Tpl2 (Tumor Progression Locus 2) Phosphorylation at Thr^{290} Is Induced by Lipopolysaccharide via an Iκ-B Kinase-β-dependent Pathway and Is Required for Tpl2 Activation by External Signals*

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The serine-threonine protein kinase encoded by the tumor progression locus 2 (Tpl2) proto-oncogene transduces Toll-like receptor and death receptor signals in a variety of cell types. Here we show that Tpl2 undergoes phosphorylation at Thr^{290} both in cells overexpressing Tpl2 and in cells stimulated with lipopolysaccharide (LPS) or tumor necrosis factor-α and that phosphorylation on this site parallels Tpl2 activation. Reconstitution of Tpl2^{-/-} macrophages with wild type Tpl2 or Tpl2 T290D restored ERK activation by LPS, whereas reconstitution of the same cells with Tpl2 T290A did not, suggesting that phosphorylation at Thr^{290} is required for the physiological activation of Tpl2 by external signals. Both the wild type Tpl2 and the kinase-inactive mutant Tpl2 K167M undergo Thr^{290} phosphorylation, suggesting that Thr^{290} may be a site of trans-phosphorylation rather than auto-phosphorylation. Pretreatment of 293 cells and primary macrophages with the Iκ-B kinase-β (IKKβ) inhibitor PS-1145 blocked Tpl2 phosphorylation at Thr^{290}, suggesting that phosphorylation depends on IKKβ, an obligatory positive regulator of Tpl2. We conclude that Tpl2 phosphorylation at Thr^{290} is induced by LPS, depends on IKKβ, and is required for the physiological activation of Tpl2 by external signals.

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Tumor progression locus 2 (Tpl2)^1 encodes a serine-threonine protein kinase that is activated by provirus integration in Moloney murine leukemia virus-induced rodent lymphomas and in mouse mammary tumor virus-induced mammary adenocarcinomas (1, 2). Provirion integration always occurs in the last intron of the gene and gives rise to a truncated mRNA that is translated into a protein in which 43 C-terminal amino acids encoded by the last exon are replaced by seven amino acids encoded by intron sequences (1). The latter protein exhibits enhanced kinase activity and is highly oncogenic (3).

Overexpression of Tpl2 in a variety of cell types activates all of the mitogen-activated protein kinase pathways (4–6), nuclear factor-activated T cells, and NF-κB (7–10) and promotes cell proliferation (1, 11). Moreover, the expression of the tumor-specific truncated form of Tpl2 transforms cells in culture (6) and is highly oncogenic in animals (3). Despite the profound effects of Tpl2 overexpression, Tpl2 knock-out mice appear healthy. However, these mice are resistant to LPS/d-galactosamine-induced shock (12), a rapidly developing syndrome that depends on TNF-α, and to high-dose LPS-induced shock,^2^ a slowly developing inflammatory syndrome that is mediated by TNF-α, IL-1β, interferon-γ, and other proinflammatory molecules (13, 14). The same mice are resistant to TNF-α-induced inflammatory bowel disease (15). These findings suggested that Tpl2 plays an obligatory role in the transduction of LPS and TNF-α signals, a hypothesis that has now been amply confirmed. Studies from this laboratory have indeed shown that Tpl2 is required for the transduction of LPS and CD40L signals that activate ERK in macrophages and B cells and TNF-α signals that also activate ERK in macrophages (12, 16, 17). Our more recent studies have also shown that, in mouse embryo fibroblast, Tpl2 is required for the transduction of TNF-α signals that activate in addition to ERK, c-Jun NH₂-terminal kinase, and NF-κB. Studies based on these observations confirmed that Tpl2 is activated by both LPS (Refs. 18, 19, and this report) and TNF-α (20).

^2^ Tpl2 activation by LPS or anti-CD40 antibodies depends on TRAF6. Thus, anti-CD40 antibodies promote the association of endogenous Tpl2 and TRAF6 with CD40. Moreover, overexpression of TRAP6 in Tpl2^{+/−} fibroblasts and keratinocytes promotes the activation of ERK, whereas overexpression of TRAF6 in Tpl2^{−/−} cells does not (17). More recent studies addressed the role of RIP1 and TRAF2 on Tpl2 activation by either TNF-α or LPS. These studies revealed that the activation of Tpl2 by TNF-α depends on RIP1 and TRAF2, because treatment of RIP1^{−/−} and TRAF2^{−/−} mouse embryo fibroblasts with TNF-α does not activate Tpl2. In addition, they showed that Tpl2

[^2]: C. D. Dimitriu and P. N. Tsichlis, unpublished data.

[^3]: S. Das, J. Cho, I. Lambertz, M. A. Kelliher, A. G. Eliopoulos, K. Du, and P. N. Tsichlis, submitted for publication.
activation by LPS or TNF-α depends on the activation of IKKβ (19, 20). Because inactive Tpl2 in unstimulated cells is stoichiometrically bound to NF-κB/p105 and Tpl2 activation by external signals depends on its dissociation from p105 (9, 18), these findings were interpreted to suggest that the function of IKKβ is to promote the phosphorylation and degradation of p105 and the subsequent release of activated Tpl2 (19, 20).

In this report, we present evidence that Tpl2 undergoes phosphorylation at Thr290 in vivo and that phosphorylation is required for the enzymatic activation of the Tpl2 kinase. We also show that phosphorylation is induced by LPS and TNF-α and that it is required for the physiological activation of Tpl2 by external signals. Finally, we present evidence that Thr290 is a site of trans-phosphorylation rather than auto-phosphorylation and that its modification depends on IKKβ, an obligatory positive upstream regulator of Tpl2 (19, 20). Of the 52- (p52) and the 58-kDa (p58) isoforms of Tpl2, the p58 isoform is targeted preferentially for phosphorylation at Thr290, suggesting that the two isoforms are functionally distinct. These data collectively show that phosphorylation at Thr290 is required for Tpl2 activation by external signals. In addition, they show that IKKβ is required for the activation of Tpl2, not only because it phosphorylates p105 and targets it for degradation but also because it promotes the phosphorylation of Tpl2 at Thr290. Therefore, these data provide a novel link between IKKβ and Tpl2 activation by external signals.

**MATERIALS AND METHODS**

**Cell Culture**—Bone marrow-derived macrophages (BMDM) from Tpl2−/− and Tpl2+/− mice (12) were infected with a pZIP-NeoSV40 large T antigen retrovirus harvested from virus-producing Y2 cells (kindly provided by Anthony DeFranco, University of California San Francisco). To infect primary BMDMs, 1 × 106 cells were resuspended in 2 ml of virus supernatants. 2 h later, they were cultured in macrophage media (Dulbecco’s modified Eagle’s medium supplemented with 20% fetal bovine serum, 30% L929-conditioned media, non-essential amino acids, penicillin, and streptomycin). Because G418 resistance gene, they could not be G418-selected. Therefore, to isolate cultures of large T antigen-expressing cells, we selected for cells surviving long term culture. Two months were generally sufficient to isolate fully infected immortalized lines. The immortalized macrophage cell lines were maintained in macrophage media. RAW264.7 cells, Y2 cells, 293 cells, L929 cells (12), and Phoenix cells (kindly provided by Garry Nolan, Stanford University) were maintained in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum, non-essential amino acids, penicillin, and streptomycin. To inhibit IKKβ, 293 cells transiently transfected with Tpl2 constructs were treated with PS-1145 (Millennium Pharmaceuticals Inc.) 1.5 h prior to harvesting and RAW264.7 cells were treated with PS-1145 (50 μM) 1.5 h prior to LPS (1 μg/ml from Salmonella enteritidis, purchased from Sigma) stimulation. Alternatively, IKKβ was knocked down by siRNA (see below).

**Expression Plasmids, Site-directed Mutagenesis, and Retrovirus Constructs**—Wild type Tpl2, C-terminally tagged with the HA epitope, was cloned by PCR in the polylinker of pCMV5 between EcoRI and BamHI. Tpl2 mutants were generated by site-directed mutagenesis of this plasmid and their expression was confirmed by sequencing. Wild type Tpl2 and Tpl2 mutants of Tpl2 (Thr→Ala and Thr→Asp) were also expressed in the EcoRI site of the pMSCV-based retrovirus vector pMigR1 (kindly provided by Warren Pear, University of Pennsylvania).

**Transfections, Retrovirus Infections, and Small Interfering RNA Experiments**—Transfection of 293 cells was carried out in 6-well plates (1 μg of DNA/plate) or 100-mm plates (8 μg of DNA/plate). Plasmid DNA was mixed with FuGene 6 (Roche Applied Science) in Opti-MEM I (Invitrogen) containing 25 min of room temperature. The mixture was added to 40% confluent 293 cell monolayers. Cells were harvested 48 h later. Cultures were serum-starved for 18 h prior to harvesting in all of the experiments in which ERK phosphorylation was the end point. Retrovirus packaging and infection were carried out as follows. pMigR1 retrovirus constructs of wild type Tpl2.HA, Tpl2 T290A.HA, and Tpl2 T290D.HA were transfected into 293-Phoenix cells in combination with pSVG and pC-L Amphi. The culture medium was changed 24 h later. Viral supernatants were collected at the 72-h time point, and they were concentrated 30-fold by ultracentrifugation (20,000 rpm in the SW 40Ti rotor for 3 h at 4 °C). Immortalized macrophages were infected with the concentrated viruses using the spinfection method (21). Efficiencies of infection achieved by this method were reproducibly higher than 50%. Cells were serum-starved 48 h after infection, and they were supplemented with LPS at the 72-h time point. Three double-stranded Stealth™ IKKβ RNA oligonucleotides and one control oligonucleotide were synthesized (Invitrogen) and tested in RAW264.7 macrophages. One of them (sense 5′-GCCAGGAGGAGCUCCGAGAUA-3′) was successful in knocking down mouse IKKβ by 70%. This siRNA was used for subsequent experiments. All of the siRNAs (150 nm) were transfected into RAW264.7 cells using Lipofectamine 2000 (Invitrogen). Efficiencies of siRNA transfer were monitored using siGLO siRNA purchased from Dharmacon. Cell lysates were harvested 72 h after transfection.

**Phosphopeptide Mapping**—Tryptic phosphopeptide mapping was performed as described previously (22, 23). pCMV5 constructs of wild type Tpl2.HA, Tpl2-T290A.HA, and Tpl2-T290D.HA were transfected into 293 cells, and they were metabolically labeled with [32P]orthophosphate (0.5 μCi/ml) (PerkinElmer Life Sciences). Immunoprecipitated [32P]-labeled Tpl2 proteins were resolved in SDS-PAGE, transferred to polyvinylidene difluoride membranes, and visualized by autoradiography. Tryptic elution from the membranes by 0.5% polyvinylpyrrolidone-360 in 0.1 M acetic acid for 30 min at 37 °C, the samples were digested with 20 μg of TPCK-treated trypsin (Sigma) in 50 mM NH4HCO3, pH 8.0, for 5 h at 37 °C. The resulting peptides were dried and, following a 2-h treatment with performic acid (1 volume of 50% H2O2, pH 1.9), the resulting peptides were spotted onto nitrocellulose plates, and they were separated in the first dimension by electrophoresis at pH 1.9 and in the second dimension by thin layer chromatography in phosphochromatography buffer (37.5% n-butyl alcohol, 25% pyridine, and 7.5% acetic acid in H2O).

**Antibodies, Immunoprecipitations, and Western Blotting**—Phospho-p44/p42 MAPK (Thr202/Tyr204) and phospho-MEK1/2 (Ser217/Ser221) antibodies as well as the corresponding antibodies that recognize “total” i.e. both phosphorylated and non-phosphorylated forms of these kinases, were purchased from Cell Signaling Technology. Anti-HA monoclonal and polyclonal antibodies were from Covance. Anti-NF-κB/p105 antibody was from Delta Biologics. Anti-Tpl2 (M20) antibody and anti-Tpl2-agarose-conjugated antibody were from Santa Cruz Biotechnology. The phospho-Thr290-specific anti-Tpl2 antibody was raised by immunizing rabbits with the peptide CKDLRGT(PO4)EIYMSPE. Anti-rabbit IgG horseradish peroxidase and anti-mouse IgG horseradish peroxidase were purchased from Amersham Biosciences. Protein G-Sepharose was purchased from Invitrogen. Phosphospecific antibodies were diluted in 5% bovine serum albumin in TBS (Tris-buffered saline) supplemented with 0.1% v/v Tween 20 (TBS-T), whereas all of the other antibodies were diluted in 5% milk in TBS-T.

Cells were lysed in Triton X-100 lysis buffer (20 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1 mM Na2EDTA, 1 mM EGTA, 1% Triton X-100, 2.5 mM sodium pyrophosphate, 1 mM β-glycerophosphate, 1 mM Na3VO4, 1 μg/ml leupeptin, and 1 mM phenylmethylsulfonyl fluoride). The protein concentration in the soluble fraction, isolated by centrifugation of the total lysates (12,000 rpm for 10 min at 4 °C), was determined using the Bradford assay (Bio-Rad). For immunoblotting, 50–100 μg of protein was separated by SDS-PAGE, transferred onto polyvinylidene difluoride membranes (0.45 μm, Millipore), and blocked for 1 h at room temperature with 5% nonfat milk dissolved in TBS-T. Following three washes with TBS-T, membranes were incubated overnight at 4 °C with primary antibody, washed in TBS-T, and incubated for 1 h at room temperature with secondary antibody followed by enhanced chemiluminescence (ECL, Amersham Biosciences). Protein bands were quantitated using a densitometer (Bio-Rad GS-800, Quantity one). For immunoprecipitation, lysates (500 μg-1.5 mg) were preclared with Sepharose G-beads and immunoprecipitated overnight using a monoclonal anti-HA (Tpl2.HA) antibody. Immunoprecipitates were washed three times with lysis buffer and re-suspended in 50 μl of loading buffer containing 1% sodium dodecyl sulfate. Aliquots of 20 μl were resolved in SDS-PAGE, and blots were probed for Thr290-phosphorylated or total Tpl2.

**In Vitro Kinase Assays**—293 cells transiently transfected with Tpl2.HA constructs and immortalized Tpl2−/− murine macrophages and RAW264.7 cells expressing stably near physiological levels of Tpl2.HA were lysed in Triton X-100 lysis buffer. Tpl2 immunoprecipitated from cell lysates was washed three times in lysis buffer and once in kinase buffer (25 mM Tris-HCl, pH 7.5, 5 mM β-glycerophosphate, 2
Tpl2 may be activated by LPS. These data suggested that the Tpl2 isoform is rapidly degraded upon activation as previously described. Immunoprecipitates used for the in vitro kinase reaction with an anti-HA antibody detected the 58- and 52-kDa Tpl2.HA isoforms. The 58-kDa isoform is rapidly degraded upon activation as previously described. In vitro kinase assays on Tpl2 immunoprecipitated from lysates of Tpl2.HA-expressing RAW264.7 cells harvested before and after LPS stimulation. The substrate was GST-MEK1. Probing a Western blot of the products of the kinase reaction with the phospho-MEK1/2 antibody. Phosphorylation in Tpl2 was visualized by autoradiography. In some of the experiments, phosphorylated GST-MEK1 was detected by probing Western blots of the products of the kinase reaction with the phospho-MEK1/2 antibody.

RESULTS

The Tpl2 Kinase Is Activated by LPS and TNF-α—Our earlier studies had shown that Tpl2 transduces LPS signals that activate ERK in macrophages and that these signals are required for the induction of TNF-α, cyclooxygenase-2, and other proinflammatory molecules. These data suggested that Tpl2 may be activated by LPS. In vitro kinase assays on Tpl2 immunoprecipitated from LPS-stimulated RAW264.7 macrophages indeed showed that the activity of the Tpl2 kinase, which is very low prior to stimulation (0 min), increases rapidly following stimulation (Fig. 1A). More recent studies have shown that Tpl2 is also activated by TNF-α in both macrophages and primary mouse embryo fibroblasts.

Most of our earlier studies on Tpl2 had been carried out on primary peritoneal or BMDM. Recently, we established immortalized macrophages harvested before and at the indicated time points after LPS stimulation. A Western blot of the same immunoprecipitates was probed with the anti-Tpl2 antibody. Lysates of immortalized macrophages harvested before and after LPS stimulation were also subjected to SDS-PAGE and immunoblotted using anti-phospho-ERK and total ERK antibodies.

TPL2 Is Phosphorylated at Thr<sup>290</sup> in Vivo—To address the potential role of activation loop phosphorylation in Tpl2 activation, we generated Tpl2 mutants in which phosphorylatable residues in this region were replaced one at a time with alanine or with the phosphomimetic aspartic acid (Fig. 2A). The construction of these mutants, fused at their C terminus to a HA epitope tag along with expression constructs of HA-tagged wild type Tpl2, were transiently transfected into 293 cells. Transfected cells were metabolically labeled with [32P]orthophosphate and subjected to SDS-PAGE, they were digested with trypsin. The resulting tryptic peptides were subjected to two-dimensional separation (electrophoresis versus thin layer chromatography), and they were detected by autoradiography. The results were interpreted to suggest that Tpl2 undergoes phosphorylation at multiple sites. Because the Tpl2 T290A mutant lacked phosphopeptide 8 (Fig. 2B), we conclude that Thr<sup>290</sup> is likely to be one of the Tpl2 phosphorylation sites.

TPL2 T290A Is Inactive, whereas Tpl2 T290D Exhibits Weak Kinase Activity—Western blots of 293 cells transiently transfected with HA-tagged wild type Tpl2, were transiently transfected into 293 cells. Transfected cells were metabolically labeled with [32P]orthophosphate and subjected to SDS-PAGE, they were digested with trypsin. The resulting tryptic peptides were subjected to two-dimensional separation (electrophoresis versus thin layer chromatography), and they were detected by autoradiography. The results were interpreted to suggest that Tpl2 undergoes phosphorylation at multiple sites. Because the Tpl2 T290A mutant lacked phosphopeptide 8 (Fig. 2B), we conclude that Thr<sup>290</sup> is likely to be one of the Tpl2 phosphorylation sites.

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unable to activate ERK, whereas substitution of Thr\textsuperscript{278} with either residue gave rise to mutants that were as active as the wild type protein. On the other hand, substitution of Thr\textsuperscript{290} with alanine had the opposite effect than its substitution with aspartic acid. Thus, whereas a T290A mutant failed to induce phosphorylation of ERK, a T290D mutant induced ERK phosphorylation, although weakly, suggesting that phosphorylation at Thr\textsuperscript{290} may contribute to the activation of the Tpl2 kinase (Fig. 2C). To address this hypothesis, we carried out Tpl2 in vitro kinase assays using Tpl2 immunoprecipitated from 293 cells transiently transfected with HA-tagged constructs of wild type Tpl2.HA (WT), kinase-inactive Tpl2 (K167M.HA), and Tpl2 mutants at potential activation loop phosphorylation sites were probed with anti-HA (Tpl2), anti-phospho-ERK, and anti-total ERK antibodies. Whereas a T290A mutant failed to induce phosphorylation of ERK, a T290D mutant induced ERK phosphorylation, although weakly, suggesting that phosphorylation at Thr\textsuperscript{290} may be required for Tpl2 activation. The posttranslational modifications responsible for the slow migrating Tpl2 bands have not been determined. D, 293 cells were transiently transfected with wild type Tpl2.HA (WT), Tpl2 K167M.HA, Tpl2 T290A.HA, and Tpl2 T290D.HA or the empty vector. Cell lysates were probed with antibodies against HA (Tpl2) and against phosphorylated or total ERK. The same lysates were used to carry out in vitro kinase assays. The end point of these assays was the auto-phosphorylation of Tpl2 and phosphorylation of GST-MEK1. IP, immunoprecipitated.
Recent studies provided evidence that IKKβ is required for Tpl2 activation by LPS and TNF-α, rather than LPS, revealed that TNF-α also induces phosphorylation of Tpl2 at Thr290 (data not shown).

To determine whether the p58 isoform is rapidly degraded following treatment with LPS, as expected. Finally, probing for phosphorylated and total ERK showed that ERK phosphorylation by LPS is restored inTpl2−/− macrophages reconstituted with wild type Tpl2 and Tpl2 T290D but not Tpl2 T290A. A Tpl2−/− Mac cell line was infected with pMigR1 retrovirus constructs of wild type Tpl2.HA (WT), Tpl2 T290A.HA, and Tpl2 T290D.HA. Alternatively, the same cells were infected with the empty vector. Tpl2 expression was monitored by probing infected cell lysates with anti-HA antibody. Total ERK and phosphorylated ERK were monitored by probing the same lysates with anti-total ERK and anti-phospho-ERK antibodies.

**Fig. 3.** ERK phosphorylation by LPS is restored in Tpl2−/− macrophages reconstituted with wild type Tpl2 and Tpl2 T290D but not Tpl2 T290A. A Tpl2−/− Mac cell line was infected with pMigR1 retrovirus constructs of wild type Tpl2.HA (WT), Tpl2 T290A.HA, and Tpl2 T290D.HA. Alternatively, the same cells were infected with the empty vector. Tpl2 expression was monitored by probing infected cell lysates with an anti-HA antibody. Total ERK and phosphorylated ERK were monitored by probing the same lysates with anti-total ERK and anti-phospho-ERK antibodies.

**Fig. 4.** Characterization of the Thr290-specific Tpl2 phospho-antibody. A and B, wild type Tpl2.HA (WT), Tpl2 K167M.HA (kinase-inactive), Tpl2 T290A.HA, and Tpl2 T290D.HA were immunoprecipitated with a mouse monoclonal antibody from 293 cells transiently transfected with the corresponding constructs. The immunoprecipitates (IP) were probed with a rabbit polyclonal antibody raised against a phosphorylated Tpl2 peptide encompassing Thr290 (CDKLRGT(PO4)EIYMSPE) or with a rabbit polyclonal anti-HA antibody. The lower band in the Western blot probed with the anti-phospho Thr290 antibody is a nonspecific band and not p52 Tpl2 isoform (see “Results” for details). C, HA-tagged constructs of wild type Tpl2 (WT), Tpl2 T290A, and Tpl2 T290D along with the empty vector were transiently transfected into 293 cells. A Western blot of Tpl2 HA immunoprecipitated from transfected cell lysates was probed with the phospho-Thr290-specific anti-Tpl2 antibody (top panel). The same blot was stripped, incubated with alkaline phosphatase at 37 °C overnight, and reprobed with the phospho-Thr290-specific anti-Tpl2 antibody (lower panel).

**Fig. 5.** Tpl2 undergoes phosphorylation at Thr290 following stimulation with LPS. Wild type Tpl2.HA was immunoprecipitated (IP) with a monoclonal anti-HA antibody from lysates of LPS-stimulated RAW264.7 cells engineered to express near physiological levels of Tpl2.HA (RAW264.7 Tpl2.HA). The immunoprecipitates were probed with the Thr290-specific phospho-Tpl2 antibody (upper panel) or with a polyclonal anti-HA antibody (second panel from the top). The same lysates were probed with antibodies against phosphorylated and total ERK. IB, immunoblotted.
Phosphorylation at Thr\textsuperscript{290} Is Required for Tpl2 Activation

Expression of Tpl2 in vivo and that phosphorylation at this site is required for the enzymatic activity of overexpressed Tpl2, as well as for the physiological activation of Tpl2 by external signals. In unstimulated cells expressing physiological levels of Tpl2, the Tpl2 kinase was inactive and lacked detectable phosphorylation at Thr\textsuperscript{290}. However, both kinase activity and phosphorylation were induced upon stimulation with LPS or TNF-α. When overexpressed in 293 cells, wild type Tpl2 underwent phosphorylation at Thr\textsuperscript{290} and it was catalytically active in the absence of stimulation. Upon transient transfection into 293 cells, the kinase-inactive mutant Tpl2 K167M also underwent phosphorylation at Thr\textsuperscript{290}, suggesting that Thr\textsuperscript{290} is a site of trans-phosphorylation rather than auto-phosphorylation. Additional studies revealed that inhibition of IKKβ, a positive upstream regulator of Tpl2, blocks the phosphorylation of Tpl2 at Thr\textsuperscript{290}, suggesting that phosphorylation depends on the activity of IKKβ. The finding that Tpl2 undergoes phosphorylation at Thr\textsuperscript{290} in vivo is in agreement with a recent report (24) that appeared while this work was being prepared for publication. However, the published report (24) failed to show that phosphorylation is induced by LPS or TNF-α and that it is required for the physiological activation of Tpl2 by external signals. Moreover, it did not address the relationship of Tpl2 phosphorylation at Thr\textsuperscript{290} with upstream signaling. All of these issues are addressed in the present study.

In unstimulated cells, endogenous Tpl2 is stoichiometrically bound to NF-κB/p105 (9). Bound Tpl2 is stable but inactive (18, 25). When overexpressed in transiently transfected 293 cells, a fraction of Tpl2 remains unbound. Despite the fact that this applies to wild type Tpl2 as well as to the mutants Tpl2 T290D and Tpl2 T290A, only wild type Tpl2 and the T290D mutant were catalytically active upon overexpression. Given the fact that, upon overexpression, Tpl2 or perhaps the unbound fraction of Tpl2 undergoes phosphorylation at Thr\textsuperscript{290}, these findings suggest that phosphorylation at Thr\textsuperscript{290} is required for the catalytic activity of the Tpl2 kinase.

Expression of wild type Tpl2 and Tpl2 T290D to near physiological levels in Tpl2\textsuperscript{-/-} macrophages restored ERK activation by LPS, whereas expression of Tpl2 T290A did not. These data demonstrate conclusively that phosphorylation of Tpl2 at Thr\textsuperscript{290} is required for the physiological activation of Tpl2 by external signals. However, because the expression of Tpl2 T290D to near physiological levels in Tpl2\textsuperscript{-/-} macrophages did not activate ERK in the absence of LPS stimulation, these data also suggest that phosphorylation at Thr\textsuperscript{290} is necessary but
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phorylation and degradation of p105, which ultimately leads to the release at Tpl2 from its inhibitor. The data in this report provide evidence that IKKβ promotes the activation of Tpl2 by promoting not only the phosphorylation of p105 but also the phosphorylation of Tpl2. Therefore, these data provide an additional link between IKKβ and Tpl2 activation in cells stimulated with LPS. Overall, the data presented in this report identify Tpl2 phosphorylation at Thr290 as a required step in Tpl2 activation by external signals. Based on these data, we propose the model of Tpl2 activation and signaling shown in Fig. 7. According to this model, IKKβ contributes to the activation of Tpl2 by external signals not only by promoting the phosphorylation and degradation of p105, as previously suggested, but also by promoting the phosphorylation of Tpl2 at Thr290. Based on data in this report and the results of additional studies from this laboratory (26), Tpl2 phosphorylation at Thr290 is required for the enzymatic activation of Tpl2, the release of Tpl2 from p105, and the subsequent degradation of Tpl2 via the proteasome in response to external signals.

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[Image 59x460 to 306x737]
Tpl2 (Tumor Progression Locus 2) Phosphorylation at Thr^{290} Is Induced by Lipopolysaccharide via an Iκ-B Kinase-β-dependent Pathway and Is Required for Tpl2 Activation by External Signals

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