Redox regulation of nuclear factor κB (NF-κB) has been described, but the molecular mechanism underlying such regulation has remained unclear. We recently showed that a novel disulfide reductase, TRP14, inhibits tumor necrosis factor α (TNFα)-induced NF-κB activation, and we identified the dynein light chain LC8, which interacts with the NF-κB inhibitor IκBα, as a potential substrate of TRP14. We now show the molecular mechanism by which NF-κB activation is redox-dependently regulated through LC8. LC8 inhibited TNFα-induced NF-κB activation in HeLa cells by interacting with IκBα and thereby preventing its phosphorylation by IκB kinase (IKK), without affecting the activity of IKK itself. TNFα induced the production of reactive oxygen species, which oxidized LC8 to a homodimer linked by the reversible formation of a disulfide bond between the Cys residues of each subunit and thereby resulted in its dissociation from IκBα. Butylated hydroxyanisol, an antioxidant, and diphenyleneiodonium, an inhibitor of NADPH oxidase, attenuated the phosphorylation and degradation of IκBα by TNFα stimulation. In addition, LC8 inhibited NF-κB activation by other stimuli including interleukin-1β and lipopolysaccharide, both of which generated reactive oxygen species. Furthermore, TRP14 catalyzed reduction of oxidized LC8. Together, our results indicate that LC8 binds IκBα in a redox-dependent manner and thereby prevents its phosphorylation by IKK. TRP14 contributes to this inhibitory activity by maintaining LC8 in a reduced state.

Dyneins are large multi-component complexes that function as microtubule-based molecular motors both in the cytoplasm and in flagella (1). Cytoplasmic dyneins participate in a variety of intracellular motile processes including mitosis and vesicular transport, whereas axonal dyneins provide motive force for the beating of cilia and flagella. The 8-kDa dynein light chain (LC8, also known as DLC8 or DLC1) was originally identified in flagellar dynein of Chlamydomonas (2) and was subsequently found to be a component of cytoplasmic dynein motor (3). LC8 is widely expressed and highly conserved among species, with the Chlamydomonas and human proteins sharing 93% sequence identity (2–4). It also serves essential cellular functions. For instance, in Drosophila, a partial loss-of-function mutation in LC8 results in pleiotropic morphogenetic defects in bristle and wing development, female sterility, and disruption of sensory axon projections (5, 6). Furthermore, a null mutation results in massive cell death via the apoptotic pathway and consequent embryonic death. In addition to being an essential component of the dynein motor complex, LC8 binds to a large number of proteins with diverse biological functions (7). For example, LC8 associates with and inhibits the activity of neuronal nitric-oxide synthase, giving rise to its alternative designation as PIN (protein inhibitor of neuronal nitric-oxide synthase) (8). It also binds to IκBα (9), an inhibitor of NF-κB; to Bim (Bcl-2-interacting mediator of cell death) and Bmf (Bcl-2-modifying factor) (10), both of which are proapoptotic members of the Bcl-2 family of proteins; to p21-activated kinase 1 (12, 13); and to p53-binding protein 1 (14).

The transcription factor NF-κB is a key regulator of immune and inflammatory responses and exists as a homo- or heterodimer composed of members of the Rel/NF-κB family of proteins, including RelA (p65), RelB, c-Rel, NF-κB1 (p105/p50), and NF-κB2 (p100/p52) (15). The most common form of NF-κB in mammalian cells is the heterodimer composed of RelA (p65) and p50 (16). Under basal conditions, NF-κB is present in the cytoplasm as an inactive complex with the inhibitor protein IκB. However, in response to a variety of stimuli, including TNFα, interleukin (IL)-1β, and lipopolysaccharide (LPS), IκBα is phosphorylated at residues Ser32 and Ser36 by IκB kinase, which ubiquitinates at residues Lys31 and Lys32.
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ing in its degradation by the 26 S proteasome (15). The degrada-
tion of IkBα exposes the nuclear localization signal of NF-κB, result-
ing in its translocation to the nucleus, where it regulates the transcrip-
tion of various target genes that control the immune system, cell growth, and inflammation (17).

Recent findings suggest that ROS such as H₂O₂ activate NF-κB (18). The observations that potent NF-κB activators such as TNFα, IL-1β, and LPS trigger the production of ROS (19–22) and that a broad range of antioxidants inhibit NF-κB activation (23–25) suggest that most NF-κB inducers generate ROS, which mediate NF-κB activation cascade. The molecular mechanisms underlying these observations remain poorly understood, however, and the contribution of redox regulation to NF-κB activation remains unclear because of some conflicting reports (26, 27).

We recently showed that a novel disulfide reductase, TRP14, inhibits TNFα-induced NF-κB activation by suppressing the phosphorylation of IkBα, and we identified LC8 as a potential substrate of TRP14 (28). The proteins β-arrestin and κB-Ras inhibit NF-κB by interacting with IkBα and IkBβ, respectively (29–31), indicating that IkBα-binding proteins are potentially important regulators of NF-κB function. We have therefore investigated whether LC8 might serve as a molecular intermediary that links the disulfide reductase activity of TRP14 to NF-κB regulation. We now show that LC8 inhibits IkBα phosphorylation by IKK through its redox-dependent interaction with IkBα and that TRP14 regulates this inhibitory activity by maintaining LC8 in a reduced state.

**EXPERIMENTAL PROCEDURES**

Reagents and Antibodies—LPS, butylated hydroxyanisol (BHA), diphenylethydiionion (DPI), NADPH, and dithiothre-
titol (DTT) were obtained from Sigma; 4′,6-diamidino-2-phen-
ylindole and 5-(and-6)-chloromethyl-2′,7′-dichlorodihydro-
fluorescein diacetate (CM-H₂DCFDA), acetyl ester were from Molecular Probes; MG132 was from Calbiochem; glutathione-Sepharose and nickel-chelating Sepharose were from Amersham Biosciences; and TNFα and IL-1β were from R & D Systems. Normal rabbit IgG was from Caltag Laboratories; mouse monoclonal and rabbit polyclonal antibodies to LC8 were from BD Biosciences and Phoenix Pharmaceuticals, respectively; rabbit antibodies to phosphorylated (p-) IkBα, to p-IKKα/β, and to IKKβ were from Cell Signaling Technology; a monoclonal antibody to p65 and rabbit polyclonal antibodies to IkBα and to IKKγ were from Santa Cruz Biotechnology; a monoclonal antibody to β-actin was from Abcam; rabbit polyclonal antibody to Mn²⁺-dependent superoxide dismutase (MnSOD) was from Upstate Biotechnology; a monoclonal antibody to the FLAG epitope was from Sigma; and a rat antibody to the hemagglutinin (HA) epitope was from Roche Applied Sci-
ce. Horseradish peroxidase-conjugated goat antibodies to rabbit or mouse IgG were from Amersham Biosciences Bio-
sience, and Alexa Fluor 488-conjugated monoclonal IgG was from Molecular Probes.

Cloning and Mutagenesis of Human LC8 cDNA—A mamma-
lian expression vector for LC8, pFLAG-LC8, was described pre-
viously (28). Cysteine mutants of LC8 (C2S, C24S, and C56A, in which Cys², Cys²⁴, and Cys⁶⁴ are individually replaced by serine or alanine) were generated with the use of a site-directed mutagenesis kit (Stratagene) and complementary primers containing a 1-bp mismatch that converts the codon for cysteine to one for serine or alanine. To express HA-LC8 or LC8-FLAG proteins, cDNAs for wild-type LC8 or cysteine mutants of LC8 were cloned into the Xbal and BamHI sites of pCGN or into the EcoRI and BamHI sites of pFLAG-CMV5.1, respectively. For expression of LC8 in bacteria, the human LC8 gene was cloned into the NdeI and EcoRI sites of pET15b or pET17b.

Preparation of Recombinant Proteins—Escherichia coli
BL21(DE3) transformed with pET17b-LC8 was cultured at 37 °C in LB medium supplemented with ampicillin (100 μg/ml). Isopropyl-β-D-thiogalactopyranoside was added to the culture at a final concentration of 0.4 mm when the optical density at 600 nm had reached 0.5. After incubation for an additional 3 h, the cells were harvested by centrifugation and stored at −70 °C until use. The frozen cells were suspended in a solution containing 20 mM Tris-HCl (pH 8.0), 1 mM EDTA, and 1 mM 4-(2-
aminooethyl)benzene-sulfonfonyl fluoride (AEBSF) and were dis-
rupted by sonication. After the removal of debris by centrifugation, the remaining soluble fraction was applied at a flow rate of 2 ml/min to a DEAE-Sepharose column that had been equilibrated with a solution containing 20 mM Tris-HCl (pH 8.0) and 1 mM EDTA. The flow-through fraction was col-
clected and then applied to a gel filtration column (G3000SW; Tosoh Bioscience) that had been equilibrated with a solution containing 50 mM HEPES-NaOH (pH 7.0) and 0.1 mM NaCl. The fractions containing LC8 were pooled and dialyzed against 10 mM HEPES-NaOH (pH 7.0). For the bacterial expression of IkBα, a DNA fragment encoding human IkBα was amplified by PCR from HeLa cell cDNA and cloned into the NdeI and BamHI sites of pET14b. Hisα-tagged IkBα was purified from lysates of the transformed E. coli cells by affinity chromatogra-
phy on nickel-chelating Sepharose.

In Vitro Kinase Assay—The phosphorylation of IkBα (1 μg) was performed for 30 min at 30 °C with an IKK immune com-
plex in a final volume of 40 μl containing 50 mM Tris-HCl (pH 7.5), 1 mM EDTA, 5 mM DTT, 1 mM MgCl₂, 1 mM Na₃VO₄, 5 mM glycerophosphate, 100 μM ATP, and 10 μCi of [γ-³²P]ATP. IkBα and LC8 were incubated for 30 min at 30 °C before the addition of the IKK complex. The IKK complex was isolated by immunoprecipitation with antibody to IKKγ and protein A-Sepharose from HeLa cells that had been exposed to TNFα (20 ng/ml) for 10 min. The kinase reaction mixtures were frac-
tionated by SDS-PAGE on a 4–20% gradient gel, and the radio-
activity associated with IkBα band was quantified with the use of a phosphorimaging device (Molecular Dynamics).

NF-κB Reporter Assay—NF-κB activity was measured with the use of a dual luciferase reporter assay system. The cells were transfected for 24 h with 0.25 μg of pNF-κB-Luc (NF-κB reporter plasmid; Stratagene), 0.25 μg of pRL-SV40 (internal control), and either pFLAG-CMV2 or pFLAG-LC8. The total amount of plasmid DNA was adjusted with pFLAG-CMV2. A dual luciferase assay was subsequently performed with a kit (Promega). The activity of firefly luciferase was normalized by that of the Renilla enzyme and was then expressed as fold increase relative to the normalized value for cells transfected with pFLAG-CMV2.
Depletion of LC8 by RNA Interference—A small interfering RNA (siRNA) corresponding to nucleotides 315–333 (relative to the translation initiation site) of human LC8 cDNA (5'-GGACT-GGAGCGCTAAATTCC-3') was synthesized with T7 RNA polymerase (32) and was introduced into HeLa cells with the use of Oligofectamine (Invitrogen) as described (28).

RNA Isolation, Reverse Transcription, and Real Time PCR Analysis—Cells that had been stimulated with TNFα (20 ng/ml) for 1 h were harvested, and total RNA was isolated1 with the use of the TRIzol reagent (Invitrogen) and quantified by measurement of absorbance at 260 nm. Reverse transcription was performed with 2 μg of total RNA and Moloney murine leukemia virus reverse transcriptase (Promega) for 1 h at 42 °C followed by 10 min at 70 °C, and the resulting cDNAs were first confirmed the interaction between LC8 and IKK—Although LC8 was previously shown to interact physically with the regulatory domain of IKKα (9), the physiological function of this association has remained unknown. We further confirmed the interaction between LC8 and IkBα in HeLa cells by immunoprecipitation with antibody to IkBα. Not only transiently expressed FLAG-LC8 but also endogenous LC8 was transiently expressed FLAG-LC8 but also endogenous LC8 was immuno precipitated together with IkBα (Fig. 1A). To examine whether LC8 inhibits IkBα phosphorylation by IKK, we assayed the kinase activity of the immunoprecipitated IKK complex with recombinant IkBα as the substrate in the presence of LC8. LC8 indeed inhibited the phosphorylation of IkBα by the IKK complex (Fig. 1B).

LC8 Expression Inhibits TNFα-induced NF-κB Activation—Given that LC8 inhibited IKK-mediated phosphorylation of IkBα in vitro, we next investigated the effect of forced expression of LC8 on the NF-κB signaling pathway in HeLa cells. Expression of FLAG-LC8 inhibited NF-κB activation by TNFα in a concentration-dependent manner (Fig. 2A) as well as attenuated the TNFα-induced nuclear translocation of RelA (p65) (Fig. 2B). To determine which step of the NF-κB signaling pathway is influenced by LC8, we transfected cells with an expression vector for LC8 together with those for IKKβ or p65. LC8 inhibited the increase in NF-κB activity induced by IKKβ overexpression but not that attributable to overproduction of p65 (Fig. 2C), indicating that LC8 inhibits the NF-κB signaling pathway at a step between IKK activation and p65 nuclear translocation. However, the IKK complexes immunoprecipitated from cells transfected with the LC8 expression vector or the corresponding empty vector showed no difference in kinase activity measured in vitro with recombinant IkBα as the substrate (Fig. 2D). These results thus suggested that the binding of LC8 to IkBα inhibits its phosphorylation by IKK, without perturbing the activity of IKK itself, in TNFα-treated cells.

Depletion of LC8 Promotes TNFα-induced NF-κB Activation—To investigate the role of endogenous LC8 in TNFα-induced NF-κB activation, we depleted HeLa cells of LC8 by RNA interference and then monitored the expression of endogenous MnSOD, which is induced in response to NF-κB activation (33). The amount of LC8 was reduced by >90% after transfection of cells for 60 h with an siRNA specific for LC8 mRNA, compared with that apparent in cells transfected with a control RNA. Such depletion of LC8 resulted in a ~2-fold increase in the abundance of MnSOD in unstimulated cells and a 1.8-fold increase in TNFα-stimulated cells (Fig. 3A). We also determined the effect of LC8 depletion on the transcriptional induction of the NF-κB target genes for IkBα, cyclooxygenase-2, and IL-8 by reverse transcription and real time PCR analysis. Depletion of LC8 markedly increased the amounts of the target gene mRNAs under both basal and TNFα-stimulated conditions (Fig. 3B). In addition, depletion of LC8 promoted the nuclear translocation of p65 stimulated by TNFα (Fig. 3C). These data
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FIGURE 2. Inhibition of TNFα-induced NF-κB activation by LC8 overexpression. A, HeLa cells were transfected for 24 h with either pFLAG-CMV2 (2 μg) or the indicated amounts of pFLAG-LC8, with 0.25 μg of pNF-κB-Luc (NF-κB reporter plasmid), and with 0.25 μg of pRL-SV40 (internal control). The cells were incubated in the absence or presence of TNFα (20 ng/ml) for 6 h, after which the luciferase activities of cell lysates were measured with a dual luciferase assay system as described under “Experimental Procedures” (lower panel); the data are the means ± S.D. of values from three independent experiments. Cell lysates from a representative experiment were also subjected to immunoblot analysis with antibody to LC8 (upper panel). B, HeLa cells transfected with either pFLAG-CMV2 (Mock) or pFLAG-LC8 for 24 h were grown on coverslips, incubated in the absence or presence of TNFα (10 ng/ml) for 15 min, and fixed with 4% paraformaldehyde. They were then subjected to immunofluorescence staining with antibody to p65 as well as to staining of nuclei with 4',6-diamidino-2-phenylindole. C, HeLa cells were transfected with 1 μg of expression vectors for IKKβ or p65 as well as with pNF-κB-Luc, pRL-SV40, and the indicated amounts of pFLAG-LC8. They were harvested after 36 h for assay of luciferase activities. The data are the means ± S.D. of values from three independent experiments. Cell lysates were subjected to reverse transcription and real time PCR analysis to quantify the relative amounts of IkBα, cyclooxygenase-2, and IL-8 mRNAs. The data were normalized by the corresponding amount of β-actin mRNA and are the means ± S.D. of values from three independent experiments. C. HeLa cells were transfected as in A, incubated in the absence or presence of TNFα (10 ng/ml) for 7 min, fixed with 4% paraformaldehyde, and subjected to immunofluorescence staining as in Fig. 2B. D and E, HeLa cells transfected with LC8 siRNA as in A were incubated with TNFα (20 ng/ml) for the indicated times, after which cell lysates were subjected to immunoblot analysis with antibodies to p-IkBα, IkBα, p-IKKα/β, IKKβ, LC8, and β-actin (D). The chemiluminescence signals for p-IkBα were quantified and normalized by those of β-actin (E).

indicated that depletion of LC8 augments TNFα-induced NF-κB activation.

In response to activation signals such as TNFα, IkBα is phosphorylated by IKK and subsequently degradated by the ubiquitin-proteasome system. We therefore examined the effect of LC8 depletion on IkBα phosphorylation and degradation in HeLa cells stimulated with TNFα. Transfection of cells with LC8 siRNA substantially increased the extent of serine phosphorylation of IkBα compared with that apparent in cells transfected with a control RNA, and this effect was accompanied by an increase in the rate of IkBα degradation (Fig. 3, D and E). In contrast, depletion of LC8 had little effect on the phosphorylation status of IKK. These results suggested that LC8 blocks IkBα phosphorylation by IKK but does not inhibit the activity of IKK itself, consistent with the results shown in Fig. 2D.

LC8 Inhibits NF-κB Activation by Other Stimuli That Activate IKK—Our results suggested that LC8 inhibits NF-κB activation by interacting with IkBα and thereby preventing its phosphorylation by IKK. Given that IL-1β and LPS also induce IkBα phosphorylation by IKK during activation of NF-κB (15,
FIGURE 4. Inhibition by LC8 of NF-κB activation induced by IL-1β or LPS. A, HeLa, HEK293, and Raw 264.7 cells were transfected for 24 h with pNF-κB-Luc and pR-L404V as well as with pFLAG-CMV2 (Mock) or pFLAG-LC8. HeLa cells were then stimulated with TNFα (10 ng/ml) for 12 h, HEK293 cells were incubated with IL-1β (4 ng/ml) for 8 h, and Raw 264.7 cells were exposed to LPS (100 ng/ml) for 12 h. The cells were then harvested for determination of MnSOD expression as in Fig. 3A. Depletion of LC8 by RNA interference increased MnSOD expression in unstimulated or IL-1β-stimulated HEK293 cells (Fig. 4C). These results thus suggested that LC8 blocks 1κBα phosphorylation by IKK in the canonical NF-κB activation pathway.

LC8 Forms a Reversible Intermolecular Disulfide Bond between Cys² Residues on Oxidation—LC8 was identified as a potential substrate for the novel disulfide reductase TRP14 in a substrate-trapping experiment on the basis of its formation of a mixed disulfide linkage with TRP14 (28), suggesting that LC8 likely forms a disulfide bond on oxidation. LC8 exists as a dimer under physiological conditions (35), and the human protein contains three cysteine residues (Cys², Cys²⁴, and Cys⁵⁶). To determine whether LC8 forms a disulfide bond on exposure of cells to H₂O₂, we analyzed its redox status by nonreducing SDS-PAGE after modification of free thiol groups with AMS. Alkylation of thiol groups with AMS increases the molecular mass of the host protein by 490 Da/thiol, resulting in a mobility shift on SDS-PAGE that allows determination of the number of oxidized cysteine residues (36). AMS modification of LC8 in unstimulated HeLa cells gave rise to two LC8 bands with markedly retarded electrophoretic mobility in nonreducing SDS-PAGE gels (Fig. 5A); the lower of these two bands appeared to correspond to a fully reduced form of LC8 (LC8-AMS²), whereas the upper band seemed to represent an intermolecular disulfide-linked form (AMS²-LC8-S-S-LC8-AMS²). The intensity of the upper band was increased by exposure of the cells to H₂O₂ in a concentration-dependent manner. Under reducing conditions, the upper band was shifted to a position below the lower band (LC8-AMS²) that appeared to correspond to AMS²-LC8-SH (Fig. 5A). These results indicated that oxidation of LC8 results in the reversible formation of an intermolecular disulfide bond.

We next investigated which of the three cysteine residues (Cys², Cys²⁴, or Cys⁵⁶) of LC8 contribute to formation of the intermolecular disulfide bond. We substituted each cysteine residue with either serine or alanine by site-directed mutagenesis and then analyzed the oxidation status of the cysteine mutants in cells exposed to H₂O₂. Given that LC8 forms an intermolecular disulfide bond, we monitored the levels of disulfide-linked dimer by nonreducing SDS-PAGE after masking of free sulfhydryl groups with N-ethylmaleimide to prevent random disulfide bond formation. The C24S and C56A mutants formed the intermolecular disulfide bond, as did wild-type LC8, whereas the C2S mutant did not (Fig. 5B), suggesting that two Cys² residues form the disulfide linkage on oxidation of LC8 to the homodimer. It was possible that the environment surrounding the Cys² residue of recombinant LC8 was affected by the NH₂-terminal HA tag, which may have led to artifactual results. To exclude this possibility, we examined the oxidation status of COOH-terminally tagged LC8 (LC8-FLAG). Substitution of Cys² with serine also abolished intermolecular disulfide bond formation by LC8-FLAG (Fig. 5C).
Redox Regulation of NF-κB Activation through LC8

Interaction between LC8 and IkBα Is Redox-dependent—In addition to LC8 being identified as a potential substrate of TRP14, this disulfide reductase was shown to inhibit the TNFα-induced activation of NF-κB (28). Our results showing that LC8 inhibits NF-κB by interacting with IkBα and thereby preventing its phosphorylation by IKK thus implicated LC8 as a molecular intermediary that links the reductase activity of TRP14 to NF-κB regulation. Given that TNFα also increases the intracellular generation of ROS (20), we examined whether TNFα induces LC8 oxidation. Stimulation of HeLa cells with TNFα resulted in a 2.2-fold increase in the intracellular level of ROS (Fig. 6A) as well as increased the amount of oxidized LC8 (Fig. 6C). This latter finding is consistent with the previous observation that LC8 formed a mixed disulfide intermediate with the C46S mutant of TRP14 in cells treated with TNFα (28). ROS generated in response to TNFα stimulation thus oxidized LC8 to an intermolecular disulfide-linked dimer that might then be reduced by TRP14.

To determine whether the association of LC8 with IkBα is redox-dependent, we examined the interactions of oxidized or reduced LC8 with His6-IkBα with the use of a pulldown assay using nickel-chelating agarose. The proportion of LC8 precipitated with His6-IkBα was markedly greater for the reduced form than for the oxidized form (Fig. 6C), suggesting that LC8 indeed interacts with IkBα in a redox-dependent manner. To examine whether the redox-dependent association of LC8 with IkBα also occurs in cells, we treated HeLa cells with H2O2 or TNFα in the presence of the proteasome inhibitor MG132 and then subjected cell lysates to immunoprecipitation with antibody to IkBα. Interaction between LC8 and IkBα was decreased greatly by exposure of cells to H2O2 and only slightly less so by treatment with TNFα, despite precipitation of similar amounts of IkBα from control and stimulated cells (Fig. 6D). These results suggested that ROS generated in response to TNFα stimulation oxidize LC8 and thereby induce its dissociation from IkBα.

Given that LC8 also inhibited NF-κB activation by LPS and IL-1β, both of which have been shown to trigger ROS generation (21, 22, 37), we also examined whether these NF-κB activators induced ROS production in the cells studied here. Indeed, LPS and IL-1β induced 10.2- and 3.5-fold increases, respectively, in the intracellular ROS level compared with that in control cells (Fig. 6, E and F). These results thus suggested that the inhibition by LC8 of NF-κB activation induced by LPS or IL-1β might also be regulated in a redox-dependent manner.

IkBα Phosphorylation Is Regulated in a Redox-dependent Manner—Our previous (28) and present results implicated TRP14 as an inhibitor of IkBα phosphorylation and showed that LC8 inhibits IKK-mediated IkBα phosphorylation through redox-dependent interaction with IkBα, respectively, in the pathway of TNFα-induced NF-κB activation. Together with the identification of LC8 as a potential substrate of TRP14 (28), these results suggested that IkBα phosphorylation may be regulated in a redox-dependent manner during TNFα-induced NF-κB activation. We therefore investigated the effect of the antioxidant BHA on TNFα-induced IkBα phosphorylation. The extent of TNFα-induced serine phosphorylation of IkBα was decreased in cells pretreated with BHA compared with that in cells not exposed to the antioxidant (Fig. 7, A and B), and this effect was accompanied by a decrease in the rate of IkBα degradation (Fig. 7, A and C).

TNFα is known to produce ROS by activating NADPH oxidases (Noxs) in neutrophils, endothelial cells, and fibroblasts...
Redox Regulation of NF-κB Activation through LC8

We previously identified LC8 as a potential substrate of a novel disulfide reductase, TRP14, that regulates TNFα-induced NF-κB activation (28). We have now shown that LC8 interacts with IκBα in a redox-dependent manner and that TRP14 regulates the redox status of LC8. In cells exposed to TNFα or H2O2, LC8 was shown to form a reversible intermolecular disulfide linkage between the Cys residues of each subunit of the homodimer and to dissociate from IκBα. All NF-κB activators examined in the present study (TNFα, IL-1β, and LPS) generated ROS, and TNFα-induced IκBα phosphorylation was inhibited by both the antioxidant BHA and the NADPH oxidase inhibitor DPI. TRP14 also catalyzed the reduction of oxidized LC8. These observations suggest that IκBα phosphorylation is regulated in a redox-dependent manner by LC8, whose interaction with IκBα depends on its redox status, which in turn is modulated by cellular antioxidants such as TRP14.

Recently it has been reported that the ligated TNFα receptor forms a signaling complex harboring TRADD (TNF receptor-associated death domain protein), RIP1 (receptor interacting protein 1), NOXO1, the small GTPase Rac1, and Nox1, leading to the production of ROS in TNFα-stimulated mouse fibroblasts (19). TNFα is also known to be a potent activator of Nox2 in neutrophils and endothelial cells (38–40). On the basis of these observations together with our findings, we propose a tentative model for the redox regulation of NF-κB activation.

FIGURE 7. Effects of BHA and DPI on the phosphorylation and degradation of IκBα during NF-κB activation by TNFα. HeLa cells were incubated in the absence or presence of 100 μM BHA (A–C) or 10 μM DPI (D–F) for 30 min and were then stimulated with TNFα (20 ng/ml) in the continued absence or presence of the chemicals for the indicated times, after which cell lysates were prepared and subjected to immunoblot analysis with antibodies to p-IκBα, to IκBα, or to tubulin (loading control) (A and D). The chemiluminescence signals corresponding to p-IκBα (B and E) and to IκBα (C and F) were quantified, normalized by those of tubulin, and plotted.

FIGURE 8. Regulation of the redox status of LC8 by TRP14. A, reduction of oxidized LC8 by TRP14 was assayed at 30 °C by monitoring A290 in a 0.2-ml reaction mixture containing 50 mm HEPES-NaOH (pH 7.0), 1 mM EDTA, 0.2 mM NADPH, 100 mM TrxR1, 170 μM oxidized LC8, and 10 μM TRP14 or TRP14-C43S. Oxidized LC8 was prepared as in Fig. 6C. A reaction mixture lacking TrxR1 served as a control. B, reduction of oxidized LC8 by TRP14 was initiated by the addition of oxidized LC8 to a reaction mixture containing 50 mM HEPES-NaOH (pH 7.0), 1 mM EDTA, 0.2 mM NADPH, 100 mM TrxR1, and 5 μM TRP14 or TRP14-C43S. The reaction was terminated after incubation for 30 min at 30 °C. As a positive control, 1 mM DTT was used for the reduction of oxidized LC8 under the same conditions. The redox status of LC8 was analyzed as in Fig. 6C. Ox and Re indicate the oxidized and reduced forms of LC8, respectively.

(19, 38–40). DPI, an inhibitor of flavin-containing enzymes, is widely used to inhibit Nox activity in cells. We therefore examined the effect of DPI on the serine phosphorylation and degradation of IκBα induced by TNFα (Fig. 7, D–F). The results were similar to those obtained in cells treated with BHA, suggesting that Nox-derived ROS are involved in the TNFα-induced phosphorylation of IκBα.

TRP14 Regulates the Redox Status of LC8—We previously showed that LC8 forms a mixed disulfide intermediate with the C46S mutant of TRP14, but not with wild-type TRP14, in cells treated with H2O2 or TNFα (28), suggesting that, under conditions of oxidative stress, intracellular LC8 forms a disulfide that is reduced by TRP14. We examined whether oxidized LC8 is reduced by TRP14 in the presence of rat thioredoxin reductase 1 (TrxR1) and NADPH by monitoring NADPH oxidation at 340 nm. Oxidized LC8 was indeed reduced by TRP14 but not by the catalytically inactive mutant TRP14-C43S (Fig. 8A). To confirm the reduction of oxidized LC8 by TRP14, we analyzed the redox status of LC8 by nonreducing SDS-PAGE after incubation of oxidized LC8 with TRP14. TRP14, but not TRP14-C43S, converted the oxidized LC8 dimer to the reduced monomer (Fig. 8B). These results thus suggest that TRP14 maintains LC8 in a reduced form and thereby contributes to the inhibition of NF-κB.

DISCUSSION

Although inhibitory effects of antioxidant proteins on NF-κB activation have been described (28, 41), little is known of the underlying molecular mechanisms. We have now revealed the molecular mechanism for redox-dependent regulation of NF-κB activation through LC8. We first showed that LC8 is a novel inhibitor of NF-κB. LC8 was thus found to prevent IκBα phosphorylation by IKK through interaction with IκBα, resulting in inhibition of NF-κB activation induced by TNFα. LC8 also inhibited NF-κB activation by other stimuli, including IL-1β and LPS that induce IκBα phosphorylation by IKK. LC8 thus likely serves as a common inhibitor in the canonical pathway of NF-κB activation mediated by IKK-dependent serine phosphorylation of IκBα.

We previously identified LC8 as a potential substrate of a novel disulfide reductase, TRP14, that regulates TNFα-induced NF-κB activation (28). We have now shown that LC8 interacts with IκBα in a redox-dependent manner and that TRP14 regulates the redox status of LC8. In cells exposed to TNFα or H2O2, LC8 was shown to form a reversible intermolecular disulfide linkage between the Cys residues of each subunit of the homodimer and to dissociate from IκBα. All NF-κB activators examined in the present study (TNFα, IL-1β, and LPS) generated ROS, and TNFα-induced IκBα phosphorylation was inhibited by both the antioxidant BHA and the Nox inhibitor DPI. TRP14 also catalyzed the reduction of oxidized LC8. These observations suggest that IκBα phosphorylation is regulated in a redox-dependent manner by LC8, whose interaction with IκBα depends on its redox status, which in turn is modulated by cellular antioxidants such as TRP14.
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FIGURE 9. Model for the redox regulation of TNFα-induced NF-κB activation through LC8. See text for details.

through LC8 (Fig. 9). In resting cells, LC8 binds IκBα and inhibits IκBα phosphorylation by the IKK complex. Exposure of cells to TNFα triggers activation of the IKK complex and the production of ROS by activating a Nox enzyme. The transient ROS oxidize LC8 to a homodimer linked by a reversible intermolecular disulfide bond between the Cys^2 residues of each subunit, resulting in a conformational change and the dissociation of LC8 from IκBα. The released IκBα is then phosphorylated by the IKK complex and degraded by the 26 S proteasome. TRP14 contributes to LC8-mediated inhibition of NF-κB by maintaining LC8 in the reduced form, with TrxR1 and NADPH as a cellular source of reducing power. Antioxidants such as BHA and the Nox inhibitor DPI appear to inhibit IκBα phosphorylation by scavenging ROS and prohibiting ROS production, respectively. Given that IL-1β and LPS also triggered ROS generation and activated NF-κB in a manner sensitive to LC8, activation of NF-κB by these stimuli might also conform to this model. It has been reported that IL-1β and LPS induce the activation of Noxa2 and Noxa4, respectively (42, 43).

Our data support the notion that LC8 is a novel inhibitor of NF-κB and a target of redox regulation during NF-κB activation. In addition to the typical pathway of NF-κB activation elicited by TNFα, IL-1β, or LPS, atypical activation pathways have been described for UV, hypoxia reoxygenation, or peroxanate. UV induces phosphorylation of IκBα at a cluster of COOH-terminal sites by casein kinase II and subsequent IκBα degradation (44). Activation of NF-κB by hypoxia-reoxygenation or peroxanate depends on phosphorylation of IκBα at Tyr^22 (45, 46). In addition, ROS have been increasingly recognized as critical components in the cellular response to stress-induced injury such as that caused by ischemia reperfusion or UV irradiation. It will therefore be of interest to determine whether LC8 also plays an inhibitory, redox-dependent role in atypical pathways of NF-κB activation.

Various proteins are known to interact with their binding partners in a redox-dependent manner. For example, reduced Trx1 associates with apoptosis signal-regulating kinase 1 and thereby inhibits its activity, but oxidized Trx1 does not (47, 48).

Such redox-dependent interaction is likely due to a conformational change according to redox status. Given that the two Cys^2 residues are positioned distant from each other in the crystal structure of the LC8 dimer (49), formation of an intermolecular disulfide bond between these two residues likely induces a large conformational change that results in disruption of the LC8-IκBα complex. Structural comparisons of reduced and oxidized LC8 proteins will likely provide insight into the redox-dependent interaction between LC8 and IκBα.

LC8 proteins of mammals, including human, cow, rat, mouse, and rabbit, contain three cysteine residues (Cys^2, Cys^24, and Cys^56). Both Cys^24 and Cys^56 are conserved from fruit fly to human, but Cys^2 is present only in mammalian LC8 proteins, suggesting that the redox-dependent association of LC8 with IκBα is likely limited to mammals. Two members of the LC8 family, LC8a (also known as LC8 or DLC1) and LC8b (also known as DLC2), are present in several mammalian species, and a third member, LC8c, is listed in the human sequence data base (4). Two proapoptotic members of the Bcl-2 family of proteins, Bim and Bmf, interact with LC8 family members (50). Whereas Bim is normally sequestered by the microtubule-associated dynein motor complex through its interaction with LC8a, Bmf is sequestered by the actin-based myosin V motor through association with LC8b. LC8b shares 93% sequence identity with LC8a but contains Ser^2 instead of Cys^2. It will therefore be of interest to examine whether LC8-mediated regulation of the proapoptotic activity of Bim is redox-dependent.

NF-κB plays a key role in osteoclast differentiation and chronic inflammatory diseases such as rheumatoid arthritis, asthma, inflammatory bowel disease, and psoriasis (51–53). It has also been implicated in other diseases including atherosclerosis, Alzheimer disease, and cancer (54–56). Given that total loss of LC8 function in mice, but Cys2 is present only in mammalian LC8 proteins, suggesting that the redox-dependent association of LC8 with IκBα is likely limited to mammals. Two members of the LC8 family, LC8a (also known as LC8 or DLC1) and LC8b (also known as DLC2), are present in several mammalian species, and a third member, LC8c, is listed in the human sequence data base (4). Two proapoptotic members of the Bcl-2 family of proteins, Bim and Bmf, interact with LC8 family members (50). Whereas Bim is normally sequestered by the microtubule-associated dynein motor complex through its interaction with LC8a, Bmf is sequestered by the actin-based myosin V motor through association with LC8b. LC8b shares 93% sequence identity with LC8a but contains Ser^2 instead of Cys^2. It will therefore be of interest to examine whether LC8-mediated regulation of the proapoptotic activity of Bim is redox-dependent.

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