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Molecular mechanisms involved in the regulation of cytokine production by muramyl dipeptide.

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Running title: Mechanisms of NOD2/RIP2 signaling

Synopsis Muramyl dipeptide (MDP), a component of peptidoglycan, interacts with NOD2 stimulating the NOD2/RIP2 complex to activate signaling pathways important for anti-bacterial defence. Here we demonstrate that the protein kinase activity of RIP2 has two functions, namely to limit the strength of downstream signaling and to stabilise the active enzyme. Thus pharmacological inhibition of RIP2 kinase with either SB 203580 or PP2 induces a rapid and drastic decrease in the level of the RIP2 protein, which may explain why these RIP2 inhibitors block MDP-stimulated downstream signaling and the production of IL-1 β and TNF α . We also show that RIP2 induces the activation of the protein kinase TAK1, that a dominant negative mutant of TAK1 inhibits RIP2-induced activation of JNK and p38 α MAPK, and that signaling downstream of NOD2 or RIP2 is reduced by the TAK1 inhibitor 5Z-7-oxozeaenol or in TAK1-deficient cells. We also show that MDP activates ERK1/ERK2 and p38 α MAPK in human PBMC and that both MAP kinases and TAK1 activity are required for MDP-induced production of IL-1 β and TNF α in these cells. Taken together, our results indicate that the MDP-NOD2/RIP2 and LPS-TLR4 signaling pathways converge at the level of TAK1 and that many subsequent events that lead to the production of pro-inflammatory cytokines are common to both pathways.

Key words: NOD2, RIP2, MDP, TAK1, pro-inflammatory cytokines

Abbreviations:- CK1, casein kinase 1; ERK, extracellular signal regulated kinase; GAK, cyclin G-associated kinase; HEK, human embryonic kidney; JNK, c-Jun N-terminal kinase; KI, kinase inactive; MKK1, MAPK kinase-1; IL, interleukin; LPS, lipopolysaccharide; MAPK, mitogen-activated protein kinase; MDP, muramyl dipeptide; M-Tri_{DAP}, muramyl-NAc-L-Ala-D-Glu-meso-diaminopimelic acid; NF κ B, nuclear factor κ B; NOD, nucleotide-binding oligomerisation domain; PBMC, peripheral blood mononuclear cells; RIP2, receptor interacting protein 2; TAK1, TGF β -activated kinase-1; TAB, TAK1-binding protein; TLR, Toll-like receptor; TNF, tumour necrosis factor, Tpl2, tumour progression locus 2; TRAF6, TNF receptor associated factor 6.

Introduction

The Nucleotide binding and Oligomerisation Domain (NOD) proteins NOD1 and NOD2 are the intracellular receptors for peptidoglycan, a major component of bacterial cell walls. The minimal sub-fragments of peptidoglycan recognised by NOD1 and NOD2, are muramyl-NAc-L-Ala-D-Glu-meso-diaminopimelic acid (M-Tri_{DAP}) and muramyl dipeptide (MDP), respectively, which bind to the C-terminal leucine rich repeat (LRR) domains of these proteins. NOD1 and NOD2 interact with a protein kinase, termed receptor-interacting protein 2 (RIP2, also called RICK, RIPK2 and CARDIAK), which is required for “downstream” signaling. NOD1 and NOD2 acting via RIP2, switch on the transcription factor NF κ B and the mitogen-activated protein kinases (MAPKs) termed p38 α MAPK, c-Jun N-terminal kinase (JNK) and extracellular signal-regulated kinase 1 (ERK1) and ERK2 [1-3], which stimulate production of the pro-inflammatory cytokines that mount innate immune responses to combat invading bacteria.

The NOD1/NOD2-RIP2 signaling pathway is thought to be used for host defence against intracellular bacteria that have evaded the Toll-like receptor (TLR)-mediated defence mechanisms in the plasma membrane, such TLR4 which responds to bacterial lipopolysaccharide (LPS). The NOD1/NOD2 signaling pathway may also provide a defence against gram-positive bacteria whose cell walls do not contain LPS [1]. The importance of NOD2 in protection against inflammatory bowel disease has been highlighted by the finding that particular mutations in NOD2 are associated with the susceptibility to Crohn’s disease [1, 3-7]. Significantly, Peripheral Blood Mononuclear Cells (PBMC) from these patients do not produce the cytokines interleukin 1 β (IL-1 β), IL-10 or tumour necrosis factor α (TNF α) in response to either MDP or M-Tri_{DAP}, demonstrating that NOD2 is also required for signaling by NOD1 in these cells [8]. However, NOD2 clearly plays additional roles in regulating systemic responses to pathogens, because NOD2-deficient mice are protected against LPS-induced septic shock, even though LPS-induced signaling pathways and pro-inflammatory cytokine production are apparently normal [3]. RIP2-deficient mice are

also protected against LPS-induced septic shock and have impaired ability to defend against infection by *Listeria monocytogenes* [9].

How the MDP-NOD2-RIP2 signaling module actually switches on “downstream” signaling is unclear because, surprisingly, when over-expressed in HEK 293 cells, a catalytically inactive (“kinase inactive”, KI) mutant of RIP2 was reported to be as effective as wild type (WT) RIP2 in activating NF κ B-dependent gene transcription and c-Jun N-terminal kinase (JNK) [10] or in triggering apoptosis [11]. Thus the protein kinase activity of RIP2 is thought not to be essential for the MDP-induced activation of these signaling pathways. These observations raised the question of how RIP2 switches on “downstream” signaling events and what function its associated kinase activity might have.

In this paper we demonstrate that the protein kinase activity of RIP2 plays at least two roles in the MDP-NOD2 signaling system. First, we find that KI-RIP2 is even more effective than WT-RIP2 in activating NF κ B-dependent gene transcription, c-Jun N-terminal kinases 1 and 2 (JNK1/JNK2) and p38 α mitogen-activated protein kinase (p38 α MAPK), suggesting that RIP2 kinase activity functions to limit the strength of “downstream” signaling. Second, we find that RIP2 kinase activity is required to maintain RIP2 expression levels in transfected HEK 293 cells, which may explain our finding that pharmacological inhibition of the endogenous RIP2 kinase activity suppresses the MDP-induced activation of NF κ B. We also find that, when over-expressed, RIP2 interacts with and activates TGF β -activated kinase 1 (TAK1) and that MDP-NOD2 or RIP2 induced NF κ B gene transcription does not occur when TAK1 is inhibited or in TAK1-deficient cells. Finally, we find that the MDP-induced signaling and production of IL-1 β and TNF α in human PBMC is attenuated by pharmacological inhibition of p38 α MAPK, MAPK kinase-1 (MKK1) or TAK1. Taken together, our results suggest that the signaling pathways by which MDP-NOD2 and LPS-TLR4 induce the production of IL-1 β and TNF α converge at the level of TAK1.

Experimental

Materials

PD 184352 synthesised by an improved method [12] and BIRB 0796 synthesised from 4,4-dimethyl-3-exopentanenitrile [13] were provided by Natalia Shpiro and Rudolfo Marquez. SB 203580 was purchased from Promega, the Src family kinase inhibitors PP1 and PP2 from Calbiochem, the TAK1 inhibitor (5Z-7-oxo-zeaenol) from AnalytiCon Discovery GmbH, LPS and muramyl dipeptide free of LPS from Invivogen and the MEF2 nucleofection kit from Amaxa.

DNA constructs

DNA encoding human RIP2 (NP_003812) was amplified from IMAGE clone 4026156 (Geneservice) with oligonucleotides MP1155 and MP1160 using the GC Rich PCR System (Roche), cloned into a pCR2.1 vector. The construct was sequenced then ligated into the BamH1 /Not1 sites in the pCMV HA-1 vector. A vector in which Asp164 was changed to Ala (pCMV HA-RIP2[D164A]) was created using the Quickchange Mutagenesis kit (Stratagene) with oligonucleotides MP2345 and MP2346 and a vector in which Thr95 was changed to Met (pCMV HA-RIP2[T95M]) was created using oligonucleotides MP2847 and MP2848. Mouse NOD2 (BC044774) was amplified from IMAGE clone 5324763 as described above with oligonucleotides MP1879 and MP1880 and cloned into the BamH1 site of pCMV HA-1.

The oligonucleotide sequences were:-

MP1155 - GCGCGGCCCGCCAACGGGGAGGCCATCTGCAGC

MP1160 - GCGGCCGCTTACATGCTTTTATTTTGAAGTAAATTTAAAGATG
GTGATCTAGAAA

MP1879 - GCGGATCCATGTGCTCACAGGAAGAGTTCCAGGC

MP1880 - GCGGATCCTCACAACAAGAGTCTGGCGTCCCTG

MP2345 - CATGTTAAGATTGCAGCTTTTGGTTTATCAAAG

MP2346 - CTTTGATAAACCAAAAGCTGCAATCTTAACATG

MP2847 - TTTTGGGAATAGTTATGGAATACATGCCAAAT

MP2848 - ATTTGGCATGTATTCCATAACTATTCCCAAAAA

DNA encoding a ConA basal promoter into which three copies of the NF κ B DNA response element had been inserted (termed 3x κ B luciferase reporter construct) and constructs encoding gag-pol and VSV-G for the production of Lentiviruses were provided by Ron Hay, College of Life Sciences University of Dundee [14]. The TAK1 short hairpin (shRNA) lentiviral plasmid was from Sigma and the pRL-TK vector driving *Renilla* luciferase from Promega.

Production of lentiviruses and infection

Lentiviruses carrying a TAK1 shRNA plasmid (TRCN0000001558, Sigma) were produced using a gag-pol construct and a VSV-G encoded plasmid by triple transfection as described [15]. To create stable cell lines 200 μ l viral supernatant were used to infect HEK 293 cells on a 10 cm² dish. After 48 h, 3 μ g puromycin was added to the medium for selection. Stably transfected cells were used for experiments.

Cell culture, transfection and NF κ B reporter gene assay

Human embryonic kidney (HEK) 293 cells that stably express the IL-1 receptor (termed IL-1R cells, a gift from Tularik Inc., USA) and mouse embryonic fibroblasts from TAK1^{+/+} and TAK1^{-/-} mice [16] were cultured in 10 cm² dishes in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% (v/v) foetal calf serum (FCS). The HEK 293 cells were transfected with DNA vectors mixed with polyethylenimine [17], while mouse embryonic fibroblasts were transfected with the Amaxa MEF2 kit according to the manufacturer's instructions. RAW 264.7 cells were maintained in RPMI 1640 medium (Invitrogen) supplemented with 100 U/ml penicillin and 100 μ g/ml streptomycin, 1 mM sodium pyruvate, 2 mM L-glutamine and 10% heat-inactivated foetal bovine serum (FBS). Transient transfection of the RAW 264.7 cell line was achieved by electroporation. For this, cells were harvested, washed twice in Opti-MEM (Invitrogen) and resuspended at 4 x 10⁷ cells/ml in Opti-MEM. 0.5 ml of cell suspension was then mixed with plasmid DNA and the cell-

DNA mixture added to a 0.4 cm electrode gap electroporation cuvette and shocked in a BioRad GenePulser II (300V, 950 μ FD) at 21°C. Cells were resuspended immediately in 1 ml of pre-warmed growth medium and aliquots (1.6×10^6 cells) added to 6 well plates and treated as described in Results.

For the measurement of NF κ B-dependent luciferase gene expression, cells were lysed in Passive Lysis Buffer (Promega) and luciferase activity measured using a Dual-Luciferase Reporter Assay System (Promega) according to the manufacturer's instructions. Firefly luciferase activity was normalised on the basis of *Renilla* luciferase activity.

Antibodies

Anti-TAK1, the antibody recognising TAB1 phosphorylated at Thr431 [18] and an antibody that recognises TAK1 phosphorylated at Thr187 (raised against the peptide IQTHMT*NNKGS, where T* is phospho-threonine) were raised in sheep. The antibody that recognises TAK1 phosphorylated at Thr187 was affinity purified on a phospho-peptide antigen-agarose column and used for immunoblotting in the presence of 10 μ g/ml of the unphosphorylated peptide immunogen. Antibodies recognising the active phosphorylated forms of JNK, p38 α MAPK and ERK1/ERK2 were from Cell Signaling Technologies, anti-TAK1 from Santa Cruz, anti-HA and anti-myc from Sigma Chemical Company and anti-human IL-1 β from R&D Systems.

Immunoblotting

Cells were extracted in "standard lysis buffer" (50 mM Tris-HCl pH 7.5, 0.1 mM EGTA, 1 mM EDTA, 1% (w/w) Triton X-100, 1 mM Na₃VO₄, 50 mM NaF, 5 mM sodium pyrophosphate, 0.27 M sucrose, 0.1% mercaptoethanol, 'complete' proteinase inhibitor cocktail (Roche). After centrifugation for 15 min at 18,000 x g the supernatant (termed cell extract) was decanted and protein concentrations measured by the Bradford method.

Isolation of peripheral blood mononuclear cells (PBMC) and stimulation with MDP and LPS

PBMC were isolated from buffy coats (provided by the Ninewells Hospital Blood Transfusion Centre, Dundee, Scotland) and diluted with an equal volume of RPMI 1640, 25 mM HEPES, L-Glutamine (Invitrogen). The suspensions were subjected to density centrifugation over Ficoll-Paque (GE Healthcare) and the PBMC fraction was washed three times with RPMI 1640, 25 mM HEPES, L-Glutamine before the cells were plated in the same medium. The cells were then treated as described under Results.

Expression, purification and assay of RIP2

RIP2 was expressed from a baculovirus vector into insect Sf21 cells as a His₆-tagged fusion protein and purified by chromatography on nickel nitrilo-triacetate agarose. The purified RIP2 was diluted in 50 mM Tris/HCl pH 7.5, 0.1% (v/v) 2-mercaptoethanol, 0.1 mM EGTA, 1 mg/ml bovine serum albumin and assayed for 10 min at 30°C in a reaction volume of 50 µl containing 50 mM Tris/HCl pH 7.5, 0.1% (v/v) 2-mercaptoethanol, 0.1 mM EGTA, 1 mg/ml myelin basic protein, 10 mM magnesium acetate, 0.1 mM [γ ³²P]-ATP (50-1000 cpm/pmol). The reactions were initiated by the addition of ATP after incubating the other components for 5 min at room temperature. Reactions were stopped by spotting 40 µl of assay mixture on to 1.5 cm x 1.5 cm square of Whatman P81 paper, which were washed three times in 75 mM phosphoric acid and once in acetone before air drying and measurement of ³²P-radioactivity incorporated by Cerenkov counting.

Results

Catalytically inactive RIP2 is more effective than wild type RIP2 in switching on downstream signaling pathways.

The over-expression of either WT-RIP2 or the catalytically inactive mutant RIP2[D164A] (termed KI-RIP2) in 293 cells induced NF κ B-dependent gene transcription and phosphorylation of the activation loops of p38 α MAPK and JNK1/JNK2 (Fig 1A), as expected from previous studies. However, we noticed that inactive RIP2 was expressed at a far lower level than the wild type protein (Fig 1A), suggesting that the catalytically inactive protein might be even more effective in switching on downstream signaling pathways. In order to investigate this possibility, we therefore decreased the amount of DNA encoding WT-RIP2 that we transfected into the cells until the level of expression of the protein became similar to that of KI-RIP2. These experiments revealed that KI-RIP2 was indeed far more effective than the WT-RIP2 in switching on JNK and p38 α MAPK activation and NF κ B-dependent gene transcription (Fig 1B). For example, transfection of 0.25 μ g of DNA encoding WT-RIP2 induced a 3-4 fold lower activation of NF κ B-dependent gene transcription or phosphorylation of JNK and p38 α MAPK than did transfection of 1 μ g of DNA encoding KI-RIP2, even though the expression of the WT-RIP2 protein was higher than that of the KI-RIP2 protein under these conditions.

The protein kinase activity of RIP2 controls RIP2 stability.

The observation that KI-RIP2 (RIP2[D164A]) (Fig 1A) and another catalytically inactive mutant RIP2[K47A] (results not shown) were expressed at a greatly reduced level compared to WT-RIP2 (Fig 1A) suggested that RIP2 kinase activity might also control the level of expression of the RIP2 protein. However, it remained entirely possible that these amino acid substitutions had affected the translation, stability or degradation of the protein in a way that was unrelated to the loss of protein kinase activity caused by the mutations. In order to distinguish between these possibilities we therefore exploited the protein kinase inhibitor SB 203580. This compound is a relatively specific inhibitor of p38 α MAPK [19, 20], but

was later found to inhibit RIP2 kinase activity *in vitro* with even greater potency ([21], see Fig 2A). We therefore transfected HEK 293 cells with DNA encoding either WT-RIP2 or KI-RIP2 and then exposed the cells to SB 203580. Strikingly, SB 203580 reduced the level of expression of WT-RIP2 to that of the KI-RIP2. Moreover, SB 203580 did not affect the level of expression of the catalytically inactive mutant (Fig 2B).

SB 203580 not only inhibits p38 α MAPK and RIP2, but also two other protein kinases (CK1 and cyclin G-associated protein kinase (GAK)) [21]. It could therefore be argued that the effect of SB 203580 to reduce the level of expression of the RIP2 protein was caused not by the inhibition of RIP2 kinase activity, but by the inhibition of p38 α MAPK, CK1 or GAK. In order to address this possibility we repeated the experiment using BIRB 0796, a much more potent inhibitor of p38 α MAPK than SB 203580 [22, 23], which does not inhibit RIP2 (Fig 2A), CK1 or GAK (L. Cummings, H. McLaughlan and P. Cohen, unpublished results) *in vitro*, even at concentrations 1000-fold higher than those required to inhibit p38 α MAPK. In contrast to SB 203580, BIRB 0796 did not affect the level of expression of wild type RIP2 (Fig 2C), implying that it was the inhibition of RIP2 kinase activity by SB 203580, and not the inhibition of p38 α MAPK, that had caused the expression of RIP2 to decline.

The p38 α MAPK can be largely desensitised to inhibition by SB 203580 by mutating Thr106 to Met, the larger side chain of Met preventing access of SB 203580 to a hydrophobic pocket near the ATP-binding site [24, 25]. Since RIP2 possesses a threonine residue at the equivalent position, it seemed likely that the presence of this residue was critical for the sensitivity of RIP2 kinase activity to the drug. We therefore mutated Thr95 of RIP2 to Met and examined whether SB 203580 was now able to reduce the level of expression of RIP2. These experiments demonstrated that SB 203580 decreased the level wild type RIP2 but not RIP2[T95M] (Fig 2C). Taken together, these results establish that it is the protein kinase activity of RIP2 that maintains the level of expression of activated RIP2 kinase, at least when over-expressed in HEK 293 cells.

SB 203580 and PP2, but not BIRB 0796, suppresses MDP-NOD2-induced activation of NF κ B in HEK 293 cells and RAW 264.7 cells.

In order to investigate whether the endogenous RIP2 kinase activity is required for MDP-induced signaling, we initially performed experiments in HEK 293 cells. Although these cells do not express NOD2 and therefore do not respond to MDP, sensitivity to MDP can be conferred by transfection with DNA encoding NOD2 [26]. When relatively large amounts of NOD2 DNA (10 ng per 10 cm² dish) were transfected, NF κ B-dependent reporter gene expression was activated but little further stimulation by MDP could be detected (Fig 3A), presumably because NOD2 possesses some intrinsic ability to signal, even in the absence of MDP, which in over-expression experiments is sufficient to activate the pathway. However, when the amount of NOD2 DNA transfected was reduced to 1 ng, there was little activation of NF κ B transcription in the absence of MDP, but MDP could now induce a 5- to 10-fold increase in NF κ B-dependent gene transcription (Fig 3A).

We found that PP1 and PP2, which are potent inhibitors of Src family protein kinases, are even more potent inhibitors of RIP2. The IC₅₀ value for inhibition of RIP2 in vitro was 10-20 nM for PP2 (Fig 3B) and PP1 (data not shown), about 2-fold lower than the IC₅₀ for inhibition of Src and the related Lck (data not shown). These observations led us to study the effect of PP2, as well as SB 203580, on MDP-induced signaling. Both of these compounds, but not BIRB 0796, suppressed MDP-stimulated NF κ B gene transcription (Fig 3C), indicating that this signaling pathway requires RIP2 kinase activity. In contrast, neither SB 203580 nor PP2 inhibited IL-1 β -stimulated NF κ B gene transcription in HEK 293 cells that stably express the IL-1 receptor (termed IL-1R cells) (Fig 3D) or I κ B degradation (Fig 3E), indicating that the effects of SB 203580 and PP2 are specific for MDP-signaling and that RIP2 kinase activity is not rate limiting for IL-1-stimulated NF κ B gene transcription or I κ B degradation. Moreover, at the concentrations used in these experiments, SB 203580 and BIRB 0796, but not PP2, attenuated the phosphorylation of TAB1 (Fig 3E), a

well authenticated physiological substrate of p38 α MAPK [18]. Thus the effects of SB 203580 and PP2 are not mediated by the inhibition of p38 α MAPK.

To investigate whether SB 203580 inhibits MDP signaling in a cell that normally expresses NOD2 and RIP2, we carried out further experiments in RAW 264.7 cells, a transformed mouse macrophage cell line. In these cells, MDP (presumably acting through the endogenous NOD2 and RIP2) stimulated NF κ B-dependent reporter gene transcription, which was suppressed by SB 203580, but not by BIRB 0796 (Fig 3F). In contrast, LPS-stimulated NF κ B-dependent gene transcription was unaffected by either SB 203580 or BIRB 0796 (Fig 3F).

TAK1 is required for signaling downstream of NOD2 and RIP2.

The protein kinase TAK1 is known to play an essential role in mediating the LPS-induced activation of NF κ B, JNK and p38 α MAPK and the production of pro-inflammatory cytokines in vivo [16, 27-29], raising the question of whether TAK1 was also essential for MDP-NOD2/RIP2 signaling to NF κ B and MAP kinases. Consistent with the potential involvement of TAK1, this protein kinase has been reported to interact with NOD2 in co-transfection experiments [30]. These investigators also reported that the MDP-induced activation of NF κ B-dependent gene expression was suppressed by the over-expression of a catalytically inactive “dominant negative” form of TAK1. Moreover, they also observed that the over-expression of NOD2 inhibited TAK1-induced NF κ B activation in RIP2-deficient fibroblasts. Based on these experiments, they concluded that the NOD2 signaling pathway antagonises TAK1-mediated signaling and vice versa [30].

We have confirmed the previous report that NOD2 interacts with TAK1 (Fig 4A) and, in addition, found that RIP2 interacts with TAK1 in co-transfection experiments (Fig 4B). This interaction between RIP2 and TAK1 is likely to be direct and not indirect via NOD2, because HEK 293 cells do not express NOD2. Importantly, we also found that the endogenous TAK1 (measured by the phosphorylation of Thr187 [31]) and JNK were activated by transfection with DNA encoding either WT-RIP2 or KI-RIP (Fig 4C) and that a catalytically inactive TAK1

mutant suppressed the RIP2-induced phosphorylation (activation) of endogenous JNK and p38 α MAPK (Fig 4D). Therefore, in contrast to the conclusions reached earlier [30], our experiments suggested that TAK1 was not a negative regulator of NOD2-RIP2 signaling, but instead might be essential for signaling through this pathway. In order to investigate whether this was the case, we first reduced the level of expression of TAK1 by 80-90% in HEK 293 cells by introducing a vector expressing a short hairpin RNA (Fig 5A). This suppressed by 65-70% the activation of NF κ B induced by either the over-expression NOD2- or RIP2 (Fig 5B) or by MDP in the presence of low levels of transfected NOD2 (Fig 5C). In parallel experiments RIP2-induced JNK activation was similarly reduced by shRNA knockdown of TAK1 (results not shown).

In order to confirm these results by an independent method, we studied the effect of 5Z-7-oxo-zeaenol, which has been reported to be a potent and relatively specific inhibitor of TAK1 [32]. These experiments showed that this compound suppressed MDP-stimulated NF κ B gene transcription by about 70% when included in the cell culture medium at 1 μ M (Fig 5D). We found that this concentration was needed to completely suppress IL-1-stimulated activation of JNK, p38 α MAPK and ERK1/ERK2 in IL-1R cells (data not shown).

We then studied the effects of NOD2 and RIP2 on NF κ B gene transcription in immortalised embryonic fibroblasts from wild type mice and TAK1^{-/-} mice. These experiments showed that transfection with DNA expressing either NOD2 or RIP2 stimulated NF κ B-dependent gene transcription in the wild type cells, but no stimulation occurred in the TAK1-deficient cells (Fig 5E).

MDP-induced production of interleukin-1 β in peripheral blood mononuclear cells (PBMC) is prevented by specific inhibition of TAK1, p38 α MAPK or MKK1/MKK2.

The experiments described above indicated that TAK1 represents an important point of convergence of the LPS-TLR4 and MDP-NOD2 signaling pathways, suggesting that many subsequent downstream events might be common to both pathways. We therefore investigated the signaling pathways required for the MDP-

induced production of the pro-inflammatory cytokines IL-1 β and TNF α in human PBMC, a more physiological system in which the cells are known to respond particularly well to this agonist [8].

To examine whether TAK1 activity is required for signaling by MDP in human PBMC, we studied the effect on the TAK1 inhibitor 5Z-7-oxo-zeaenol. When added to the culture medium at 1 μ M, this compound prevented both the MDP-stimulated and LPS-stimulated secretion of IL-1 β (Fig 6A) and TNF α (Fig 6B) and the MDP- or LPS-induced production of pro-IL-1 β (Fig 6C) in human PBMC.

Two protein kinases activated downstream of TAK1 and required for the LPS-induced production of a number of pro-inflammatory cytokines are p38 α MAPK [20] and tumour progression locus 2 (Tpl2, also called COT) [33]. The p38 α MAPK activates MAPK-activated protein kinase 2 (MAPKAP-K2), which is required for the LPS-induced production of several pro-inflammatory cytokines [34], while Tpl2 activates the MAP kinase kinases MKK1 and MKK2 and hence ERK1/ERK2 [33]. We found that, as for LPS, MDP induced the activation of ERK1/ERK2 within 60 min in human PBMC (Fig 7A) and that this was prevented by PD 184352 (Fig 7B), a potent and specific inhibitor of the MKK1/MKK2 [35, 36]. PP2, a potent inhibitor of RIP2 (Fig 3B), suppressed the MDP-induced, but not LPS-induced activation of ERK1/ERK2, indicating that RIP2 kinase activity is required for MDP signaling to ERK1/ERK2 in human PBMC (Fig 7C).

Like LPS, MDP stimulated the secretion IL-1 β and TNF α in human PBMC, and this was suppressed by PD 184352 or the p38 α MAPK inhibitor BIRB 0796 (Figs 8A and 8B). The BIRB 0796 was used at a concentration of 0.1 μ M, at which it inhibits p38 α / β MAPKs specifically, but does not inhibit RIP2 (Fig 2A) or many other protein kinases tested [22]. PD 184352 (Fig 8C) or BIRB 0796 (Fig 8D) also prevented the MDP- or LPS-induced expression of pro-IL-1 β , demonstrating that these drugs exert their effects by blocking pro-IL-1 β expression, rather than just IL-1 β secretion.

It should be mentioned that PBMC from different human donors showed large quantitative differences in the amount of IL-1 β or TNF α produced in response to MDP or LPS, although sensitivity to BIRB 0796 and PD 184352 was unaltered. However, in two donors, MDP-stimulated production of IL-1 β in PBMC was only inhibited 50% and 70%, respectively, by BIRB 0796 and 90% and 70%, respectively, by PD 184352, whereas in other donors these inhibitors caused almost complete suppression (Christine Lang, unpublished experiments).

Discussion

At the time RIP2 was first identified it was noticed that, when over-expressed in HEK 293 cells, a catalytically inactive (KI, “kinase inactive”) mutant was as effective as the wild type protein in activating JNK or NF κ B [10] or inducing the apoptosis of MCF7 breast sarcoma cells [11]. These observations raised the question of what role the protein kinase activity of RIP2 might have. Here, we demonstrate that KI-RIP2 is much more effective than WT-RIP2 in activating JNK and NF κ B in transfected HEK 293 cells (Figs 1 and 2), indicating that the protein kinase activity functions to limit the strength of signaling under these conditions. The molecular events that underlie this action are unknown, but since HEK 293 cells do not express NOD2, it cannot be explained by RIP2 exerting a negative feedback control on NOD2. Thus mechanisms involving a RIP2 kinase-catalysed enhancement of the interaction of NOD2 with an inhibitory protein, such as Erbin [37, 38], appear to be excluded. RIP2 is known to phosphorylate itself at multiple sites [39], raising the possibility that the autophosphorylated protein kinase is less effective than the unphosphorylated form of the enzyme in activating TAK1 and/or other proteins that drive “downstream” signaling events.

We have also shown that the protein kinase activity of RIP2 is required to maintain its level of expression in transfected HEK 293 cells. Thus KI-RIP2 was expressed at much lower levels than WT-RIP2, while pharmacological inhibition with

SB 203580 (Figs 2B and 2C) or PP2 (data not shown) reduced the expression of WT-RIP2 to a level similar to that of KI-RIP2, without affecting the level of expression of KI-RIP2. Moreover, SB 203580 had a much smaller effect on the expression of a RIP2 mutant that is less sensitive to SB 203580 (Fig 2C). Interestingly, the SB 203580-induced decrease in expression of RIP2 is quite fast, being complete within 30-60 min, but preliminary experiments indicate that this cannot be prevented by either the proteasome inhibitor MG 132, or a specific inhibitor of caspase 1 (M. Windheim and P. Cohen, unpublished results), a proteinase that interacts with and is activated by NOD2 [40]. Thus the mechanism that leads to the disappearance of activated RIP2 has still to be clarified.

Importantly, we found that the MDP-stimulated activation of NF κ B gene transcription in NOD2-transfected HEK 293 cells or in untransfected RAW 264.7 cells was prevented by SB 203580 or PP2, but not by the much more potent p38 α MAPK inhibitor BIRB 0796, which does not inhibit RIP2 (Fig 3). Our working hypothesis is that MDP induces the active conformation of RIP2, which then phosphorylates itself or another regulatory protein, leading to stabilisation of the activated enzyme. Pharmacological inhibition of RIP2 kinase prevents this stabilisation and the MDP-induced downstream signaling does not occur because the level of expression of RIP2 falls below the threshold needed for signaling to occur. We assume that KI-RIP2 is able to trigger MDP-dependent NF κ B gene transcription in transfected cells, because it is expressed at supra-physiological levels under these conditions, well in excess of those needed to support signaling. To our knowledge, the present study represents the first evidence that the protein kinase activity of RIP2 is required for signaling “downstream” of MDP-NOD2. Interestingly, knock-in mice that express the catalytically inactive RIP2[K47A] mutant instead of WT-RIP2 also exhibit decreased expression of the mutant enzyme [41]. It would clearly be of considerable interest to know whether MDP is capable of stimulating NF κ B-dependent gene transcription and pro-inflammatory cytokine production in these animals, but this was not investigated in the published study [41].

In contrast to the inhibition of MDP-stimulated NF κ B gene transcription by SB 203580 and PP2, LPS-stimulated and IL-1 stimulated NF κ B reporter gene transcription in RAW 264.7 (Fig 3F) and IL-1R cells (Fig 3D), respectively, were unaffected by SB 203580 and PP2. Moreover, PP2 suppressed MDP-induced, but not LPS-induced activation of ERK1/ERK2 in human PBMC (Fig 7C) Thus, although LPS stimulates RIP2 kinase activity [39, 41], RIP2 kinase activity is not rate limiting for LPS-induced activation of NF κ B and ERK1/ERK2. Other investigators also reported that RIP2 was not required for signalling through LPS-TLR4 or other TLRs, while this paper was undergoing revision [42].

TAK1 is thought to play an essential role in the LPS-induced activation of signaling pathways and the production of pro-inflammatory cytokines [16, 27-29] and, in this paper, we demonstrate that TAK1 is also required for MDP-NOD2/RIP2 signaling (Figs 4, 5) and proinflammatory cytokine production (Fig 6). These results imply that TAK1 represents one of the key points at which the LPS-TLR4 and MDP-NOD2 signaling pathways converge (Fig 9), explaining why “downstream” events, such as the activation of NF κ B, JNK, p38 α MAPK and ERK1/ERK2, and the production of IL-1 β and TNF α are common to both pathways (Figs 4-8). Consistent with a key role for TAK1, it has been reported that the over-expression of NOD2 or RIP2 activates IKK β by inducing the phosphorylation of Ser177 and Ser 181 [43], the sites phosphorylated by TAK1. Moreover, as for LPS, the activation of p38 α MAPK and ERK1/ERK2 are both required for the MDP-induced production of Pro-IL-1 β , secreted IL-1 β or secreted TNF α in human PBMC (Fig 8), which express endogenous levels of NOD2 and RIP2.

RIP2 is also known to be required for the signalling by M-Tri_{DAP}-NOD1 (see Introduction). During the revision stage of this paper, other investigators reported that dominant negative TAK1 and siRNA knockdown of TAK1 reduced M-Tri_{DAP}-NOD1-induced activation of JNK and IL-8 secretion [44]. This study indicates that TAK1 lies downstream of RIP2 in the NOD1 as well as the NOD2 signaling pathway.

A major outstanding question concerns the mechanism by which RIP2 triggers the activation of TAK1 and other “downstream” signaling events. The activation of TAK1 and IKK β by TNF α , IL-1 β or LPS is thought to be initiated by the formation of Lys63-linked polyubiquitin (K63-pUb) chains that become linked covalently to the protein kinases RIP [45, 46] and the E3 ligase TRAF6 [47]. These K63-pUb scaffolds interact with the NEMO regulatory subunit of the IKK complex and the TAB2/TAB3 regulatory subunits of the TAK1 complex, which may co-localise TAK1 and IKK β and so facilitate the TAK1-catalysed phosphorylation and activation of IKK β . The catalytic domain of RIP is most similar to RIP2, and like RIP2, the catalytic activity of RIP does not appear to be required for signalling by TNF α [48], raising the question of whether RIP2 might also trigger downstream signaling by a mechanism that requires the Lys63-linked polyubiquitination of one or more proteins. Indeed, it has been reported that NEMO undergoes Lys63-linked polyubiquitination when co-transfected with either NOD2 or RIP2 in HEK 293 cells [43]. Moreover, these effects were suppressed by co-transfection with vectors expressing the Lys63-specific deubiquitinating enzyme CYLD [43]. These observations have provided the first indication that Lys63-linked polyubiquitination of NEMO may be critical for the NOD2/RIP2-induced activation of IKK β , which can lead to the activation of ERK1/ERK2 [49, 50], as well as the activation of NF κ B. However, as the NEMO-IKK β complex does not participate in the activation of p38 α MAPK or JNK (Fig 8), it seems likely that additional devices are needed to mediate the NOD2/RIP2-induced activation of these MAPKs.

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Figure legends

Figure 1. RIP2 activates JNK, p38 α MAPK and NF κ B-dependent gene transcription in transfected HEK 293 cells.

(A) Cells were co-transfected with 1 μ g of plasmid RIP2 DNA encoding either HA-tagged wild type (WT) or kinase-inactive (KI) RIP2 and 0.1 μ g of DNA encoding the 3x κ B luciferase reporter construct (see Methods). After 48 h, the cells were extracted in Passive Lysis Buffer (Promega) and NF κ B-dependent luciferase reporter gene expression was measured (uppermost panel). In identical experiments carried out in parallel, the cells were extracted with standard cell lysis buffer and the extract (20 μ g protein) immunoblotted with anti-HA antibodies to detect the HA-tagged RIP2 and with phospho-specific antibodies that recognise the active phosphorylated forms of JNK1 and JNK2 (p-JNK1/2) and p38 α MAPK (p-p38 α MAPK). The extracts were also immunoblotted with antibodies that recognise glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as a loading control. The error bars in the upper panel and results in the lower panel show the standard deviation between duplicate experiments.

Similar results were obtained in several other experiments. (B) The experiment was carried as in A, except that the amounts of plasmid DNA encoding HA-tagged WT-RIP2 were varied as indicated.

Figure 2. The RIP2 inhibitor SB 203580 decreases the protein expression levels of WT-RIP2 but not KI-RIP2 in transfected HEK 293 cells.

All experiments were carried out at least twice with similar results. (A) Effect of SB 203580 and BIRB 0796 on the activity of RIP2 in vitro. RIP2 was assayed as described under Experimental at the indicated concentrations of SB 203580 or BIRB 0796. The IC_{50} value for inhibition by SB 203580 was 43 nM. (B) Cells were transfected with DNA constructs encoding HA-tagged versions of either WT-RIP2 or KI-RIP2 (1 μ g of plasmid RIP2 DNA). After 24 h the cells were incubated for a further 24 h with (+) or without (-) 10 μ M SB 203580, then extracted with standard lysis buffer. The level of transfected RIP2 in the extract was analysed by immunoblotting with an anti-HA antibody. (C) Cells were transfected for 48 h with a construct encoding HA-tagged WT-RIP2 or RIP2[T95M] (1 μ g of plasmid RIP2 DNA). The cells were incubated with 10 μ M SB 203580 or 0.1 μ M BIRB 0796 for 1 h before the end of transfection period, lysed and 20 μ g of extract protein was analysed by immunoblotting with an anti-HA antibody. The extracts were also immunoblotted with antibodies that recognise GAPDH as a loading control.

Figure 3. The RIP2 kinase inhibitors SB 203580 and PP2 suppress MDP-NOD2-induced activation of NF κ B

All experiments were performed in duplicate and similar results were obtained in at least two independent experiments. (A) HEK 293 cells were co-transfected with 0.1 μ g of DNA encoding 3x κ B luciferase reporter construct and 0.1 μ g pTK-RL plasmid DNA (encoding *Renilla* luciferase) plus 0.8 μ g of empty pCMV5 vector (to bring the final amount of vector to 1.0 μ g as used for all experiments) and the indicated amounts of plasmid DNA encoding HA-tagged NOD2. After 24 h, cells were

incubated without (unfilled bars) or with (filled bars) 1 μ M MDP. 24 h later, the cells were lysed and NF κ B-dependent luciferase reporter gene expression was analysed. The error bars show the standard deviation between duplicate experiments. (B) Effect of PP2 on the activity of RIP2 in vitro. RIP2 was assayed as described under Experimental at the indicated concentrations of PP2. The IC₅₀ value for inhibition by PP2 was 10 nM. (C) Experiments were performed as in (A) using 1 ng DNA encoding HA-NOD2 for transfection. 1 h before stimulation with (filled bars) or without (unfilled bars) MDP the cells were incubated without (control) or with 10 μ M SB 203580, 0.1 μ M BIRB 0796 or 1 μ M PP2. The error bars show the standard deviation between duplicate experiments. (D) 293 IL-1R cells were transfected with the 3x κ B luciferase reporter construct and pTK-RL plasmid DNA as in (A), treated with the indicated inhibitors as in (C) and stimulated with 5 ng/ml IL-1 β instead of MDP. The error bars show the standard deviation between duplicate experiments. (E) 293 IL-1R cells were incubated without (control) or with 10 μ M SB 203580, 0.1 μ M BIRB 0796 or 1 μ M PP2 1 h before stimulation with 5 ng/ml IL-1 β for 20 min. Cells were lysed and 20 μ g of extract protein was analysed by immunoblotting with antibodies recognizing phosphorylated TAB1 (pThr431) and p38 α MAPK as well as I κ B α and GAPDH as a loading control (F) RAW 264.7 cells were transiently co-transfected via electroporation with 50 μ g of DNA encoding 3x κ B luciferase reporter construct and 5 μ g pTK-RL plasmid DNA (encoding *Renilla* luciferase) (see legend to Fig 1A). Cells were left to recover for 4 h in RPMI 1640 supplemented with 10% heat-inactivated foetal bovine serum (FBS), and the medium replaced with RPMI 1640 without FBS. The cells were then incubated for 60 min with (+) or without (-) 5 μ M SB 203580 or 0.1 μ M BIRB 0796, then stimulated with (+) or without (-) 1 μ M MDP or 100 ng/ml LPS or left untreated. After 24 h, the cells were extracted in 0.5 ml of Passive Lysis Buffer (Promega) and 20 μ l aliquots of the extract were analysed for NF κ B-dependent luciferase reporter gene expression. The results are given as standard deviation for three different experiments each performed in duplicate.

Figure 4. RIP2 and NOD2 interact with and activate TAK1.

(A) HEK 293 cells were co-transfected for 48 h with 3.75 μ g of plasmid DNA encoding GST-tagged NOD2 (or GST as a control) and 3.75 μ g of DNA encoding myc-tagged WT-TAK1 or an empty vector (as a control) and then lysed. An aliquot of the cell extract (0.5 mg protein) was added to 20 μ l of glutathione Sepharose beads and, after 1 h incubation at 4°C, the beads to which GST-NOD2 or GST were now bound were collected (GST-pull down (PD)), washed once with 1 ml of lysis buffer, twice with 1 ml of lysis buffer containing 500 mM NaCl and once with 1 ml 10 mM Tris-HCl pH 8. After denaturation in 1% (w/v) SDS, the proteins released were subjected to SDS-PAGE and immunoblotted with anti-myc antibodies (IB: myc) to detect TAK1. Further aliquots of the cell extract (20 μ g protein) were also immunoblotted with the antibodies indicated. (B) Same as A, except that HA-RIP2 replaced GST-NOD2 and, after cell lysis, an aliquot of the cell extract (0.5 mg protein) was added to 5 μ g of anti-HA antibody. Following incubation for 45 min at 4°C, 20 μ l of Protein G Sepharose was added, and after another 45 min at 4°C, the HA-immunoprecipitates (IP: HA) were collected, washed as described in A and immunoblotted with anti-myc antibodies (IBM) to detect TAK1. (C) Same as B, except that 7.5 μ g of DNA encoding HA-WT-RIP2 or HA-KI-RIP2 was transfected into HEK 293 cells, and after immunoprecipitation of the TAK1 complex (using antibodies raised against the TAB1 regulatory subunit), followed by washing as in A and immunoblotting was performed with antibodies that recognise TAK1 phosphorylated at Thr187 (p-TAK1) and antibodies that recognise the phosphorylated and unphosphorylated forms of TAK1 equally well (TAK1). Further aliquots of the cell extract (20 μ g protein) were immunoblotted with antibodies that recognise the active phosphorylated forms of JNK1 and JNK2, anti-HA antibodies (to detect RIP2) and anti-GAPDH antibodies as a loading control. (D) HEK293 cells were co-transfected with vectors expressing HA-RIP2 and myc-tagged WT-TAK1 or KI-TAK1. After cell lysis, the extracts were then immunoblotted with the antibodies indicated.

Figure 5. TAK1 is required for NOD2/RIP2 signaling.

All experiments were carried at least twice with similar results. (A) Control HEK 293 cells or cells depleted of TAK1 by transfection with a short hairpin RNA (shRNA) were generated as described under Experimental. The cells were lysed, TAK1 immunoprecipitated as described in the legend of Fig 4 and the pellet extracted in SDS and immunoblotted with anti-TAK1 antibodies. (B) HEK 293 cells were co-transfected with 0.1 μ g of DNA encoding 3x κ B luciferase reporter construct (see Methods), 0.1 μ g pTK-RL plasmid DNA (encoding *Renilla* luciferase) plus 0.8 μ g of empty pCMV5 vector (grey bars) or plasmids encoding GST-NOD2 (filled bars) or HA-tagged WT-RIP2 (unfilled bars). 48 h later, the cells were lysed and NF κ B-dependent luciferase reporter gene expression was analysed. The error bars show the standard deviation between duplicate experiments. (C) Experiments were performed as described in the legend of Fig 3A using 1 ng DNA encoding HA-NOD2 or empty vector DNA for transfection of control HEK 293 cells or cells depleted of TAK1. After 24 h, cells were incubated without (unfilled bars) or with (filled bars) 1 μ M MDP. 24 h later, the cells were lysed and NF κ B-dependent luciferase reporter gene expression was analysed. The error bars show the standard deviation between duplicate experiments. (D) Experiments were performed as in (C). 1 h before stimulation with (+) or without (-) MDP the cells were incubated without (control) or with 1 μ M 5Z-7-oxo-zeaenol, a TAK1 inhibitor. The error bars show the standard deviation between duplicate experiments. (E) Wild type or TAK1^{-/-} mouse embryonic fibroblasts (MEFs) were co-transfected with 0.5 μ g of DNA encoding 3x κ B luciferase reporter construct (see Methods), 0.5 μ g pTK-RL plasmid DNA (encoding *Renilla* luciferase) plus 4 μ g of empty pCMV5 vector (grey bars) or plasmids encoding GST-NOD2 (filled bars) or HA-tagged WT RIP2 (open bars) using the Amaxa Nucleofection MEF2 kit, according to the manufacturer's instructions. 48 h later, the cells were lysed and NF κ B-dependent luciferase reporter gene expression was analysed. The error bars show the standard deviation between duplicate experiments.

Figure 6. The TAK1 inhibitor 5Z-7-oxo-zeaenol suppresses MDP-stimulated production of IL-1 β and TNF α .

Human peripheral blood mononuclear cells were resuspended in RPMI 1640 medium without FCS. Cells were grown in 24 well plates (3×10^7 cells per well) and incubated for 60 min with (+) or without (-) 1 μ M 5Z-7-oxo-zeaenol, then stimulated with 50 nM MDP or 100 ng/ml LPS (+) or left unstimulated (-). After 16 h the cells were pelleted by centrifugation for 2 min at 3000 x g and the supernatants collected. (A, B) Aliquots of the supernatant were diluted appropriately and the concentration of IL-1 β (A) or TNF α (B) determined by ELISA (R&D systems) as described by the manufacturer. The results are presented as the standard deviation for duplicate determinations on one donor. Similar results were obtained in experiments with a second donor. (C) The cell pellet from A was lysed and levels of pro-IL-1 β and mature IL-1 β in the extracts analysed by immunoblotting with an IL-1 β -specific antibody. The results shown are representative of experiments with cells derived from two independent donors.

Figure 7. MDP induces the phosphorylation of ERK1 and ERK2 by activating MKK1. Each results shown is representative of experiments with cells derived from at least two independent donors. Human peripheral blood mononuclear cells (PBMC) resuspended in RPMI 1640 without FCS were kept in 10 cm² dishes and left to recover for 3 h. (A) PBMC were stimulated with (+) or without (-) 50 nM MDP or 100 ng/ml LPS, then lysed at the times indicated. Levels of phosphorylation of ERK1 and ERK2 (pERK1, pERK2) were analysed by immunoblotting with a phospho-specific antibody. (B) The experiment was carried out as in A, except that the PBMC were incubated for 60 min with (+) or without (-) 2 μ M PD 184352, before stimulation with (+) or without (-) 50 nM MDP or 100 ng/ml LPS. After 60 min, cells were lysed and the levels of phosphorylated ERK1/ERK2 were analysed as in (A). (C) The experiment was carried out as in B, except that PBMC were incubated for 60 min with (+) or without (-) 1 μ M PP2 instead of 2 μ M PD 184352, prior to stimulated with MDP or LPS.

Figure 8. MDP-induced secretion of IL-1 β and expression of pro-IL-1 β is prevented by inhibition of MKK1 or p38 α MAPK. Human peripheral blood mononuclear cells were resuspended in RPMI 1640 medium without FCS. (A, B) Cells were grown in 24 well plates (3×10^7 cells per well) and incubated for 60 min with (+) or without (-) 2 μ M PD 184352 or 0.1 μ M BIRB 0796, then stimulated with 50 nM MDP or 100 ng/ml LPS (+) or left unstimulated (-). After 16 h the cells were pelleted by centrifugation for 2 min at 3000 x g and the supernatants collected. Aliquots were diluted appropriately and the concentration of IL-1 β (A) and TNF α (B) determined by ELISA (R&D systems) as described by the manufacturer. The results are presented as the standard deviation for duplicate determinations on one donor. Similar results were obtained in experiments with a second donor. (C, D) Cells were grown in 10 cm² dishes (1×10^8 cells per dish) and incubated for 60 min with (+) or without (-) 2 μ M PD 184352 or 0.1 μ M BIRB 0796, and then stimulated with MDP or LPS as described in A, B. After 16 h, cells were lysed and levels of pro-IL-1 β and mature IL-1 β in the extracts analysed by immunoblotting with an IL-1 β -specific antibody. The results shown are representative of experiments with cells derived from two independent donors.

Figure 9. Signaling pathways required for the MDP- and LPS-induced the production of pro-inflammatory cytokines. The results described in this paper indicate that MDP- and LPS-stimulated signaling converges at the level of TAK1 and that MKK1 and that the activation of MKK1 and p38 α MAPK is required for the production of IL-1 β . The targets for the drugs used in this study (SB 203580, PP2, BIRB 0796, 5Z-7-oxo-zeaenol and PD 184352) are indicated. NF κ B and JNK may promote IL-1 β and TNF α production by stimulating transcription of their genes, as indicated by the arrow with the dashed line. The abbreviations are defined in the text.

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Figure 1

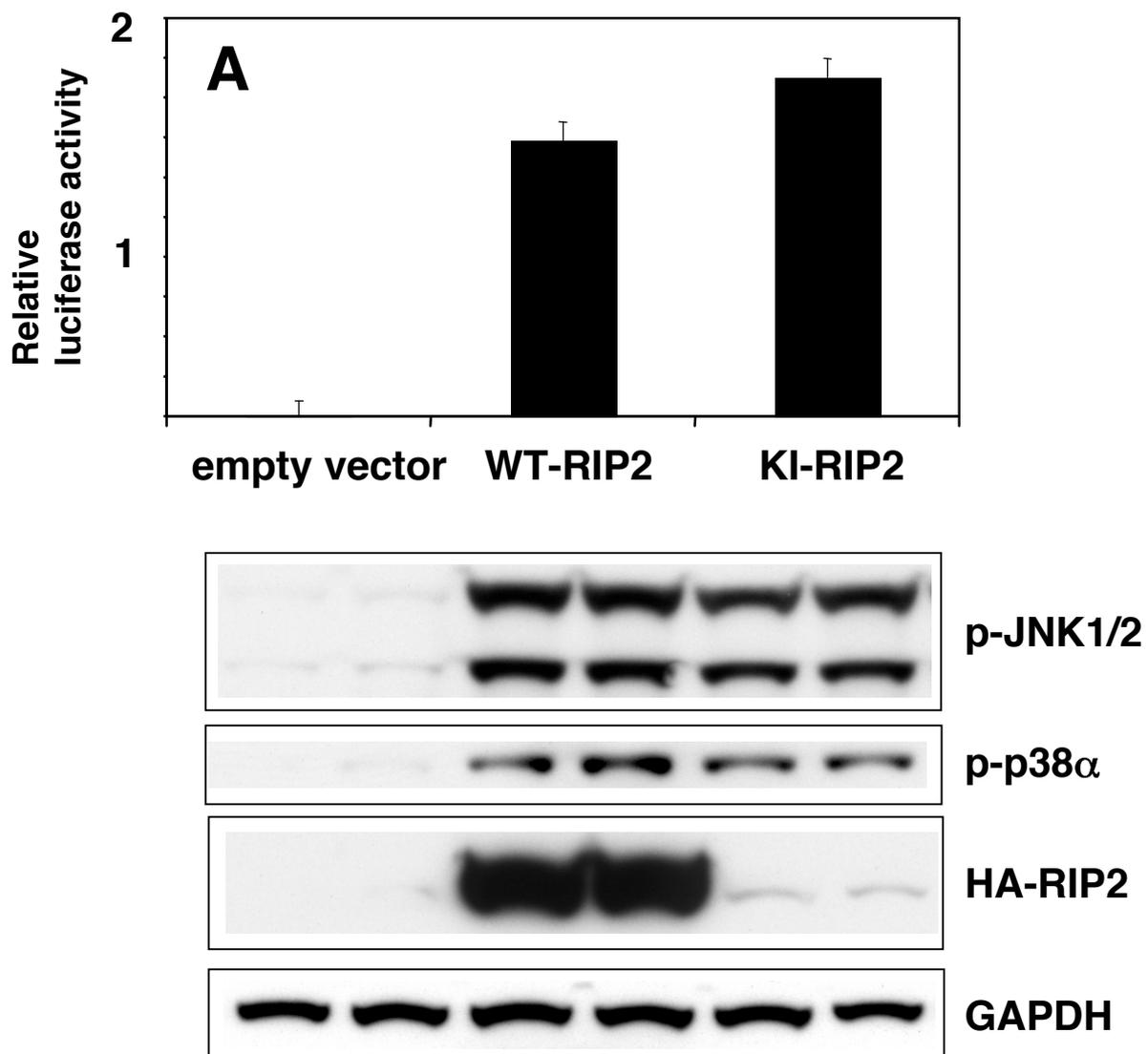


Figure 1

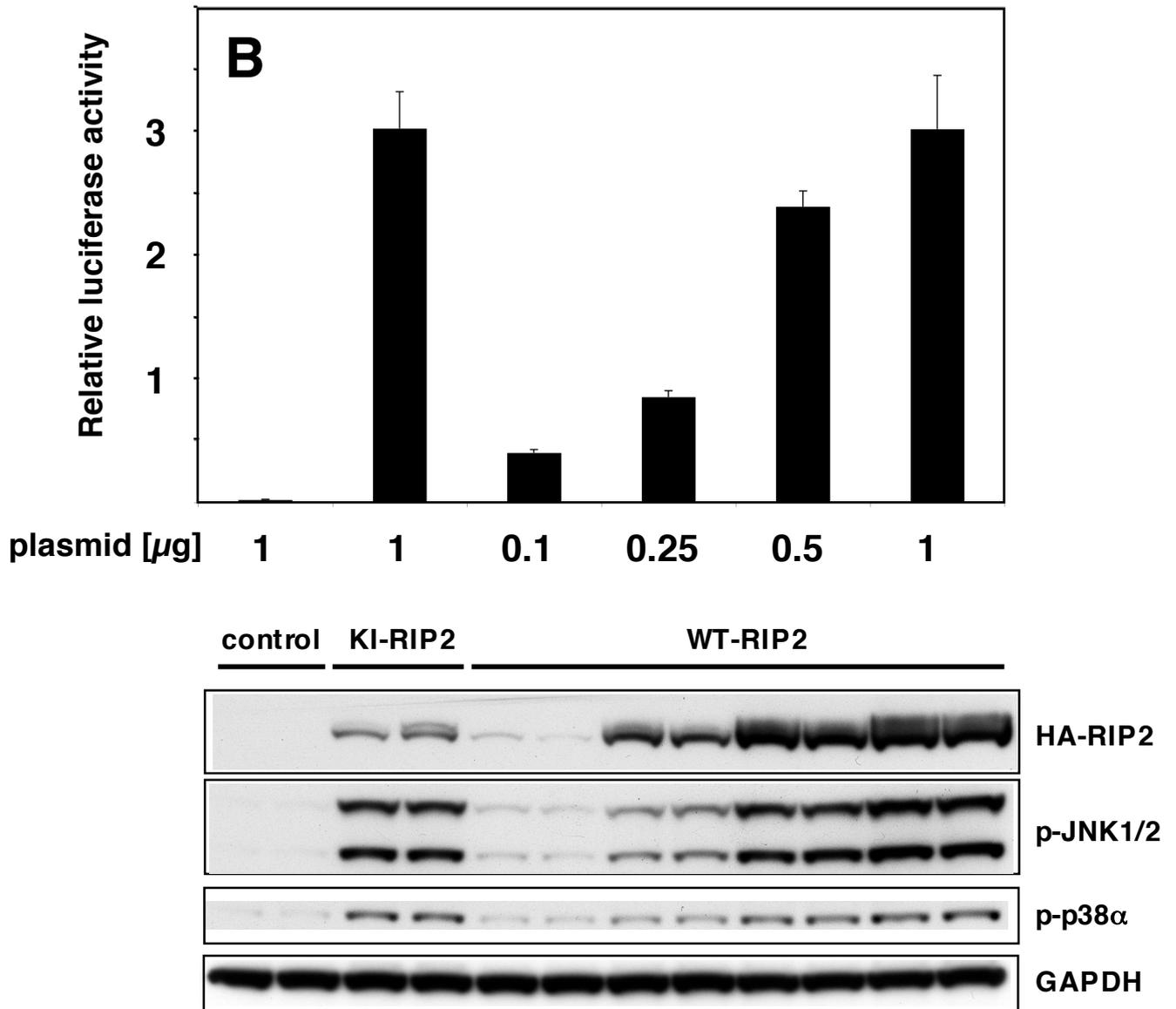


Figure 2

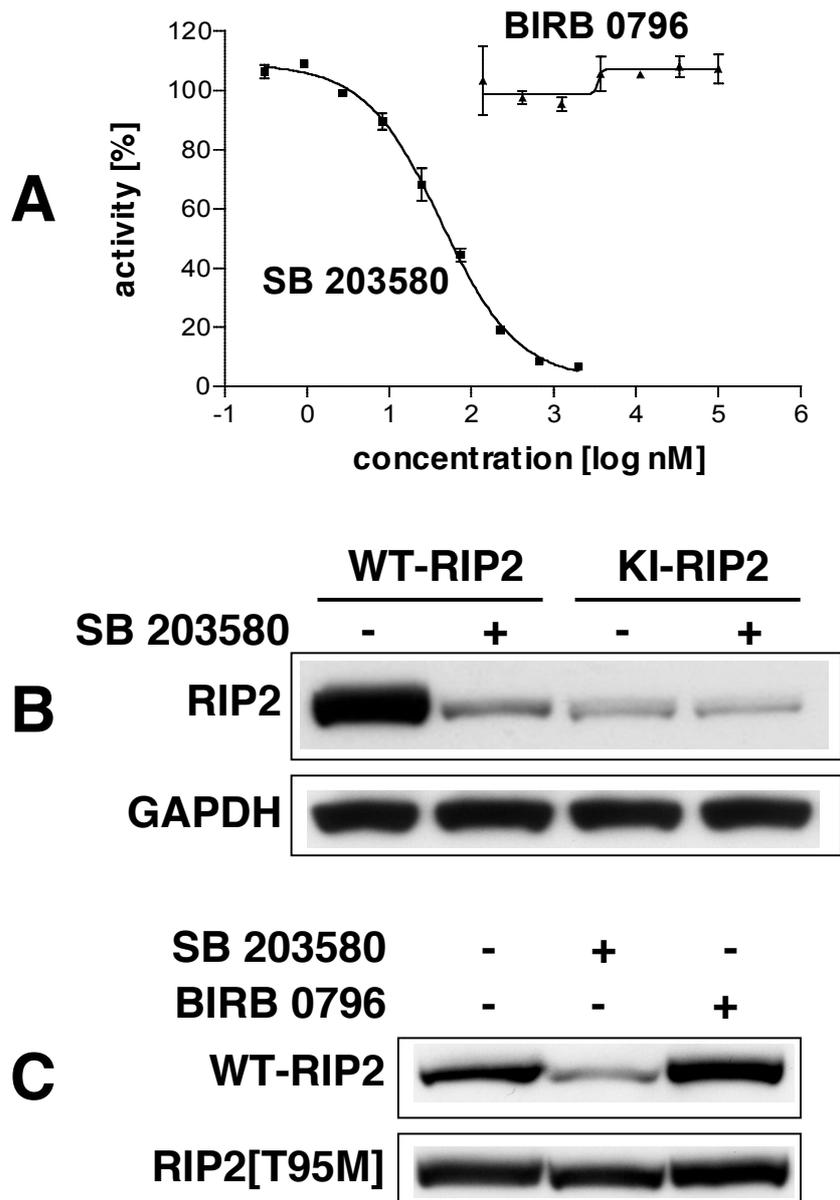


Figure 3

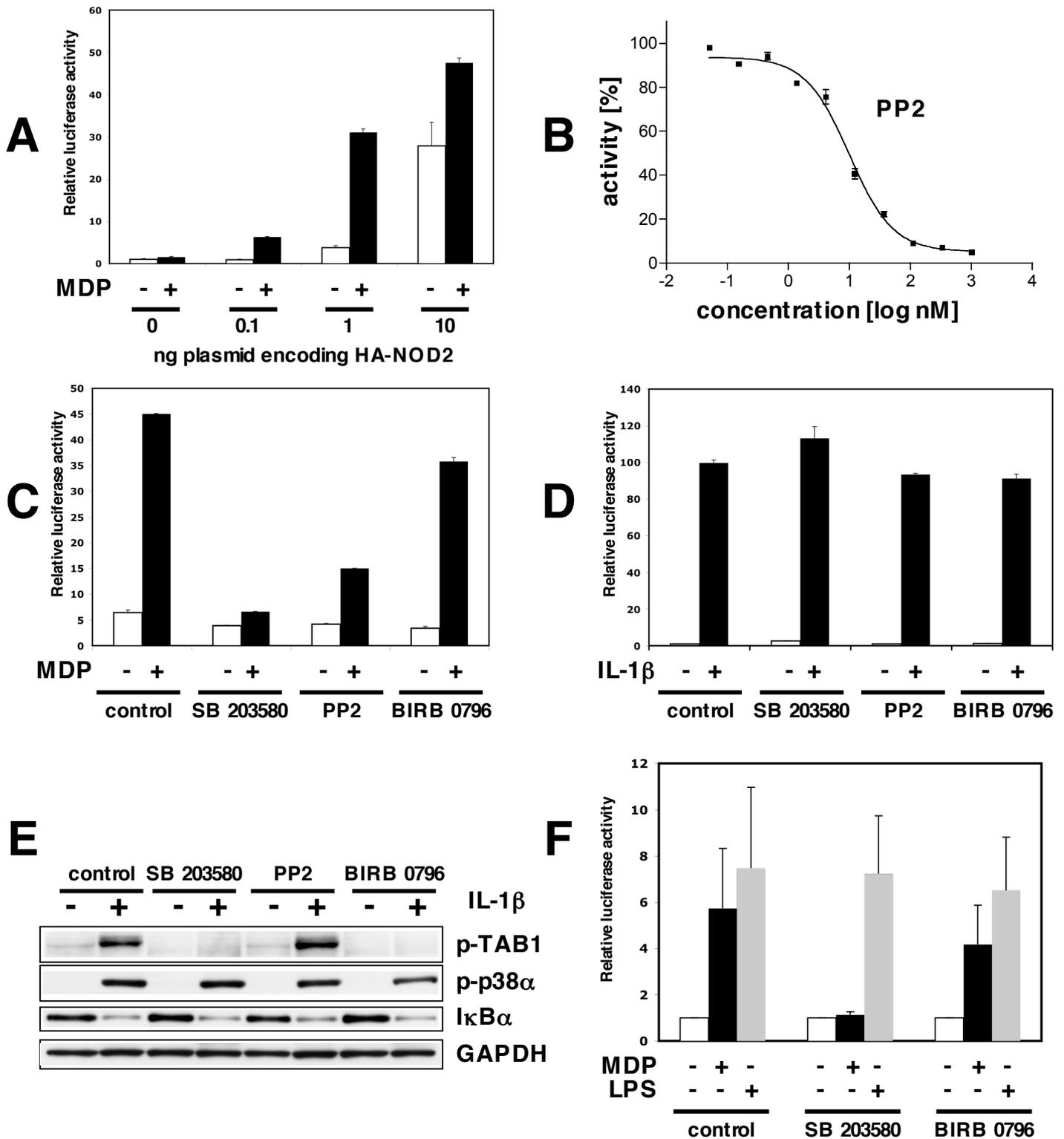


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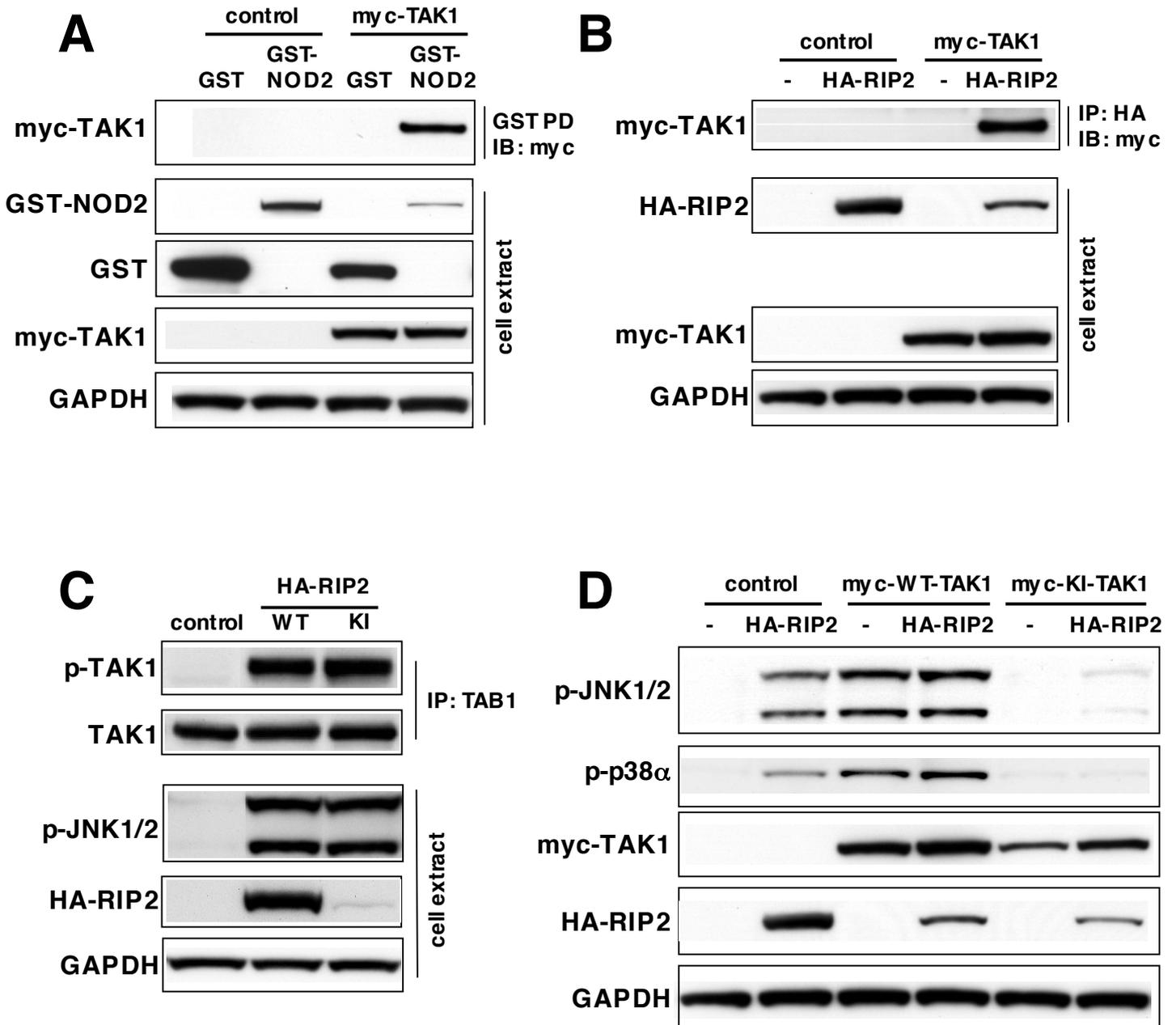


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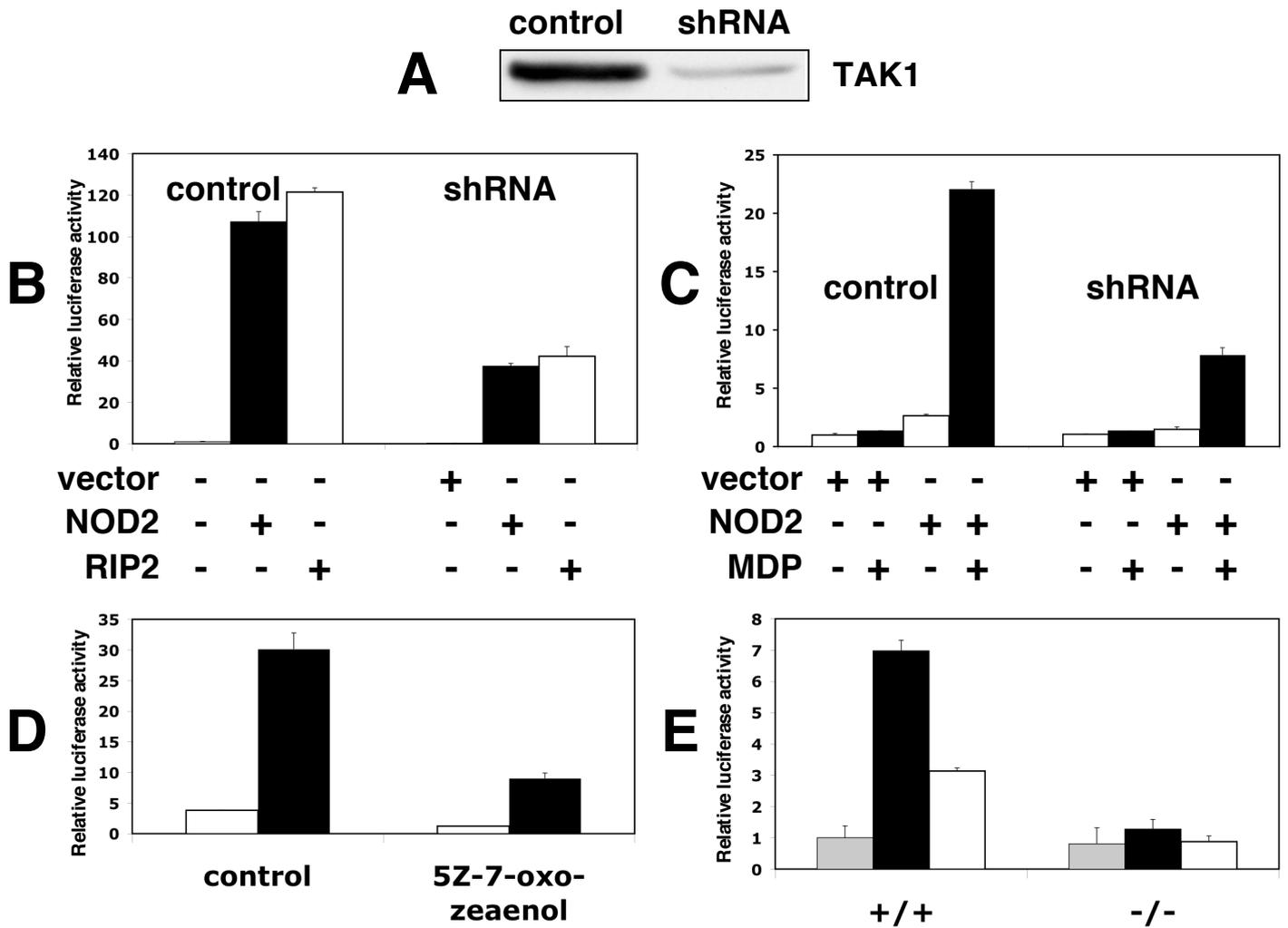


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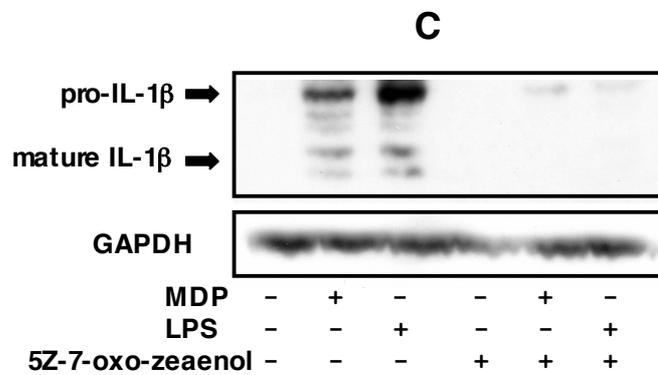
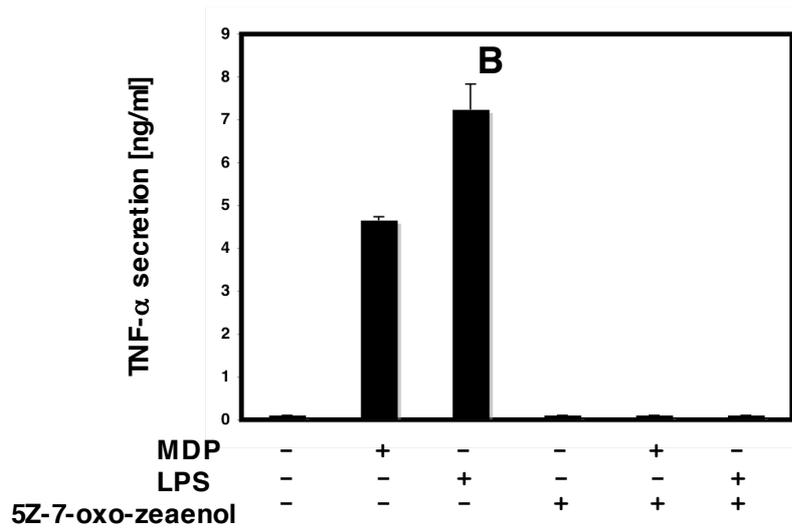
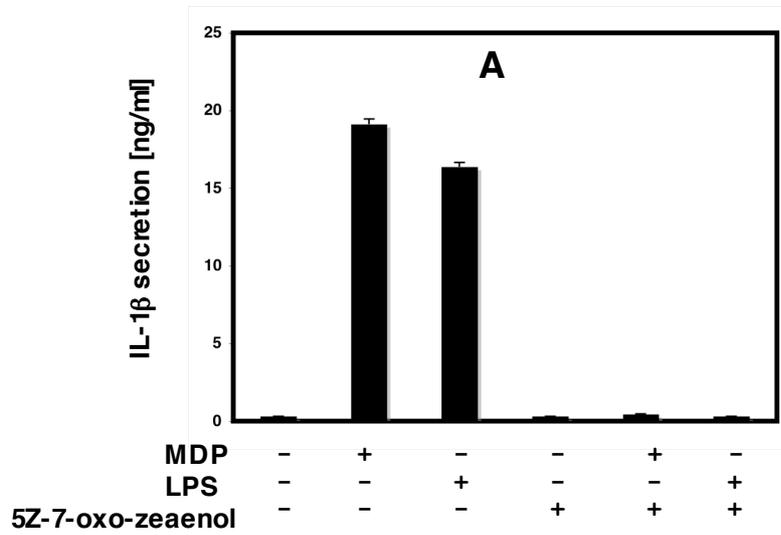
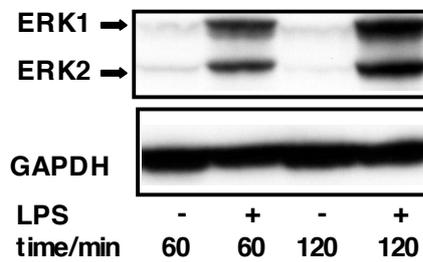
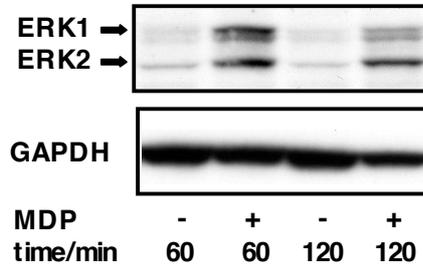
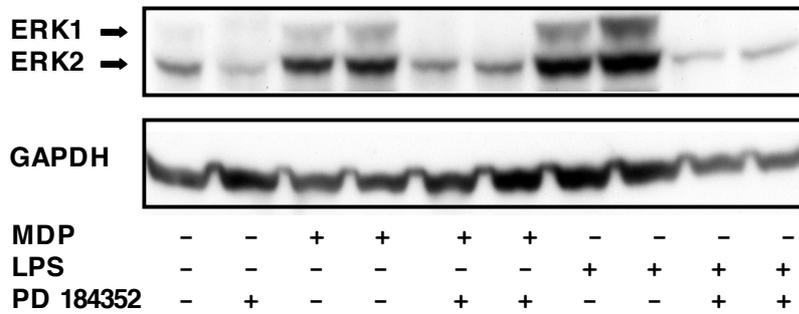


Figure 7

A



B



C

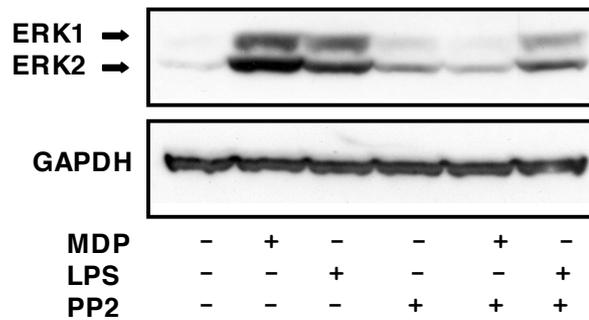


Figure 8

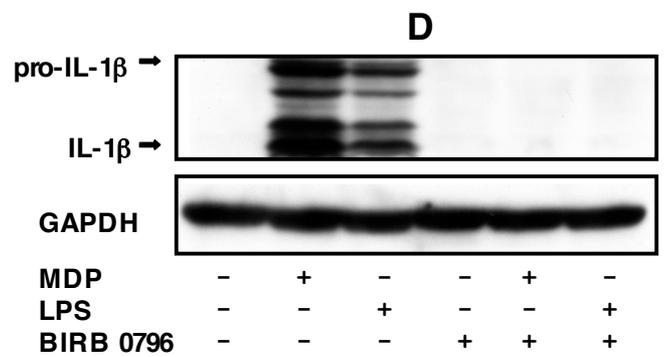
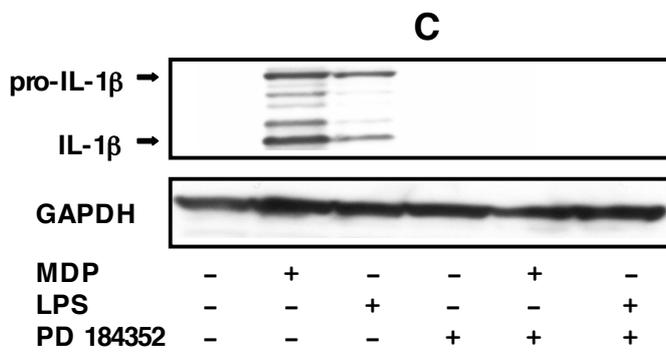
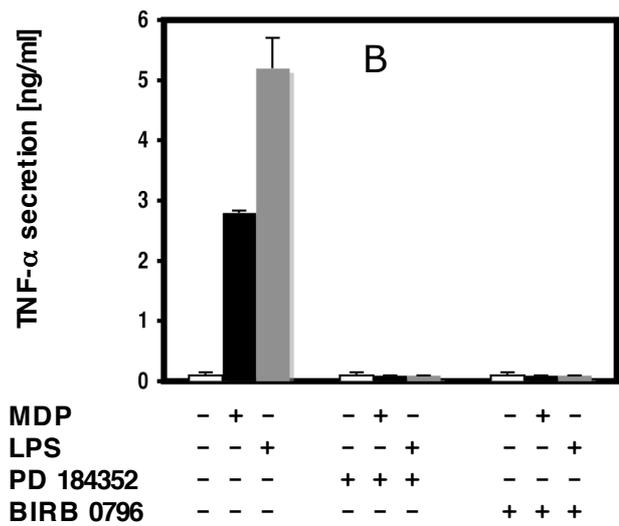
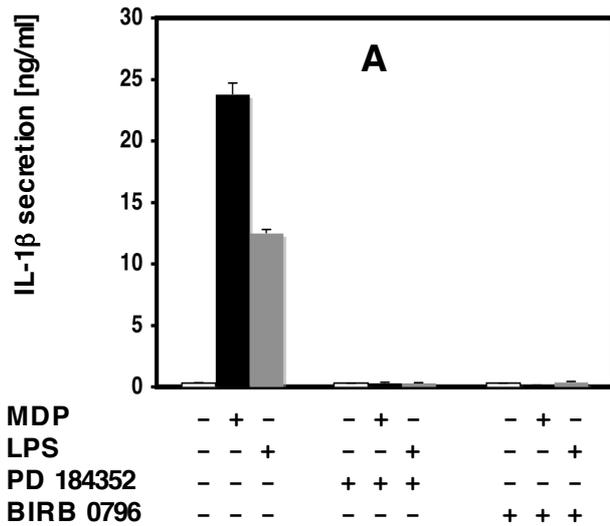


Figure 9

