new lines of evidence that underscore the pathophysiological relevance and specificity of this post-translational modification to IKKβ. We demonstrate that (i) monoubiquitinated IKKβ is integrated into higher order complexes containing the IKKγ regulatory subunit, (ii) IKKγ is conjugated to polyubiquitin rather than monoubiquitin in Tax-expressing cells, and (iii) Tax targets endogenous IKKβ for monoubiquitination in the absence of IKK subunit overexpression (Fig. 1). Thus, IKKβ joins a small but growing roster of cellular proteins that are modified by the addition of a single ubiquitin unit in a signal-dependent manner (20).

In order to understand the regulatory potential of monoubiquitin in chronic IKKβ signaling, we introduced a comprehensive set of Lys→Arg mutations in IKKβ and then monitored the kinase for monoubiquitination in Tax-expressing cells. Replacement of the T loop-proximal residue Lys-163 with Arg (mutant K163R) yielded a ubiquitination-defective (UD) mutant of IKKβ, whereas conservative Lys→Arg mutations at other potential Ub acceptors in the leucine zipper, helix-loop-helix, and intervening regions of IKKβ failed to block this modification step (Fig. 2). More importantly, the K163R mutant retained the capacity to associate with IKKγ and was targeted for Tax-induced phosphorylation at Ser-177/Ser-181 (Figs. 2E and 4A). These biochemical experiments confirm the structural integrity of Lys-163 as a UD mutant of IKKβ and reinforce the assignment of Lys-163 as the major Ub acceptor site. K163R was also defective for ubiquitination mediated by chronic TNF-R1 signaling, suggesting that monoubiquitin conjugation to Lys-163 in IKKβ is a common step in multiple pathways for NF-κB induction (Fig. 3).

Recent structural studies indicate that the spacing between a substrate's inducible phosphorylation motif and its Ub acceptor sites is a key determinant of ubiquitination efficiency (23). Consistent with these
spatial constraints, Lys-163 lies in close proximity to the T loop phosphoacceptors of IKKβ. This configuration is highly reminiscent of the distribution of modification sites involved in the phosphorylation-dependent mechanism for ubiquitination of IkBa, a downstream target of IKKβ (6). By analogy with IkBa (7), phosphorylation of IKKβ at Ser-177/Ser-181 may generate a substrate recognition motif, permitting the Ub-conjugating machinery to dock with and monoubiquitinate the kinase at nearby Lys-163. This mechanistic model for IKKβ monoubiquitination may have broader relevance, because Lys-163 is highly conserved among other cellular enzymes under T loop control, including members of the MAP kinase family of signal transducers (11, 27).

To determine whether monoubiquitination of IKKβ regulates its kinase action on downstream substrates, we monitored the phosphorylating activity of K163R in cells expressing either Tax or TNF-R1. In the presence of Tax, which interfaces directly with IKKβ (24), K163R mediated efficient phosphorylation of IKKβ and Tax (Fig. 4A). In contrast, the UD mutant was defective for sustained T loop phosphorylation in cells expressing TNF-R1, thus attenuating IKKβ and IkBa kinase activity (Fig. 4B). In subsequent studies, we found that the requirement for Ub attachment at Lys-163 was circumvented by RIP, a downstream effector of TNF-R1 that binds directly with the kinase complex, such as Tax and RIP. Further resolution of the cytoplasmic tail of TNF-R1. In contrast, the Ub modification is largely served among other cellular enzymes under T loop control, including members of the MAP kinase family of signal transducers (11, 27). These agonist-specific effects suggest that monoubiquitination facilitates persistent activation of IKKβ via its stable receptor to the cytoplasmic tail of TNF-R1. In contrast, the Ub modification is largely dispensable for persistent activation of IKKβ by agonists that associate directly with the kinase complex, such as Tax and RIP. Further resolution of this issue awaits in vivo gene targeting studies with the UD mutant of IKKβ identified in the present study.

In response to cellular stimulation with TNF, IKKβ phosphoacceptor, a cluster of serines located near its C terminus (11). Unlike T loop phosphorylation of IKKβ at Ser-177/Ser-181, which greatly stimulates kinase activity, modification of these TNF-responsive serines may influence the duration of IKKβ signaling (11). Our phosphopeptide mapping data provide evidence that the latter phosphorylation step is facilitated by Ub attachment to IKKβ. Two distinct subsets of C-terminal phosphopeptides in IKKβ were revealed in these studies (Fig. 5). In vivo phosphorylation of one subset was unaffected by the K163R mutation and fully recapitulated in vitro. This biochemical phenotype is in keeping with IKKβ autophosphorylation as reported by Delhase et al. (11). However, in vivo phosphorylation of the second subset was altered by the K163R mutation, indicating the involvement of monoubiquitin. These second-order phosphorylation sites in IKKβ were also resistant to in vitro modification, suggesting a requirement for accessory factors.

The precise mechanism underlying Ub-mediated phosphorylation of IKKβ remains unclear. Our findings may reflect the emerging role of monoubiquitin as an adaptor that mediates protein/protein interactions (28). Assuming that IKKβ-conjugated Ub provides an interaction surface, at least two mechanistic models can be postulated. In one model, formation of IKKβ-Ub conjugates enables the catalytic domain of IKKβ to functionally engage its C-terminal phosphorylation targets. Alternatively, Ub attachment to IKKβ facilitates the recruitment of a novel IKKβ kinase, either directly or indirectly via a Ub receptor. Consistent with the latter prospect, recent studies have identified a Ub-binding motif in MEKK1, a MAP kinase kinase kinase (29). Members of this enzyme family have been implicated previously in TNF-induced activation of IKK (30).

In vitro stimulation of mammalian cells with the cytokine TNF leads to rapid T loop phosphorylation and activation of IKKβ, followed by dephosphorylation and inactivation within 30–60 min (11). Preliminary experiments indicate that this transient response does not enable the stable formation and/or detection of IKKβ-Ub conjugates (supplemental Figs. 1 and 2). In contrast, IKKβ-Ub conjugates were readily detected after reinforced expression of TNF-R1, which evokes sustained T loop phosphorylation and activation of IKKβ (19). This persistent pattern of IKKβ signaling has been implicated in chronic inflammatory disease and cancer (31, 32). The capacity of TNF-R1 to trigger IKKβ monoubiquitination was fully recapitulated by the Tax oncoprotein, a pathophysiologic agonist that mediates persistent IKK signaling via a direct binding mechanism (19). Accordingly, formation of stable IKKβ-Ub conjugates may be contingent upon sustained T loop phosphorylation, persistent IKK kinase activity, or the action of a late gene product that is selectively expressed under chronic inducing conditions.

In summary, we report here that chronic phosphorylation of the T loop at Ser-177/Ser-181 in IKKβ leads to monoubiquitination at nearby Lys-163. This close spatial relationship may be attributed to docking of the relevant Ub-conjugating machinery at the T loop phosphoacceptors in IKKβ. We find that Ub modification is required for sustained IKKβ signaling from the proinflammatory receptor TNF-R1 but not for acquisition of the deregulated IKKβ phenotype in cells expressing the Tax oncoprotein. Our studies also indicate that monoubiquitination regulates the phosphorylation status of TNF-responsive serines located in the C-terminal region of IKKβ (11). Interference with Ub attachment selectively alters the phosphopeptide fingerprint of IKKβ following in vivo but not in vitro phosphorylation, suggesting the involvement of a kinase other than IKKβ itself. These findings highlight a novel mechanism for post-translational cross-talk between monoubiquitination and phosphorylation that may play an important regulatory role in IKK signaling during a chronic inflammatory response.

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