A *Legionella* type IV effector activates the NF-κB pathway by phosphorylating the IκB family of inhibitors

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Communicated by Jack E. Dixon, Howard Hughes Medical Institute, Chevy Chase, MD, June 27, 2009 (received for review June 9, 2009)

NF-κB is critical in innate immune defense responses against invading microbial pathogens. *Legionella pneumophila* infection of lung macrophages causes Legionnaire’s disease with pneumonia symptoms. A set of NF-κB-controlled genes involved in inflammation and anti-apoptosis are up-regulated in macrophages upon *L. pneumophila* infection in a *Legionella* Dot/Icm type IV secretion system-dependent manner. Among ~100 Dot/Icm substrates screened, we identified LegK1 as the sole *Legionella* protein that harbors a highly potent NF-κB-stimulating activity. LegK1 does not affect MAPK and IFN pathways. Activation of the NF-κB pathway by LegK1 requires its eukaryotic-like Ser/Thr kinase activity and is independent of upstream components in the NF-κB pathway, including TRAFs, NIK, MEKK3, and TAK1. Cell-free reconstitution revealed that LegK1 stimulated NF-κB activation in the absence of IKKα and IKKβ, and LegK1 efficiently phosphorylated IkBα on Ser-32 and Ser-36 both in vitro and in cells. LegK1 seems to mimic the host IKK as LegK1 also directly phosphorylated other IκB family of inhibitors including p100 in the noncanonical NF-κB pathway. Phosphorylation of p100 by LegK1 led to its maturation into p52. Thus, LegK1 is a bacterial effector that directly activates the host NF-κB signaling and likely plays important roles in modulating macrophage defense or inflammatory responses during *L. pneumophila* infection.

Hijacking the NF-κB signaling by injected bacterial effectors serves as a key virulence mechanism for many bacterial pathogens (5, 6). For example, *Yersinia* YopJ acts as an acetyltransferase to block phosphorylation of IKK (7, 8); *Salmonella* AviR and SscL deubiquitinates IkBε to prevent its degradation (9–11); VP1686, a *Vibrio* type III effector, directly binds RelA and attenuates the DNA-binding activity of NF-κB (12); the secreted chlamydial protease (CT441) specifically cleaves RelA to block NF-κB activation (13). *Legionella pneumophila* infection of alveolar lung macrophages causes Legionnaire’s disease. *L. pneumophila* resides and multiplies inside a decorated ER-like membrane-bound vacuole (14). Pathogenesis of *L. pneumophila* relies on a specialized translocation system known as the Dot/Icm type IV secretion system (TFSS). Vacuoles containing *L. pneumophila* TFSS-deficient mutant is quickly fused with and degraded within the lysosome (15), and a number of genetically highly redundant *L. pneumophila* TFSS effectors are involved in interfering with the host vesicular-trafficking pathway (16). *L. pneumophila* also actively manipulates the host innate immune system (17). Recent studies suggest that *L. pneumophila* triggers PAMP-independent, but TFSS-dependent, activation of the host NF-κB signaling and up-regulation of anti-apoptotic genes (18, 19). However, no *L. pneumophila* effectors are demonstrated to directly modulate the host innate immune pathway. In this study, we identify LegK1, a *L. pneumophila* eukaryotic-like Ser/Thr kinase that potently and specifically activates the host NF-κB signaling in its kinase activity-dependent manner. LegK1 is translocated into host macrophages by the *L. pneumophila* TFSS. Cell-free reconstitution, together with RNAi and knockout mouse embryonic fibroblast (MEF) experiments, reveals that LegK1-induced NF-κB activation does not require the host TRAF2/6, TAK1, NIK, and MEKK3. Surprisingly, LegK1-induced phosphorylation of IkBα also bypasses the host IKKs, and purified LegK1 directly phosphorylates IkBα and other IkB family of inhibitors including p100 in vitro. Phosphorylation of p100 by LegK1 is IKKe-independent and induces processing of p100 into p52, indicating that the noncanonical NF-κB pathway might also be a potential target of LegK1. LegK1 functionally mimics the host IKKs and represents a bacterial effector that activates the host NF-κB signaling.

**Results**

**Identification of a *Legionella* Ser/Thr Kinase LegK1 That Induces a Potent and Specific Activation of the NF-κB Pathway in Eukaryotic Cells.** *L. pneumophila* infection of macrophages activates the host NF-κB pathway and triggers anti-apoptotic signaling in its TFSS-
dependent manner (18, 19). In view of this phenomenon as well as the important role of NF-κB in host defense against pathogen infection, we performed a gain-of-function screen to search for potential L. pneumophila type IV effectors capable of stimulating the host NF-κB signaling. Among ~100 known or putative L. pneumophila type IV effectors (http://microbiology.columbia.edu/shuman/effectors.html), only LegK1 (ORF name: lpg1483) induced a reproducible and significant NF-κB-specific luciferase reporter activation when ectopically expressed in HEK 293T cells (Fig. S1). SdhA and SidF, two effectors previously proposed to have anti-apoptotic functions during L. pneumophila infection (20, 21), did not activate the NF-κB reporter. Notably, LegK1-induced NF-κB activation was comparable to, if not more potent than, that induced by TNFx treatment (Fig. 1A). Consistent with the luciferase assay, evident phosphorylation of the endogenous IκBα on Ser-32 and Ser-36 in 293T cells and nuclear localization of p65 (RelA) in HeLa cells occurred in response to LegK1 expression (Fig. 1B and C).

LegK1 contains 529 amino acids and harbors a typical eukaryotic-like Ser/Thr kinase domain (residues 82–364). An inactive kinase with a mutation (K121A) in LegK1 ATP binding site failed to induce the NF-κB activation in all three assays noted above (Fig. 1A–C), indicating a strict requirement of the kinase activity. The genome of L. pneumophila encodes three eukaryotic-like Ser/Thr kinases (LegK1, LegK2, and LegK3) (22). Expression of LegK2 or LegK3 had no effects on the NF-κB pathway, suggesting that induction of the NF-κB activation is a unique feature of LegK1 (Fig. 1A).

We also examined whether LegK1 could regulate other innate immune signaling that involves extensive phosphorylation events. Consistent with genetic data from Shin et al. (17), expression of LegK1 did not affect mitogen-activated protein kinases (MAPKs) activation including Erk1/2, JNK, and p38. Using the IFN-β (IFN-β) promoter-driven luciferase reporter or a more general IFN-stimulated response element (ISRE) luciferase reporter, we found that LegK1 did not activate the IFN-responsive gene transcription (Fig. S2A and B). Interestingly, the promoter of IFN-β also contains an NF-κB-responsive domain known as PRDII that positively regulates IFN-β promoter activation (23). Although LegK1 did not stimulate IRF3-controlled PRDII-luciferase reporter (Fig. 1D), activation of PRDII-luciferase by LegK1 was observed (Fig. 1E). Moreover, neither LegK2 nor LegK3 could trigger activation of any of these IFN promoter-driven luciferase report (Fig. 1 D and E and Fig. S2). These results further substantiates that LegK1, an eukaryotic-like Ser/Thr kinase from L. pneumophila, although does not interfere with the host MAPK (Erk1/2, JNK, and p38) and IFN signaling, harbors a highly potent and specific activity of inducing NF-κB activation.

**LegK1 Is Translocated into Host Macrophages via the Dot/Icm TFSS.**

The presence of the eukaryotic-specific Ser/Thr kinase domain strongly indicates that LegK1 is a translocated effector that functions inside host macrophages. To confirm this prediction, the TEM1 (β-lactamase) reporter system (24) was used to visualize Dot/Icm-dependent translocation of LegK1. Differentiated human U937 monocytes with no translocated TEM1, such as in the case of no infection or infection with L. pneumophila expressing TEM1-GST, developed green fluorescence at 520 nm due to the FRET dye CCF2/AM loaded to cells (Fig. 2). In contrast, evident emission of a blue fluorescence at 450 nm was observed in cells infected with wild-type L. pneumophila (Lp02) expressing the TEM1-LegK1 fusion protein (Fig. 2). The blue fluorescence, generated by β-Lactamase-mediated cleavage of CCF2/AM and the consequent disruption of FRET, is an indication of translocated TEM1 fusion proteins. When the TEM1-LegK1 fusion protein was expressed in the DotA mutant strain (Lp03), infected U937 cells only emitted the green fluorescence, similarly as that seen in control cells (Fig. 2). Another known Dot/Icm-translocated substrate LegAS4 (25), was included in this assay as a positive control.

These analyses firmly establish that LegK1 is translocated into host macrophages in a Dot/Icm TFSS-dependent manner during L. pneumophila infection.
proteins, and TRAF2, TRAF6, and TAK1/TAB1 expression plasmids were used as positive controls. (Fig. 3A and B). Downstream of TRAFs are the TAK1 complex, MEKK3, or NIK, which directly or indirectly mediates phosphorylation and activation of IKK. Knockdown of NIK did not affect LegK1-induced NF-κB activation; LegK1 triggered a comparable level of NF-κB reporter activation in MEKK3−/− and the control wild-type MEF cells (Fig. 3C). Moreover, TAK1-specific siRNA treatment largely abrogated the NF-κB reporter activation induced by overexpression of TRAF2 or TAK1, but not that by LegK1 (Fig. 3D). Activation of the TAK1 complex could also stimulate the JNK pathway (26), but expression of LegK1 did not induce the JNK-specific luciferase reporter activation and stimulate JNK phosphorylation (Fig. 3E and F). These results suggest that signaling components upstream of the IKK complex including TRAF2, TRAF6, TAK1, MEKK3, and NIK are not involved in LegK1-induced NF-κB activation.

**Reconstitution of LegK1-induced IκBα Phosphorylation in Cell-Free Extracts.** Cell-free reconstitution is a powerful approach in helping to decipher the biochemical mechanism used by bacterial effectors that modulate the host MAPK signaling (27). Addition of purified TRAF6 into cell-free extracts resulted in robust IκBα phosphorylation (28) (Fig. 4A), recapitulating the NF-κB signal transduction downstream of receptor activation. We found that addition of bacterially expressed and purified recombinant LegK1, but not the K121A mutant protein, into HeLa S3 S100 extracts also triggered evident and dose-dependent phosphorylation of IκBα on Ser-32 and Ser-36 (Fig. 4A and Fig. S3). Similar results were obtained with central IKK complex. TRAF2/5 mediates TNFα/TNFR-induced NF-κB activation whereas TRAF6 transduces signaling downstream of IL-1/IL-1R. RNAi knockdown of TRAF2 and TRAF6 largely abolished exogenous TRAF2 and TRAF6-triggered NF-κB activation, respectively, but had little effects on LegK1-induced NF-κB activation. (Fig. 3A and B). Downstream of TRAFs are the TAK1 complex, MEKK3, or NIK, which directly or indirectly mediates phosphorylation and activation of IKK. Knockdown of NIK did not affect LegK1-induced NF-κB activation; LegK1 triggered a comparable level of NF-κB reporter activation in MEKK3−/− and the control wild-type MEF cells (Fig. 3C). Moreover, TAK1-specific siRNA treatment largely abrogated the NF-κB reporter activation induced by overexpression of TRAF2 or TAK1, but not that by LegK1 (Fig. 3D). Activation of the TAK1 complex could also stimulate the JNK pathway (26), but expression of LegK1 did not induce the JNK-specific luciferase reporter activation and stimulate JNK phosphorylation (Fig. 3E and F). These results suggest that signaling components upstream of the IKK complex including TRAF2, TRAF6, TAK1, MEKK3, and NIK are not involved in LegK1-induced NF-κB activation.

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activation, addition of TRAF6 induced IKK indicated. mutant), and the reactions were analyzed by immunoblotting using antibodies as incubated with a buffer control, recombinant TRAF6, or bacterially purified LegK1 (WT or the KA

52x249 460 80 25 5 15 30 60 5 15 30 60 min

Fig. 4. Reconstitution of LegK1-induced NF-κB activation in cell-free extracts and IKK-independent NF-κB activation by LegK1. (A) Activation of the NF-κB pathway by recombinant TRAF6 in cell-free extracts. HeLa S100 were incubated with a buffer control, recombinant TRAF6, or bacterially purified LegK1 (WT or the KA mutant) in the presence of ATP regeneration system at 30 °C. Samples were analyzed by immunoblotting using antibodies recognizing IκBα or phospho-Ser-32 IκBα. Left and Right show the dose dependency and time-course analysis, respectively. (B) Activation of the NF-κB pathway by recombinant LegK1 in extracts from IKK knockout MEF cells. Experiments were performed and data are presented similarly as shown in (A) except that indicated wild-type, IKKα−/−, IKKβ−/−, or IKKαβ−/− (double knockout) MEF cells were used. (C) Luciferase assays of LegK1-induced NF-κB activation in IKKαβ−/− MEF cells. (D) Effects of LegK1 expression on phosphorylation of IKKα and IKKβ in cells. HEK 293T cells were transfected with EGFP-LegK1 (WT or the KA mutant), NIK, or TAK1/TAB1 as indicated. Cell lysates were subjected to immunoprecipitation by NEMO antibody. Total cell lysates (Input) or the immunoprecipitates (NEMO-IP) were analyzed by immunoblotting using indicated antibodies. (E) Effects of LegK1 expression on phosphorylation of NEMO and IKKβ in cell-free extracts. HeLa S100 was incubated with a buffer control, recombinant TRAF6, or LegK1 (WT or the KA mutant), and the reactions were analyzed by immunoblotting using antibodies as indicated.

extracts prepared from 293T, human THP1 monocytes, or MEF cells (Fig. S3). These data suggest that LegK1-induced NF-κB activation does not require the host transcription and further indicate that the target of LegK1 is likely a core and universal component in the NF-κB pathway.

LegK1-Induced IκBα Phosphorylation and NF-κB Activation Are Independent of the IKK Complex. Taking advantage of the reconstitution in cell-free extracts, we then investigated whether LegK1 was capable of inducing IκBα phosphorylation in the absence of IKKs. S100 cell extracts were prepared from wild-type (WT), IKKα−/−, IKKβ−/−, or IKKαβ−/− (double knockout) MEFs. Agreeing with the requirement of IKKβ (not IKKα) for TRAF6-mediated NF-κB activation, addition of TRAF6 induced IκBα phosphorylation only in WT and IKKα−/− MEF extracts, but not in IKKβ−/− and IKKαβ−/− MEF extracts (Fig. 4B). Significantly, robust IκBα phosphorylation appeared in any of the four extracts when supplemented with recombinant LegK1 (Fig. 4B). This in vitro result indicates that neither IKKα nor IKKβ is essential for LegK1 to induce IκBα phosphorylation. Further supporting this idea, expression of LegK1 in IKKα−/−/β−/− MEF cells triggered potent activation of the NF-κB luciferase reporter in its kinase activity-dependent manner (Fig. 4C). Similar results were obtained with 293T cells treated with IKKα and IKKβ siRNAs, suggesting that the IKK-independent function of LegK1 is not an effect specific to knockout MEF cells.

IKKα and/or IKKβ are phosphorylated at two serine residues in the kinase activation loop upon upstream stimulation of the NF-κB pathway. This was confirmed here by phospho-IKKα/β immunoblotting of NEMO immunoprecipitates from cells expressing NIK or the TAK1/TAB1 complex (Fig. 4D). In contrast, no phosphorylations of IKKα or IKKβ were observed in NEMO immunoprecipitates from cells expressing LegK1 (Fig. 4D). Furthermore, phosphorylation of IKK accompanied IκBα phosphorylation in the cell-free extracts upon TRAF6 addition, but neither IKKα nor IKKβ was found to be phosphorylated in LegK1-supplemented HeLa S100 cell extracts despite marked phosphorylation of IκBα (Fig. 4E). These data suggest that IKKα/IKKβ and their activities are not required for LegK1-induced NF-κB activation and that LegK1 hijacks the NF-κB signaling downstream of IKKs.

LegK1 Directly and Specifically Phosphorylates IκBα in Vitro. IκBα is only known to be phosphorylated by either IKKα or IKKβ. We then explored the hypothesis that IκBα is the direct target of LegK1. In vitro LegK1 kinase assay was carried out by using GST-IκBα purified from E. coli as the substrate. As shown in Fig. 5A, recombinant LegK1 efficiently phosphorylated GST-IκBα, but not myelin basic protein (MBP), as detected by incorporation of 32p radioactivity and immunoblotting using phospho-Ser-32 specific IκBα antibody. Conversely, a mixture of the constitutive active

Fig. 5. Recombinant LegK1 directly phosphorylates IκBα in vitro. (A) In vitro phosphorylation of IκBα by recombinant LegK1 and its truncation mutants. Purified GST-IκBα or myelin basic protein (MBP) was used as the substrate in the in vitro kinase assay by using indicated purified kinases. Autoradiography (Upper) shows incorporation of phosphates into the substrate and immunoblotting using the pser32-IκBα antibody (middle) reflects the site-specific phosphorylation. Lower shows the relative level of LegK1 added into each reaction. (B) NF-κB luciferase assays of LegK1 kinase activation loop mutant (SY/AA, LegK1 S252A/Y256A) (B) or truncation mutants of LegK1 (D). HEK 293T cells were transfected with LegK1 or its variants as indicated, and the relative NF-κB lucerase activity is shown. (C) Phosphorylation of IκBα induced by truncation mutants of LegK1 in HeLa S100 extracts.
MEK1 mutant (MEK1 R4F) and its substrate kinase Erk2 induced phosphorylation of MBP, but not GST-IkBα. This suggests that LegK1 could specifically and directly phosphorylate IkBα in vitro.

To gain further insights into the mechanism of LegK1 function, we constructed several truncation and point mutants of LegK1 and analyzed their phosphorylation toward IkBα and induction of NF-κB activation. Substitutions of both Ser-252 and Tyr-256 into alanine in LegK1 did not affect its NF-κB induction activity (Fig. 5B). Ser-252 and Tyr-256 are the only two phosphate-acceptor residues within the region in LegK1 equivalent to the kinase activation loop, suggesting that LegK1 is either constitutively active or regulated by means other than phosphorylation of the activation loop. In the in vitro kinase assay (Fig. 5A), full-length LegK1 and LegK1 (1–386) phosphorylated IkBα with a comparable efficiency when analyzed by 32P incorporation. Interestingly, when phosphorylation of Ser-32 in IkBα was examined, full-length LegK1 appeared to have a much stronger activity than LegK1 (1–386). Similar results were obtained with LegK1-induced phosphorylation of IkBα in cell-free extract (Fig. 5C). Consistently, LegK1 (1–386) was slightly less active in inducing NF-κB luciferase reporter activation (Fig. 5D). Moreover, deletions of the N-terminal prekinase region in LegK1 as short as 45 residues largely abolished in vitro phosphorylation of IkBα (Fig. 5A). LegK1 (46–529) and LegK1 kinase domain alone (86–386) completely lost the ability to activate the NF-κB-controlled luciferase reporter (Fig. 5D). Taken together, these data suggest that the C-terminal postkinase region in LegK1 is not absolutely required for phosphorylating IkBα but likely contributes to specification of phosphorylation sites, whereas the N-terminal 45 residues are strictly required for IkBα phosphorylation and are likely responsible for a productive IkBα binding.

Phosphorylation of p100 by LegK1 Induces p100 Processing and Activates the Noncanonical NF-κB Pathway in Vitro. We further examined whether LegK1 could induce p100 phosphorylation and processing of p52. (A and B) Processing of p100 into p52 induced by LegK1 expression. HEK 293T (A) or indicated MEF cells (wild-type or IKKα/β knockout) (B) were transfected with Myc-p100 together with indicated expression plasmids. Shown are immunoblots of the total cell lysates using Myc or indicated antibodies. (C) Phosphorylation of endogenous p100 induced by recombinant LegK1 in cell-free extracts. Cell extracts were prepared from HeLa S3 or MEF cells and reactions were analyzed by immunoblotting using antibodies specific for p100 or p100 phosphorylated at Ser-866 and Ser-870. (D) Direct phosphorylation of purified p100 by LegK1. In vitro kinase assay was carried out as that shown in Fig. S4 except that bacterially purified GST-p100C (the C-terminal domain, amino acids 755–900) was used as the substrate.

Discussion

L. pneumophila infection stimulates both the apoptotic pathway and nonapoptotic cell death through mechanisms not well understood. TFSS-dependent caspase-3 activation and bacterial flagellin-stimulated caspase-1 activation are reported (30–32). The pore forming activity of L. pneumophila and some unknown Dot/Icm-translocated substrates contributes to the nonapoptotic cell death (16, 33). Premature death of infected macrophages is detrimental to intracellular replication and L. pneumophila likely has evolved strategies to attenuate the host cell death. In fact,
that activation of the NF-κB pathway (Fig. S1). Not surpris-
ingly and similar to other known Legionella effectors, deletion of LegK1 in L. pneumophila had no notable effects on its intracel-
lar replication (Fig. S5). However, macrophages infected with
LegK1-null mutant strain were slightly more sensitive to stauro-
sorine-induced apoptosis, suggesting that LegK1-stimulated
NF-κB signaling might contribute to prevent premature macro-
phage apoptosis induced by L. pneumophila itself or cytokines
released by other cells upon infection. LegK1 might also be
involved in modulating other aspects of macrophage inflamma-
tory responses. This hypothesis is particularly intriguing given
that the noncanonical NF-κB pathway is not known to regulate
innate immunity so far. Further careful studies on in vivo mouse
infection model are needed to examine and dissect the function
of LegK1 in L. pneumophila pathogenesis. It is worth to mention
that activation of the NF-κB pathway by LegK1 is extremely
robust, and such high potency is often seen with bacterial
effectors as they are usually injected into host cells with limited
amounts.

Pathogenic organisms including viruses, bacteria and parasites
have evolved diverse strategies to manipulate the host NF-κB
signaling, usually at the level or upstream of IKK. Direct modula-
tion of the core NF-κB signaling components by secreted effectors
have been documented for several bacterial pathogens including
Yersinia spp. (7, 8), Salmonella typhimurium (9–11), Vibrio parah-
aeolyticus (12), and Chlamydia trachomatis (13). All these NF-
κB-modulating bacterial effectors are inactive in nature, and
instead LegK1 represents the first one capable of activating the host
NF-κB pathway. LegK1 is also unique in that it can promote
processing and maturation of p100 and induce noncanonical NF-κB
activation, at least in vitro. The activity of LegK1 appears to be
specific to the IκB family of NF-κB inhibitors as neither the MAPK
nor the IFN pathway was activated by LegK1. Among the 1xB
family of proteins, only those containing a typical DSGXXS/T motif
(1xBα, 1xBβ, p100, and 1xBε, but not 1xBζ and Bcl-3) are substrates
of LegK1. This is much similar to properties of the host IKK that
phosphorylates the serine or threonine residues within the
DSSGXXS/T motif. Thus, LegK1 likely represents a bacterial mimic
of the host IKK. Different from IKK, LegK1 does not require
phosphorylation of its “activation” loop to become activated, which
allows the bacteria to achieve host-dependent NF-κB signaling.

Materials and Methods
cDNAs for all of the candidate putative effectors listed in Fig. S1
were amplified from L. pneumophila genomic DNA and inserted into mammalian ex-
pression vector pCS2 with an N-terminal EGFP tag. cDNA for TRAF2, TRAF6, p100, TAK1, and TAB1 were amplified from a Rela cDNA library and cloned into pCS2–3–Flag or pCS2–6–Myt. For translocation assay, LegK1 or the indicated gene was cloned into pB908 and with its N terminus fused with
β-lactamase. NF-κB and JNK luciferase reporter plasmids were described pre-
niously (27). Truncation and deletion mutants were constructed by standard
PCR cloning strategy. All of the point mutants were generated by the
QuickChange Site-Directed Mutagenesis Kit (Stratagene). All of the plasmids
were verified by DNA sequencing. The rest of information about cell culture and
protein expression, luciferase reporter and immunofluorescence assays, bac-
terial infection and TEM translocation assays, cell-free extract reconstitu-
tion, recombinant protein purification, and in vitro kinase assay is presented
in SI Materials and Methods.

ACKNOWLEDGMENTS. We thank Zhao-qing Luo (Purdue University, West
Lafayette, IN) for providing Legionella strains, Zhijian Chen (University of Texas
Southwestern Medical Center, Dallas) for TRAF6 baculoviral P3X3-4 (Har-
vard University, Boston) and Christine Ehrhardt (University of Muenster, Ger-
many) for PRD-1 luciferase reporters, Zheng-Gang Liu (National Institutes of Health,
Bethesda, NIH) for NIK constructs, John Hiscott (McGill University, Montreal) for GFP–
IRE3 and IRE3-Luc plasmid, Tatsushi Muta (Tohoku University, Japan) for IκBα,
DNA, Alain Inoue (Institut Pasteur, France) for IκBζ and IκBε cDNAs, Kunitada
Shimotohno (Kyoto University, Japan) for Bcl-3 plasmid, and Aaron Ciechanover
(Technion-Israel Institute of Technology, Haifa, Israel) for p10S (DNA). We are
grateful to Inder Verma (Salk Institute, La Jolla, CA) and Bing Su (Yale University,
New Haven, CT) for IKK knockout MEFs and MEKKS 3/7+ MEF, respectively.
This work was supported by Chinese Ministry of Science and Technology “863” Grant
2008AA022939 (to F.S.).