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Lysine 63-linked Ubiquitination Modulates Mixed Lineage Kinase-3 Interaction with JIP1 Scaffold Protein in Cytokine-induced Pancreatic β Cell Death*[S]

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Background: Cytokines activate MLK3 to compromise mitochondrial integrity and promote pancreatic β cell death.
Results: Results suggest a model in which IL-1β/TRAF6-mediated Lys-63-linked ubiquitination of MLK3 dissociates monomeric MLK3 from JIP1 for subsequent dimerization and activation.
Conclusion: IL-1β-stimulated Lys-63-linked ubiquitination activates MLK3, most likely by altering the dynamics of MLK-JNK-JIP module.
Significance: These findings can be exploited for therapeutic intervention in diabetes.

The mixed lineage kinase MLK3 plays a crucial role in compromising mitochondrial integrity and functions as a proapoptotic competence factor in the early stages of cytokine-induced pancreatic β cell death. In an effort to identify mechanisms that regulate MLK3 activity in β cells, we discovered that IL-1β stimulates Lys-63-linked ubiquitination of MLK3 via a conserved, TRAF6-binding peptapeptide motif in the catalytic domain of the kinase. TRAF6-mediated ubiquitination was required for dissociation of inactive monomeric MLK3 from the scaffold protein IB1/JIP1, facilitating the subsequent dimerization, autophosphorylation, and catalytic activation of MLK3. Inability to ubiquitinate MLK3, or the presence of A20, an upstream Lys-63-linked deubiquitinase, strongly curtailed the ability of MLK3 to affect the proapoptotic translocation of BAX in cytokine-stimulated pancreatic β cells, an early step in the progression toward β cell death. These studies suggest a novel mechanism for MLK3 activation and provide new clues for therapeutic intervention in promoting β cell survival.

Autoimmune destruction of pancreatic β cells is induced by a combined effect of a potent mix of cytokines, including IL-1β, TNF-α[2], and IFN-γ. Building on reports showing that MLK3 directs cytokine-mediated activation of the stress kinase JNK (1–4), we have reported previously that MLK3 functions as an important competence factor in the induction of cytokine-mediated β cell death. Of the cytokines that affect the autoimmune response in pancreatic β cells, IL-1β is by far the most potent inducer of MLK3. IL-1β stimulates MLK3 early in the apoptotic cascade of events and ultimately instigates a conformational change in the proapoptotic BCL-2 family protein BAX, mitochondrial outer membrane permeabilization, and compromise in mitochondrial integrity. MLK3 mediates its effects, at least in part, by the induction of TRB3, a well characterized inhibitor of the prosurvival kinase AKT (5). In the β cell, the overall effect of MLK3 is to disable cellular defense mechanisms, damage mitochondria, and thereby increase the potency of the late effect of cytokines.

Signaling via the TNF-α and IL-1/TLR receptors is characterized by an intricate cross-talk between posttranslational events of phosphorylation and ubiquitination (6). Upon binding to its cognate extracellular receptor, TNF-α/IL-1β induce the intracellular docking of downstream adaptor proteins, ultimately engaging tumor necrosis factor receptor-associated factors TRAF2 or TRAF6 (7–9). The TRAF protein interaction domain enables TRAF2/6 to function as adaptor proteins through structurally distinct subdomains (10, 11). TRAF adaptors also participate in cytokine signaling via the N-terminal Really Interesting New Gene (RING) domain, which functions as an E3 ligase and recruits the E2 ligase Uev13 for the atypical polyubiquitination of downstream signaling effectors (11). Notwithstanding exceptions, the isopeptide linkage at lysine 63 (Lys-63) in the ubiquitin (ub) moiety (12, 13) directs substrates to non-degradative fates such as cell signaling, protein sorting, or protein targeting (14). Mechanistically, Lys-63 recognition motifs are thought to facilitate cytokine signaling by nucleating the assembly of multiprotein complexes, as seen in the activation of NFκB, downstream of Toll-like receptor (TLR)/IL-1 receptor (IL-1R) signaling (12, 15, 16).

Lys-63-linked ubiquitination has emerged as an increasingly important modification in signaling processes (17, 18). The central role of TRAF proteins in cytokine signaling underscores the crucial role of this atypical ubiquitin modification in inflammation (19). A recent report demonstrating MLK3 as a target for Lys-63-linked polyubiquitination (2) and, more importantly, the increasing involvement of MLK3 in pathophysiologic conditions (20–24) prompted us to examine mechanisms

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[2] The abbreviations used are: TNF-α, tumor necrosis factor α; TLR, Toll-like receptor; TRAF, tumor necrosis factor receptor-associated factor; ub, ubiquitin; IRAK, Interleukin-1 receptor-associated kinase; MAP3K, mitogen-activated protein kinase kinase kinase; MKK, Mitogen-activated protein kinase kinase.

by which such an ubiquitination event regulates MLK3 activation in the pancreatic β cell.

In this study we report that IL-1β stimulation of pancreatic β cell lines resulted in Lys-63-linked ubiquitination, possibly at lysine 264 (Lys-264) in the catalytic domain of MLK3. Lys-63-linked ubiquitination of MLK3 appears to decrease the interaction of MLK3 with scaffold protein IB1/JIP1(islet-brain-1/JNK interacting protein 1, further referred to as JIP1), facilitating dimerization, autophosphorylation, and activation of the kinase. Inhibiting MLK3 ubiquitination also diminished the potency of MLK3 to compromise mitochondria resulting from a conformational change and activation of BAX. Our results are supported by published reports that demonstrate the efficacy of TNFAIP3/A20, a potent Lys-63 deubiquitinase of TRAF6 (25, 26), in preventing β cell death (27). Although many of the cytoprotective effects of A20 have been attributed to its ability to inhibit cytokine-induced NFκB, our study suggests that MLK3 may be an additional target for the protective action of A20 on pancreatic β cell death.

EXPERIMENTAL PROCEDURES

Reagents—Antibodies used include rabbit anti-HA (Santa Cruz Biotechnology, Santa Cruz, CA); rabbit anti-MLK3, pMLK3, JNK, pJNK, GST, TRAF6, Myc, and FLAG (Cell Signaling Technology, Beverly, MA); and mouse anti-JIP1 for Western blotting and mouse anti-BAX clone 6A7 (both from BD Biosciences) for immunostaining. Mouse anti-HA-agarose beads (Sigma) and glutathione-Sepharose beads (Amersham Biosciences-Pharmacia) were used for immunoprecipitation and pull-down experiments. Fluorescence and Western blotting employed fluorescent or HRP-conjugated secondary antibodies (Jackson ImmunoResearch Laboratories, West Grove, PA), the latter detected using Supersignal chemiluminescence reagents (Pierce). Other reagents include CEP11004 (Cephalon, Inc., Frazer, PA), IL-1β (Peprotech, Rocky Hill, NJ), and On-Target Plus Smartpool human TRAF6 siRNA (Dharmacon, Lafayette, CO). Reagents for *in vitro* ubiquitination, including recombinant UbE1, UbeH13-Uev1a heterodimer complex, Myc-ubiquitin, Ub conjugation buffer, and Mg-ATP mix cofactor mix were purchased from Boston-Biochem (Cambridge, MA).

Human Splenocyte and Islet Coculture—Human splenocyte and islet coculture was performed on the basis of our published protocol developed for mouse tissue, with some modifications (23). Briefly, human spleen was dissected into small 1-cm^2 cubes, crushed, and passed through a 50-mesh screen. Red blood cells were lysed in 0.15 M NH_4Cl, and the splenocytes were frozen in RPMI supplemented with 90% FBS and 10% dimethyl sulfoxide. Prior to receipt of the human islets, splenocytes were thawed and plated at 9000 cells/mm^2 in RPMI supplemented with 10% heat-inactivated FBS and stimulated for 2 days with plate-bound anti-CD3 and exogenous anti-CD28 antibodies (10 μg/ml and 1 μg/ml respectively; BD Biosciences). Stimulated human splenocytes secreted a full array of cytokines (supplemental Table 1), and no difference in stimulation was observed between fresh or frozen splenocytes (data not shown). The human islets were rested overnight upon their receipt and then cultured in transwell filters in the presence of unstimulated or stimulated splenocytes with or without 500 nM CEP11004 pretreatment. After 48 h of coculture, the islets were fixed, spun into pellets, and embedded in agarose plugs. Individual plugs from each treatment were laid out on a grid and processed for paraffin embedding in a single block.

Human Tissue—Human islets were obtained from the Integrated Islet Distribution Program. Islets were handpicked and cultured overnight in CRML supplemented with 10% FBS. Prior to stimulation with cytokines, islets were cultured for at least 8 h in RPMI 1640 supplemented with 10% FBS. Human spleen was provided by the National Disease Research Interchange.

Plasmids and Constructs—The PEBG-MLK3-WT (Dr. Ajay Rana, Loyola University, IL), PEBG-JNK (Dr. Tom Roberts, Harvard Medical School, MA), and Myc-JIP1 (Dr. Roger Davis, University of Massachusetts, MA) plasmids were gifts. All MLK3 and ubiquitin point mutants were generated using the QuikChange mutagenesis protocol (Stratagene, Inc.) using PEBG-MLK3-WT and pcDNA3-Myc-Ubiquitin as template, respectively. For HA-tagged MLK3 constructs, BamH1 fragments coding full-length wild-type or mutant plasmids were excised from PEBG vectors and subcloned into pcDNA3-HA, and the orientation was checked prior to use. Plasmids purchased from Addgene include FLAG-TRAF6 (John Kyriakis Laboratory, Boston, MA) and pcDNA3-EGFPC1-A20 (Addgene/Hong-bing Shu Laboratory, Wuhan University, China). To generate Myc-A20, full-length A20 was excised from pcDNA3-EGFPC1-A20 using BglII and SalI enzymes and cloned into the BamHI and XhoI sites of pcDNA3-Myc. Myc-TRAF6-ΔN, (amino acids 295–518) was generated by PCR with EcoRI and BglII flanking sites using pcDNA3-FLAG-TRAF6 as a template and inserted in-frame into the pcDNA3-Myc plasmid.

Immunofluorescence—For immunofluorescence, Min6 or HepG2 cells were fixed in 4% paraformaldehyde for 5 min, permeabilized with 0.1% Triton X, and primary antibodies were visualized with species-specific secondary antibodies conjugated to fluorescent probes.

Cell Culture and Transfection—Min6 cells (passages 15–18 only) were grown in DMEM containing 25 mM glucose supplemented with 4% heat-inactivated FBS and 50 μM β-mercaptoethanol. HepG2 cells were grown in a 1:1 mix of DMEM and F12K and 5% heat inactivated FBS. Adherent QBI-HEK293 cells (Q-biogene, now MP Biomedicals) and HeLa cells were cultured in DMEM containing 25 mM glucose supplemented with 10% FBS. For conditioned media, briefly, 0.9×10^6/cm^2 splenocytes were plated in RPMI supplemented with 10% heat-inactivated FBS and stimulated with plate-bound anti-CD3 and exogenous anti-CD28 antibodies (10 μg/ml and 1 μg/ml, respectively; BD Biosciences) for 2 days. Conditioned medium was cleared by centrifugation prior to use. Transfections for GST pull-down, immunoprecipitation assays, and BAX translocation were performed in Min6 or HepG2 cells using Lipofectamine 2000 (Invitrogen) according to the instructions of the manufacturer and were treated as described 48 h post-transfection.

GST Pull-down Assay and Immunoprecipitation—Mammalian expression vectors encoding GST, HA, or Myc-tagged fusion proteins were expressed in Min6 or HepG2 cells fol-

lowed by GST pull-downs with glutathione-Sepharose (Amersham Biosciences-Pharmacia) or immunoprecipitations as described (28). Min6 cells were used wherever possible. However, because of the susceptibility of Min6 cells, some experiments consisting of MLK3 expression along with proapoptotic JNK were conducted in HepG2 cells. JIP1-related experiments were conducted in HepG2 cells to avoid artifacts because of high levels of endogenous JIP1.

In Vitro Ubiquitination—The FLAG TRAF6 and HA-MLK3 or GST-MLK3 constructs were expressed in HEK293 cells, harvested in NETN (150 mM NaCl, 1 mM EDTA, 50 mM Tris (pH 8.0), and 0.5% Nonidet P-40) with a full complement of protease and phosphatase inhibitors. The proteins were purified by immunoprecipitation or GST pull-down. GST-MLK3 was eluted using 10 mM glutathione, and FLAG-TRAF6 was eluted using 200 μg/ml 3× FLAG peptide (Sigma) according to the instructions of the manufacturer. Proteins were filtered, and quality was assessed by Western blotting. Ubiquitin assays were performed in 20- to 30-μl reactions using 0.2 μg of UbE1, 0.5 μg of UbeH13-Uev1a heterodimer complex, 100 μM Myc-ubiquitin, Ub conjugation buffer, and Mg-ATP cofactors according to the instructions of the manufacturer. The reactions were performed in solution using 0.2–0.5 μg of GST-MLK3 and equal amounts of FLAG-TRAF6 or HA-MLK3 protein bound to beads. Reactions were incubated at 30 °C for 2–3 h. Reactions were stopped by heat inactivation at 100° for 5 min, and MLK3 proteins were immunoprecipitated using anti-GST antibodies in radioimmune precipitation assay buffer (50 mM Tris (pH 8.0), 150 mM NaCl, 0.1% SDS, 0.5% Na deoxycholate, and 1% Triton X-100) supplemented with protease and phosphatase inhibitors and 10 mM NEMA (*N*-methyl maleimide) was and resolved using SDS-PAGE. For JIP1 binding, ubiquitinated proteins were immunoprecipitated using 50 mM Tris (pH 8.0), 400 mM NaCl, 1% Triton X-100, washed 5 times, and then incubated with Myc-JIP1 protein extracts for protein-protein interactions performed in NETN in the presence of protease, phosphatase, and deubiquitinase inhibitors. Immunoprecipitates were also assessed to ascertain complete dissociation with TRAF6 and also assessed for ubiquitination by SDS-PAGE and Western blotting.

In Vivo Ubiquitination—Cells were transfected with wild-type or mutant HA-MLK3 along with specific Myc-ub constructs as indicated in Min6 or 293 cells as described. 40–48 h post-transfection, cells were treated with IL-1β as described, harvested in 1% SDS, 50 mM Tris (pH 8.0), and 1 mM EDTA containing phosphatase inhibitors and 10 mM *N*-methyl maleimide deubiquitinase inhibitor. Lysates were boiled, cooled on ice, sonicated, and precleared with Sepharose beads. The supernatants were diluted 5-fold with dilution buffer (150 mM NaCl, 20 mM Tris, pH 8.0, and 1 mM EDTA) with a full complement of phosphatase and protease inhibitors. Additionally, deubiquitinase inhibitor (*N*-methyl maleimide) was used for immunoprecipitation with agarose-conjugated anti-HA antibodies. The immunoprecipitates were resolved by SDS-PAGE and probed using anti-Myc antibodies. Blots were stripped and probed using anti-HA antibodies for HA-MLK3 input.

TRAF6 siRNA—TRAF6 knockdown was accomplished using Smartpool siRNA mix (Dharmacon). siRNA duplexes were verified in both HeLa and 293 cells using Western blots for endogenous TRAF6 following transfection using Lipofectamine 2000.

Microscopy and Image Acquisition—Fluorescent images were acquired on a Nikon Eclipse E800 microscope. All images were assembled in Photoshop CS4 (Adobe Systems, Inc., San Jose, CA). Quantification was performed on ten randomly selected fields of view using the ×60 objective from three separate experiments. Total cell counts for the transfected population expressing the epitope tag were obtained using DAPI (1 μg/ml) nuclear counterstain.

Statistics—Differences between means were examined using analysis of variance followed by a Bonferroni *post hoc* comparison. Densitometry was used to compare the untreated sample to the mean fold difference in response to IL-1β, and significance was tested using the one-sample *t* test. In all cases, $p \leq 0.05$ was considered significant. Analysis was performed using GraphPad Prism software.

RESULTS

MLK3-dependent BAX Conformational Change in Human Islets—The therapeutic promise of targeting MLK3 in treating some human diseases prompted us to examine whether MLK3 inhibition can prevent cytokine-induced damage of human islets. We have shown previously that a splenocyte-islet coculture system of activated splenocytes with isolated mouse islets mimics the milieu encountered by islets during the autoimmune destruction process. Using this system we have demonstrated a crucial role for MLK3 in cytokine-induced destruction of islets. A mouse splenocyte isolation protocol was adapted for isolation of splenocytes from cadaveric donor spleens, and the splenocytes were activated using plate-bound anti-CD3 antibodies and exogenous anti-CD28 antibodies. After 48 h, human donor islets were incubated in transwells and inserted into microwells containing activated splenocytes so that the system ensured a free exchange of secreted cytokines without direct contact with the splenocytes (23). Conditioned medium from activated splenocytes was used for a multiplex ELISA assay to ascertain a strong induction of cytokines, including IL-1β, TNF-α, and IFN-γ, the three requisite cytokines for induction of β cell death *in vitro* (supplemental Table 1). Human islets were cocultured with splenocytes for an additional 24 h in the presence and absence of CEP11004 (Cephalon), a well characterized inhibitor of mixed-lineage kinases. Each group of islets was fixed and pelleted, and sections of the paraffin-embedded islets were used for immunostaining using a monoclonal antibody against conformationally altered BAX.

Mitochondrial membrane permeabilization represents a commitment point for the induction of apoptosis (29–31). We have reported previously that cytokine stimulation of β cells triggers MLK3-dependent conformational change in BAX (23). This structural change exposes a stretch of seven amino acids normally buried in a hydrophobic pocket under quiescent conditions (32) and marks a critical transition toward BAX insertion in the mitochondrial membrane to initiate apoptosis. Using a monoclonal (clone 6A7) antibody that specifically detects this exposed region, we examined the status of BAX in

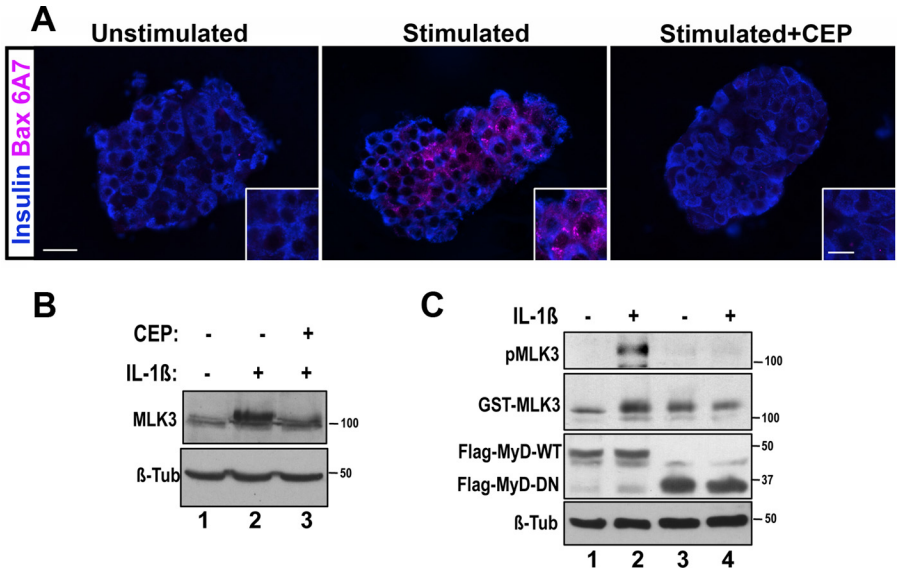


FIGURE 1. **MLK3 is activated by IL-1B via the IL-1R/TLR receptor and is required for cytokine-dependent conformational change of BAX in human islets.** *A*, human islets cocultured for 24 h with unstimulated (*left panel*) and stimulated splenocytes pretreated with DMSO (*center panel*) or with CEP11004 (*CEP*) (*right panel*) were used to detect conformationally altered BAX using monoclonal antibody clone 6A7 as described. *Scale bar* = 20 μm. *B*, Western blotting of extracts (60 μg of protein) from human islets treated for 15 min with 20 ng/ml IL-1β using anti-MLK3 antibodies and β-tubulin used as a control. *A* and *B* were repeated 5 times. *C*, Min6 cells were cotransfected (data shown is from one of two experiments) with a mammalian vector for GST-MLK3-WT and either FLAG-MyD88-WT (*lanes 1* and *2*) or FLAG-MyD88-dominant negative (*DN*, *lanes 3* and *4*). Following IL-1β treatment, Western blot analyses of GST pull-downs were probed for pMLK3 and total MLK3. Anti-FLAG Western blot analyses of 8% input of total cellular lysates show expression of MyD88-WT and DN, and β tubulin was used a control.

islets from the human splenocyte and islet coculture. As shown in Fig. 1*A*, activated splenocytes elicit a strong signal for BAX6A7 staining (*magenta*) in insulin-positive cells (*blue*), and this response was inhibited by the presence of the MLK3 inhibitor. These data closely reflect our observations in mouse islets as well and indicate an important role for MLK3 in compromising mitochondrial integrity of human β cells.

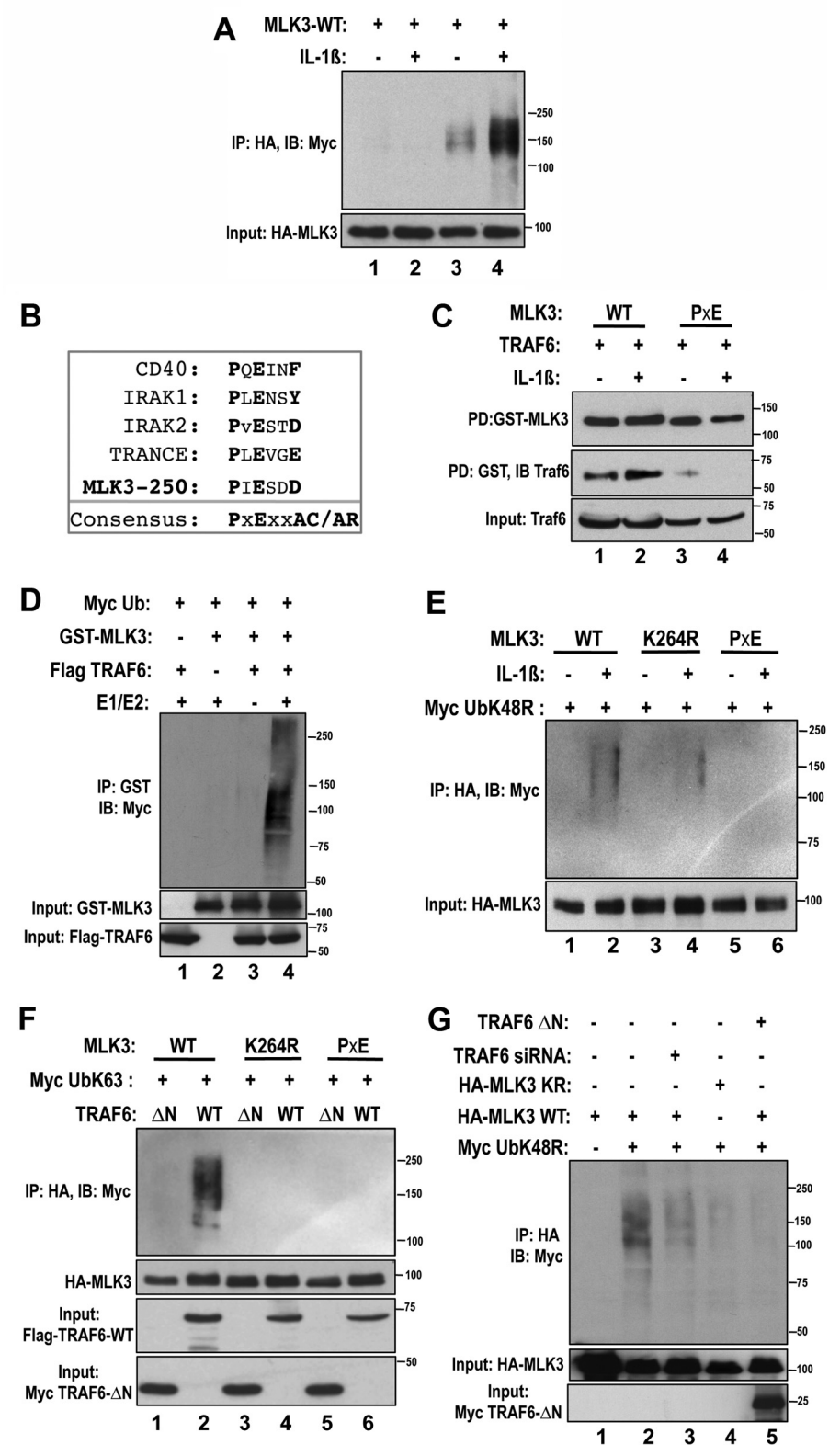
IL-1β Activates MLK3 via MyD88—MLK3 activation is associated with an increased stability of the protein (33) and, as shown here, IL-1β induces total MLK3 protein levels in human islets (Fig. 1*B*). Stimulus-mediated induction of total MLK3 protein levels has been clearly demonstrated elsewhere and has been attributed to a stabilizing feed-forward loop initiated by MLK3 activation and reinforced by up-regulation and activation of other components of the JNK module (33). Accordingly, CEP11004-dependent inhibition of MLK3 activity was sufficient to prevent the induction of total MLK3 protein levels in response to IL-1β. Thus, our results presented in Fig. 1*B* are consistent with previous reports (23, 33).

The IL-1β receptor signals via a cascade of events involving sequential docking of adaptors including MyD88, IRAKs, and TRAF6 (34). Upon ligand binding, the IL-1β receptor complex engages the MyD88 adaptor protein via a homotypic interaction of the TLR domain (35). MyD88, in turn, recruits IL-1 receptor-associated kinases IRAK4 and IRAK1 (35, 36) and promotes TRAF6-dependent activation of downstream kinases (8, 37–39). To ascertain that stimulation of MLK3 occurs via the canonical IL-1β pathway, we examined whether the expression of dominant negative MyD88 could inhibit MLK3 activation. We expressed tagged MLK3 along with wild-type MyD88 or a truncated dominant negative form of MyD88 that consists of only the TLR domain. The truncated MyD88 adaptor fails to recruit downstream effectors of the signaling cascade, unproductively binds the IL-1 receptor complex, and terminates signaling (40). Such an approach was taken largely because of the difficulty of homogenous expression of exogenous DNA in highly differentiated β cell lines or islets. As shown in Fig. 1*C*, IL-1β stimulation of transfected Min6 insulinoma cells augmented MLK3 protein levels and induced MLK3 phosphorylation in the presence of wild-type MyD88, which was abrogated in the presence of the dominant negative form, suggesting that IL-1β signals to MLK3 protein via a conserved signaling pathway. Our data are consistent with previous reports showing that genetic ablation of MyD88 protects β cells from streptozotocin (41). Also, similar to our approach here, functional inhibition using a dominant negative MyD88 protected pancreatic β cells from IL-1β-induced inflammation (42).

TRAF6-dependent, Lys-63-linked ubiquitination of MLK3—Although MyD88 is the immediate downstream adaptor of IL-1β signaling, TRAF6 acts at a more distal step and catalyzes the atypical Lys-63-linked ubiquitination. We examined whether IL-1β stimulates Lys-63-linkage-specific ubiquitination of MLK3 in Min6 β cells. We coexpressed HA-tagged MLK3-WT with two different Myc-tagged ubiquitin constructs in Min6 cells and harvested the cells for immunoprecipitation using anti-HA beads after a brief (15-min) incubation with IL-1β. For the Myc-ub constructs, arginine substitution at Lys-48 (Ub-K48R) was used to detect Lys-63-linked ubiquitination without interference from the more common Lys-48-linkage, and the Lys-63 mutant (Ub-K63R) was used to demonstrate specificity for the Lys-63-linkage. This approach was used because MLK3 has been shown to undergo Lys-48 as well as Lys-63-linked ubiquitination (2), and preliminary use of wild-type ubiquitin resulted in a very high basal signal, making it impossible to discern the specific nature of the ubiquitination event. Myc-antibody Western blot analyses of HA-MLK3

immunoprecipitates showed a robust conjugation with Myc-Ub-K48R (Fig. 2*A*, *lanes 3* and *4*) but displayed low to undetectable levels of Myc-Ub-K63R incorporation (*lanes 1* and *2*). This modification of MLK3 by Lys-63-linked ubiquitination supports our previously published findings that IL-1β is a potent inducer of MLK3 protein in primary islets and insulinoma cells (23).

TRAF6 serves as a downstream mediator of IL-1β signaling and is known to catalyze Lys-63-linked ubiquitination of substrates via an N-terminal RING domain, whereas the C-termi-



nal TRAF domain interacts with substrate proteins containing a pentapeptide consensus motif (P*X*E*XX*Ac/Ar) where proline and glutamic acid are invariant amino acids in positions 1 and 3 and position 5 may be an aromatic or acidic residue (43). A closer scrutiny of the MLK3 protein sequence led us to identify a perfect TRAF6-interacting motif (P*X*E*XX*D) starting at amino acid 251 in the human MLK3 protein sequence (Fig. 2*B*). Evolutionary conservation of the TRAF6 interaction motif in MLK3 protein reinforces the functional significance of this interaction, and alanine substitution mutations at proline 251 and glutamic acid 253 (referred to as P*X*E) were sufficient to abolish basal and IL-1β-induced TRAF6-MLK3 interaction (Fig. 2*C*). Accordingly, consistent with published reports (2), we found that the C-terminal TRAF domain of TRAF6 interacts with MLK3, whereas the N-terminal RING domain of the protein serves as an E3 ub-ligase (not shown). Confirmation of MLK3 and TRAF6 colocalization *in vivo* further underscores the biological relevance of this interaction and activity (supplemental Fig. S1).

Direct interaction between MLK3 and TRAF6 coupled with the ability of IL-1β to induce Lys-63-linked ubiquitination prompted us to examine whether MLK3 is a direct substrate for TRAF6 ub-ligase activity. Using a cell-free system, we reconstituted the ubiquitin reaction *in vitro* as indicated using purified GST-MLK3, Myc-ubiquitin with UBE1, and UbeH13-Uev1a E2 complex (Boston Biochem), along with purified FLAG-TRAF6 as the E3 ligase. The reactions were used for immunoprecipitation of GST-MLK3 and resolved using SDS-PAGE. Anti-Myc antibody Western blot analyses of the reactions showed the characteristic smear of Myc-ubiquitin (Fig. 2*D*, compare *lane 4* with *lanes 1–3*) only in the presence of all the requisite components for the reaction, thus demonstrating that MLK3 is a direct target for TRAF6-mediated ubiquitination *in vitro*.

Although TRAF6 functions importantly in multiple signaling events, a clear consensus for TRAF6-mediated ubiquitination has not been defined. Some TRAF6 substrates reveal conservation of hydrophobic amino acids in the minus 1 and plus 1, 4, and 6 positions flanking ubiquitin acceptor lysine residues (44, 45). By this criterion, lysines 130, 180, and 264 appeared to be candidate ub-acceptor sites. Compared with wild-type MLK3, the Lys-130 and Lys-180 mutants showed no appreciable difference in IL-1β-stimulated ubiquitination (supplemental Fig. S2). By contrast, examination of IL-1β-induced ubiquitination of MLK3 in Min6 cells transfected with Myc-Ub-K48R and HA-tagged MLK3 constructs showed that an arginine substitution mutant of lysine 264 (K264R) resulted in a decreased IL-1β-dependent ubiquitination of MLK3 (Fig. 2*E*, *lanes 3* and *4*), whereas the P*X*E mutant appeared to abrogate ubiquitination.

To further ascertain Lys-63-linked MLK3 ubiquitination, we refined the experiment slightly. One difference was the use of Myc-Ub-K63-only as the ubiquitin donor. As the name suggests, the ubiquitin construct is mutated for all lysines except the Lys-63 lysine residue and could yield a much better readout of TRAF6 action on MLK3. To obtain more robust signals and overcome the poor transfectability of Min6 cells, we augmented the reactions with coexpression of FLAG-TRAF6, whereas TRAF6-ΔN served as a negative control. Anti-Myc Western blot analyses of HA-MLK3-WT immunoprecipitates displayed robust ubiquitination in the presence of WT-TRAF6, whereas TRAF6-ΔN completely abrogated Lys-63-linked ubiquitination of MLK3 (Fig. 2*F*, *lanes 1* and *2*). More importantly, under identical conditions, MLK3-K264R (Fig. 2*F*, *lanes 3* and *4*) and MLK3-PXE (*lanes 5* and *6*) both displayed a marked loss of Lys-63-linked ubiquitination.

Finally, we examined whether knockdown of TRAF6 could impact MLK3 ubiquitination. 293 cells, which express high levels of endogenous TRAF6, were transfected with HA-MLK3 and Myc-Ub-K48R. A strong basal ubiquitination of MLK3 (Fig. 2*G*, *lanes 1* and *2*) was observed. However, cotransfection with 100 nM small interference RNA duplexes specifically targeted to TRAF6 substantially attenuated MLK3 ubiquitination (Fig. 2*G*, *lane 3*). TRAF6 siRNA was examined previously for efficient knockdown of total levels of TRAF6 (supplemental Fig. S3). TRAF6 siRNA attenuated ubiquitination to similar levels obtained upon coexpression of TRAF6-ΔN or K264R MLK3 (Fig. 2*G*, *lanes 5* and *4*, respectively). Taken together, these data support the authenticity of TRAF6 as an E3 ligase for MLK3 ubiquitination.

MLK3 Ubiquitination Impacts Kinase Activation—Activation of potentially death-inducing kinases such as MLK3 is apt to be tightly regulated, and activation is usually triggered by well orchestrated signaling mechanisms. However, overexpression leads to constitutive activity of MLK3, perhaps by bypassing regulatory control mechanisms (33). We examined whether the K264R and P*X*E mutations impacted functionality of the kinase. MLK3 WT, K264R, and P*X*E were each expressed in 293 cells, chosen for the high degree of transfection efficiency, and the extracts were used to assess the activation of endogenous JNK. Not surprisingly, WT MLK3 activated endogenous JNK (Fig. 3*A*, *lane 1*), as judged by Western blotting using anti-p-

FIGURE 2. **Direct interaction with TRAF6 leads to TRAF6-dependent Lys-63-linked ubiquitination of MLK3 at lysine 264.** *A*, HA immunoprecipitates (*IP*) from IL1-β-stimulated (15 min) Min6 cells cotransfected with HA-MLK3 and either Myc-Ub-K63R (*lanes 1* and *2*) or Myc-Ub-K48R (*lanes 3* and *4*) were assessed for ubiquitination using anti-Myc Western blotting (*IB*). Total levels of MLK3 in each lane are shown. *B*, presence of a consensus TRAF6 binding motif in the catalytic domain of MLK3. *C*, GST pull-downs (*PD*) of MLK3-WT (*lanes 1* and *2*) from cells cotransfected with FLAG-TRAF6 show IL-1β induced interaction with MLK3-WT but not with MLK3 mutated for the TRAF6 binding consensus site (P*X*E, *lanes 3* and *4*). Total pull-down levels of GST-MLK3 constructs and input of FLAG-TRAF6 are shown. *D*, TRAF6-mediated ubiquitination of GST-MLK3 was performed *in vitro* in a cell-free reconstituted system using eluted Myc-Ub, purified GST-MLK3, purified FLAG-TRAF6, and recombinant E1 and E2 enzymes as described. Controls, including no MLK3 protein (*lane 1*), TRAF6 (*lane 2*), or E1/2 ubiquitin ligases (*lane 3*), were each used to show specific ubiquitination of MLK3 in the presence of all components. GST immunoprecipitates were subjected to SDS-PAGE and immunoblotting for anti-Myc antibodies. Inputs for MLK3 and TRAF6 are shown. Data from one of eight experiments is presented. *E*, HA immunoprecipitates from IL-1β-stimulated Min6 cells cotransfected with Myc-Ub-K48R along with HA-MLK3-WT (*lanes 1* and *2*), K264R (*lanes 3* and *4*), or P*X*E (*lanes 5* and *6*) were assessed for ubiquitination using anti-Myc-antibodies. Inputs of HA-MLK3 constructs are shown. *F*, a similar experimental setup as in *E*, except cells were cotransfected with Myc-Ub-K63 only in the presence of FLAG-TRAF6-WT (*lanes 2*, *4*, and *6*) or dominant negative Myc-TRAF6-ΔN (*lanes 1*, *3*, and *5*). Inputs of HA-MLK3 and TRAF6 constructs are shown. *G*, *in vivo* ubiquitination of MLK3-WT (*lanes 1–3* and *5*) and MLK3-K264R (*lane 4*) in adherent HEK-293 cells cotransfected with Myc-Ub-K48R (*lanes 2*-5) and either TRAF6 siRNA (*lane 3*) or TRAF6-ΔN (*lane 5*). Inputs of HA-MLK3 and TRAF6 are shown. *E* and *G* represent results from one of at least three experiments.

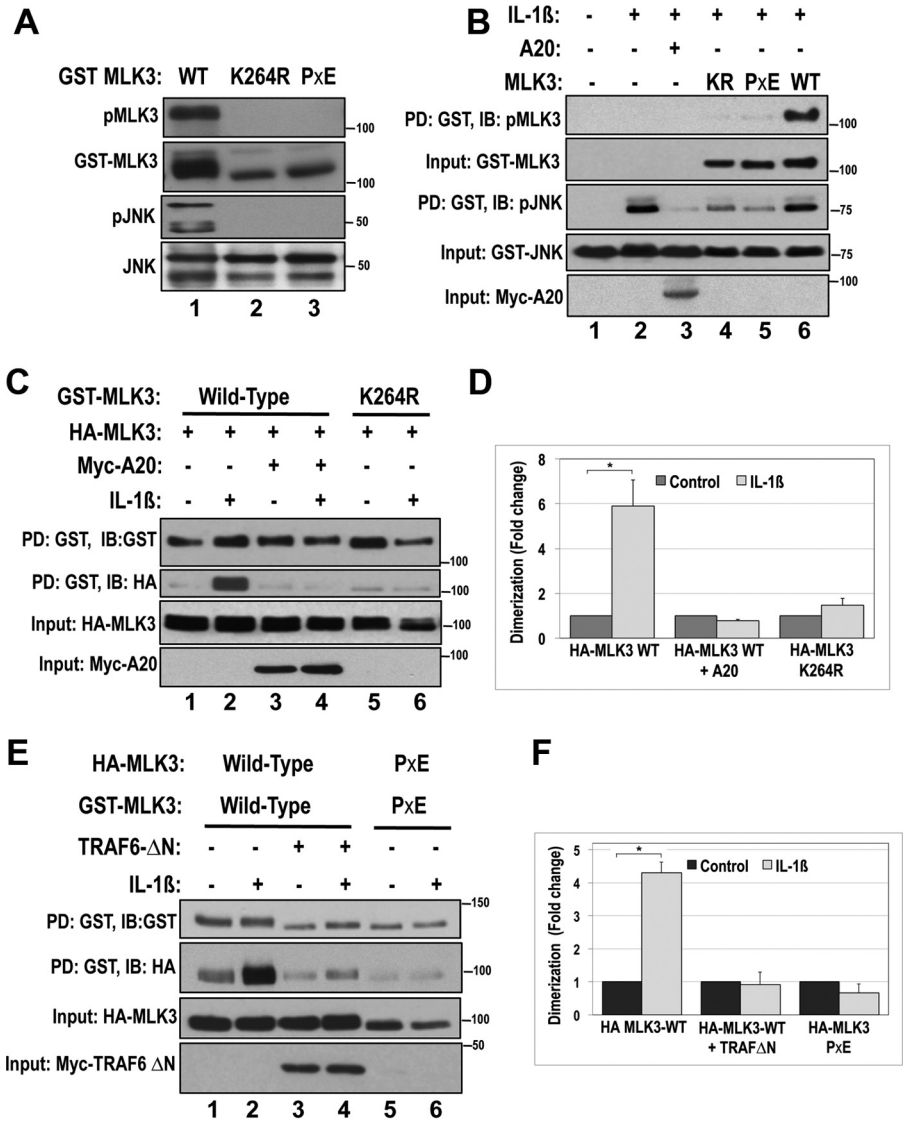


FIGURE 3. **Lys-63-linked ubiquitination facilitates dimerization and autophosphorylation of MLK3.** *A*, total cell lysates from HEK293 cells transfected with GST-MLK3-WT (*lane 1*), K264R (*lane 2*), or P*X*E (*lane 3*) were analyzed by immunoblotting (*IB*) using the indicated antibodies ($n = 3$). *B*, pull-downs (*PD*) of GST-JNK were performed using IL-1β-treated Min6 cells (*lanes 2–6*) followed by Western blotting with antibodies as shown (one of five experiments). Cells were transfected with GST-JNK along with a control vector (*lanes 1–3*), Myc-A20 (*lane 3*), or GST-MLK3 constructs mutated for the ubiquitin acceptor site (*KR*, *lane 4*) or for the TRAF6 binding motif (P*X*E, *lane 5*). Inputs of Myc-A20, total MLK3, and JNK proteins are shown. MLK3-WT (*lane 6*) was used here for comparison with the respective MLK3 mutants. *C*, HepG2 cells were transfected with HA-MLK3, along with GST-MLK3-WT (*lanes 1–4*) or GST-MLK3-K264R ub acceptor mutant (*lanes 5* and *6*) and treated with vehicle (*lanes 1*, *3*, and *5*) or IL-1β (*lanes 2*, *4*, and *6*) for 15 min prior to GST pull-downs, followed by Western blotting for HA-MLK3. A20 was used as a control (*lanes 3* and *4*) for the effects of ubiquitination. Inputs for Myc-A20 and HA-MLK3 are shown. *D*, densitometry from three independent experiments normalized to untreated controls and presented as the means ± S.E. fold difference. *, $p = 0.05$ compared with control. *E*, identical experimental conditions as in *C*, except that A20 was substituted by dominant negative Myc-TRAF6-ΔN. To study the effect of TRAF6-binding mutation on protein dimerization, P*X*E mutants of both dimer partners, *i.e.* HA and GST-MLK3, were used (*lanes 5* and *6*). *F*, densitometry from three independent experiments were normalized to untreated control samples and presented as the means ± S.E. fold difference. *, $p = 0.01$ compared with control.

JNK antibody. In contrast, each of the mutants failed to activate JNK (Fig. 3*A*, *lanes 2* and *3*). Unlike Min6 cells, we found that each of the mutants was inherently unstable in 293 cells, showing less expression despite transfection of 2–3 times the DNA of MLK3-WT. It is noteworthy that the P*X*E motif and the Lys-264 residue are both located in the kinase domain of MLK3 and that even in the absence of upstream signaling, each mutation renders MLK3 inactive.

Having observed the near total lack of activity of each of the mutants, we asked whether inhibiting ubiquitination in general and K264R and P*X*E mutations in particular could exert a dominant negative effect on IL-1β-dependent JNK activation in Min6 cells. The general relevance of Lys-63-ubiquitination for JNK activation was assessed in the presence of A20 (TNFAIP3), a well characterized deubiquitinase known to selectively edit Lys-63-ub linkages. A20 gene expression is stimulated by cytokine signaling, and its ubiquitin editing function exerts a negative feedback regulation of TRAF6 and other proteins in IL-1/TLR receptor signaling (26). A20 is known to limit activation of NFκB (25, 46), and TRAF6 is a critical target for A20 (25). We performed pull-down assays from cells transfected with GST-JNK and examined its phosphorylation status in the presence of

a variety of constructs. Additional experiments were performed to ensure complete recovery of GST-JNK and absence of GST-pJNK signal in the post-pull-down supernatant (supplemental Fig. S4).

Results for these experiments showed that phosphorylation of GST-JNK is strongly induced upon treatment with IL-1β (15 min) and that this effect is almost completely inhibited upon the cotransfection of A20 (Fig. 3*B*, *lanes 1–3*), confirming a crucial role for TRAF6-dependent ubiquitination in IL-1β-induced activation of JNK. Under the same conditions, and similar to A20, the K264R and P*X*E mutants of MLK3 also attenuated IL-1β-induced activation of JNK (Fig. 3*B*, *lanes 4* and *5*). This inability to activate JNK correlated with the absence of autophosphorylation for each of the MLK3 mutants. In contrast, the presence of wild-type MLK3 did not show any induction beyond what was observed for IL-1β alone (Fig. 3*B*, *lane 6*). These striking results lead us to conclude that in Min6 insulinoma cells, IL-1β activation of JNK requires MLK3 and that an inability to interact with or be ubiquitinated by TRAF6 exerts a dominant negative effect on IL-1β-mediated activation of JNK. The putative Lys-63-ub acceptor site at Lys-264 in the MLK3 protein is strategically located between the TRAF6 binding site at amino acids 251–255 and the autophosphorylation sites at Thr-277 and Ser-281. Also, the TRAF6 interaction motif and the potential site at Lys-264 are both immediately adjacent to the kinase activation loop (amino acids 267–291). Taken together, our data demonstrate that TRAF6-mediated Lys-63-linked ubiquitination requires an intact lysine at position 264. However, additional experiments are required to clarify whether Lys-264 is a direct ub acceptor site or whether K264R mutation plays a secondary role in preventing Lys-63-linked ubiquitination of MLK3.

Inhibition of Ubiquitination Attenuates MLK3 Dimerization—In Fig. 3*B* we noted that compared with WT-MLK3, the MLK3-P*X*E and MLK3-K264R mutants showed a marked attenuation of autophosphorylation. Having found that interaction with TRAF6 and the consequent Lys-63-linked ubiquitination are crucial for the activation of MLK3, we sought to identify the mechanism by which ubiquitination facilitates MLK3 activation. Studies have shown that MLK3 activation is preceded by homodimerization and transphosphorylation of each dimer partner so that the inability to dimerize can interfere with MLK3 activation (47–49). To address this question, we used GST-MLK3 in pull-down assays from cells cotransfected with HA-MLK3. We found that in the basal state, MLK3 showed very low levels of dimerization. Dimerization was strongly induced (5- to 8-fold) by a 15-min treatment with IL-1β, and the coexpression of A20 (Fig. 3*C*, *lanes 1–4*) abolished IL-1β-mediated dimerization of MLK3. Similar attenuation of HA-MLK3 binding by GST-MLK3-K264R (Fig. 3*C*, *lanes 5* and *6*) suggests that the putative Lys-63-linked ubiquitination of MLK3 at Lys-264 impacts dimerization. Data from an average of three experiments are represented in graphical format in Fig. 3*D*. Having seen these results, we also examined whether IL-1β-induced MLK3 dimerization would be impacted by its ability to interact with the E3 ub ligase TRAF6. Using an experimental design similar to that in Fig. 3*C*, we found that the IL-1β-induced dimerization was markedly decreased in the presence of TRAF6-ΔN, which acts as a dominant negative inhibitor of TRAF6 action (Fig. 3*E*, *lanes 1–4*). IL-1β induced dimerization was also attenuated upon mutation of the TRAF6 interaction motif of the dimer pair (Fig. 3*E*, *lanes 5* and *6*). Taken together, these data suggest that TRAF6-mediated Lys-63-linked ubiquitination of MLK3 drives dimerization and, thereby, the autophosphorylation and activation of MLK3. Fig. 3*F* depicts in graphical format an average of three experiments of the above data.

Lys-63-linked Ubiquitination Regulates the Dynamics of MLK3 Interaction with the Scaffold Protein JIP1—Cytokine-dependent activation of JNK is achieved by the assembly of distinct signaling modules containing a MAP3K, MAP2K, and JNK. The MLK3-MKK7-JNK-JIP1 module is uniquely responsive to cytokines like IL-1β and TNF-α (4). Pancreatic β cells express significant levels of JIP1 (50), and activation of JNK has been shown to be sensitive to the absolute amounts of JIP1 (50, 51), which is consistent with the behavior of a scaffold protein that stoichiometrically interacts with members of the JNK cascade. Dual leucine zipper bearing kinase (DLK), also a JIP1-interacting MAP3K of a mixed-lineage kinase family, has been shown to interact with JIP1 in its monomeric form, which in turn restricts the high affinity and energetically favored interaction of the DLK dimer pair (52). After confirming colocalization of MLK3 and JIP1 *in vivo* (supplemental Fig. S5), we first examined whether IL-1β regulated the dynamics of MLK3 interaction with endogenous JIP1. HA-tagged MLK3 was expressed in Min6 cells, and the lysates were harvested following a brief stimulation with IL-1β and were used for immunoprecipitation of HA-MLK3 under native conditions. Western blot analyses of the immunoprecipitates were probed using anti-JIP1 antibodies. Our data show that MLK3 binds endogenous JIP1 in untreated cells, but little JIP1 binding was detected upon IL-1β treatment (Fig. 4*A*).

Having observed the dissociation of HA-MLK3 from endogenous JIP1, coupled with the reported regulation of JIP1-MLK3 interaction (53), we asked whether IL-1β-induced ubiquitination of MLK3 affects the JIP1-MLK3 interaction. Using a stoichiometric excess of Myc-JIP1, we found that MLK3 in its basal state was almost completely bound to JIP1 and was inactive, as judged by the absence of MLK3 phosphorylation. A short 15-min IL-1β treatment induced a 7- to 8-fold dissociation of MLK3 from JIP1 that was accompanied by robust autophosphorylation of MLK3 kinase (Fig. 4*B*, *lanes 1* and *2*). Similar to WT-MLK3, both the K264R and P*X*E mutants of MLK3 showed a strong interaction with JIP1 in the unstimulated state, suggesting that these mutations do not impact the basal monomeric association with JIP1 (Fig. 4*B*, *lanes 1*, *3*, and *5*).

However, in contrast with WT-MLK3, the K264R and P*X*E mutants of MLK3 remained bound to JIP1 even in the presence of IL-1β (Fig. 4*B*, *lanes 4* and *6*). Accordingly, and as seen in earlier figures, neither the K264R nor the P*X*E mutant of MLK3 showed autophosphorylation. These data demonstrate a potentially critical and regulatory role for MLK3 ubiquitination in kinase activation. Furthermore, our results suggest that IL-1β-induced ubiquitination is required for dissociation of MLK3 from JIP1, leading to activation of the kinase. Quantification of the loss of binding from three independent experiments reveals

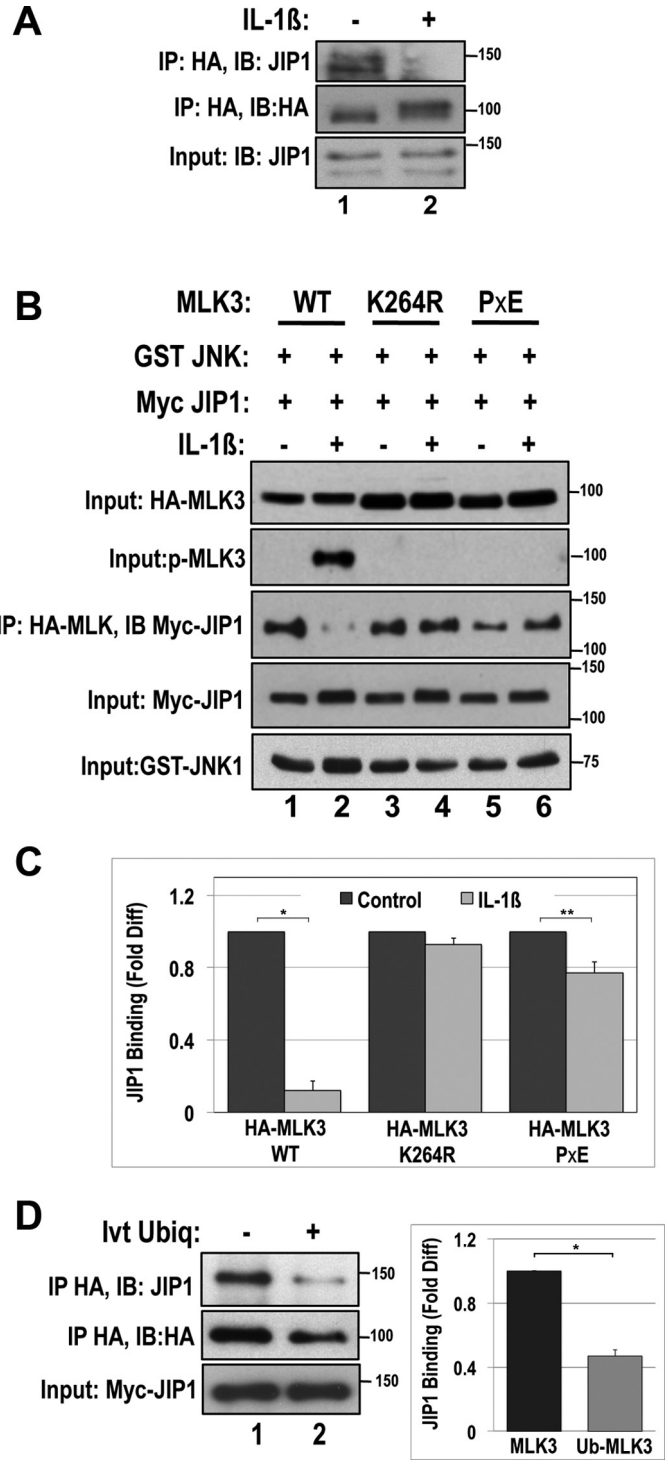


FIGURE 4. **Lys-63-linked ubiquitination dissociates MLK3-JIP1 binding and enables autophosphorylation of MLK3.** *A*, Western blotting (*IB*) of endogenous JIP1 ($n = 4$) was performed on HA-MLK3-WT immunoprecipitates (*IP*) from Min6 cells treated for 15 min with vehicle (*lane 1*) or IL-1β (*lane 2*). Total MLK3 and JIP1 input are shown ($n = 3$). *B*, HepG2 cells were transfected with equal amounts of GST-JNK and HA-MLK3-WT, K264R, or P*X*E mutants (*lanes 1 and 2*, 3 and *4*, and *5* and *6*, respectively) with a 3-fold excess of Myc-JIP1 and treated with vehicle (*lanes 1*, *3*, and *5*) or IL-1β for 15 min (*lanes 2*, *4*, and *6*). Lysates were used for HA immunoprecipitation, followed by Western blot analyses for interacting Myc-JIP1. Inputs were probed for pMLK3, and the blots were stripped and reprobed for total HA-MLK3 levels. Inputs were also probed for total JIP1 and JNK expression. *C*, densitometric analysis from three independent experiments were normalized to untreated control samples and presented as the means ± S.E. fold difference. *, $p = 0.002$; **, $p = 0.03$ compared with controls). *D*, mock-treated (*lane 1*) or *in vitro* ubiquitinated (*Ivt Ubiq*) HA-MLK3-WT (*lane 2*) were immunoprecipitated, followed by incubation with HEK293 cell lysates transfected with Myc-JIP and examined by immunoblotting using JIP1 antibodies. Total MLK3 and Myc-JIP1 inputs are shown. Densitometry from three independent experiments were normalized to non-ubiquitinated MLK3 and presented as the means ± S.E. fold difference. *, $p = 0.002$ compared with non-ubiquitinated MLK3.

a dramatic drop in JIP1 binding by the WT-MLK3 constructs but not the two mutants (Fig. 4*C*). Although the above data suggest that IL-1β-induced dissociation of MLK3 from JIP1 is preceded by the ubiquitination of MLK3 *in vivo*, we examined whether ubiquitination sterically hinders the interaction between MLK3 and JIP1. MLK3 was ubiquitinated *in vitro*, similar to the reaction described in Fig. 2*D*, and allowed to bind Myc-JIP1 *in vitro*. As shown in Fig. 4*D*, across three experiments we observed a small but significant 2-fold loss of JIP1 binding upon MLK3 ubiquitination *in vitro*. Although this difference is not as robust as that seen *in vivo* (Fig. 4, *B* and *C*), it is possible that additional cellular events or additional proteins may influence the dynamics of JIP1-MLK3 interaction. As a scaffold protein, JIP1 can bind multiple cellular proteins and can be dynamically modified by post-translational events, which can critically determine JIP1-mediated protein interactions.

Ubiquitination of MLK3 Impacts BAX Conformational Change—Finally, we examined whether ubiquitination also contributes to MLK3 action in a biological context. We have shown previously that a key effect of MLK3 is to induce a conformational change in BAX in both human (Fig. 1*A*) and mouse primary islets (23) and Min6 cells (supplemental Fig. 6). BAX conformational change has been consistently linked to mitochondrial translocation of BAX, which induces outer membrane permeabilization (29–31). In this study, we have used BAX conformational change as an important readout for MLK3 effects on the β cell. We examined whether the MLK3-K264R mutant or the presence of A20 affect BAX conformational change. The cells were transfected with functionally inactive, dimerization-defective MLK3 (HA-MLK3-ΔCATΔLZ) as an inactive cotransfection control, ub acceptor mutant HA-MLK3-K264R, or with the deubiquitinase Myc-A20. As described earlier, cytokine stimulation was induced by using conditioned medium from stimulated mouse splenocytes. Following an 8-h treatment of Min6 cells with conditioned medium from unstimulated or stimulated mouse splenocytes, the cells were stained for expression of endogenous, conformationally altered BAX6A7 (Fig. 5*A*, *first column*) or for the epitope tag of the transfected gene (*second column*, stained *green* for HA or Myc epitope).

Min6 cells are highly inefficient at DNA uptake, with only a 2–3% success rate of transfection efficiency. The impact of the transfected, epitope-tagged gene was assessed by its colocalization with the BAX6A7 signal. Such a colocalization would suggest the inability of the expressed, tagged protein to rescue mitochondrial compromise. By contrast, an exclusion of BAX6A7 expression from the epitope-expressing cells would suggest a protective effect of the expressed protein against the cytokine stimulus. For clarity, a higher magnification of the cells and a schematic of epitope-expressing cells (Fig. 5*A*, *green*)

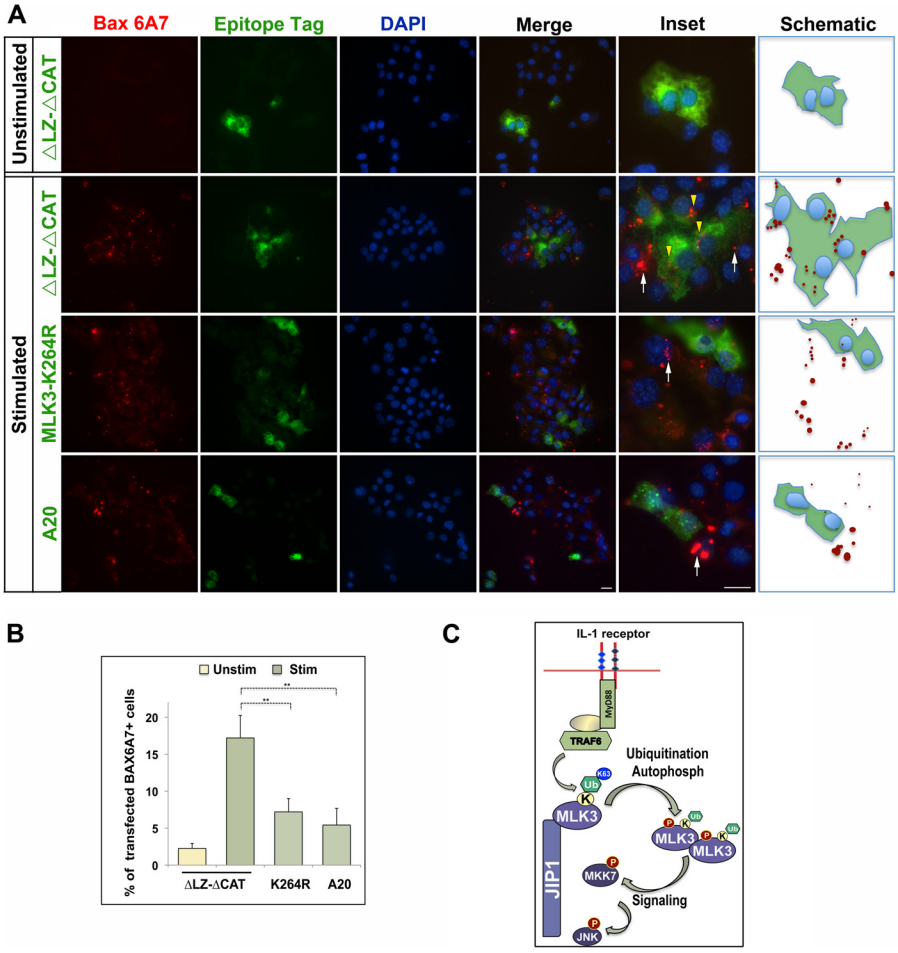


FIGURE 5. **Inhibition of Lys-63-linked ubiquitination of MLK3 attenuates cytokine-dependent conformational change of BAX in Min6 insulinoma cells.** *A*, Min6 cells were transfected with catalytically inactive and dimerization-incompetent HA-MLK3-ΔLZ-ΔCAT, HA-MLK3-K264R, or the deubiquitinase Myc-A20 and treated with conditioned medium from inactive splenocytes (*first row*, *unstimulated*) or conditioned medium from activated splenocytes (*second*, *third*, and *fourth rows*, *stimulated*) as described. Cells were fixed and stained for expression of HA-MLK3 or Myc-A20 (*green*) using anti-HA or anti-Myc epitope antibodies. Cells were also stained for conformational change in endogenous BAX using 6A7 antibody (*red*) raised against the hydrophobic region of BAX exposed prior to mitochondrial outer membrane localization. A higher-magnification *inset* from the merged panel is included, and for clarity schematic representations of the transfected cells (*green*) and BAX-6A7 (*red*) in the magnified areas are shown. *Yellow arrowheads* show expression of BAX-6A7 in transfected cells, whereas *white arrows* show the exclusion of BAX-6A7 from transfected cells. *Scale bar* = 10 μm. *B*, quantification (means ± S.E. of three independent experiments) of transfected cells (epitope tag-positive) coexpressing BAX-6A7. *, $p < 0.05$ *versus* MLK3-ΔLZ-ΔCAT in stimulated media as tested by analysis of variance followed by Bonferroni *post hoc* test. *C*, an illustration for context and likely progression of Lys-63-linked ubiquitination of MLK3 is shown.

and BAX6A7-positive cells (*red*) are shown in the *fourth* and *fifth columns*, respectively. In each case, cells were scored for the number of transfected cells coexpressing the epitope-tagged protein and BAX6A7 and graphically represented as the percent of total cells.

Cells treated with conditioned medium from unstimulated splenocytes showed little expression of BAX6A7 (Fig. 5*A*, *first row*). By contrast, in the presence of cytokine-rich conditioned medium from stimulated splenocytes, BAX6A7 was induced in both untransfected (*white arrows*) and also the HA-MLK3-ΔCATΔLZ-tranfected cells (kinase- and dimerization-deficient MLK3, used here as an inactive transfection control) (Fig. 5*A*, *second row*, *yellow arrowheads*). However, we found that BAX6A7 was largely excluded from cells expressing the ub acceptor mutant HA-MLK3-K264R (Fig. 5*A*, *third row*). These data were similar to the exclusion of BAX6A7 from Myc-A20-expressing cells (Fig. 5*A*, *first row*). In each case, the percentage of transfected cells positive for BAX6A7 was calculated, and the graphic representation of data from an average of three experiments is included in Fig. 5*B*.

These data are supported by experiments in additional cell types. Punctate localization for BAX was also observed in HeLa and HepG2 cells in the presence of WT-MLK3 but not in the presence of the MLK3-K264R or Myc-A20 (supplemental Figs. 7, A and B, respectively). The effect of MLK3 on altering BAX to the conformation required for mitochondrial translocation was consistent with the lower survival rate (TUNEL assay) of Min6 cells expressing WT-MLK3 compared with MLK3-K264R or coexpression of A20 (supplemental Fig. 8). These data are also consistent with the effect of MLK3-K264R and A20 on IL-1β-mediated activation of JNK. Taken together, these results suggest that the overexpression of K63-ubiquitin editing activity and, more importantly, the specific failure to induce Lys-63-linked ubiquitination of MLK3, have the potential to protect the β cell from the proapoptotic effects of cytokines.

DISCUSSION

Apoptosis marks a turning point in the autoimmune destruction of pancreatic β cells and for the induction of type 1 diabetes. We have reported previously that in response to an influx of cytokines, the early activation of MLK3 compromises mitochondrial integrity. Mitochondria damaged by MLK3 action are more vulnerable to the late effects cytokines, which enable the progression toward β cell apoptosis (23). In this study, we have focused our efforts on understanding receptor-mediated signaling events that lead to MLK3 activation. Our results show that IL-1β stimulates MLK3 activity by promoting atypical Lys-63-linked ubiquitination via the E3 ligase activity of TRAF6. Furthermore, ubiquitination of MLK3 appears to facilitate the dimerization and autophosphorylation of MLK3.

JNK signaling has been shown to occur in the context of a three-tiered signaling module consisting of a MAPKKK, MKK, and JNK scaffolded by a single protein (54). The existence of multiple candidate proteins for participation at each level of the module suggests that signal-specific activation of JNK could be achieved via unique signaling modules. In this regard, targeted deletion of MKK7 or MLK3 was found to attenuate JNK activation by cytokines like TNF-a and IL-1β (4, 22). Other studies reveal that JIP1 selectively associates with MLK3 and MKK7 kinases for the activation of the JNK stress kinase response (55). Thus, although the possibility exists for MLK3 to form stimulus-specific modules with other signaling components, IL-1β appears to preferentially employ the MLK3-MKK7-JNK-JIP1 complex.

Studies on the dynamics of MLK-JIP1 interaction suggest that MLK activation occurs off the JIP1 scaffold. Dissociation from the JIP1 scaffold releases the conformational constraints imposed on MLK3, permitting the energetically favored dimeric state and ensuing activation of the kinase by cross-phosphorylation of the dimer pair (52). Proximity of the newly released active MLK3 is thought to enable phosphorylation of JIP1-bound MKK7 and the subsequent activation of JNK. Other reports suggest that active JNK itself phosphorylates JIP1, reducing its affinity for MLK3, and thereby sustains its activation (52, 56). Phosphorylation of MLK3 and the ensuing association of JNK with the JIP1 scaffold both appear to be critical for maintenance of free and active MLK (52). Furthermore, the status of JIP1 phosphorylation also contributes to its interaction with the MLK family of kinases (52, 57).

Although the impact of MLK3 dissociation from JIP1 is clear, the specific events that initiate dissociation of MLK3 from JIP1 remain unclear. An obvious candidate is the possible phosphorylation of JIP1 by specific incoming signals, a hypothesis that merits further examination. The more likely scenario is that multiple events are triggered by the incoming cytokine signal, which in turn initiate the dissociation of MLK3 from JIP1.

The results presented in this study suggest that TRAF6-mediated, Lys-63-linked ubiquitination of MLK3 can serve as one such trigger for IL-1β-induced activation of the MLK-JNK pathway. Our assertion is supported by multiple observations including a) the inability of P*X*E, the TRAF6-interaction mutant, and the K264R substitution mutant of MLK3 to dissociate from JIP1 in response to IL-1β treatment; b) the P*X*E and K264R mutants are catalytically inactive; c) the dominant negative inhibition of TRAF6 action inhibits IL-1β-mediated MLK3 dimerization and dissociation from JIP1; and d) A20, a specific Lys-63-linked deubiquitinase of TRAF6, alters the behavior of wild-type MLK3 to resemble that of the P*X*E and K264R MLK3 mutants.

Mutational analysis of a few candidate lysines showed that Lys-264, located in the kinase domain, could serve as an ub acceptor site in the MLK3 protein. It is also plausible that the position of the lysine within a few amino acids of the DFG motif in the activation loop may somehow alter the structure of the MLK3, precluding its ubiquitination. Interestingly, the MAP3K TAK1, also a target for TRAF6, has been shown to undergo Lys-63-linked ubiquitination at Lys-158, which is in close proximity to the activation loop of the kinase (58, 59). Lys-264 in MLK3 is located in kinase subdomain 7, just preceding the activation loop of MLK3 catalytic domain. The structure of the MLK kinase domain (60) predicts that a lysine at this position is likely to be exposed and surface-accessible for TRAF6-mediated ubiquitination. Nevertheless, future studies, including structural analyses, will unambiguously reveal whether lysine 264 in the kinase domain of MLK3 is a *bona fide* ub acceptor site.

In addition to providing insight into the mechanism of MLK3 activation, understanding the dynamics of the MLK3-JIP-JNK module in the β cell also provides some clarity on the mechanisms regulating β cell death. JIP1 expression is highly enriched in the pancreatic β cell and neurons, as reflected by its alternate name: islet-brain 1 (IB1) (61). Mutations in IB1/JIP1 have been linked to familial autosomal dominant diabetes, and haploinsufficiency of JIP1 is associated with an increase in JNK activity (50). These results are consistent with our data and suggest that appropriate levels of JIP1 are required to sequester MLK3 in its monomeric inactive state. In contrast, overexpression of JIP1 enhances β cell survival, possibly by altering the stoichiometric balance with other components of the MLK3-MKK7-JNK-JIP1 module and, thus, disrupting cytokine-dependent JNK signaling (50).

Understanding IL-1β signaling in β cells is also merited because of the paradoxical endogenous production of IL-1β by the islet (62, 63). IL-1β activates MLK3 via the canonical IL-1/TLR signaling pathway and engages the MyD88 adaptor (42) which, in turn, recruits IL-1 receptor-associated kinases IRAK4 and IRAK1 (35, 36), whose phosphorylation induces the recruitment and Lys-63-linked autoubiquitination of TRAF6. Activated IRAK/TRAF6 complexes are thought to dissociate from the receptor complex for downstream Lys-63-linked ubiquitination of TRAF6 substrates (34). Consistent with this mechanism, our data show that MLK3 activation is attenuated upon overexpression of dominant negative MyD88.

Beyond the immediate dynamics of the MLK3-JIP1 signaling module, the biomedical significance of this study is underscored by results showing a significant decrease in cytokine-induced proapoptotic BAX conformational change upon expression of MLK3-K264R, a putative ub acceptor site mutant, or the presence of A20/TNFAIP3, a Lys-63-linkage-specific deubiquitinase. As reported in our previous study, BAX conformational change is the most proximal and reliable functional

readout of MLK3 activation in β cells. A20 belongs to the ovarian tumor domain family of deubiquitinases (46) and is induced by cytokine-mediated transcriptional activation of NFκB (64) to restrict cytokine action in a negative feedback loop (65). Unlike other substrates of A20, we were unable to show a direct association of MLK3 and A20. However, A20 does interact with TRAF6, and consistent with the ability of A20 to deubiquitinate TRAF6 (26), our studies showed that the presence of A20 prevented MLK3 dissociation from JIP1 and also influenced the subsequent events of dimerization and autophosphorylation-dependent activation of the MLK3/JNK cascade. A20-mediated inhibition of MLK3 also compared well with the repressive effect of dominant negative TRAF6.

Published reports indicate a potent protective effect of A20 against cytokine-induced inflammation and apoptosis of the β cell (27, 66). Genetic ablation of murine A20 expression results in cachexia (67) and the persistent inflammation of multiple organs (65). In humans, A20 gene mutations are associated with several autoimmune disorders, including type 1 diabetes (68, 69), which highlights the significance of our study and provides important clues for therapeutic intervention.

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