Macrophages and B-cells from Tpl2 knock-out mice exhibit a restricted defect in lipopolysaccharide and death receptor signaling that is limited to the activation of ERK. Here we show that Tpl2−/− MEFs exhibit defects in ERK, JNK, and NF-κB activation, or ERK activation only when stimulated with tumor necrosis factor-α (TNF-α) or interleukin-1β, respectively. In addition, we show that the activation of Tpl2 by TNF-α depends on signals transduced by both TRAF2 and RIP1. Activated Tpl2 phosphorylates MKK4/SEK1 upstream of JNK and stimulates NF-κB DNA binding and transcriptional activity by mechanisms that are independent of the nuclear translocation of p50 and p65. Tpl2-transduced TNF-α signals instead promote the phosphorylation of p65 at Ser276 and modulate the spectrum of proteins associated with p65. Phosphorylation stimulates the transcriptional activity of NF-κB but does not affect its ability to bind DNA, which may be affected by the composition of the nuclear NF-κB complexes. These data confirm that defects caused by a single mutation may be cell-type and signal-specific and delineate the role of Tpl2 in the transduction of TNF-α signals that activate JNK and NF-κB in MEFs.

Tpl2 is a serine-threonine protein kinase that was cloned based on its ability to induce T-cell lymphomas when activated by proviral insertion in rodents (1, 2). When overexpressed, it activates ERK, JNK, p38 MAPK, and ERK5 as well as NF-κB and NFAT (3–7). The physiological role of Tpl2 in the hematopoietic system appears to be more restricted, as macrophages from Tpl2−/− mice stimulated with lipopolysaccharide (LPS), tumor necrosis factor-α (TNF-α), and anti-CD40 antibodies are defective in ERK activation but not in the activation of other MAPKs and NF-κB (8, 9). Similarly, Tpl2−/− B-cells also exhibit a restricted defect in ERK activation when stimulated with anti-CD40 antibodies (9).

Tpl2 regulates both innate and adaptive immunity and plays a critical role in the response to inflammatory signals. Indeed, mice that lack Tpl2 secrete low levels of TNF-α when exposed to LPS and are resistant to LPS/galactosamine-induced endotoxin shock (8). Moreover, ablation of Tpl2 protects from TNF-α-induced inflammatory bowel disease (IBD) (10) and arthritis.2 It is interesting to note that although Tpl2 is required for the development of full-blown TNF-α-induced arthritis and IBD, the pathobiology of the two syndromes may depend on signals originating in different cell types. Specifically, whereas IBD depends on the activity of CD8+ T-cells (10), collagen-induced arthritis, a TNF-α-dependent process (11, 12), can develop in the absence of B- and T-cells (13). Instead, it appears to depend on the function of non-lymphoid cells, perhaps the synovial fibroblasts. It is therefore important to define the signaling role of Tpl2 in different cell types.

Our previous studies had shown that Tpl2 is required for the transduction of TNF-α signals that are induced via ligation of TNFR1 (9). Binding of soluble TNF-α to TNFR1 promotes the release of the receptor-bound silencer of death domains and the recruitment of the death domain-containing adaptor protein TRADD (14). TNFR1 bound to TRADD then serves as an assembly platform for the binding of the TNF receptor-associated factor-2 (TRAF2) and the death domain containing serine-threonine protein kinase RIP1 (receptor-interacting kinase-1) (14, 15).

TRAF2 is obligatory for JNK activation as has been clearly demonstrated by measuring the signaling output of TNF-α in TRAF2−/− MEFs (16). However, the pathway from TRAF2 to JNK, including the role MAP3Ks may play, remains largely unresolved (17, 18). Regarding NF-κB, TRAF2 is not required but contributes to its activation by TNF-α. This is underscored by the fact that TRAF2 ablation combined with the ablation of TRAF5 dramatically impairs NF-κB activation by TNF-α, despite the fact that the effect of ablation of either TRAF2 or TRAF5 alone on NF-κB activation is minimal (19).

RIP1 is essential for TNF-α-induced NF-κB and p38 MAPK activation, whereas its role in the activation of JNK has been controversial (15, 20–23). RIP1 recruits MEKK3 and the IκB kinases (IKKs) into a signaling complex that is formed after TNF-α stimulation. Assembly of this complex promotes the phosphorylation and activation of the IKKs and the MAP2Ks that activate p38 MAPK. The activated IKK complex phos-
phosphorylates IkB-α resulting in its degradation via the proteasome. IkB-α degradation releases NF-κB from a trimeric p65-p50-IκB-α cytoplasmic complex. Once released, NF-κB translocates into the nucleus, binds the κB consensus sequences in the promoters of numerous genes, and activates their transcription (14, 24). In addition to signals that regulate its translocation into the nucleus, p65 is also regulated by phosphorylation (25–27). One of the p65 phosphorylation sites was mapped at Ser276 in the Rel homology domain (27, 28). At this site in response to different signals (27, 29). Phosphorylation at Ser276 significantly enhances the transcriptional activity of NF-κB (28, 29). It was originally thought that phosphorylation at this site enhances NF-κB DNA binding (30). However, more recent studies challenged the earlier data (29).

In this paper we present evidence thatTpl2 is obligatory for the activation of ERK, JNK, and NF-κB by TNF-α in MEFs. The differences between hematopoietic cells and MEFs with regard to the TNF-α output suggest that the signaling role of Tpl2 depends on the cell type. In addition, we show that Tpl2 is obligatory only for the activation of ERK in IL-1β-stimulated MEFs, suggesting that its signaling role is also stimulus-specific. Finally, we present data that place Tpl2 downstream of Tpl2 and NF-κB DNA signaling in MEFs.

MATERIALS AND METHODS

Cell Culture and Retroviral Infections—MEFs from 13.5-day-old wild type and Tpl2/−/− C57BL6 embryos were cultured in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum (Invitrogen), penicillin and streptomycin, and non-essential amino acids as described (16). Briefly, 1 ml of the murine leukemia virus, also encoding Tpl2. Experiments were typically carried out using wild type and kinase-inactive (K167M) Tpl2 in MEFs. Cells were lysed using Nonidet P-40 lysis buffer (50 mM HEPES, pH 7.4, 100 mM NaCl, 2 mM EDTA, 0.5% Nonidet P-40, 10% glycerol, 50 mM β-glycerophosphate, 1 mM sodium orthovanadate, 1 mM phenylmethylsulfonfyl fluoride, 1 mM NaF, 10 μg/ml leupeptin, and 5 μg/ml aprotinin). Endogenous JNK was immunoprecipitated by incubating the lysates with a rabbit monoclonal antibody and protein G-agarose beads (Invitrogen) overnight at 4 °C. Following washing, protein complexes bound to the beads were denatured by adding sample buffer, and they were resolved in SDS-PAGE.

In Vitro Kinase Assays—JNK in vitro kinase assays were carried out using 25 ng/ml of TNF-α (or TNF-α or anisomycin-stimulated, wild type and Tpl2/−/− MEFs. Cells were lysed using Nonidet P-40 lysis buffer (500 mM LiCl, 100 mM Tris-Cl, pH 7.6, 0.1% Triton X-100, 1 mM dithiothreitol), and 2 times with the kinase assay buffer (25 mM Tris-Cl, pH 7.5, 5 mM β-glycerophosphate, 2 mM dithiothreitol, 0.1 mM sodium orthovanadate, 10 mM MgCl₂, 10 μg/ml aprotinin). Bead-bound kinase was then incubated in the kinase buffer with 5 μg of purified GST-ε-Jun fusion protein (Cell Signaling Technology Inc) and [γ-32P]-ATP (PerkinElmer Life Sciences) at 30 °C for 30 min. The reaction was terminated by adding sample loading buffer. Protein samples were electrophoresed in SDS-PAGE, and transferred to nylon membranes.

Tpl2 kinase assays were carried out using the same general strategy with the following exceptions. Tpl2 was incubated with 0.4 μg of the purified substrate GST-MEK1 (US Biologicals) and cold ATP (0.5 mM) for 20–30 min. After 30 min. As the reactions were terminated, the samples were electrophoresed in SDS-PAGE, transferred to nylon membranes, and probed with the phospho-specific MEK1 antibody. As MEFs express very low levels of endogenous Tpl2, they were infected with pMSVC retroviral constructs of Tpl2-HA and Tpl2 K167M-HA. Infected cells express HA-tagged Tpl2 at levels sufficiently high to easily detect it, but low enough to prevent its constitutive activation.

Electrophoretic Mobility Shift Assays (EMSAs)—NF-κB EMSAs were carried out using standard protocols (16). Briefly, nuclear extracts of MEFs or 3T3 fibroblasts were incubated with 32P-labeled κB consensus oligonucleotides (5′-AGTTAGGGGACTTCCAGGC-3′ and 5′-TCAACTTCCCTGTAAAGGTTCCG-3′) (Promega Inc.) in a binding buffer (50 mM HEPES, 250 mM KCl, 0.5 mM EDTA, 50% glycerol, 1 mM dithiothreitol, 5 mM MgCl₂, 0.4 μg/ml single-stranded DNA) at 30 °C for 30 min. DNA-protein complexes were resolved in a 6% non-denaturing polyacrylamide gel at 200 volts for 2 h. For supershift and cold oligonucleotide competition, nuclear extracts were preincubated with the respective antibody or with a 100-fold excess of unlabeled cold oligonucleotides prior to incubation with the labeled ones.

 Luciferase Reporter Assay—Luciferase reporter assay was done as described previously (22). MEFs were transiently transfected with a β-galactosidase luciferase reporter construct (Promega Inc.) using FuGENE (Roche Diagnostics). Twenty-four hours post-transfection, cells were stimulated with TNF-α (10 ng/ml) in media supplemented with 0.5% fetal bovine serum. Twelve hours later, the cells were lysed in reporter lysis buffer and the luciferase reporter activity was measured by a luminometer using a luciferase reporter assay kit (Promega Inc.).

RESULTS

Tpl2 Transduces TNF-α Signals That Activate ERK and JNK, but Not p38 MAPK in MEFs—TNF-α activates ERK, JNK, and p38 MAPK in wild type MEFs (14, 32). To determine with a horseradish peroxidase-conjugated IL-6 antibody. To measure antibody binding, horseradish peroxidase substrate was added to the wells. Optical density at 450 nm was measured after 30 min of incubation.

Immunostaining was carried out as described (31). Briefly, unstimulated and TNF-α-stimulated wild type and Tpl2/−/− MEFs, fixed in 4% paraformaldehyde, were first incubated with an anti-p65 antibody overnight at 4 °C. After washing with phosphate-buffered saline, the cells were incubated with a phycoerythrin-conjugated secondary antibody for 1 h at room temperature. Nuclei were counterstained with 4,6-diamidino-2-phenylindole. Stained cells were visualized by fluorescent microscopy.

Metabolic Labeling, Immunoprecipitation, and SDS-PAGE—MEFs were washed twice with methionine and cysteine-free Dulbecco’s modified Eagle’s medium and then incubated in the same media for 20 min. Subsequently, cells were cultured for 6 h in the same media in the presence of [35S]methionine (PerkinElmer Life Sciences).

Nuclear extracts were prepared as described above. p65 was immunoprecipitated by incubating the extracts with an anti-p65 antibody and protein G-agarose beads (Invitrogen) overnight at 4 °C. Following washing, protein complexes bound to the beads were denatured by adding sample buffer, and they were resolved in SDS-PAGE.

In vitro transfection and luciferase reporter activity was measured by a luminometer using a luciferase reporter assay kit (Promega Inc.).
the physiological role of Tpl2 in MAPK activation by TNF-α in these cells, Western blots of lysates from Tpl2+/+ and Tpl2−/− MEFs harvested before and after TNF-α stimulation, were probed with antibodies against phosphorylated and total ERK, JNK, and p38 MAPK. The results (Fig. 1A), showed that while the phosphorylation of ERK and JNK was impaired in Tpl2−/− cells, the phosphorylation of p38 MAPK was normal. In vitro kinase assays confirmed that the kinase activity of JNK immunoprecipitated from TNF-α-stimulated Tpl2−/− MEFs is significantly lower. The defect in JNK activation in Tpl2−/− cells was specific for TNF-α as other stress signals such as anisomycin activated JNK equally in both wild type and knock-out cells.

To further explore the specificity of Tpl2 in the transduction of TNF-α signals, we examined the activation of ERK, JNK, and NF-κB in TNF-α-stimulated Tpl2+/+ and Tpl2−/− macrophages and the activation of MAP kinases and NF-κB in IL-1β-treated Tpl2+/+ and Tpl2−/− MEFs. Fig. 1B shows that Tpl2 is indeed required only for ERK activation by TNF-α in macrophages. The signaling defect in Tpl2−/− macrophages stimulated with TNF-α is therefore, similar to the signaling defect in Tpl2+/− macrophages stimulated with LPS. Fig. 1C shows that whereas ERK phosphorylation is impaired in IL-1β-treated Tpl2−/− MEFs, IL-1β-induced phosphorylation of JNK and p38 MAPK and activation of NF-κB is normal in these cells. We conclude that the Tpl2 signaling defect is cell-type and stimulus-specific.

Tpl2 Is Required for the Activation of NF-κB by TNF-α in MEFs—TNF-α also activates NF-κB in wild type MEFs (14, 32). To investigate the role of Tpl2 in NF-κB activation by TNF-α, we performed EMSA using nuclear extracts of unstimulated and TNF-α-stimulated wild type and Tpl2−/− MEFs. The induction of NF-κB DNA-protein complexes resolved on EMSA was significantly weaker in Tpl2−/− MEFs (Fig. 2A). Preincubation of the nuclear extracts with an excess of cold κB consensus oligonucleotides completely obliterated DNA binding, whereas preincubation with a polyclonal p65 antibody supershifted the DNA-protein complex (Fig. 2B).

To confirm that Tpl2 is required for the activation of NF-κB, we carried out luciferase reporter assays on TNF-α-stimulated Tpl2+/+ and Tpl2−/− MEFs. The results showed that TNF-α induced a 2–4-fold increase in reporter activity in wild type MEFs and a minimal increase in Tpl2−/− MEFs (Fig. 2C).

TRAF2 and RIP1 Are Required for the Activation of Tpl2 by TNF-α—TRAF2−/− MEFs exhibit a major block in the activation of JNK and a minor block in the activation of NF-κB by TNF-α (15, 16). RIP1−/− MEFs, on the other hand, exhibit a major block in the activation of NF-κB and p38 MAPK (20, 21). Given that Tpl2 is required for the activation of both JNK and NF-κB, we examined whether it functions downstream of TRAF2 and RIP1. To this end, we carried out Tpl2 in vitro kinase assays using lysates of TRAF2−/+ and TRAF2−/− 3T3 fibroblasts as well as RIP1−/+ and RIP1−/− MEFs. Because the endogenous levels of Tpl2 in fibroblasts are low and Tpl2 undergoes rapid degradation following TNF-α stimulation (33), we expressed low levels of wild type Tpl2-HA from a retroviral promoter construct into wild type as well as the TRAF2−/−and RIP1−/− fibroblasts. A kinase-inactive form of Tpl2 (K167M) was expressed as a control in wild type fibroblasts. Tpl2 immunoprecipitated with an anti-HA antibody from wild type and mutant cells before and 10 min after stimulation with TNF-α was used to carry out in vitro kinase assays using purified GST-MEK1 as the substrate. Phosphorylated substrate was electrophoresed in SDS-PAGE, transferred to nylon membranes, and probed with a phospho-specific MEK1 antibody. The results showed induction of MEK1 phosphorylation by wild type Tpl2 pulled down from TNF-α-stimulated wild type fibroblasts but not from similarly treated TRAF2−/− and RIP1−/− fibroblasts (Fig. 3A). The kinase-inactive Tpl2 immunoprecipitated from TNF-α-stimulated wild type fibroblasts failed to phosphorylate MEK1 in the in vitro kinase assay, thus confirming that phosphorylation was not because of a Tpl2-associated kinase (supplemental materials Fig. 2). These results suggest that both TRAF2 and RIP1 are required for the activation of Tpl2 by TNF-α in MEFs.

If RIP1 is required for Tpl2 activation, and Tpl2 is required for JNK activation, one would expect that optimal JNK activation will depend on RIP1. Previous studies addressing the role of RIP1 in JNK activation had produced conflicting results (20, 23). We therefore revisited its role by probing Western blots of lysates derived from unstimulated and TNF-α-stimulated RIP1−/+ and RIP1−/− MEFs with antibodies against total JNK and JNK phosphorylated at Thr183 and Tyr185. Lysates from cells stimulated with IL-1β were used as controls. The results showed that RIP1−/− cells exhibit a partial defect in JNK phosphorylation in response to TNF-α but not in response to IL-1β (Fig. 3B) and provided support to the study showing that RIP1 contributes to the activation of JNK by TNF-α.

Tpl2 Transduces TNF-α Signals That Activate MKK4/SEK1 Upstream of JNK—It was earlier reported that activation of JNK/SAPK in MEFs in response to TNF-α requires two upstream kinases, MKK7, which phosphorylates JNK preferentially on Thr, and MKK4, which phosphorylates JNK preferentially on Tyr (34). It has been proposed that whereas MKK7 is obligatory for JNK activation by TNF-α, MKK4/SEK1 is required only for optimal JNK activation (35). Our data that JNK phosphorylation and activation were reduced but not abolished in TNF-α-treated Tpl2−/− MEFs suggested that Tpl2 may be required for the activation of MKK4/SEK1. To test this hypothesis, we examined the phosphorylation of MKK4/SEK1, in total lysates of untreated and TNF-α-treated Tpl2+/+ and Tpl2−/− MEFs by Western blotting. The results showed that the phosphorylation of MKK4/SEK1 in Tpl2−/− MEFs was impaired (Fig. 4).

The Regulation of NF-κB by Tpl2 in TNF-α-stimulated MEFs Does Not Depend on the Phosphorylation of IκB and the Nuclear Translocation of NF-κB—NF-κB activation depends on the phosphorylation and activation of the IKK complex by upstream kinases. Activated IκKβ phosphorylates IκB-α and marks it for degradation via the proteasome. This, in turn, releases the p50 and p65 subunits of NF-κB that translocate into the nucleus and bind DNA to activate transcription (14, 24). To determine whether Tpl2 regulates these events, we first addressed its role in the TNF-α-induced degradation of IκB-α. As shown in Fig. 5A, a, TNF-α stimulation induced rapid degradation of IκB-α in both wild type and Tpl2−/− MEFs. Interestingly, regeneration of IκB-α, beginning at 30 min from the start of the stimulation, was more robust in wild type MEFs. As IκB-α is an NF-κB target (24), its weak regeneration in the Tpl2−/− MEFs suggests a role of Tpl2 in the regulation of NF-κB. To further investigate whether Tpl2 plays a role in IKK signaling, we examined the phosphorylation of IκB-α upon TNF-α stimulation. To prevent degradation of IκB-α, MEFs were first treated with the proteasome inhibitor MG132. TNF-α stimulation applied 45 min after exposure to MG132 induced phosphorylation of IκB-α in both wild type and Tpl2−/− MEFs within 10 min from the start of the stimulation (Fig. 5A, b). These results combined, confirmed that IKK signaling remains intact in Tpl2−/− MEFs.

It has been reported that signal-induced degradation of IκB-α leads to automatic and almost immediate translocation of NF-κB into the nucleus without the need of additional modifications (14, 24). As a result, nuclear translocation of NF-κB
**Fig. 1.** The role of Tpl2 in the transduction of TNF-α and IL-1β-induced MAPK activation signals in MEFs. A, Tpl2 transduces TNF-α signals that activate ERK and JNK, but not p38 MAPK. a, cell lysates harvested at the indicated time points after stimulation with TNF-α (10 ng/ml) were probed with antibodies against phosphorylated ERK (upper panel) or total ERK (lower panel). b1, cell lysates harvested before and after stimulation with TNF-α (10 ng/ml), as indicated, were probed with antibodies against phosphorylated JNK (upper panel) or total JNK (lower panel). b2, JNK was immunoprecipitated from cell lysates harvested before and after TNF-α stimulation (50 ng/ml), and the immunoprecipitates were used to carry out in vitro kinase assays with GST-c-Jun as the substrate. Anisomycin treatment was used as a control. c, cell lysates harvested before and after TNF-α stimulation (10 ng/ml) were probed with antibodies against phosphorylated p38 MAPK (upper panel) or total p38 MAPK (lower panel). B, Tpl2 is required for the transduction of TNF-α signals that activate ERK but not JNK and NF-κB in macrophages. a1 and a2, Tpl2+/− and Tpl2−/− macrophages were harvested before and at the indicated time points after stimulation with TNF-α. Cell lysates were probed with antibodies against phosphorylated or total ERK (a1) or with antibodies against phosphorylated or total JNK (a2). b, nuclear lysates of similarly treated Tpl2+/− and Tpl2−/− macrophages were harvested before and at the indicated time points after stimulation with TNF-α. Nuclear lysates were employed for EMSAs, using an NF-κB-specific 32P-labeled double-stranded oligonucleotide as a probe. C, Tpl2 transduces IL-1β signals that are required for the activation of ERK but not for the activation of JNK, p38 MAPK, and NF-κB in MEFs. Cell lysates harvested before and after stimulation with IL-1β (10 ng/ml), as indicated, were probed with antibodies against phosphorylated ERK (a, upper panel) or total ERK (a, lower panel), phosphorylated JNK (b, upper panel) or total JNK (b, lower panel), and phosphorylated p38 MAPK (c, upper panel) or total p38 MAPK (c, lower panel). Nuclear lysates of similarly treated Tpl2−/− and Tpl2−/− MEFs were employed for EMSAs, using an NF-κB-specific 32P-labeled double-stranded oligonucleotide as a probe (d). WT, wild type.
signals in LPS-stimulated macrophages (37), we proceeded to
that the activation of MSK1 depends on Tpl2/ERK-transduced
and Tpl2

MEFs stimulated with TNF-

potential of NF-

homology domain (25–27) (Fig. 6). These data combined suggest that the differences in NF-κB DNA binding and transcriptional activity between wild type and Tpl2

MEFs are not the result of the differential effects of Tpl2 on IKK signaling.

Fig. 2. Tpl2 is required for the activation of NF-κB by TNF-α in MEFs. A, NF-κB EMSAs of nuclear extracts of wild type and knock-out MEFs stimulated with TNF-α. Nuclear extracts of wild type and Tpl2

MEFs treated with TNF-α and harvested at the indicated time points were incubated with 32P-labeled κB consensus oligonucleotides and the resulting DNA-protein complexes were resolved by nondenaturing polyacrylamide gel electrophoresis. B, characterization of TNF-α-induced κB DNA-protein complexes in MEFs. Nuclear extracts of wild type cells stimulated with TNF-α for 15 min were used to carry out EMSAs. In lanes 1 and 3 nuclear extracts were preincubated with 100-fold excess of cold κB oligonucleotides or with an anti-κB antibody, respectively. C, Tpl2 is required for transcriptional activation of NF-κB. Luciferase reporter assays were carried out in unstimulated and TNF-α-stimulated MEFs 36 h after transfection of a luciferase reporter construct. The data shown in the figure combine the results from three independent experiments.

determine whether its activation in TNF-α-treated MEFs also depends on Tpl2. Probing Western blots of cell lysates from unstimulated and TNF-α-stimulated MEFs, with antibodies against phosphorylated and total MSK1, revealed that MSK1 phosphorylation in response to TNF-α in MEFs was indeed also dependent on Tpl2 (Fig. 6C). This suggested that the impaired phosphorylation of p65 Ser276 in Tpl2

MEFs may be because of the impaired activation of MSK1. Consistent with this hypothesis, pretreatment of wild type MEFs with the MSK1 inhibitor Ro-31-8220 (Calbiochem) abolished TNF-α-induced phosphorylation of p65 at Ser276, whereas pretreatment with a structurally similar non-inhibitor bisindolylmaleimide V (Calbiochem) did not affect the phosphorylation (Fig. 6D, a, upper panel).

Earlier studies on the outcome of phosphorylation of p65 at Ser276 gave conflicting results. One report showed that phosphorylation of p65 at this site regulates NF-κB DNA binding (30). However, this was challenged by subsequent reports (28, 29). To revisit this question, we examined whether Ro-31-8220 inhibits NF-κB DNA binding in response to TNF-α. EMSAs using nuclear extracts from unstimulated and TNF-α-stimulated wild type MEFs pre-treated with either Ro-31-8220 or bisindolylmaleimide V showed that Ro-31-8220 does not affect NF-κB DNA binding (Fig. 6D, a, lower panel). To confirm this result, we examined the κB binding activity of nuclear extracts of p65

3T3 fibroblasts reconstituted with wild type p65 or p65 S276A and harvested before and after TNF-α treatment. The results showed that TNF-α induced comparable levels of κB DNA binding activity in fibroblasts reconstituted with the two constructs (Fig. 6D, b).
Although phosphorylation at Ser276 does not affect NF-κB DNA binding, it stimulates transactivation by NF-κB. One gene whose induction by TNF-α is regulated by p65 phosphorylation at Ser276 is IL-6 (28, 29). To determine whether IL-6 induction by TNF-α depends on Tpl2, we examined whether Tpl2−/− MEFs were defective in IL-6 induction. The results showed modest but reproducible differences in IL-6 secretion between TNF-α-treated Tpl2+/+ and Tpl2−/− MEFs. The bars in Fig. 6E show the mean ± S.D. of triplicate samples in one experiment. The experiment was repeated four times and the results were almost identical in all experiments.

**TNF-α Treatment Induces NF-κB Complexes of Different Composition in Tpl2+/+ and Tpl2−/− MEFs**—The preceding data did not reveal the mechanism by which Tpl2 regulates NF-κB DNA binding in response to TNF-α. To approach this question we examined the protein composition of nuclear NF-κB complexes induced by TNF-α in Tpl2+/+ and Tpl2−/− MEFs. We hypothesized that Tpl2 may regulate the assembly of NF-κB nuclear complexes, and that protein complexes of different composition assembled in Tpl2+/+ and Tpl2−/− cells may exhibit different DNA binding affinities. First, we metabolically labeled unstimulated and TNF-α-stimulated cells with [35S]methionine. p65 was immunoprecipitated from nuclear lysates derived from the labeled cells and resolved in SDS-PAGE. The identity of p65 was confirmed by Western blotting (Fig. 6, lower panel). The results confirmed the nuclear translocation of p65. In addition, they showed that nuclear lysates of Tpl2+/− MEFs lack a 70-kDa protein that translocates along with p65 into the nucleus in wild type cells. Instead they contain a slower migrating protein that was missing from the wild type cells. These may be different proteins or one protein that was differentially modified in the two cell types. In addition, two proteins that migrate between the 65- and 70-kDa range translocate into the nucleus only in the wild type cells (Fig. 7).

**DISCUSSION**

In this report we showed that Tpl2 is required for the activation of ERK, JNK, and NF-κB in MEFs. This is in contrast to earlier reports, which observed a narrow signaling defect limited to the activation of ERK in Tpl2−/− macrophages and B-cells in response to Toll-like receptor and death receptor stimulation (8, 9). Interestingly, Tpl2 signals induced by IL-1β in MEFs differ from Tpl2 signals induced by TNF-α, in that they are required only for the activation of ERK. These observations suggest that Tpl2 signals are cell type-specific as well as stimulus-specific. We conclude that the signaling defect caused by a single mutation in a given cell type in response to a specific signal is not sufficient to predict the phenotypic effects of the mutation in the intact animal, which may depend on the functional interaction of signaling defects caused by the mutation in different cell types.

Following binding to soluble TNF-α, TNFR1 recruits the death-domain containing adaptor protein TRADD, which in turn recruits TRAF2 and RIP1 (14), both of which are required for the activation of Tpl2 (38) (this report). TRAF2 is known to be required for JNK activation by TNF-α (16). Based on data in this report showing that RIP1 is required for Tpl2 activation and that Tpl2 is required for JNK activation by TNF-α, we predicted that JNK activation would also depend on RIP1. Because previous studies addressing the role of RIP1 in the activation of JNK by TNF-α had given conflicting results (20, 23), this question was revisited in this report. The results confirmed that optimal JNK activation indeed depends on RIP1.

TRAF2-transmitted signals activate MKK4/SEK1 and MKK7 upstream of JNK. MKK4 and MKK7 serve non-redundant functions in vivo as both are required for embryonic viability (17), as well as for optimal activation of JNK by TNF-α in MEFs (35). Data presented here indicate that Tpl2 is required for the activation of MKK4/SEK1. Because Tpl2 phosphorylates MKK4/SEK1 in vitro (39), this finding identifies MKK4 as the second documented physiological substrate of Tpl2, after MEK1. Although the role of Tpl2-transduced signals in the activation of MKK7 was not addressed in the present study, the demonstration that JNK is activated partially by TNF-α in Tpl2−/− cells suggests that Tpl2 signals may target only MKK4. The data showing that MKK4 plays an important role in JNK activation by TNF-α are in agreement with the results of other studies showing that filamin, a scaffold protein that binds MKK4, is also required for JNK activation by TNF-α (40).

The data in this report identify Tpl2 as the only known MAP3K required for the physiological activation of JNK by TNF-α in MEFs. In earlier reports, the MAP3K MKK1 was shown to play an obligatory role in TNF-α-induced activation of...
FIG. 5. Tpl2 is not required for TNF-α-induced IKK signaling in MEFs. A, TNF-α-induced phosphorylation and subsequent degradation of IκB-α is not regulated by Tpl2. a, cell lysates harvested at the indicated time points after stimulation with TNF-α (10 ng/ml) were probed with antibodies against IκB-α (upper panel) or β-actin (lower panel). b, MEFs were first treated with the proteasome inhibitor MG132 for 45 min prior to stimulation with TNF-α for 10 min. Cell lysates were probed with antibodies against phospho-IκB-α (upper panel) or total IκB-α (lower panel). B, Tpl2 does not regulate the nuclear translocation of p50 and p65. a and b, nuclear (NE), cytoplasmic (CE), and total (TE) cellular extracts from Tpl2−/− and Tpl2+/− MEFs harvested at the indicated time points after stimulation with TNF-α were probed with anti-p50 (a) or anti-p65 (b) antibodies. C, Tpl2−/− and Tpl2+/− MEFs were treated with TNF-α or they were left untreated. Fifteen minutes later, the cells were fixed in 4% paraformaldehyde and incubated with an anti-p65 primary antibody followed by a phycoerythrin (PE)-conjugated secondary antibody. Nuclei were counterstained with 4,6-diamidino-2-phenylindole (DAPI). Cells were visualized by fluorescent microscopy. WT, wild type.
Several MAP3Ks includingTpl2 have been shown to activate IKKβ when overexpressed. However, only MEKK3 is known to play a physiological role in the activation of NF-κB by TNF-α (22, 24). Signals induced by TNF-α are transmitted via RIP1 and MEKK3 to activate IKKβ. Although IKKβ is required for the activation of Tpl2 (33) (see also supplemental materials Fig. 1), we cannot exclude the possibility that Tpl2 is activated by a combination of IKKβ-dependent and -independent RIP1 signals, both of which may be necessary but not sufficient for activation (Fig. 8). Data presented in this report showed that Tpl2, a MAP3K that functions downstream of IKK-β, is required for the activation of NF-κB and that Tpl2 signals involved in the activation of NF-κB do not regulate the degradation of IκB-α and the subsequent nuclear translocation of the NF-κB subunits p50 and p65 (24). Instead, they play an obligatory role in the phosphorylation of p65 at Ser276 by MSK1, a downstream target of Tpl2 (37) and in the induction of NF-κB DNA binding in response to TNF-α. A number of reports over the past several years provided evidence that phosphorylation of p65 by upstream kinases at several sites including Ser276, regulates the transcriptional activity of NF-κB (25–27, 29). One NF-κB target gene that is induced following phosphorylation of p65 at Ser276 is the gene encoding IL-6 (28). Consistent with this observation, we detected decreased secretion of IL-6 by TNF-α-treatedTpl2−/−MEFs. Whereas phosphorylation of Ser276 induces the transactivation function of NF-κB, its role in the regulation of the DNA binding activity of NF-κB has been
controversial (28–30). In this report we presented data addressing this controversy. These data showed that whereas pretreatment with Ro-31-8220, a potent MSK1 inhibitor, completely abolishes TNF-α-induced phosphorylation of p65 at Ser276 by TNF-α, it does not affect NF-κB DNA binding activity, and that the NF-κB DNA binding activity induced by TNF-α in p65−/− fibroblasts reconstituted with p65 S276A is normal. We conclude, therefore, thatTpl2 is required for the phosphorylation of p65 at Ser276 by MSK1 and that phosphorylation of p65 at this site affects NF-κB transactivation potential but not DNA binding.

To explore the mechanism by which Tpl2 regulates the DNA binding activity of NF-κB in response to TNF-α, we hypothesized that DNA binding may be regulated by the composition of NF-κB complexes assembling in the nucleus in response to TNF-α, and we initiated studies to address the composition of such complexes in TNF-α-stimulated Tpl2+/+ and Tpl2−/− MEFs. To this end, we immunoprecipitated p65 from nuclear lysates of [32P]methionine-labeled cells, before and after stimulation with TNF-α and we examined the spectrum of proteins that associate with p65 in a TNF-α-dependent manner. The results presented in this report showed that the p65 protein complex assembled in wild type MEFs differs from the complex assembled in Tpl2−/− MEFs. TNF-α signals transduced via Tpl2 may regulate the assembly of the complex by targeting either p65 or the complex-associated proteins. To determine therefore the mechanisms and the significance of this observation, we need to identify these proteins. In addition to the ablation of Tpl2, ablation of GSK3β, ablation of Tpl2, ablation of GSK3β, ablation of JNK, and ablation of NF-κB, which in turn, may regulate NF-κB DNA binding activity. Given the strong proinflammatory role of TNF-α, these findings may have therapeutic implications for rheumatoid arthritis and other inflammatory syndromes.

Acknowledgments—We are grateful to Dr. John Kyriakis (Tufts-NEMC, Boston, MA) for helpful discussions. We thank Dr. Sankar Ghosh (Yale University School of Medicine, New Haven, CT) for providing the p65 and p65 S276A constructs. The GRASP center at Tufts-NEMC provided core services for this project.

REFERENCES

1. Patriotis, C., Makris, A., Bear, S. E., and Tsichlis, P. N. (1993) Proc. Natl. Acad. Sci. U. S. A. 90, 2251–2255
2. Makris, A., Patriotis, C., Bear, S. E., and Tsichlis, P. N. (1993) J. Virol. 67, 4283–4289
3. Tsatsanis, C., Patriotis, C., and Tsichlis, P. N. (1998) Oncogene 17, 2609–2618
4. Tsatsanis, C., Patriotis, C., Bear, S. E., and Tsichlis, P. N. (1996) Proc. Natl. Acad. Sci. U. S. A. 93, 3827–3832
5. Patriotis, C., Makris, A., Chernoff, J., and Tsichlis, P. N. (1994) Proc. Natl. Acad. Sci. U. S. A. 91, 9755–9759
6. Ceci, J. D., Patriotis, C. P., Tsatsanis, C. A., Kovatch, R., Swing, D. A., Jenkins, N. A., Tsichlis, P. N., and Copeland, N. G. (1997) Genes Dev. 11, 688–700
7. Chiariello, M., Marinissen, M. J., and Gutkind, J. S. (2000) Mol. Cell. Biol. 20, 1747–1758
8. Dumitru, C. D., Ceci, J. D., Tsatsanis, C., Kontoyiannis, D., Stamatakis, K., Lin, J. H., Patriotis, C., Jenkins, N. A., Copeland, N. G., Kollias, G., and Tsichlis, P. N. (2000) Cell 103, 1071–1083
9. Elissoupolous, A. G., Wang, C. C., Dumitru, C. D., and Tsichlis, P. N. (2003) EMBO J. 22, 3855–3864
10. Kontoyiannis, D., Boulougouris, G., Manoloukos, M., Armpka, M., Apostolaki, M., Pizarro, T., Kotlyarov, A., Forster, I., Flavell, R., Gaedeel, M., Tsichlis, P., Cominelli, F., and Kollias, G. (2002) J. Exp. Med. 196, 1563–1574
11. Mori, L., Iselin, S., De Libero, G., and Lesslauer, W. (1996) J. Immunol. 157, 3178–3182
12. Yamaguchi, N., Ohshima, S., Umesha-Sasai, M., Nishikawa, K., Kobayashi, H., Mima, T., Kishimoto, T., and Saeke, Y. (2000) J. Rheumatol. 27, 22–27
13. Ploew, D., Kontogeorgos, G., and Kollias, G. (1999) J. Immunol. 162, 1018–1023
14. Wajant, H., Pfizenmaier, K., and Scheurich, P. (2000) Cell Death Differ 7, 45–65
15. Devin, A., Cook, A., Lin, Y., Rodriguez, Y., Kelliher, M., and Liu, Z. (2000) Immunity 12, 419–429
16. Yeh, W. C., Shahinian, A., Speiser, D., Krausus, J., Billia, F., Wakenham, A., de la Pompa, J. L., Ferrick, D., Hun, B., Iszove, N., Obashi, N., Rothe, M., Goeddel, D. V., and Mak, T. W. (1997) Immunity 7, 715–725
17. Davis, R. J. (2000) Cell 103, 239–252
18. Krajewski, S., Krajewska, M., Kim, M. Y., and Reed, J. (1997)Curr. Opin. Genet. Dev. 7, 67–74
19. Chung, J. Y., Park, Y. C., Ye, H., and Wu, H. (2002) J. Cell. Sci. 115, 679–688
20. Kelliher, M. A., Grimm, S., Ishida, Y., Kuo, F., Stanger, B. Z., and Leder, P. (1996) Immunity 8, 297–303
21. Lee, T. H., Huang, Q., Oikemus, S., Shank, J., Ventura, J. J., Cusson, N., and Baldwin, A. S., Jr. (2000) Mol. Cell. Biol. 20, 8377–8385
Tpl2 Transmits TNF Signals to ERK, JNK, and NF-κB

27. Zhong, H., SuYang, H., Erdjument-Bromage, H., Tempst, P., and Ghosh, S. (1997) *Cell* 89, 413–424
28. Okazaki, T., Sakon, S., Sasazuki, T., Sakurai, H., Doi, T., Yagita, H., Okumura, K., and Nakano, H. (2003) *Biochem. Biophys. Res. Commun.* 300, 807–812
29. Vermeulen, L., De Wilde, G., Van Damme, P., Vanden Berghe, W., and Hae-geman, G. (2003) *EMBO J.* 22, 1313–1324
30. Zhong, H., Voll, R. E., and Ghosh, S. (1998) *Mol. Cell* 1, 661–671
31. Melestita, R. E., Payne, T. M., Coppens, I., and Sinai, A. P. (2003) *J. Cell Sci.* 116, 4359–4371
32. Chen, G., and Goeddel, D. V. (2002) *Science* 296, 1634–1635
33. Waterfield, M., Jin, W., Reiley, W., Zhang, M., and Sun, S. C. (2004) *Mol. Cell. Biol.* 24, 6040–6048
34. Lawler, S., Fleming, Y., Goedert, M., and Cohen, P. (1998) *Curr. Biol.* 8, 1387–1390
35. Tourrier, C., Dong, C., Turner, T. K., Jones, S. N., Flavell, R. A., and Davis, R. J. (2001) *Genes Dev.* 15, 1419–1426
36. Sizemore, N., Lerner, N., Dombrowski, N., Sakurai, H., and Stark, G. R. (2002) *J. Biol. Chem.* 277, 3863–3869
37. Eliopoulos, A. G., Dumitruc, C. D., Wang, C. C., Cho, J., and Tsichlis, P. N. (2002) *EMBO J.* 21, 4831–4840
38. Eliopoulos, A. G., Davies, C., Blake, S. S., Murray, P., Najafipour, S., Tsichlis, P. N., and Young, L. S. (2002) *J. Virol.* 76, 4567–4579
39. Salmeron, A., Ahmad, T. B., Carlile, G. W., Pappin, D., Narsimhan, R. P., and Ley, S. C. (1996) *EMBO J.* 15, 817–826
40. Marti, A., Luo, Z., Cunningham, C., Obta, Y., Hartwig, J., Stossel, T. P., Kyriakis, J. M., and Avruch, J. (1997) *J. Biol. Chem.* 272, 2620–2626
41. Xia, Y., Makris, C., Su, B., Li, E., Yang, J., Nemerow, G. R., and Karin, M. (2000) *Proc. Natl. Acad. Sci. U. S. A.* 97, 5243–5248
42. Huang, Q., Yang, J., Lin, Y., Walker, C., Cheng, J., Liu, Z. G., and Su, B. (2004) *Nat. Immunol.* 5, 98–103
43. Hoeflich, K. P., Luo, J., Rubie, E. A., Tsao, M. S., Jin, O., and Woodgett, J. R. (2000) *Nature* 406, 86–90
44. Leitges, M., Sanz, L., Martin, P., Duran, A., Braun, U., Garcia, J. F., Camacho, F., Diaz-Meco, M. T., Rennert, P. D., and Moscat, J. (2001) *Mol. Cell* 8, 771–780
45. Oliver, F. J., Menissier-de Murcia, J., Nacci, C., Decker, P., Andriantsitohaina, R., Muller, S., de la Rubia, G., Stoclet, J. C., and de Murcia, G. (1999) *EMBO J.* 18, 4446–4454
46. Kontoyiannis, D., and Kollia, G. (2000) *Arthritis Res.* 2, 342–343
47. Palladino, M. A., Bahjat, F. R., Theodorakis, E. A., and Moldawer, L. L. (2003) *Nat. Rev. Drug Discov.* 2, 736–746