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The role of TBK1 and IKK ϵ in the expression and activation of Pellino 1

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Abstract

Mammalian Pellino isoforms are phosphorylated by IRAK (interleukin receptor associated kinase) 1/IRAK4 *in vitro*, converting them into active E3 ubiquitin ligases. In the present paper we report a striking enhancement in both transcription of the gene encoding Pellino 1 and Pellino 1 protein expression when murine BMDMs (bone-marrow-derived macrophages) are stimulated with LPS (lipopolysaccharide) or poly(I:C). This induction occurs via a TRIF [TIR (Toll/interleukin-1 receptor)-domain-containing adaptor-inducing interferon- β]-dependent IRAK-independent pathway and is prevented by inhibition of the IKK [I κ B (inhibitor of nuclear factor κ B) kinase]-related protein kinases, TBK1 {TANK [TRAF (tumour-necrosis-factor-receptor-associated factor)-associated nuclear factor κ B activator]-binding kinase 1} and IKK ϵ . Pellino 1 is not induced in IRF3 (interferon regulatory factor 3)^{-/-} BMDMs, and its induction is only reduced slightly in type 1 interferon receptor^{-/-} BMDMs, identifying Pellino 1 as a new IRF3-dependent gene. We also identify Pellino 1 in a two-hybrid screen using IKK ϵ as bait, and show that IKK ϵ /TBK1 activate Pellino 1 *in vitro* by phosphorylating Ser⁷⁶, Thr²⁸⁸ and Ser²⁹³. Moreover, we show that the E3 ligase activity of endogenous Pellino 1 is activated in LPS- or poly(I:C)-stimulated macrophages. This occurs more rapidly than the increase in Pellino 1 mRNA and protein expression, is prevented by the inhibition of IKK ϵ /TBK1 and is reversed by phosphatase treatment. Thus IKK ϵ /TBK1 mediate the activation of Pellino 1's E3 ligase activity, as well as inducing the transcription of its gene and protein expression in response to TLR3 and TLR4 agonists.

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Author Contributions

Hilary Smith, Xin-Yu Liu, Liang Dai, Eddy T.H. Goh, Aye-Thu Chan, Jiajia Xi, Insaf A. Qureshi, Robert Gourlay and Simon Morton performed the experiments; Joanne Hough and Edward G. McIver developed MRT67307; Peter C.F. Cheung and Cheah-Chen Seh developed the anti-Pellino 1 antibody; Julien Lescar and Christiane Ruedl contributed new reagents and derived the immortalized bone marrow cell lines; Hilary Smith, Philip Cohen and Peter C.F. Cheung planned the experiments, analysed the data and wrote the manuscript.

Keywords

E3 ligase; interferon regulatory factor 3 (IRF3); interleukin receptor-associated kinase (IRAK); Toll-like receptor (TLR); Toll/IL-1 receptor-domain-containing adaptor-inducing interferon- β (TRIF); ubiquitin

Introduction

During infection by bacteria or viruses, components of these pathogens bind to TLRs (Toll-like receptors) in immune cells. This triggers the activation of signalling pathways that control the production of inflammatory mediators and IFNs (interferons) to combat the invading pathogen [1]. The receptors for the pro-inflammatory cytokine IL (interleukin)-1 and all TLRs, except for TLR3, signal via the adaptor protein MyD88 (myeloid differentiation factor 88) [2], which recruits the protein kinases IRAK (IL receptor associated-kinase) 1 and IRAK4 [3,4]. This enables IRAK4 to activate IRAK1 [5] and IRAK2 [6], which are thought to initiate 'downstream' signalling events. In contrast, TLR3 signals via a distinct adaptor TRIF [TIR (Toll/IL-1 receptor)-domain-containing adaptor-inducing IFN β], while TLR4 is unique in requiring both MyD88 and TRIF for signalling [7–9]. The TRIF-dependent pathway can either signal via RIP1 (receptor-interacting protein 1), which leads to activation of the transcription factor NF- κ B (nuclear factor κ B) [10,11], or via IKK [I κ B (inhibitor of nuclear factor κ B) kinase] ϵ and TBK1 {TANK [TRAF (tumour-necrosis-factor-receptor-associated factor)-associated NF- κ B activator]-binding kinase 1} [12,13], the protein kinases that activate the transcription factor IRF3 (IFN regulatory factor 3). IRF3 then stimulates transcription of the gene encoding IFN β [14–16].

Pellino was identified originally as a protein that interacts with Pelle, the orthologue of the only IRAK in *Drosophila* [17]. The three mammalian isoforms, Pellino 1, Pellino 2 and Pellino 3, [18,19] were shown subsequently to interact with, and to be phosphorylated by, IRAK1 and IRAK4 [20–22]. More recently, the IRAK1- and IRAK4-catalysed phosphorylation of Pellino isoforms was found to greatly enhance their ability to function as E3 ubiquitin ligases and to produce unanchored Lys⁶³-linked polyubiquitin chains in the presence of the E2 conjugating enzyme Ubc13–Uev1a [21,23]. IRAK1 and IRAK4 phosphorylate Pellino 1 at ten serine and/or threonine residues *in vitro*, but full activation of the E3 ligase activity of Pellino 1 can be achieved by phosphorylation of any one of the three key activating residues Ser⁷⁶, Thr²⁸⁸ and Ser²⁹³, or by the combined phosphorylation of three other residues Ser⁷⁸, Thr⁸⁰ and Ser⁸² [23]. Moreover, the co-transfection of vectors encoding IRAK1 and Pellino 2 into IRAK1-null cells led to the Lys⁶³-linked polyubiquitylation of IRAK1, but not if wild-type IRAK1 was replaced by a catalytically inactive mutant or if Pellino 2 was replaced by an E3-ligase defective mutant of this protein [21]. Taken together, these experiments indicate that, in co-transfection experiments, IRAK1 phosphorylates and activates Pellino isoforms, which then polyubiquitylate IRAK1. This suggested that one or more Pellino isoforms may contribute to the Lys⁶³-linked polyubiquitylation of IRAK1, which occurs within minutes following the stimulation of mammalian cells with IL-1 [24,25].

More recently, mice were generated that do not express Pellino 1. Interestingly, these mice did not display any defect in the activation of the canonical IKKs (IKK α and IKK β) or binding of NF- κ B to DNA in response to IL-1 in MEFs (mouse embryonic fibroblasts) or to TLR agonists that signal via MyD88 in splenocytes. Instead, the genetic deficiency of Pellino 1 was reported to attenuate the activation of the canonical IKKs and binding of NF κ B to DNA in MEFs and splenocytes in response to the ligands poly(I:C) and LPS (lipopolysaccharide) that activate TLR3 and TLR4 respectively. The Pellino 1^{-/-} mice also produced reduced serum levels of TNF α (tumour necrosis factor α), IL-6 and IL-12 following an intraperitoneal injection of poly(I:C) or LPS, explaining their resistance to poly(I:C)- or LPS-induced septic shock. These studies demonstrated a key non-redundant role for Pellino 1 in TLR3- and TLR4-mediated innate immunity [26].

Until now, the lack of any antibodies capable of detecting the endogenous Pellino isoforms has hampered the study of how these E3 ligases are regulated. In the present paper, we describe an antibody that recognizes Pellino 1 specifically and exploit it to show that the expression of the Pellino 1 protein is greatly enhanced when BMDMs (bone-marrow-derived macrophages) are stimulated with LPS or poly(I:C). We demonstrate that this occurs via a TRIF-dependent pathway that requires the IKK-related protein kinases, IKK ϵ and TBK1, and the transcription factor IRF3, but not the type 1 IFN receptor. We also show that LPS or poly(I:C) rapidly activate the E3 ligase activity of the endogenous Pellino 1 in macrophages and demonstrate that this is mediated by phosphorylation catalysed by IKK ϵ /TBK1. Taken together, our results establish that TBK1 and IKK ϵ stimulate both the activation of Pellino 1's E3 ligase activity and, via IRF3, the transcription of its gene in response to agonists that activate TLR3 and TLR4.

Experimental

Materials

The IRAK4 inhibitor [27,28] was synthesized by Dr Natalia Shpiro (MRC Protein Phosphorylation Unit, University of Dundee, UK) and the structures of the TBK1/IKK ϵ inhibitors MRT67307 [29] and BX795 [30] have been presented previously. Both compounds were dissolved and stored at -20°C as 10 mM solutions in DMSO. Poly(I:C) was purchased from Invitrogen, LPS (*Escherichia coli* strain O5:B55), poly(I:C) and R848 were purchased from Alexis Biochemicals, CpG [ODN 1826], IL-1, LPS and FLAG-ubiquitin were purchased from Sigma and IFN β was from PBL Interferon Source. The source of the LPS and poly(I:C) used in each experiment is indicated in the Figure legends.

DNA constructs

Pellino 1, Pellino 2 and Pellino 3 were subcloned into the pCMV5 vector by EcoRI/BamHI with an in frame FLAG epitope at the N-terminus. pSUPER-GFP-Pellino 1 shRNA (small hairpin RNA) (216) and pSUPER-GFP-Pellino1 shRNA(1239) target the sequences AGACCAGCATAGCATATCA and TCAAGGACCTCTAGACTAA in the human Pellino 1 coding regions respectively [31]. pSUPER-GFP-mPellino1 shRNA(216) and pSUPER-GFP-mPellino 1 shRNA(1239) target the sequences GGACCAGCATAGCATATCA and CCAAGGACCTTTAGACTAG in the mouse Pellino 1 coding region respectively. All

plasmid constructs were sequenced to verify DNA integrity. Other DNA and expression vectors have been described previously [21,23].

Protein expression and purification

GST (glutathione transferase)–IRAK1 and GST–IRAK4 expressed in insect cells and GST–Pellino 1, GST–Ubc13–Uev1a and His₆–UBE1 expressed in *E. coli* were purified as described previously [21,23,32]. His₆-tagged TBK1 and His₆-tagged IKK ϵ were expressed and purified as described in [30]. The GST tag was removed from Pellino 1, Ubc13 and Uev1a by cleavage with PreScission Protease. The protein phosphatase encoded by bacteriophage λ gt10 was obtained from New England Biolabs, and ubiquitin was purchased from Sigma.

Production and characterization of a Pellino 1-specific antibody

A polyclonal antibody was raised against Pellino 1 in rabbits by Biogenes (Germany) using as an antigen the peptide CPDQENHPSKAPVKY, corresponding to residues 4–17 of Pellino 1 preceded by a cysteine residue. The cysteine was used to couple the peptide to keyhole limpet haemocyanin prior to injection. The antibody was purified from the antiserum by affinity chromatography using the antigen peptide coupled to Sepharose. The peptide antibody recognized Pellino 1, but not Pellino 2 and Pellino 3 in transfected HEK (human embryonic kidney)-293 cells (Figure 1A). The antibody also recognized a ~50 kDa protein in murine RAW264.7 extracts and this band was no longer detected after the level of Pellino 1 had been 'knocked down' by expression of two different shRNAs targeting Pellino 1 (Figure 1B). These shRNAs had previously been found to be effective in knocking down transiently-transfected Pellino 1 in human HEK-293 cells (Figure 1C). These studies demonstrated that the ~50 kDa protein in RAW264.7 cell extracts was indeed Pellino 1.

Other antibodies

Anti-FLAG and anti- α -tubulin were purchased from Sigma, antiubiquitin was purchased from DakoCytomation, anti-actin was purchased from Santa Cruz Biotechnologies, anti-pSer³⁹⁶ of IRF3 and anti-pTyr⁷⁰¹ of STAT1 were purchased from Cell Signalling and a rabbit secondary antibody conjugated to HRP (horseradish peroxidase) was from Pierce.

Knockout mice

MyD88^{-/-} and TRIF^{-/-} mice [7] and their wild-type littermates were kindly provided by Shizuo Akira (Department of Host Defence, University of Osaka, Osaka, Japan). Bones from IRF3 knockout mice and C57BL/6J control mice were provided by the RIKEN BioResource Center through the National Bio-Resource Project of the MEXT (Ministry of Education, Culture, Sports and Technology), Japan via Christophe Desmet (University of Liège, Liège Belgium). Bones from mice lacking expression of the type 1 IFN receptor (IFN α R^{-/-} mice) were provided by Anne O'Garra (National Institute for Medical Research, London, U.K.).

Cell culture and transfection

RAW264.7 and HEK-293 cells were obtained from the American Type Culture Collection (Manassas, VA, U.S.A.) and were cultured at 37°C with DMEM (Dulbecco's modified Eagle's medium) containing 10% (v/v) FBS (foetal bovine serum) and antibiotics (100 units/ml penicillin and 100 µg/ml streptomycin) in a humidified atmosphere containing 5% CO₂. HEK-293 cells were transiently transfected with 1 µg of DNA per ml of cell culture medium by a modified calcium phosphate method [33]. The cells were harvested 36 h post-transfection and lysed with ice-cold lysis buffer [21].

Primary BMDMs were isolated from mice [34]. Cells were maintained on bacterial grade plates for 1 week in DMEM supplemented with 10% (v/v) heat inactivated FBS, 2 mM L-glutamine, 100 units/ml penicillin G, 100 µg/ml streptomycin, 0.25 µg/ml amphotericin and 5 ng/ml macrophage colony-stimulating factor. Adherent cells were then re-plated on tissue culture plastic dishes in fresh medium and used 24 h after replating.

Wild-type, MyD88^{-/-} [35], TRIF^{-/-} [36] and MyD88^{-/-}/TRIF^{-/-} [7] immortalized bone marrow cell lines were generated as described in [37] and maintained in IMDM (Iscove's modified Dulbecco's medium) supplemented with 2% (v/v) FBS, 0.03% Primatone, supplemented with IL-6 and stem cell factor. To initiate experiments, the immortalized bone marrow cells were collected, washed and re-plated in 60 mm Petri dishes at a density of 0.5×10^5 cells per plate. The cells were then incubated for 4–5 days with IMDM supplemented with 2% (v/v) FBS and 30% (v/v) L929-conditioned medium to differentiate them into macrophages.

shRNA mediated knockdown of Pellino 1

RAW264.7 macrophages were transfected with shRNAs (shRNA[216] and shRNA[1239]) contained within the pSUPER-GFP vector (Oligoengine) that knocks-down the expression of Pellino 1 in cells. The shRNAs were co-transfected with pCMV5-Pellino 1 into HEK-293 cells by a modified calcium phosphate method as described above, or transfected into RAW264.7 macrophages using LipofectamineTM (Invitrogen), according to the manufacturer's instructions. Transfected RAW264.7 macrophages expressing the plasmids were selected and grown in the presence of G418 (500 µg/ml).

Real-time PCR

Total RNA was extracted from 10⁶ RAW264.7 cells using a Qiagen RNeasy kit according to the manufacturer's instructions. 5 µg of total RNA was reverse transcribed into cDNA for 1 h at 50°C using oligo(dT) primer and reverse transcriptase in the presence of RNase inhibitor. Transcribed cDNA template (50 ng) was incubated with 200 nM primers in a total volume of 20 µl using KAPA SYBR FAST qPCR (quantitative PCR) kit. The following primers were used: Pellino 1-F 5'-CTGCGTGAAACCAGATCAGCTCAGCAGAG-3'; Pellino 1-R 5'-CCGAGCTGCATTGATCTCCTGTCTTAAAGC-3'; GAPDH-F 5'-CCATGCCATCACTGCCACCCAGAA-3'; GAPDH-R 5'-GTCCACCACCCTGTTGCTGTAGCCG-3'.

Immunoprecipitation of Pellino 1

Cell lysate protein (2 mg) was incubated for 16 h at 4°C with 1 μ g of anti-Pellino 1 antibody attached to Protein G–Sepharose (7.5 μ l of packed beads) on a rotating wheel. The beads were collected by centrifugation (for 2 min at 780 g at 4°C), washed three times in 0.5 ml of 50 mM Tris/HCl (pH 7.5), 1% (v/v) Triton X-100, 0.05% 2-mercaptoethanol and 0.2 M NaCl, and once in 50 mM Tris/HCl (pH 7.5) and 5 mM MgCl₂.

Analysis of phosphorylation by mass spectrometry and Edman sequencing

Phosphorylated proteins were separated by SDS/PAGE (12%), stained with colloidal Coomassie Blue and digested with trypsin [38]. An aliquot of the digest was analysed by LC-MS (liquid chromatography mass spectrometry) with multistage activation on a ThermoElectron LTQ-Orbitrap system coupled to a Proxeon easy-nLC (nano LC) system. The resultant MS/MS spectra were searched by using Mascot (Matrix Science) run on a local server, allowing for phosphoserine/phosphothreonine and phosphotyrosine. The mass tolerances used were $\pm$ 20 p.p.m. Edman degradation of ³²P-labelled peptides was performed by solid phase sequencing as described previously [39].

Results

Inflammatory stimuli induce the expression of Pellino 1 via a TRIF dependent pathway

Pellino was originally identified as a protein that interacts with the IRAK subfamily of protein kinases (see Introduction). We therefore initially stimulated primary BMDMs with the TLR4 agonist LPS, which signals through the adaptor protein MyD88 and the IRAK kinases, as well as via the adaptor TRIF. Stimulation with this agonist was found to induce a striking increase in the expression of the Pellino 1 protein, beginning after 2 h and reaching a maximum level after 4–6 h (Figures 2A and 2B, upper panels). This level of expression was maintained for at least 24 h (results not shown).

To investigate whether the induction of Pellino 1 resulted from the activation of the MyD88-dependent or the TRIF-dependent signalling pathway, we repeated the experiments using primary BMDMs from MyD88^{−/−} or TRIF^{−/−} mice. These experiments showed that the LPS-stimulated increase in the expression of Pellino 1 protein was similar in MyD88^{−/−} and wild-type BMDMs, but almost abolished in BMDMs from TRIF^{−/−} mice (Figures 2A and 2B, upper panels). These experiments indicated that the induction of Pellino 1 was mediated via the TRIF signalling pathway, which is not thought to involve the IRAK subfamily of protein kinases [40].

We then stimulated primary BMDMs with poly(I:C), a TLR3 agonist that only signals through the adaptor TRIF. Stimulation with poly(I:C) also strongly induced the expression of Pellino 1, in a manner similar to LPS, but with slightly delayed kinetics, beginning at 3 h and increasing up to 6 h. As expected, induction by poly(I:C) did not occur in TRIF^{−/−} BMDMs and was unaffected in MyD88^{−/−} BMDMs (Figures 2A and 2B, middle panels).

Stimulation of primary BMDMs with LTA (lipoteichoic acid), a TLR2/6 agonist that signals only through the adaptor MyD88, induced a weaker and slower increase in Pellino 1

expression, which was only revealed by a much longer exposure of the immunoblots. As expected, the weak LTA-induced expression of Pellino 1 was abolished in MyD88^{-/-} BMDMs, but unaffected in TRIF^{-/-} BMDMs (Figures 2A and 2B, lower panels).

We confirmed these findings using immortalized MyD88^{-/-} and TRIF^{-/-} macrophage cell lines, and an immortalized macrophage cell line lacking expression of both MyD88 and TRIF. These experiments showed that the induction of Pellino 1 after 6 h was decreased considerably in the TRIF^{-/-} cells and completely abolished in the TRIF^{-/-}/MyD88^{-/-} double knockout cells (Figure 2C). Taken together, these experiments demonstrate that signalling downstream of TRIF is the major contributor to the induction of Pellino 1 by LPS, but that the MyD88 pathway is also capable of inducing Pellino 1 expression to a small extent.

We also examined the induction of Pellino 1 by the TLR7 agonist R848, the TLR9 agonist CpG and IL-1 β in the RAW264.7 macrophage-like cell line. These agonists, which all signal solely through the MyD88 pathway, did not cause a significant increase in the expression of Pellino 1 (Figure 2D).

The induction of Pellino 1 is prevented by inhibitors of TBK1 and IKK ϵ and is dependent on the presence of IRF3

Signalling via TRIF leads to the activation of the transcription factor IRF3 and the production of IFN β via a pathway that requires the IKK-related protein kinases TBK1 and IKK ϵ , or to the activation of the transcription factor NF- κ B by a pathway that is independent of the IKK-related kinases [29]. To examine which arm of the TRIF signalling pathway was required for the induction of Pellino 1 we utilized MRT67307, a potent and specific inhibitor of TBK1 and IKK ϵ that completely suppresses the phosphorylation of IRF3 (Figure 3A) and the production of IFN β , without suppressing the activation of NF- κ B by ligands that activate TLR3 or TLR4 [29]. These studies showed that MRT67307 completely blocked the induction of Pellino 1, indicating a requirement for TBK1/IKK ϵ kinase activity (Figure 3A).

We then examined whether the LPS-induced increase in Pellino 1 observed at the protein level resulted from an increase in the Pellino 1 mRNA transcript. These experiments revealed that the mRNA encoding Pellino 1 was elevated greatly after 6 h (Figure 3B). Inhibition of IKK ϵ /TBK1 did not affect the decay in the levels of mRNA that had been elevated by LPS (Figure 3C), indicating that the TRIF–TBK1/IKK ϵ -dependent pathway activates transcription of the Pellino 1 gene.

To investigate whether the induction of Pellino 1 occurred through activation of the transcription factor IRF3, or another transcription factor activated downstream of TBK1/IKK ϵ , we used primary BMDMs isolated from IRF3^{-/-} mice (Figure 3D). These experiments revealed that the LPS- or poly(I:C)-stimulated induction of Pellino 1 was abolished in BMDMs from IRF3^{-/-} mice (Figure 3E).

The finding that Pellino 1 was not induced in IRF3^{-/-} macrophages did not exclude the possibility that IRF3 stimulated the production of IFN β , which then stimulated the transcription of Pellino 1. To address this issue, we carried out experiments with BMDMs

from mice that do not express the receptor for type 1 IFNs and therefore fail to respond to IFN β , as judged by the failure to stimulate STAT1 phosphorylation (Figure 3F). Stimulation of these cells with poly(I:C) or LPS showed that the induction of Pellino 1 was only reduced slightly relative to BMDMs from control mice (Figure 3G). This small reduction reflects the ability of IFN β to stimulate a weak induction of Pellino 1, by a mechanism that is independent of TBK1 and IKK ϵ (Supplementary Figure S1 at <http://www.BiochemJ.org/bj/434/bj4340537add.htm>).

Taken together, our experiments demonstrate that the TRIF-dependent signalling pathway activates TBK1/IKK ϵ , which then phosphorylate IRF3, stimulating its translocation to the nucleus where it stimulates transcription of the genes encoding IFN β and Pellino 1. IFN β can then induce a modest further increase in Pellino 1 transcription via the JAK (Janus kinase)/STAT1 pathway.

TBK1 and IKK ϵ phosphorylate and activate the E3 ligase activity of Pellino 1 *in vitro*

The finding that TBK1 and IKK ϵ control the expression of Pellino 1 was interesting because, in a separate study, we had identified Pellino 1 as an IKK ϵ -interacting protein in a yeast two-hybrid screen using IKK ϵ as bait. Four positive clones were identified as Pellino 1 after 10⁶ yeast colonies were screened from a human foetal brain cDNA library, which encoded residues 12–268, 13–266, 1–283 and 1–309 of Pellino 1. This region of Pellino 1 contains the FHA (forkhead-associated) domain, a phosphoserine/phosphothreonine-binding motif that is thought to interact with the phosphorylated form of IRAK1. Since IKK ϵ and TBK1, like IRAK1, are phosphorylated at multiple serine and threonine residues in LPS-stimulated macrophages [41], this raised the possibility that Pellino 1 might also be a substrate of IKK ϵ and/or TBK1.

The phosphorylation of Pellino 1 by IRAK4 *in vitro* can be monitored by a decrease in its mobility on SDS/PAGE (Figure 4A, lane 6). We found that incubation with MgATP and TBK1 (Figure 4A) or IKK ϵ (Figure 4B) produced a similar decrease in the electrophoretic mobility of Pellino 1 (Figures 4A and 4B, lane 2). Pre-incubation with MRT67307 completely blocked the TBK1- or IKK ϵ -induced decrease in the mobility of Pellino 1 (Figures 4A and 4B, lane 3) without affecting the IRAK4-induced band shift (Figure 4A, lane 7). Conversely, pre-incubation with an IRAK4 inhibitor, prevented the IRAK4-catalysed band shift of Pellino 1 (Figure 4A, lane 8), but not the TBK1- or IKK ϵ -catalysed decrease in mobility (Figures 4A and 4B, lane 4).

The E3 ligase activity of bacterially expressed Pellino 1 can be studied *in vitro* by its ability to form free Lys⁶³-linked polyubiquitin chains, not anchored to any other protein, in a ubiquitylation assay containing the E2-conjugating enzyme Ubc13–Uev1a [21,23]. Using this assay, we showed that the phosphorylation of Pellino 1 by TBK1 (Figure 4C) or IKK ϵ (Figure 4D) enhanced greatly the E3 ubiquitin ligase activity of Pellino 1, in a similar manner to that reported previously for IRAK1 or IRAK4 [21]. The unphosphorylated form of Pellino 1 only induced the formation of small ubiquitin oligomers after 10 or 30 min, but much larger more slowly migrating polyubiquitin chains were formed over the same period following phosphorylation by TBK1 or IKK ϵ (Figures 4C and 4D).

To establish that the activation of Pellino 1 had resulted from phosphorylation, we stopped the TBK1- and IKK ϵ -catalysed phosphorylation with MRT67307 and showed that treatment with the protein serine/threonine phosphatase, produced by bacteriophage λ gt10, increased the electrophoretic mobility of Pellino 1 to that of the unphosphorylated protein and abolished its E3 ligase activity (Figures 5A and 5B). Moreover, incubation of TBK1 or IKK ϵ with MRT67307 followed by incubation with Pellino 1 and MgATP did not cause any enhancement of the E3 ligase activity of Pellino 1 (Figures 5C and 5D). Taken together, these experiments demonstrated that the activation of Pellino 1 by TBK1/IKK ϵ had resulted from phosphorylation and not a protein–protein interaction between TBK1/IKK ϵ and Pellino 1.

TBK1 and IKK ϵ phosphorylate the key activating sites on Pellino 1 *in vitro*

We have identified previously ten serine/threonine residues on Pellino 1 that are phosphorylated by IRAK1 and IRAK4 *in vitro*. Mutagenesis studies showed that the phosphorylation of any one of the three residues Ser⁷⁶, Ser²⁸⁸ or Thr²⁹³ was capable of fully activating the E3 ligase activity of Pellino 1, and full activation could also be achieved by the combined phosphorylation of residues Ser⁷⁸, Thr⁸⁰ and Ser⁸² [23]. A Pellino 1 mutant in which all ten phosphorylation sites have been changed to alanine residues (termed 10A Pellino 1) cannot therefore be phosphorylated or activated by IRAK1 (Figure 6A, lane 6). This mutant could also not be phosphorylated by TBK1 (Figure 6A, lane 5) implying that the sites on Pellino 1 phosphorylated by TBK1 must overlap with those phosphorylated by IRAK1 and IRAK4.

To identify the sites on Pellino 1 phosphorylated by TBK1, we phosphorylated Pellino 1 with this protein kinase or IRAK1 in the presence of Mg-[γ -³²P]ATP. Following digestion with trypsin, the tryptic phosphopeptides were separated by reverse phase HPLC (Figures 6B and 6C). The major phosphopeptides were identified by mass spectrometry and the phosphorylation sites were identified by solid phase sequencing (Figure 7). The results, summarized in Supplementary Table S1 (at <http://www.BiochemJ.org/bj/434/bj4340537add.htm>), showed that the major sites phosphorylated by TBK1 were Ser⁷⁶, Thr⁸⁰, Thr²⁸⁸ and Ser²⁹³, which include the three key activating sites (Ser⁷⁶, Thr²⁸⁸ and Ser²⁹³).

In Figure 6(C), peak 2 contained a diphosphorylated peptide phosphorylated at Ser⁷⁶ and Thr⁸⁰, whereas peak 4 contained a peptide phosphorylated at Ser⁷⁶ alone (Figure 7). Peaks 5 and 6 contained diphosphorylated peptides, which were phosphorylated at Thr²⁸⁸ and Ser²⁹³ (Figure 7). In contrast the slightly latereluting tryptic peptides 5 and 6 generated after phosphorylation by IRAK1 (Figure 6B) were phosphorylated at Thr²⁸⁸ alone. Peptide 6 from the IRAK1 digest also contained a peptide phosphorylated at Thr¹²⁷, a site not phosphorylated by TBK1. Analysis of the earlier eluting peptides 1–4 confirmed that, in addition to Ser⁷⁶ and Thr⁸⁰, IRAK1 also phosphorylates Ser⁸², a residue not phosphorylated by TBK1.

TBK1 and IKK ϵ phosphorylate and activate Pellino 1 in LPS- or poly(I:C)-stimulated macrophages

To investigate whether TBK1 and IKK ϵ could activate the E3 ligase activity of endogenous Pellino 1 by phosphorylation in cells, we stimulated the RAW264.7 macrophage cell line with LPS or poly(I:C) and then measured the E3 ligase activity of Pellino 1 after immunoprecipitating it from the cell extracts. The E3 ligase activity of Pellino 1 was low in unstimulated cells, but following stimulation with LPS, an increase in activity was detectable after 15 min, which reached a maximum after 30 min (Figure 8A). Stimulation with poly(I:C) also led to an increase in the activity of Pellino 1, with maximal activation observed after 45 min (Figure 9A). The LPS- or poly(I:C)-induced activation of Pellino 1 was abolished by incubating the Pellino 1 immunoprecipitates with phage λ gt10 phosphatase, and this could be prevented by NaF, a general inhibitor of protein serine/threonine phosphatases (Figures 8B and 9B). Pre-treatment of the macrophages with the TBK1/IKK ϵ inhibitor MRT67307 prevented the LPS- or poly(I:C)-stimulated activation of Pellino 1 as well as the phosphorylation of IRF3 at Ser³⁹⁶ (Figures 8C and 9C), consistent with activation being catalysed by TBK1/IKK ϵ .

The results presented above were consistent with the endogenous Pellino 1 E3 ubiquitin ligase being activated by a TBK1/IKK ϵ -catalysed phosphorylation event, but did not establish whether this occurred as a consequence of the phosphorylation of Pellino 1 itself or an associated protein(s). In contrast with the decreased electrophoretic mobility that accompanies the phosphorylation of Pellino 1 by TBK1/IKK ϵ or IRAK1/IRAK4 *in vitro*, activation of endogenous Pellino 1 by LPS or poly(I:C) did not appear to alter its electrophoretic mobility in RAW264.7 macrophages, at least as judged by immunoblotting. This may be explained by the inability of phosphorylated Pellino 1 to be recognized by the anti-Pellino 1 antibody. However, we did notice that after stimulation of RAW264.7 macrophages for 30–60 min with LPS there was a small, but consistent decrease in the level of Pellino 1 detected by immunoblotting. Since one explanation for this apparent decrease was that unmodified Pellino 1 had been converted to a number of slower migrating phosphorylated species, each of which was present at too low an abundance to be detected by immunoblotting, we incubated cell lysates with phage λ gt10 phosphatase. This partially restored the level of the unmodified endogenous Pellino 1 to that present in unstimulated cells, but did not affect the level of endogenous Pellino 1 detected in unstimulated cells (Figure 8D). This experiment provides further evidence that the LPS-induced activation of the E3 ubiquitin ligase activity of endogenous Pellino 1 results from the direct phosphorylation of the protein.

To identify the residues that became phosphorylated in response to LPS, we immunoprecipitated endogenous Pellino 1 from LPS-stimulated RAW264.7 macrophages, digested with trypsin and analysed the resulting tryptic phosphopeptides using an Orbitrap Velos mass spectrometer. All the endogenous Pellino 1 protein was immunoprecipitated from the cell extracts, as judged by immunoblotting, but we were unable to detect a Coomassie Blue staining band corresponding to Pellino 1 after SDS/PAGE. This observation indicates that Pellino 1 is expressed at extremely low levels in unstimulated macrophages. Coupled with low levels of phosphorylation of individual serine/threonine residues in

endogenous Pellino 1 and the fact that phosphorylation of any one of three sites induces the full activation of Pellino 1 *in vitro*, this may explain why we have been unable, thus far, to identify phosphorylation sites in the endogenous protein.

Discussion

In the present study, we have shown that the expression of the Pellino 1 protein is increased greatly by TLR3 and TLR4 agonists by a TRIF–TBK1/IKK ϵ –IRF3-dependent pathway. IFN β , which is also induced by this pathway, stimulated a smaller increase in Pellino 1 expression by a TBK1/IKK ϵ -independent pathway, creating a positive autocrine loop, which may make a minor contribution to the overall increase in Pellino 1 expression. Why such a striking increase in the level of Pellino 1 is needed is unknown currently, but since Pellino 1 is an IRF3-regulated gene, we speculate that this may be required to regulate the production of IFN β and/or other IRF3/IFN-regulated genes.

The expression of IKK ϵ is also increased by LPS and poly(I:C), as well as by other MyD88-dependent TLR agonists, and for this reason has also been called IKKi (IKK-inducible). We have observed that the time course of induction of IKK ϵ in macrophages is delayed slightly compared with the induction of Pellino 1 (results not shown) and occurs via a different pathway, because it is not blocked by the IKK ϵ /TBK1 inhibitors BX795 [42] or MRT67307 [29] but is prevented by the inhibition of IKK β [29]. Therefore the induction of Pellino 1 is not a consequence of the increased expression of IKK ϵ or vice versa.

The structure of Pellino 1 consists of a FHA domain, a wing appendage that loops out from the FHA domain [43] and a C-terminal RING domain that carries the E3 ubiquitin ligase activity. FHA domains binds to phosphoserine and phosphothreonine residues in proteins, which may explain why wild-type IRAK1, which phosphorylates itself at several residues, but not a catalytically inactive mutant of IRAK1, interacts with Pellino isoforms in co-transfection experiments [22]. Our yeast two-hybrid screening using IKK ϵ as bait revealed that this protein kinase also interacts with the FHA domain of Pellino 1, but further work is needed to establish whether this interaction is phosphorylation-dependent.

The finding that IKK ϵ interacts with Pellino 1 led us to discover that IKK ϵ and TBK1 phosphorylate Pellino 1 *in vitro*, activating its E3 ligase activity. Phosphorylation occurred at the same three key activating sites that are phosphorylated by IRAK1 or IRAK4 *in vitro*, namely Ser⁷⁶ in the wing appendage and Thr²⁸⁸ and Ser²⁹³ at the start of the RING domain [23]. Studies on the specificity of IKK ϵ using small synthetic peptide substrates have shown that this protein kinase preferentially phosphorylates serine residues in which leucine, isoleucine, methionine or phenylalanine are preferred at the $n+1$ position, tyrosine, phenylalanine or tryptophan at the $n+3$ position and tyrosine, phenylalanine, proline or methionine at the $n-2$ position, where n is the site of phosphorylation [44]. The sequence surrounding Thr²⁸⁸ (Phe-Asn-Thr-Leu-Ala-Phe) conforms to this consensus, whereas the sequences surrounding Ser⁷⁶ (Gln-His-Ser-Ile-Ser-Tyr) and Ser²⁹³ (Phe-Pro-Ser-Met-Lys-Arg) each contain two of the three preferred residues. The phosphorylation of Pellino 1 by IKK ϵ /TBK1 may also be enhanced by interaction between the FHA domain of Pellino 1 and the phosphorylated forms of these protein kinases. Whether TBK1 or IKK ϵ plays the major

role in activating Pellino 1's E3 ligase activity is unclear, since the pharmacological inhibitor MRT67307 used in the present study does not discriminate between these closely related kinases.

Pellino 1^{-/-} mice were reported to produce less TNF α and IL-6 *in vivo* after injection of poly(I:C) or LPS, which may explain why these mice are resistant to poly(I:C)- or LPS-induced septic shock [26]. Embryonic fibroblasts and splenocytes from the Pellino 1^{-/-} mice showed decreased activation of the canonical IKKs and decreased binding of NF- κ B to DNA in response to poly(I:C) or LPS. They also produced decreased levels of the mRNAs encoding TNF α and IL-12 in response to LPS or poly(I:C) in MEFs and dendritic cells, and decreased secretion of IL-12 in LPS- or poly(I:C)-stimulated BMDMs [26]. From these and other experiments these investigators concluded that Pellino 1 is required for the TRIF-dependent activation of NF- κ B and that it plays a non-redundant function in this pathway that cannot be compensated for by either Pellino 2 or Pellino 3. However, we have found that pharmacological inhibition of TBK1/IKK ϵ , which blocks the poly(I:C)- or LPS-stimulated induction of Pellino 1 (Figure 3), as well as the activation of its E3 ligase activity (Figures 8 and 9), does not inhibit the poly(I:C)- or LPS-stimulated activation of IKK α /IKK β , the phosphorylation of their substrate p105 or NF- κ B-dependent gene transcription in RAW264.7 macrophages [29]. Therefore either the requirement of Pellino 1 for the TRIF-dependent activation of NF- κ B is cell specific, or Pellino 1 has a function that is independent of its E3 ligase activity. To address this issue and physiological roles of the E3 ligase activity of Pellino 1, we have recently generated knockin mouse in which wild-type Pellino 1 is replaced by an E3-ligase-deficient mutant.

The finding that LPS or poly(I:C) activate Pellino 1's E3 ligase activity via TBK1/IKK ϵ , and not via IRAK1/IRAK4, was unexpected and raises the question of how Pellino 1 and other Pellino isoforms are activated by agonists that signal via MyD88. We have recently observed that the IL-1 stimulated activation of the E3 ligase activity of endogenous Pellino 1 was not suppressed by pharmacological inhibition of TBK1/IKK ϵ (E. Goh and P. Cohen, unpublished work). Whether this is catalysed by IRAK1 and/or IRAK4 or another protein kinase(s) will require further analysis. Nevertheless, our results indicate clearly that Pellino isoforms are activated by at least two classes of protein kinases *in vivo*.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations used

BMDM	bone marrow-derived macrophage
DMEM	Dulbecco's modified Eagle's medium
FBS	foetal bovine serum
FHA	forkhead-associated
GST	glutathione transferase
HEK	human embryonic kidney
IFNβ	interferon β
IKK	I κ B (inhibitor of nuclear factor κ B) kinase
IL	interleukin
IMDM	Iscove's modified Dulbecco's medium
IRAK	IL receptor-associated kinase
IRF3	IFN regulatory factor 3
LPS	lipopolysaccharide
LTA	lipoteichoic acid
MEF	mouse embryonic fibroblasts
MyD88	myeloid differentiation factor 88
NF-κB	nuclear factor κ B
shRNA	small hairpin RNA
STAT1	signal transducer and activator of transcription 1
TBK1	TANK [TRAF (tumour-necrosis-factor-receptor-associated factor)-associated NF- κ B activator]-binding kinase 1
TLR	Toll-like receptor
TNFα	tumour necrosis factor α
TRIF	TIR (Toll/IL-1 receptor)-domain-containing adaptor-inducing interferon- β

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Figure 1. Characterization of a Pellino 1-specific antibody

(A) HEK-293 cells were transfected with plasmids encoding FLAG–Pellino 1, FLAG–Pellino 2 or FLAG–Pellino 3. Cell lysates were subjected to SDS/PAGE and immunoblotted with antibodies that recognize Pellino 1, FLAG and actin. (B) Murine RAW264.7 macrophages were transfected with plasmids shRNA(216) or shRNA(1239) or empty vector, and the cells were then selected with G418. The efficiency of knockdown of the endogenous mouse Pellino 1 was assessed by immunoblotting of cell lysates with the anti-Pellino 1 antibody. (C) The experiment was carried out as in (B), except that HEK-293 cells were transiently co-transfected with shRNA(216) or shRNA(1239) and FLAG–human Pellino 1. Knockdown of the transfected Pellino 1 was measured by immunoblotting of the cell lysates with an anti-FLAG antibody.

Figure 2. Inflammatory stimuli induce the expression of Pellino 1 predominantly via a TRIF-dependent pathway

(A and B) Primary BMDMs derived from wild-type (TRIF^{+/+}) and TRIF^{-/-} mice (A) or wild-type (MyD88^{+/+}) and MyD88^{-/-} mice (B) were stimulated with 100 ng/ml LPS (Alexis Biochemicals), 10 μ g/ml poly(I:C) (InvivoGen) or 2 μ g/ml LTA for the times indicated. The lysate from 10^5 cells was resolved by SDS/PAGE, transferred on to polyvinylidene fluoride membranes and immunoblotted with antibodies that recognize Pellino 1 or α -tubulin. (C) Immortalized macrophages from wild-type, MyD88^{-/-}, TRIF^{-/-} and MyD88^{-/-}/TRIF^{-/-} double knockout mice were stimulated for 6 h with 100 ng/ml LPS (Sigma) and immunoblotted with anti-Pellino 1 and anti-actin antibodies. (D) The RAW264.7 macrophage-like cell line was treated for the times indicated with 100 ng/ml LPS (Sigma), 100 μ g/ml poly(I:C) (Alexis Biochemicals), 10 μ M R848, 5 μ M CpG or 50 ng/ml IL-1 β . The cell lysates were subjected to SDS/PAGE and immunoblotted as in (C).

Figure 3. The induction of Pellino 1 is prevented by inhibition of TBK1 and IKKε and requires IRF3

(A) Primary wild-type BMDMs were pre-treated for 1 h without (–) or with (+) 2 μM MRT67307, then stimulated for the times indicated with 100 ng/ml LPS (Alexis Biochemicals). Cell lysates were subjected to SDS/PAGE and then immunoblotted with antibodies recognizing Pellino 1, IRF3 phosphorylated at Ser³⁹⁶ and α-tubulin. (B) Total mRNA from RAW264.7 macrophages treated for 6 h with or without 100 ng/ml LPS (Sigma) was reverse transcribed. The cDNA was subjected to real-time PCR using gene-

specific primers for Pellino 1. The level of Pellino 1 is expressed relative to the level of Pellino 1 mRNA in unstimulated cells using glyceraldehyde-3-phosphate dehydrogenase as a reference standard. The results show triplicate determinations from two independent experiments. (C) RAW264.7 macrophages were stimulated for 6 h without (open triangles) or with (open and closed circles) 100 ng/ml LPS (Sigma). Transcription was then inhibited by the addition of actinomycin D to a final concentration of 5 μ g/ml (arrow, Act) with (closed circles) or without (open circles) 1 μ M BX795, an equally potent but slightly less specific inhibitor of TBK1/IKK ϵ . The decay of mRNA was then monitored at the times indicated. Cells were harvested, the mRNA was isolated and reverse transcribed and the resulting cDNA was quantitated as in (B). The results shown are the means ($\pm$ S.D.) from duplicate samples and similar results were obtained in three separate experiments. (D) Primary BMDMs from IRF3^{+/+} and IRF3^{-/-} mice were lysed, and lysates equivalent to 10⁵ cells were subjected to SDS/PAGE and immunoblotted for the presence of IRF3 or α -tubulin as a loading control. (E) BMDMs from IRF3^{+/+} and IRF3^{-/-} mice were stimulated with 100 ng/ml LPS (Alexis Biochemicals), or 10 μ g/ml poly(I:C) (Invitrogen) for the times indicated. Following cell lysis, an aliquot of each lysate was subjected to SDS/PAGE, transferred on to polyvinylidene fluoride membrane and immunoblotted with antibodies that recognize Pellino 1 or α -tubulin. (F) IFN α β R^{+/+} or IFN α β R^{-/-} BMDMs were stimulated for 30 min with 500 units/ml IFN β and cell lysates were subjected to SDS/PAGE and immunoblotted for STAT1 α and STAT1 β phosphorylated at Tyr⁷⁰¹ or α -tubulin. (G) BMDMs from IFN α β R^{+/+} or IFN α β R^{-/-} mice were stimulated for 6 h with 100 ng/ml LPS or 10 μ g/ml poly(I:C) (InvivoGen). The cell lysates were subjected to SDS/PAGE and immunoblotted with antibodies that recognize Pellino 1 or α -tubulin.

Figure 4. TBK1 and IKKε phosphorylate Pellino 1 and enhance its E3 ubiquitin ligase activity (A) Pellino 1 (1 μ M) was incubated for 30 min at 30°C with MgATP in the presence (+) or absence (–) of 2 units/ml IRAK4 or 2 units/ml TBK1 in 50 mM Tris/HCl (pH 7.5) which had been pre-incubated in the absence (–) or presence (+) of 2 μ M MRT67307 or 10 μ M IRAK4 inhibitor. The reactions were terminated by denaturation in SDS, subjected to SDS/PAGE and the gel was stained with Coomassie Blue. The positions of unphosphorylated and phosphorylated (p) forms of Pellino 1 are indicated. (B) The experiment was carried out as in as (A), except that IKKε was used instead of TBK1. (C and D) Pellino 1 (1 μ M), Ubc13–

Uev1a (1 μ M), ubiquitin (0.1 mM), MgCl_2 (5 mM) and ATP (2 mM) were incubated for 30 min at 30°C without (–) or with (+) 2 units/ml TBK1 (C) or 2 units/ml IKK ϵ (D) and the ubiquitylation reactions initiated by the addition of E1 enzyme (0.1 μ M). The reactions were stopped after a further 0, 10, 30 or 60 min by the addition of SDS, subjected to SDS/PAGE, transferred on to polyvinylidene fluoride membranes and immunoblotted with an anti-ubiquitin antibody. For definition of enzyme units see [30].

Figure 5. The E3 ligase activity of Pellino 1 is regulated by reversible phosphorylation
 (A and B, upper panels) Pellino 1 (1 μ M) was incubated with MgATP in 50 mM Tris/HCl (pH 7.5) without (-) or with (+) 2 units/ml TBK1 (A) or 2 units/ml IKK ϵ (B) as in Figure 4. After 30 min the reactions were supplemented with (+) or without (-) MRT67307 to a final concentration of 2 μ M to inhibit TBK1/IKK ϵ . The reactions were then incubated for 30 min without (-) or with (+) bacteriophage λ gt10 phosphatase (100 units) and 1 mM MnCl₂ in a total volume of 0.02 ml. The gels were stained with Coomassie Blue and the position of phosphorylated (p) Pellino 1 is indicated. (A and B, lower panels) The reactions were carried

out as in the upper panels, except that the E3 ubiquitin ligase activity of each sample was assessed by incubation for 10 min at 30°C with Ubc13–Uev1a, E1 enzyme and ubiquitin followed by immunoblotting with anti-ubiquitin as in Figures 4(C) and 4(D). (**C** and **D**) The reactions were carried out as in Figures 4(C) and 4(D), except that, where indicated, TBK1 or IKK ϵ was incubated with 2 μ M MRT67307 before the addition of Pellino 1, to prevent phosphorylation. To aid clarity, (**A** – **D**) do not show the whole gel, but only the region with polyubiquitylated (pUb) species of >50 kDa.

Figure 6. Comparison of the phosphorylation of Pellino 1 by TBK1 and IRAK1

(A) The reaction was carried out as in Figure 4(C) and 4(D) but with either 1 μ M wild-type (WT) Pellino 1 (lanes 1–3) or a 1 μ M 10A Pellino 1 (residues 70, 76, 78, 80, 82, 86, 125, 127, 288 and 293 all changed to alanine; lanes 4–6), and with either 2 units/ml TBK1 or IRAK1. Samples of the reactions were subjected to SDS/PAGE and either immunoblotted with anti-ubiquitin (upper panel), or stained with Coomassie Blue (lower panel). To aid clarity, the Figure does not show the whole gel, but only the region with polyubiquitylated species of >50 kDa. (B and C) Separation of tryptic phosphopeptides from phosphorylated

Pellino 1. ^{32}P -labelled Pellino 1 obtained by incubation for 30 min at 30°C with Mg- $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ and either IRAK1 (**B**) or TBK1(**C**) was subjected to SDS/PAGE and the gel was stained with Coomassie Blue. The Pellino 1 was excised, digested with trypsin and subjected to reverse phase HPLC on a Vydac C_{18} column equilibrated in 0.1% trifluoroacetic acid. The column was developed with an acetonitrile gradient in 0.1% trifluoroacetic acid. ^{32}P -radioactivity in arbitrary units (au) is shown by the continuous line, and the acetonitrile gradient is indicated by the diagonal broken line.

Figure 7. Identification of the sites of Pellino 1 phosphorylated by TBK1

The indicated ^{32}P -labelled phosphopeptides from Figure 6(C) were subjected to solid-phase sequencing to identify the cycles of Edman degradation at which ^{32}P -radioactivity (filled bars) was released from the phosphopeptides present in these fractions. The sequences of each peptide are shown using the single letter code for amino acids.

Figure 8. The LPS-stimulated activation of Pellino 1 is prevented by inhibition of TBK1/IKK ϵ and reversed by phosphatase treatment

(A) RAW264.7 macrophages were stimulated with 100 ng/ml LPS (Alexis Biochemicals) for the times indicated. Pellino 1 was immunoprecipitated (see Experimental) and incubated for 60 min at 30°C in 0.02 ml of 50 mM Tris/HCl (pH 7.5), 5 mM MgCl₂, UBE1 (0.1 μ M), Ubc13–Uev1a (0.05 μ M), FLAG–ubiquitin (0.05 mM) and ATP (2 mM) in the presence of the non-specific protein kinase inhibitor staurosporine (75 μ M) [23] to prevent any phosphorylation and activation of Pellino 1 during the assay by protein kinases present in the immunoprecipitates as trace contaminants. The reactions were terminated by denaturation in

SDS, and immunoblotted with an anti-FLAG antibody (upper panel). Cell lysates were subjected to SDS/PAGE and immunoblotted with antibodies that recognize Pellino 1 or IRF3 phosphorylated at Ser³⁹⁶ (lower two panels). **(B)** RAW264.7 macrophages were stimulated with LPS for 30 min or left unstimulated (0 min) and Pellino 1 was immunoprecipitated as in **(A)**. The immunoprecipitates were then incubated for 30 min at 30°C with (+) or without (–) 100 units of phage λ gt10 phosphatase and 1 mM MnCl₂ in the presence (+) or absence (–) of 50 mM NaF. After washing three times in 50 mM Tris/HCl (pH 7.5) and 5 mM MgCl₂, the immunoprecipitated Pellino 1 was assayed for E3 ubiquitin ligase activity as in **(A)** and cell lysates immunoblotted with the antibodies used in **(A)**. **(C)** As in **(B)** except that the RAW264.7 macrophages were incubated for 1 h without (–) or with (+) 2 μ M MRT67307, before stimulation with LPS. To aid clarity, **(A–C)** do not show the whole gel, but only the region with polyubiquitylated species of >150 kDa. **(D)** RAW264.7 macrophages were stimulated with 100 ng/ml LPS (Sigma) for 60 min and the cells were lysed in the absence of any phosphatase inhibitor. Cell lysate (20 μ g protein) was then incubated for 30 min at 30°C with (+) or without (–) 300 units of phage λ gt10 phosphatase in the presence of 1 mM MnCl₂. The reactions were terminated in SDS, subjected to SDS/PAGE and immunoblotted with antibodies that recognize Pellino 1 or actin. The experiment was carried out three times with similar results.

Figure 9. The poly(I:C)-stimulated activation of Pellino 1 is prevented by inhibition of TBK1/IKK ϵ and reversed by phosphatase treatment

(A) As in Figure 8(A) except that RAW264.7 macrophages were stimulated with 50 ng/ml poly(I:C) (InvivoGen) for the times indicated. (B) As in Figure 8(B) except RAW264.7 macrophages were stimulated for 45 min with poly(I:C). (C) As in (B) except that the RAW264.7 macrophages were incubated for 1 h without (–) or with (+) 5 μ M MRT67307, before stimulation with poly(I:C). To aid clarity, (A–C) do not show the whole gel, but only the region with polyubiquitylated species of >150 kDa.