The *Yersinia enterocolitica* effector YopP inhibits host cell signalling by inactivating the protein kinase TAK1 in the IL-1 signalling pathway

Axel Thiefes^{1*}, Alexander Wolf^{1*}, Anneke Doerrie¹, Guntram A. Grassl², Kunihiro Matsumoto³, Ingo Autenrieth², Erwin Bohn², Hiroaki Sakurai⁴, Rainer Niedenthal⁵, Klaus Resch¹ & Michael Kracht¹⁺

¹Institute of Pharmacology, Medical School Hannover, Hannover, Germany, ²Institute of Medical Microbiology and Hygiene, Eberhard-Karls University, Tübingen, Germany, ³Department of Molecular Biology, Graduate School of Science, Nagoya University, Chikusa-ku, Nagoya, Japan, ⁴Division of Pathogenic Biochemistry, Institute of Natural Medicine, University of Toyama, Toyama, Japan, and ⁵Institute of Biochemistry, Medical School Hannover, Hannover, Germany

The mechanism by which YopP simultaneously inhibits mitogen-activated protein kinase (MAPK) and nuclear factor- κ B pathways has been elusive. Ectopic expression of YopP inhibits the activity and ubiquitination of a complex consisting of overexpressed TGF- β -activated kinase 1 (TAK1) and its subunit TAK1-binding protein (TAB)1, but not of MEK kinase 1. YopP, but not the catalytically inactive mutant YopP_{C172A}, also suppresses basal and interleukin-1-inducible activation of endogenous TAK1, TAB1 and TAB2. YopP does not affect the interaction of TAK1, TAB1 and TAB2 but inhibits autophosphorylation of TAK1 at Thr 187 and phosphorylation of TAB1 at Ser 438. Glutathione S-transferase-tagged YopP (GST-YopP) binds to MAPK kinase (MAPKK)4 and TAB1 but not to TAK1 or TAB2 *in vitro*. Furthermore, YopP in synergy with a previously described negative regulatory feedback loop inhibits TAK1 by MAPKK6-p38-mediated TAB1 phosphorylation. Taken together, these data strongly suggest that YopP binds to TAB1 and directly inhibits TAK1 activity by affecting constitutive TAK1 and TAB1 ubiquitination that is required for autoactivation of TAK1.

Keywords: TAK1; MEKK1; IL-1; YopP; ubiquitination

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INTRODUCTION

Interleukin-1 (IL-1) is a potent proinflammatory cytokine that induces a large number of genes during the inflammatory response (Kracht & Saklatvala, 2002). Key components of IL-1 signalling pathways, therefore, are attractive targets not only for anti-inflammatory therapy but also for anti-inflammatory virulence mechanisms of pathogenic microorganisms. After binding of IL-1 to IL-1 receptor (IL-1R) chains at the cell surface, the adaptor protein tumour necrosis factor (TNF) receptor-associated factor (TRAF)6 is recruited to the cytosolic domains of the activated IL-1R complex. Oligomerized TRAF6 then activates mitogen-activated protein kinase kinase kinase (MAPKKK) protein kinases such as transforming growth factor- β (TGF- β)-activated kinase 1 (TAK1; Ninomiya-Tsuji *et al*, 1999) or MEKK1 (mitogen-activated extracellular-signal-regulated kinase kinase 1; Xia *et al*, 2000). There is compelling evidence derived from experiments using dominant-negative mutants or antisense RNA (Thiefes *et al*, 2005), inhibitors (Ninomiya-Tsuji *et al*, 2003) and RNA interference (Takaesu *et al*, 2003), and from genetic ablation in mice (Sato *et al*, 2005), to suggest that TAK1 is a physiologically relevant MAPKKK in the IL-1 signalling pathway.

Functional TAK1 is a holoenzyme that consists of the catalytic subunit TAK1, the regulatory subunit TAK1-binding protein (TAB)1 and either TAB2 or TAB3 (Takaesu *et al*, 2000; Ishitani *et al*, 2003; Cheung *et al*, 2004). How endogenous TAK1–TAB complexes are activated is not clear, but recent evidence suggests that Lys 63-linked polyubiquitination of TRAF6 provides a docking surface for the ubiquitin-binding domains of TAB2 and TAB3, thereby recruiting TAK1 to the activated IL-1R complex (Wang *et al*, 2001; Kanayama *et al*, 2004). Then, by unknown mechanisms, TAK1 is activated by autophosphorylation at Thr 187 (Singhironunorn *et al*, 2005) and Ser 192 within the kinase activation loop (Kishimoto *et al*, 2000).

On the basis of secondary structure comparisons, the *Yersinia enterocolitica* virulence factor YopP (called YopJ in *Y. pestis* and

¹Institute of Pharmacology, Medical School Hannover, 30625 Hannover, Germany

²Institute of Medical Microbiology and Hygiene, Eberhard-Karls University, 72076 Tübingen, Germany

³Department of Molecular Biology, Graduate School of Science, Nagoya University, Chikusa-ku, Nagoya 464-8602, Japan

⁴Division of Pathogenic Biochemistry, Institute of Natural Medicine, University of Toyama, Toyama 930-0194, Japan

⁵Institute of Biochemistry, Medical School Hannover, 30625 Hannover, Germany

*These authors contributed equally to this work

+Corresponding author. Tel: +49 511 532 2800/2802; Fax: +49 511 532 4081; E-mail: kracht.michael@mh-hannover.de

Y. pseudotuberculosis) was predicted to be a cysteine protease that cleaves ubiquitin or small ubiquitin-related modifier (SUMO) conjugates of cellular proteins (Orth *et al*, 2000). *Y. enterocolitica* lacking YopP are less virulent and can be cleared by the host, demonstrating an *in vivo* immunosuppressive role of YopP (Trulzsch *et al*, 2004; Viboud & Bliska, 2005). YopP is encoded on the virulence plasmid pYV. After being injected by means of a type III secretion system into macrophages or other host cells, YopP inhibits nuclear factor- κ B (NF- κ B) and MAPK signalling simultaneously (Ruckdeschel *et al*, 1997, 1998; Orth *et al*, 1999; Viboud & Bliska, 2005). YopP binds to and deubiquitinates I κ B kinase- β (IKK β) when coexpressed with IKK β together with epitope-tagged ubiquitin (Carter *et al*, 2003). YopP also interacts with MAPK kinase (MAPKK1, MAPKK2, MAPKK3/4 and MAPKK5 (Orth *et al*, 2000). However, there is no evidence that MAPKKs require ubiquitin or SUMO conjugates for activation. Hence, the mechanism by which YopP suppresses several proinflammatory signalling pathways simultaneously has been elusive (Juris *et al*, 2002; Navarro *et al*, 2005). Considering the importance of TAK1 for activation of NF- κ B, c-Jun amino-terminal kinase (JNK) and p38 MAPK in the IL-1 signalling pathway, we set up experiments to explore whether YopP acts at the level of the TAK1 complex. We report here that YopP binds to TAB1 and inhibits endogenous TAK1 activity.

RESULTS AND DISCUSSION

To test the hypothesis of whether YopP affects host cell signalling at the level of the MAPKKK MEKK1 or TAK1, we ectopically expressed a green fluorescent protein (GFP)-tagged YopP fusion protein (GFP-YopP) in HEK293IL-1R (human embryonic kidney 293 stably expressing the IL-1 receptor) cells. GFP-YopP was expressed at a low level, but could be detected after immunoprecipitation (Fig 1A). Induction of the chemokine IL-8 requires parallel activation of NF- κ B and of at least two MAPK pathways (Hoffmann *et al*, 2002). GFP-YopP strongly suppressed transcription of an IL-8 promoter-driven luciferase reporter gene that was activated by: TAK1 coexpressed with its regulatory subunit TAB1; a truncated highly active form of MEKK1, MEKK1 Δ ; or, IL-1 treatment (Fig 1B). Interestingly, MEKK1 Δ immunoprecipitated from YopP-transfected cells was still enzymatically active (Fig 1C, lanes 1,2). Hence, suppression of MEKK1 Δ -dependent IL-8 transcription by YopP, as shown in Fig 1B, must occur by inhibition of NF- κ B and MAPK signalling downstream from MEKK1 Δ , confirming the observation by Orth *et al* (1999). By contrast, the activity of TAK1 immunoprecipitated from YopP-transfected cells was strongly suppressed (Fig 1C, lanes 3,4). *In vitro* phosphorylation of co-immunoprecipitated GFP-TAB1 by GFP-TAK1 was abolished, whereas autophosphorylation of GFP-TAK1 was significantly reduced, and 32 P-GFP-TAK1 showed a faster mobility on SDS-polyacrylamide gel electrophoresis (SDS-PAGE) *in vitro* (Fig 1C, lanes 3,4). As assessed by a specific *in vivo* ubiquitination assay, overexpressed TAK1 and TAB1 were covalently linked to coexpressed histidine (His)-tagged ubiquitin chains (Fig 1D). TAK1-ubiquitin conjugates appeared as smears on SDS-PAGE, which is compatible with TAK1 polyubiquitination, whereas ubiquitinated TAB1 appeared as a more distinct band, suggesting that TAB1 might be primarily monoubiquitinated (Fig 1D). Importantly, coexpression of YopP prevented ubiquitination of TAK1 and TAB1 in intact cells. By contrast, ubiquitination

of MEKK1 Δ was not affected by YopP (Fig 1D). Collectively, the experiments shown in Fig 1 suggested that, in addition to the previously observed mechanisms targeting inhibitor of nuclear factor- κ B kinase β (IKK β) and MAPKK, YopP can also affect specific MAPKKs such as TAK1 by directly (or indirectly) removing ubiquitin chains that are required for activation.

To confirm a role of YopP in the activation mechanism of the endogenous TAK1 kinase complex, we immunoprecipitated it by using TAB2 antibodies and analysed its composition and enzymatic activity by using western blotting and *in vitro* kinase assays. Constitutive as well as IL-1-induced kinase activity of TAK1 was strongly suppressed after YopP transfection, as shown by phosphorylation of recombinant MAPKK6 and by autophosphorylation of TAK1 at Thr 187 (Fig 2A). YopP did not affect the interaction of TAK1 with TAB1 and TAB2 (Fig 2B). A YopP mutant with a point mutation in the catalytic triad of the protease domain (Cys172Ala) failed to suppress TAK1-induced activation of IL-8 transcription (supplementary Fig 1 online), TAK1 kinase activity (Fig 3A) or TAK1-mediated phosphorylation of co-precipitated TAB1 and TAB2 (Fig 3A). Furthermore, basal and IL-1-inducible TAK1 activity was suppressed in cells infected with wild-type *Y. enterocolitica*, but not in cells infected with a strain in which YopP was replaced by an allele encoding the Cys172Ala mutant (Fig 3B). Hence, the results shown in Figs 2,3 suggest that in intact cells catalytically active YopP directly inhibits endogenous TAK1 kinase activity. As a consequence, YopP suppresses phosphorylation of TAB1, TAB2, NF- κ B, JNK and p38 (as shown in supplementary Fig 2 online).

So far, the only known negative regulatory mechanism that affects TAK1 is an autoregulatory feedback loop by which p38 MAPK-mediated phosphorylation of TAB1 dampens constitutive activity of the TAK1-TAB1 complex in unstimulated cells and shuts off IL-1-stimulated TAK1 kinase activity after an initial activation phase (Cheung *et al*, 2003). As shown in Fig 4A, lane 2 (and also supplementary Fig 2 online, lane 2), in unstimulated cells, YopP transfection resulted in the appearance of another form of TAB1 (designated form 3) with faster mobility than TAB1 from control cells (designated form 2). The faster migrating form 3 of TAB1 was also detected in cells treated with the p38 MAPK inhibitor SB203580 (Fig 4B, lane 2) or in cells lacking p38 MAPK α (Fig 4C). This suggests that TAB1 form 2 results from constitutive phosphorylation by p38 MAPK, possibly at Ser 438, which is the only known constitutively phosphorylated amino acid (Cheung *et al*, 2003). As shown in Fig 4A,B, lane 3, in IL-1-stimulated cells, TAB1 form 2 was shifted to a form with slower mobility (designated form 1), indicating that IL-1 induces further phosphorylation of TAB1, possibly at the other two p38 MAPK phosphorylation sites Ser 423 and Thr 431 (Cheung *et al*, 2003). To test this hypothesis, in haemagglutinin-tagged TAB1 (HA-TAB1), all three sites were mutated to alanine, and the mobility of TAB1 was analysed in co-transfection experiments with YopP or TAK1. As shown in Fig 4D, TAB1 form 2 was shifted to form 1 only in TAB1 versions in which Ser 438 was mutated. The mobility of the Ser438Ala mutant of TAB1 was indistinguishable from that of wild-type TAB1 after coexpression of YopP (Fig 4D, compare lane 2 with lane 10), indicating that YopP targets Ser 438 of TAB1. Interestingly, after TAK1 coexpression, the retarded form 1 of TAB1 was still detectable in all mutant TAB1 proteins, indicating that TAK1 causes phosphorylation independently of Ser 423,

Fig 1 | Ectopic expression of YopP inhibits TAK1 activity by deubiquitinating coexpressed TAK1 and TAB1. (A) HEK293IL-1R cells were transiently transfected with expression vectors for GFP or a GFP-YopP fusion protein. After 24 h, cells were lysed and the expression of GFP or GFP-YopP was analysed by immunoprecipitation (IP) followed by immunoblotting (IB) using the indicated antibodies. Arrowheads indicate GFP-YopP. (B) HEK293IL-1R cells were transiently transfected with pUHC13-3-IL-8pr and expression vectors for GFP-YopP, GFP-TAK1, GFP-TAB1 or MEKK1 Δ -BFP, as indicated. A vector encoding GFP was used to adjust the total amount of DNA in all samples. After 24 h, cells were treated with IL-1 (10 ng/ml) for 5 h (lanes 7,8) or were left untreated. Thereafter, cells were lysed and luciferase activity was determined. Mean values $\pm$ s.e.m. from two experiments are shown. Aliquots of the lysates were analysed by western blotting for expression of the transfected proteins and for endogenous TAK1 using anti-GFP or anti-TAK1 (M-579) antibodies, respectively. (C) Cells were transfected as in (B). After 24 h, GFP-TAK1 + GFP-TAB1 or MEKK1 Δ -BFP were immunoprecipitated with anti-GFP antibodies from whole-cell lysates and their activity was measured by *in vitro* kinase assay using His-MKK6 as substrate. In lane 5, the kinase reaction was performed, omitting immunoprecipitated proteins, as a control for autophosphorylation of His-MKK6. (D) HEK293IL-1R cells were transfected with expression vectors for HA-TAK1, MEKK1 Δ -BFP, Myc-TAB1, GFP-YopP and His-ubiquitin. After 24 h, cells were lysed in denaturing buffer, precipitated and redissolved in sample buffer (Input). His-tagged ubiquitinated proteins were purified by adsorption to Ni^{2+} -NTA agarose (Ni^{2+} -pull-down). Proteins were separated by SDS-polyacrylamide gel electrophoresis and detected in the input sample and in the bound fraction by western blotting using antibodies against HA, GFP or Myc. Ub_n indicates the polyubiquitinated forms of HA-TAK1 and MEKK1 Δ -BFP. Numbers indicate the relative amounts of antigen in the input samples and in the bound fractions compared with the control samples. BFP, blue fluorescent protein; GFP, green fluorescent protein; HA, haemagglutinin; His, histidine; IL-1, interleukin-1; MEKK1, MEK kinase 1; MKK6, MAPK kinase 6; TAB1, TAK1-binding protein 1; TAK1, TGF- β -activated kinase 1; TGF- β , transforming growth factor- β , YopP, *Yersinia* outer membrane protein P.

Thr 431 and Ser 438 (Fig 4D, lanes 6,9,12). Inhibition of p38 MAPK by SB203580 increased IL-1-induced autophosphorylation of TAK1 at Thr 187 (Fig 4B, lane 4), whereas YopP co-transfection suppressed

IL-1-induced phosphorylation of Thr 187 (Fig 4A, lane 4). Hence, although both YopP and SB203580 affect basal TAB1 phosphorylation at Ser 438, they affect activation of TAK1 differently.

Fig 2 | YopP inhibits autophosphorylation of TAK1 at Thr 187 without affecting the TAK1-TAB1-TAB2 interaction. HEK293IL-1R cells were transiently transfected with empty vector, expression vectors for GFP-YopP or left untransfected (Untransf.), as indicated. After 48 h, cells were stimulated with IL-1 (50 ng/ml) for 10 min or were left untreated. (A) The TAK1 kinase complex was immunoprecipitated (IP) using anti-TAB2 (K-20) antibodies, and its activity was measured *in vitro* using His-MKK6 as a substrate (upper panel). An aliquot of the immunoprecipitates was analysed by immunoblot for phosphorylation of TAK1 at Thr 187 and to confirm equal co-precipitation of TAK1 (M-579; lower panel). Numbers indicate the relative His-MKK6 phosphorylation by TAK1 compared with the unstimulated control (set as 1). (B) Anti-TAB2 (K-20) immune complexes from cells transfected as in (A) were analysed by western blotting for the presence of TAK1 (M-579), TAB1 (C-20) or TAB2, as indicated by arrowheads. GFP, green fluorescent protein; His, histidine; MKK6, MAPK kinase 6; TAB, TAK1-binding protein; TAK1, TGF- β -activated kinase 1; YopP, *Yersinia* outer membrane protein P.

We used this information to design further experiments to assess whether YopP acts at the level of TAK1 or downstream from TAK1 by affecting the p38-TAB1 feedback mechanism. When HA-TAK1 was co-purified with Myc-TAB1, strong TAK1 kinase activity and autophosphorylation at Thr 187 could be detected *in vitro* (Fig 4E, lane 5). Coexpression of YopP increased the mobility of Myc-TAB1, resulting in form 3 (Fig 4E, lanes 2,6), and inhibited HA-TAK1 (Fig 4E, lane 6). By contrast, coexpression of an active form of MAPKK6 and of p38 MAPK decreased the mobility of Myc-TAB1, resulting in form 1 (Fig 4E, lanes 3,7), and inhibited HA-TAK1 (Fig 4E, lane 7). Moreover, MAPKK6-p38 MAPK in synergy with the coexpressed YopP suppressed TAK1

Fig 3 | Inhibition of TAK1 by YopP requires Cys 172 and occurs in infected cells. (A) HEK293IL-1R cells were transiently transfected with empty vector or with expression vectors for GST-YopP or GST-YopP_{C172A}, as indicated. After 48 h, cells were stimulated with IL-1 (50 ng/ml) for 10 min or were left untreated. The TAK1 kinase complex was immunoprecipitated (IP) using anti-TAB2 (K-20) antibodies and was subjected to an *in vitro* kinase reaction to detect *in vitro* phosphorylation of endogenous TAB1, TAK1, TAB2 and His-MKK6 (as indicated by arrowheads). An aliquot of the immune complexes was analysed for equal co-precipitation of TAK1 by immunoblotting. Equal expression of GST-YopP proteins was analysed by immunoblotting of lysates. (B) HeLa cells were infected with wild-type *Yersinia enterocolitica* or with *Y. enterocolitica* in which YopP was replaced by YopP_{C172A}, or were left uninfected. After 1 h, cells were stimulated with IL-1 (50 ng/ml) for 10 min or were left untreated. TAB2 immune complex protein kinase assays were performed as described in (A) and equal co-precipitation of TAK1 was confirmed by immunoblotting. Numbers in (A,B) indicate the relative His-MKK6 phosphorylation by TAK1 compared with the unstimulated control (set as 1). GST, glutathione S-transferase; His, histidine; IL-1, interleukin-1; MKK6, MAPK kinase 6; TAB, TAK1-binding protein; TAK1, TGF- β -activated kinase 1; YopP, *Yersinia* outer membrane protein P.

Fig 4 | YopP acts in synergy with p38 mitogen-activated protein kinase to suppress TAK1 activity. (A) HEK293IL-1R cells were transiently transfected with empty vector or with expression vectors for GFP-YopP. After 48 h, cells were stimulated with IL-1 (50 ng/ml) for 10 min or were left untreated. Modifications of TAB1 and TAK1 in cell lysates were analysed by anti-TAB1 antibodies (C-20) and by P-Thr 187-specific anti-TAK1 antibodies. Equal loading of lanes was confirmed by reprobing with anti-TAK1 antibodies (M-579). (B) HEK293IL-1R cells were pretreated with 2 μ M SB203580 for 30 min followed by stimulation with IL-1 (50 ng/ml) for 10 min, as indicated. TAB1 and TAK1 modifications were analysed as described in (A). (C) Lysates of wild-type(+/+) and p38 MAPK α -deficient (-/-) embryonic fibroblasts were analysed for the modified TAB1 forms as described in (A). (D) HEK293IL-1R cells were transfected with expression vectors for HA-TAK1, GFP-YopP, HA-TAB1 wild type, or versions in which Ser 423/Thr 431/Ser 438 (STS) were mutated to alanine as indicated. After 24 h, cells were lysed and the expression of HA-TAB1 and HA-TAK1 was analysed by immunoblotting (IB) using the indicated antibodies. (E) HEK293IL-1R cells were transfected with expression vectors for Myc-TAB1 and HA-TAK1. In addition, plasmids encoding p38 MAPK and an active form of MKK6 (MKK62E) were co-transfected as indicated. Cells were lysed and Myc-TAB1 was immunoprecipitated by anti-Myc antibodies. Half of the immune complexes were analysed by IB to detect the three modified forms of Myc-TAB1, HA-TAK1 or P-Thr 187-TAK1. The other half was subjected to *in vitro* kinase assays using His-MKK6 as a substrate to measure TAK1 kinase activity (lower panel). Equal expression of Myc-TAB1, HA-TAK1, p38 MAPK and MKK6 in cell lysates was confirmed by western blotting (data not shown). Black arrowheads indicate the forms of TAK1 and TAB1 (numbered 1-3) with different mobility on SDS-polyacrylamide gel electrophoresis. Numbers indicate the relative His-MKK6 kinase activity of the immune complexes compared with the activity of HA-TAK1 in the absence of GFP-YopP or p38/MKK62E. GFP, green fluorescent protein; HA, haemagglutinin; IL-1, interleukin-1; MAPK, mitogen-activated protein kinase; MKK6, MAPK kinase 6; TAB1, TAK1-binding protein 1; TAK1, TGF- β -activated kinase 1; TGF- β , transforming growth factor- β ; YopP, *Yersinia* outer membrane protein P.

kinase activity (Fig 4E, lane 8). This clearly shows that YopP or MAPKK6-p38 MAPK inhibits TAK1 by two different mechanisms. MAPKK6-p38 indirectly inhibits TAK1 by promoting the hyperphosphorylated form 1 of TAB1, which was previously found to inhibit TAK1 (Cheung *et al*, 2003). By contrast, YopP inhibits autophosphorylation of TAK1 at Thr 187 (Figs 2A,3A,4), which indirectly results in the suppression of TAK1-MAPKK4/6-p38-mediated TAB1 phosphorylation.

YopP also inhibits monoubiquitination of TAB1 (Fig 1D), the contribution of which to TAK1 activation still needs to be clarified. However, TAB1 might be important in recruiting YopP to the TAK1 complex as glutathione S-transferase (GST)-YopP bound to cellularly expressed TAB1, but not to TAK1 or TAB2 *in vitro*

(Fig 5). In these assays, GST-YopP also bound to MAPKK4 (Fig 5), as described previously (Orth *et al*, 1999).

Speculation

While our manuscript was being reviewed, Haase *et al* (2005) reported that, in *Yersinia*-infected cells, YopP affects the recruitment of TAK1 by TAB2 and thereby inhibits TAK1. Thus, although our data support the observation that YopP inhibits TAK1, we propose a different mechanism of inhibition on the basis of the observation that it is the enzymatic activity rather than the interaction of TAK1-TAB1-TAB2 that is targeted by YopP.

In addition, in contrast to recent reports on overexpressed TRAF6 (Haase *et al*, 2005; Zhou *et al*, 2005), we did not find that

Fig 5 | YopP interacts with TAB1 but not with TAK1 or TAB2. Lysates from HEK293IL-1R cells that were transiently transfected with expression vectors for Myc-MKK4, HA-TAK1, Myc-TAB1 and T7-TAB2 were mixed with equal amounts of bacterially expressed GST or GST-YopP. Proteins bound to GST or GST-YopP were affinity-purified on GSH-Sepharose (pulldown) and were analysed by immunoblotting using the indicated antibodies against epitope tags. Expression of transfected proteins was verified by analysing duplicate samples of lysates in parallel. GFP, green fluorescent protein; GST, glutathione *S*-transferase; HA, haemagglutinin; MKK4, MAPK kinase 4; TAB, TAK1-binding protein; TAK1, TGF- β -activated kinase 1; TGF- β , transforming growth factor- β ; YopP, *Yersinia* outer membrane protein P.

YopP deubiquitinates IL-1-activated endogenous TRAF6 (supplementary Fig 3 online). These discrepancies point to different effects of YopP on the various TRAF6 and TAK1 complexes and might be resolved by future investigations analysing the effects of YopP on individually reconstituted protein complexes in cell-free systems. In the IL-1 pathway, the data presented here place the inhibitory mechanism by which YopP affects TAK1 downstream from TRAF6 and upstream from MAPKK6/p38 MAPK at the level of TAK1 autophosphorylation. In this way, YopP can act in synergy with the p38 MAPK pathway to downregulate innate immune signalling.

METHODS

Cells, bacterial strains and materials. HEK293IL-1R and mouse embryonic fibroblasts lacking p38 MAPK have been described (Ninomiya-Tsuji *et al*, 1999; Thiefes *et al*, 2005). HeLa cells were from American Type Culture Collection (Manassas, VA, USA). *Y. enterocolitica* serotype O:8, clinical isolate WA-314 pYVO8+Y, was used as the wild-type strain. To generate strain Δ YopP/YopP_{C172A}, YopP was deleted by homologous recombination and then complemented with plasmid PO8 encoding YopP_{C172A} (kindly provided by Klaus Ruckdeschel, Hamburg). Antibodies against the following proteins or peptides were used: TAK1 (M-579), TAB1 (C-20), TAB2 (K-20), ubiquitin (P4D1; all from Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA), Myc (9E10, Boehringer, Roche Applied Science, Mannheim, Germany), HA (12CA5, Roche Applied Science, Mannheim, Germany), GFP (Roche), T7 (Novagen, Madison, WI, USA), P-p65 NF- κ B (3,031), P-JNK (9,251), JNK (9,252; all from Cell Signaling Technology Inc., Danvers, MA, USA), P-p38 (44-684, Biosource International, Camarillo, CA, USA), p38 (Thiefes *et al*, 2005), P-TAK1 (Singhirunnusorn *et al*, 2005), TAB1 (Ninomiya-Tsuji

et al, 1999) and TRAF6, TAB2 (Takaesu *et al*, 2000). The anti-YopP antiserum was raised against GST-YopP in rabbits.

Plasmids. Expression vectors for p38 MAPK, MAPKK62E, GFP-TAK1 (Holtmann *et al*, 1999, 2001), GFP-TAB1, HA-TAB1, HA-TAB1 mutated in Ser 423 and Thr 431 (ST/AA; Sakurai *et al*, 2000; Singhirunnusorn *et al*, 2005), His₆-MAPKK6, Myc-TAB1, HA-TAK1, T7-TAB2 (Ninomiya-Tsuji *et al*, 1999; Takaesu *et al*, 2000) and pUHC13-3-IL-8pr encoding IL-8 promoter luc (Hoffmann *et al*, 2005) have been described. pCS3MT-MAPKK4 encoding Myc-MAPKK4 and pDest26-His₆-ubiquitin were kind gifts from Pamela Holland (USA) and Rainer Niedenthal (Germany), respectively. pQB150fA1-MEKK1 Δ -BFP, pGEX-4T3-YopP, pCDNA3.1-GFP-YopP and pEBG-YopP were obtained by standard PCR-based cloning techniques. Ser 438 in HA-TAB1 and HA-TAB1(ST/AA) was mutated to Ala to generate HA-TAB1(STS/STA) and HA-TAB1(STS/AAA). Cys 172 was mutated to Ala to generate pEBG-YopP_{C172A}.

Transfections and reporter gene assays. Calcium phosphate transfections and reporter gene assays were performed as described previously (Hoffmann *et al*, 2005). For the experiments shown in Figs 2–5A, cells (4.5×10^5 cells per six wells) were transfected with 3 μ g of expression plasmid for GFP-YopP or empty vector and 6 μ l of Roti[®]-Fect (Roth, Karlsruhe, Germany). After 24 h, cells were treated as described in the figure legends.

Cell lysis, immunoprecipitations and *in vitro* kinase assays. Cell lysis, immunoprecipitations, radioactive kinase assays were performed essentially as described by Cheung *et al* (2003) and as given in the supplementary information online.

***In vivo* ubiquitination assays.** Cells were transfected with expression vectors for HA-TAK1, Myc-TAB1 or His₆-ubiquitin and GFP-YopP. Cells were lysed in denaturing buffer and proteins bound to Ni²⁺-NTA agarose were detected by western blotting.

GST pulldown assays. Cells were transfected with expression vectors for HA-TAK1, Myc-TAB1, T7-TAB2 or Myc-MAPKK4. Proteins bound to immobilized GST or to GST-YopP were detected by western blotting.

Western blotting. Western blotting was performed as described previously (Hoffmann *et al*, 2005). Quantification of signals was achieved using ImageJ 1.36b (<http://rsb.info.nih.gov/ij/>).

For detailed information on methods and materials, see the supplementary information online.

Supplementary information is available at *EMBO reports* online (<http://www.emboreports.org>).

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REFERENCES

- Carter RS, Pennington KN, Ungurait BJ, Arrate P, Ballard DW (2003) Signal-induced ubiquitination of I κ B kinase- β . *J Biol Chem* **278**: 48903–48906
- Cheung PC, Campbell DG, Nebreda AR, Cohen P (2003) Feedback control of the protein kinase TAK1 by SAPK2a/p38 α . *EMBO J* **22**: 5793–5805
- Cheung PC, Nebreda AR, Cohen P (2004) TAB3, a new binding partner of the protein kinase TAK1. *Biochem J* **378**: 27–34
- Haase R, Richter K, Pfaffinger G, Courtois G, Ruckdeschel K (2005) *Yersinia* outer protein P suppresses TGF- β -activated kinase-1 activity to impair innate immune signaling in *Yersinia enterocolitica*-infected cells. *J Immunol* **175**: 8209–8217

- Hoffmann E, Dittrich-Breiholz O, Holtmann H, Kracht M (2002) Multiple control of interleukin-8 gene expression. *J Leukoc Biol* **72**: 847–855
- Hoffmann E, Thiefes A, Buhrow D, Dittrich-Breiholz O, Schneider H, Resch K, Kracht M (2005) MEK1-dependent delayed expression of Fos-related antigen-1 counteracts c-Fos and p65 NF- κ B-mediated interleukin-8 transcription in response to cytokines or growth factors. *J Biol Chem* **280**: 9706–9718
- Holtmann H, Winzen R, Holland P, Eickemeier S, Hoffmann E, Wallach D, Malinin NL, Cooper JA, Resch K, Kracht M (1999) Induction of interleukin-8 synthesis integrates effects on transcription and mRNA degradation from at least three different cytokine- or stress-activated signal transduction pathways. *Mol Cell Biol* **19**: 6742–6753
- Holtmann H et al (2001) The MAPK kinase kinase TAK1 plays a central role in coupling the interleukin-1 receptor to both transcriptional and RNA-targeted mechanisms of gene regulation. *J Biol Chem* **276**: 3508–3516
- Ishitani T, Takaesu G, Ninomiya-Tsuji J, Shibuya H, Gaynor RB, Matsumoto K (2003) Role of the TAB2-related protein TAB3 in IL-1 and TNF signaling. *EMBO J* **22**: 6277–6288
- Juris SJ, Shao F, Dixon JE (2002) *Yersinia* effectors target mammalian signalling pathways. *Cell Microbiol* **4**: 201–211
- Kanayama A, Seth RB, Sun L, Ea CK, Hong M, Shaito A, Chiu YH, Deng L, Chen ZJ (2004) TAB2 and TAB3 activate the NF- κ B pathway through binding to polyubiquitin chains. *Mol Cell* **15**: 535–548
- Kishimoto K, Matsumoto K, Ninomiya-Tsuji J (2000) TAK1 mitogen-activated protein kinase kinase is activated by autophosphorylation within its activation loop. *J Biol Chem* **275**: 7359–7364
- Kracht M, Saklatvala J (2002) Transcriptional and post-transcriptional control of gene expression in inflammation. *Cytokine* **20**: 91–106
- Navarro L, Alto NM, Dixon JE (2005) Functions of the *Yersinia* effector proteins in inhibiting host immune responses. *Curr Opin Microbiol* **8**: 21–27
- Ninomiya-Tsuji J, Kishimoto K, Hiyama A, Inoue J, Cao Z, Matsumoto K (1999) The kinase TAK1 can activate the NIK-I κ B as well as the MAP kinase cascade in the IL-1 signalling pathway. *Nature* **398**: 252–256
- Ninomiya-Tsuji J, Kajino T, Ono K, Ohtomo T, Matsumoto M, Shiina M, Mihara M, Tsuchiya M, Matsumoto K (2003) A resorcylic acid lactone, 5Z-7-oxozeaenol, prevents inflammation by inhibiting the catalytic activity of TAK1 MAPK kinase. *J Biol Chem* **278**: 18485–18490
- Orth K, Palmer LE, Bao ZQ, Stewart S, Rudolph AE, Bliska JB, Dixon JE (1999) Inhibition of the mitogen-activated protein kinase superfamily by a *Yersinia* effector. *Science* **285**: 1920–1923
- Orth K, Xu Z, Mudgett MB, Bao ZQ, Palmer LE, Bliska JB, Mangel WF, Staskawicz B, Dixon JE (2000) Disruption of signaling by *Yersinia* effector YopJ, a ubiquitin-like protein protease. *Science* **290**: 1594–1597
- Ruckdeschel K, Machold J, Roggenkamp A, Schubert S, Pierre J, Zumbihl R, Liautard JP, Heesemann J, Rouot B (1997) *Yersinia enterocolitica* promotes deactivation of macrophage mitogen-activated protein kinases extracellular signal-regulated kinase-1/2, p38, and c-Jun NH₂-terminal kinase. Correlation with its inhibitory effect on tumor necrosis factor- α production. *J Biol Chem* **272**: 15920–15927
- Ruckdeschel K, Harb S, Roggenkamp A, Hornef M, Zumbihl R, Kohler S, Heesemann J, Rouot B (1998) *Yersinia enterocolitica* impairs activation of transcription factor NF- κ B: involvement in the induction of programmed cell death and in the suppression of the macrophage tumor necrosis factor α production. *J Exp Med* **187**: 1069–1079
- Sakurai H, Miyoshi H, Mizukami J, Sugita T (2000) Phosphorylation-dependent activation of TAK1 mitogen-activated protein kinase kinase by TAB1. *FEBS Lett* **474**: 141–145
- Sato S, Sanjo H, Takeda K, Ninomiya-Tsuji J, Yamamoto M, Kawai T, Matsumoto K, Takeuchi O, Akira S (2005) Essential function for the kinase TAK1 in innate and adaptive immune responses. *Nat Immunol* **6**: 1087–1095
- Singhrrunusorn P, Suzuki S, Kawasaki N, Saiki I, Sakurai H (2005) Critical roles of threonine 187 phosphorylation in cellular stress-induced rapid and transient activation of transforming growth factor- β -activated kinase 1 (TAK1) in a signaling complex containing TAK1-binding protein TAB1 and TAB2. *J Biol Chem* **280**: 7359–7368
- Takaesu G, Kishida S, Hiyama A, Yamaguchi K, Shibuya H, Irie K, Ninomiya-Tsuji J, Matsumoto K (2000) TAB2, a novel adaptor protein, mediates activation of TAK1 MAPKKK by linking TAK1 to TRAF6 in the IL-1 signal transduction pathway. *Mol Cell* **5**: 649–658
- Takaesu G, Surabhi RM, Park KJ, Ninomiya-Tsuji J, Matsumoto K, Gaynor RB (2003) TAK1 is critical for I κ B kinase-mediated activation of the NF- κ B pathway. *J Mol Biol* **326**: 105–115
- Thiefes A et al (2005) Simultaneous blockade of NF κ B, JNK, and p38 MAPK by a kinase-inactive mutant of the protein kinase TAK1 sensitizes cells to apoptosis and affects a distinct spectrum of tumor necrosis target genes. *J Biol Chem* **280**: 27728–27741
- Trulzsch K, Sporleder T, Igwe EI, Russmann H, Heesemann J (2004) Contribution of the major secreted Yops of *Yersinia enterocolitica* O:8 to pathogenicity in the mouse infection model. *Infect Immun* **72**: 5227–5234
- Viboud GI, Bliska JB (2005) *Yersinia* outer proteins: role in modulation of host cell signalling responses and pathogenesis. *Annu Rev Microbiol* **59**: 69–89
- Wang C, Deng L, Hong M, Akkaraju GR, Inoue J, Chen ZJ (2001) TAK1 is a ubiquitin-dependent kinase of MKK and IKK. *Nature* **412**: 346–351
- Xia Y, Makris C, Su B, Li E, Yang J, Nemerow GR, Karin M (2000) MEK kinase 1 is critically required for c-Jun N-terminal kinase activation by proinflammatory stimuli and growth factor-induced cell migration. *Proc Natl Acad Sci USA* **97**: 5243–5248
- Zhou H, Monack DM, Kayagaki N, Wertz I, Yin J, Wolf B, Dixit VM (2005) *Yersinia* virulence factor YopJ acts as a deubiquitinase to inhibit NF- κ B activation. *J Exp Med* **202**: 1327–1332