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O-GlcNAcylation of TAB1 modulates TAK1-mediated cytokine release

Since Advance Online Publication, Fig. 2G has been corrected to show the correct (ETD) fragmentation spectrum that was used to map the TAB1 O-GlcNAc site and as referred to in the figure legend.

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Transforming growth factor (TGF)-β-activated kinase 1 (TAK1) is a key serine/threonine protein kinase that mediates signals transduced by pro-inflammatory cytokines such as transforming growth factor-β, tumour necrosis factor (TNF), interleukin-1 (IL-1) and wnt family ligands. TAK1 is found in complex with binding partners TAB1–3, phosphorylation and ubiquitination of which has been found to regulate TAK1 activity. In this study, we show that TAB1 is modified with *N*-acetylglucosamine (*O*-GlcNAc) on a single site, Ser395. With the help of a novel *O*-GlcNAc site-specific antibody, we demonstrate that *O*-GlcNAcylation of TAB1 is induced by IL-1 and osmotic stress, known inducers of the TAK1 signalling cascade. By reintroducing wild-type or an *O*-GlcNAc-deficient mutant TAB1 (S395A) into *Tab1*$^{-/-}$ mouse embryonic fibroblasts, we determined that *O*-GlcNAcylation of TAB1 is required for full TAK1 activation upon stimulation with IL-1/osmotic stress, for downstream activation of nuclear factor κB and finally production of IL-6 and TNFα. This is one of the first examples of a single *O*-GlcNAc site on a signalling protein modulating a key innate immunity signalling pathway.
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Introduction

Transforming growth factor (TGF)-β-activated kinase 1 (TAK1), also known as mitogen-activated protein kinase kinase kinase 7 (MAP3K7), is a member of the mitogen-activated protein kinase (MAPK) family (Yamaguchi *et al*, 1995). TAK1 has a key role in the production of tumour necrosis factor (TNF)α and other inflammatory mediators by activating several MAPKs, such as p38α MAPK, c-Jun N-terminal kinases (JNK1/JNK2), ERK1/2 and the transcription factor nuclear factor κB (NFκB) (Wang *et al*, 2001; Sato *et al*, 2005; Shim *et al*, 2005) via the signalling pathways shown in Figure 1A. TAK1 is essential in several cytokine-mediated innate immunity signal transduction cascades, including the TNFα, interleukin-1 (IL-1) and TGF-β pathways, as well as signalling downstream of Toll-like receptors and NOD1/2 (Shibuya *et al*, 1996; Ninomiya-Tsuji *et al*, 1999; Hasegawa *et al*, 2008) (Figure 1A). In these pathways, various pro-inflammatory cytokines and endotoxins trigger TAK1 activity, leading to its autophosphorylation and subsequent recruitment to the IκB kinase (IKK) complex, ultimately resulting in activation of the transcription factor NFκB (Adhikari *et al*, 2007) (Figure 1A). The native forms of TAK1 comprise the catalytic kinase subunit in complex with a regulatory subunit TAB1 (TAK1-binding protein 1, a pseudophosphatase; Conner *et al*, 2006) (Figure 1B), and either of two homologous proteins, TAB2 or TAB3 (Shibuya *et al*, 1996; Ishitani *et al*, 2003; Cheung *et al*, 2004). The activation of TAK1 by lipopolysaccharide (LPS) or IL-1 is triggered by the Lys^{63}-linked poly-ubiquitination of TNF receptor-associated factor 6 (TRAF6), which binds to the C-terminal zinc-finger motifs of TAB2 and TAB3, stimulating autophosphorylation and activation of TAK1 (Wang *et al*, 2001).

TAK1 activity is also subject to regulation by a feedback loop in which p38α MAPK suppresses the activation of TAK1 by phosphorylation of TAB1 at Ser423 and Thr431 (Cheung *et al*, 2003). Disruption of the *Tab1* gene in mice is embryonic lethal with several developmental phenotypes, including cardiovascular and lung dysmorphogenesis (Komatsu *et al*, 2002). Studies with *Tab1*-deficient mouse embryonic fibroblasts (*Tab1*$^{-/-}$ MEFs) suggest that TAB1 plays several roles in the regulation of the TAK1 complex, namely to recruit p38α MAPK to the TAK1 complex for the phosphorylation of TAB3, to suppress the dephosphorylation of TAB3, and to induce TAK1 catalytic activity (Mendoza *et al*, 2008). TAB1 is a crucial mediator in TAK1 signalling as *Tab1*$^{-/-}$ MEFs do not activate TAK1 in response to IL-1 and TNFα (Mendoza *et al*, 2008). MEKK3 is maintained in an inactive state by interaction with TAK1 in unstimulated cells, preventing basal NFκB signalling. Pro-inflammatory activation of TAK1 leads to disruption of MEKK3–TAK1 complexes via TAB1, allowing both TAK1 and MEKK3 to transduce biochemical signals (Di *et al*, 2008).

Protein glycosylation with *N*-acetylglucosamine (*O*-GlcNAcylation) is an abundant post-translational modification of serines/threonines occurring on nuclear and cytoplasmic proteins (reviewed in Love and Hanover, 2005; Hart *et al*, 2007). As with phosphorylation, modification by *O*-GlcNAc is dy-

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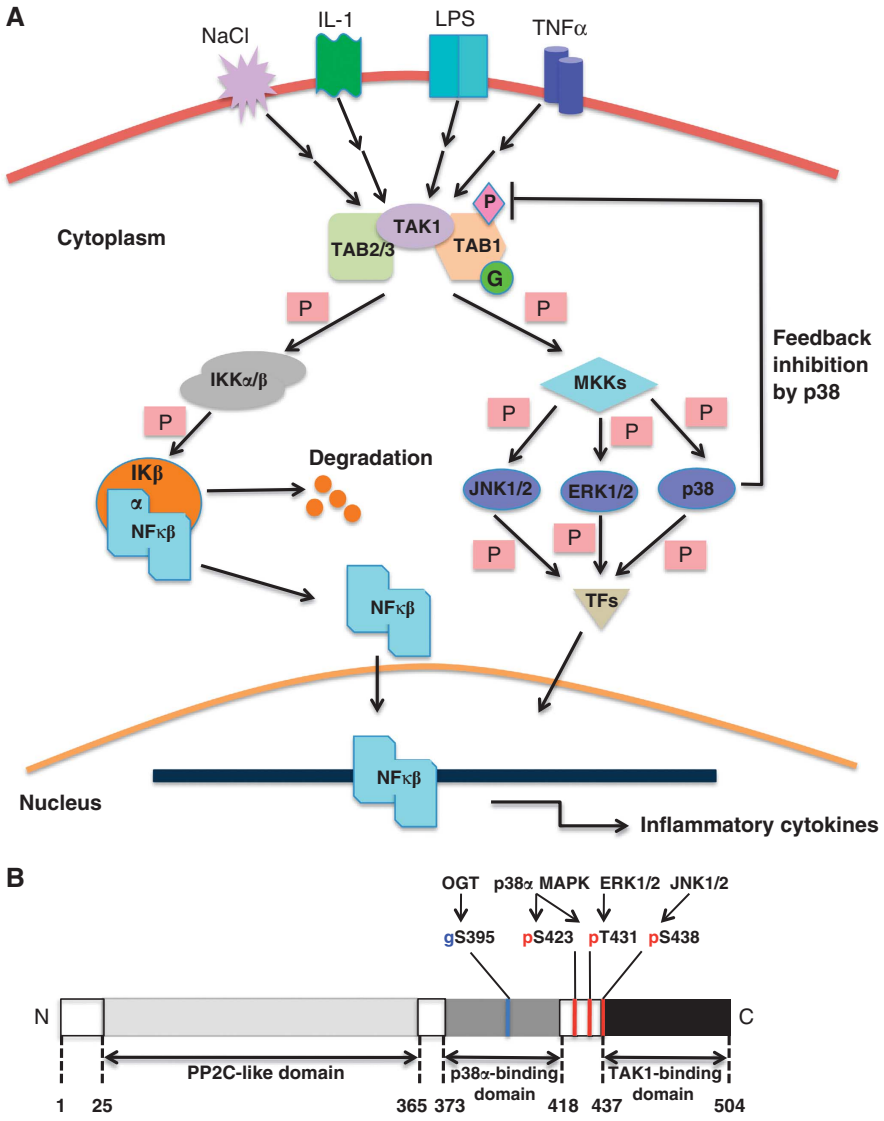

Figure 1 Stimulation and activation of the TAK1 pathway and TAB1 domain structure. (**A**) TAK1 is a key player in the cascades of cellular responses evoked by changes in the environment, as its activity is regulated by growth factors, pro-inflammatory cytokines and pathogen-derived molecules. TAK1 activity is modulated via its binding partners TAB1 and TAB2/3, phosphorylating (denoted by P) and activating a number of other important protein kinases; the p38α MAPK, c-jun N-terminal kinase (JNK) through MKK (MAPK kinase) and IKK complex. Extracellular signal-regulated kinase (ERK1/2) and p38α MAPK exert their effects at the post-translational level on transcription factors (TFs), whereas IKKβ controls the transcription of genes encoding inflammatory mediators by regulating cellular localization of NFκB. Transcriptional responses initiated by these pathways are of fundamental importance for the whole organism as they determine cell fate and protect cells from pathogens and changes in osmolarity. This study shows that TAB1, a key regulator of TAK1, is also *O*-GlcNAcylated (denoted by G). (**B**) Schematic diagram of the full-length TAB1 domain structure (amino acids, 1–504). TAB1 consisted of a PP2C-like pseudophosphatase domain, a p38α-binding domain, and TAK1-binding domain. TAB1 is phosphorylated by p38α MAPK at Ser423 (S423) and Thr431 (T431) and by both ERK1/2 and JNK1/2 at Ser438 (S438). This study shows that Ser395 (gS395) is the single *O*-GlcNAc site on the TAB1 protein.

namic (Kreppel *et al*, 1997; Comer and Hart, 2000; Zachara and Hart, 2002), giving rise to functionally distinct protein species and there is evidence to suggest that *O*-GlcNAc may show interplay with protein phosphorylation (Zeidan and Hart, 2010). *O*-GlcNAcylation is implicated in virtually all cellular processes examined, for instance gene expression (Comer and Hart, 1999), protein turnover (Hart *et al*, 2007), and also in regulating cellular responses to insulin (Vosseller *et al*, 2002; Copeland *et al*, 2008), cell-cycle control (Slawson *et al*, 2005), stress protection (Zachara *et al*, 2004) and calcium cycling (Clark *et al*, 2003). The enzymes responsible for the attachment (*O*-GlcNAc transferase, OGT) and removal (*O*-GlcNAcase, OGA) of this sugar moiety have been found in the nucleus and the cytoplasm of cells. The genes encoding these enzymatic activities have been cloned and characterized (Kreppel *et al*, 1997; Lubas and Hanover, 2000; Gao *et al*, 2001). The N-terminus of OGT contains multiple tetratricopeptide repeats thought to mediate protein–protein interactions that are critical for substrate recognition (Kreppel and Hart, 1999; Lubas and Hanover, 2000; Clark *et al*, 2003). Inactivation of the OGT gene in mouse cells has shown that OGT is required for embryonic stem cell viability and mouse ontogeny (Shafi *et al*, 2000). In addition, dysfunctional protein *O*-GlcNAcylation/phosphorylation appears to have a role in the pathology of type II diabetes (Hanover *et al*, 1999) and Alzheimer's disease (Griffith and Schmitz, 1995;

Hanover *et al*, 1999). Recent evidence supports a central role for *O*-GlcNAc modification in the regulation of immune cells, particularly in the activation processes of T and B-lymphocytes and possible increased nuclear translocation and activity of nuclear factor of activated T cells and NFκB (Golks and Guerini, 2008).

Here we demonstrate that *O*-GlcNAcylation of a single residue (Ser395) on TAB1 modulates TAK1 activation in response to IL-1 stimulation or osmotic stress. TAB1 *O*-GlcNAcylation induces a substantial increase in TAK1 autophosphorylation and activation, phosphorylation of IKK, translocation of NFκB and ultimately cytokine production.

Results

TAB1 is O-GlcNAcylated on Ser395

Many cytoplasmic and nuclear proteins such as transcription factors, RNA polymerase II, oncoproteins, nuclear pore proteins, viral proteins, and tumour suppressor proteins have been found to be modified by *O*-GlcNAc at serine and threonine residues (Hart *et al*, 2007; Lazarus *et al*, 2009). During reanalysis of a previously reported phosphosite mapping study of TAB1 (Cheung *et al*, 2003), a peptide with an increase in mass of 203 was observed, suggesting possible *O*-GlcNAc modification. To investigate whether TAB1 is an *O*-GlcNAc modified protein, full-length TAB1 was produced recombinantly in *Escherichia coli* and was *O*-GlcNAcylated *in vitro* using recombinant human *O*-GlcNAc transferase (hOGT). This resulted in a TAB1 protein species that was recognized by the anti-*O*-GlcNAc antibody CTD110.6 (Figure 2A). This was further confirmed using an alternative method for *O*-GlcNAc detection, involving chemoenzymatic labelling of the *O*-GlcNAc residue. Here, the *O*-GlcNAc moiety on the protein is labelled with UDP-GalNAz using a mutant galactosyltransferase GalT1 Y289L (mGalT1) with an azide derivative of UDP-GalNAc (UDP-GalNAz) as donor substrate, followed by labelling with biotin alkyne (Khidekel *et al*, 2003). After *in vitro O*-GlcNAcylation, TAB1 was subjected to mGalT1 labelling and then detected by probing with streptavidin-conjugated HRP (Figure 2B).

To confirm TAB1 as a *bona fide* OGT substrate with dynamic *O*-GlcNAc modification *in vivo*, we studied TAB1 *O*-GlcNAcylation in human embryonic kidney 293 (HEK293) cells overexpressing the IL-1 receptor (IL-1R cells) treated with/without the potent and specific OGA inhibitor GlcNAcstatin (Dorfmueller *et al*, 2006). TAB1 was immunoprecipitated from lysates and probed with the anti-*O*-GlcNAc antibody CTD110.6 (Figure 2C). TAB1 possesses a basal level of *O*-GlcNAcylation when grown in Dulbecco's modified Eagle's medium (DMEM) media containing 25 mM glucose (high glucose DMEM), increased by treatment with GlcNAcstatin (Figure 2C). To further confirm the *O*-GlcNAc signal on TAB1, TAB1 immunoprecipitated from IL-1R cells was treated with C*p*OGA (Rao *et al*, 2006), a promiscuous bacterial OGA, which removed *O*-GlcNAc from TAB1 without alteration in protein levels (Figure 2D). The *O*-GlcNAc signal on TAB1 could also be blocked by competition with free GlcNAc when the CTD110.6 antibody was pre-incubated with 500 mM *N*-acetylglucosamine (Figure 2D).

To identify the site(s) of TAB1 *O*-GlcNAcylation, recombinant *O*-GlcNAcylated TAB1 was analysed by mass spectrometry. In an initial experiment, *O*-GlcNAcylated TAB1 was trypsin digested and analysed by liquid chromatography-mass spectrometry (LC-MS). A single peptide VYPVSVPYSSAQSTSK (amino acids 387–402) was identified as carrying an additional molecular weight of 203. Two parallel approaches were then employed to identify the *O*-GlcNAcylation site(s) in this peptide. First, amino acids that could be putative targets for *O*-GlcNAcylation (part of PVS motif, similarity to target sequences of proline-directed kinases (Hart *et al*, 1996) were mutated to alanine. A TAB1 triple mutant (S391A/S395A/S396A) or the individual TAB1 mutants S391A, S395A and S396A were expressed as GST-tagged proteins in IL-1R cells treated with GlcNAcstatin. After 48 h, these GST fusion proteins were pulled down and blotted for *O*-GlcNAc. The triple mutant as well as the single S395A TAB1 mutant showed complete absence of an *O*-GlcNAc signal (Figure 2E), suggesting that S395 is the single *O*-GlcNAc site on TAB1. In a parallel approach, the TAB1 *O*-GlcNAc site was studied by collision-induced dissociation (CID) and electron transfer dissociation (ETD) liquid chromatography (LC) tandem mass spectrometry (MS/MS) experiments. TAB1 was *O*-GlcNAcylated *in vitro*, trypsin digested, and analysed by LC-MS/MS. The tryptic peptide VYPVSVPYSSAQSTSK ($Mw_{calc} = 1901.9$ Da) containing a HexNAc ($+203.1$ Da) was detected after 25.5 min as $[M + HexNAc + 2H]^{2+}$ m/z 951.904. While the CID spectrum (Figure 2F) did not fully define the site of modification, the ETD spectrum contained the critical fragment ions to unambiguously define S395 as the only *O*-GlcNAc modified site on TAB1 (Figure 2G).

O-GlcNAcylation of TAB1 is inducible

As shown previously (Figure 2C), levels of *O*-GlcNAc on TAB1 appear to be increased in the presence of the potent OGA inhibitor GlcNAcstatin. We next investigated stress-induced changes in the *O*-GlcNAc levels of TAB1 in wild-type (WT) MEFs. To enable these experiments, we attempted to generate a site-specific TAB1 S395 *O*-GlcNAc antibody. Although several attempts have been made to generate such site-specific *O*-GlcNAc antibodies recently, most of these still recognize a range of *O*-GlcNAc proteins (Teo *et al*, 2010). The only site-specific *O*-GlcNAc antibodies known to date are gThr58 on c-Myc (Kamemura *et al*, 2002) and gSer1011 on IRS1, gSer347 on CKII (Teo *et al*, 2010) and gSer400 on Tau (Yuzwa *et al*, 2010). Using a classical approach, we synthesized the TAB1-derived glycopeptide CVSVPYS (*O*-GlcNAc)SAQSTSKTS, exploiting the additional N-terminal cysteine for coupling to KLH. Serum generated from rabbits immunized with this antigen contained antibodies that were capable of recognizing *O*-GlcNAcylated TAB1, but not the *O*-GlcNAc-deficient TAB1 S395A mutant or the TAB1 triple mutant (S391A/S395A/S396A) (Figure 3A). Interestingly, while hyperglycaemic conditions resulted in an increase in global *O*-GlcNAc levels and also increased *O*-GlcNAcylation of TAB1 in MEFs, stimulation with IL-1 and NaCl, known to specifically activate the TAK1 signalling pathway (Ninomiya-Tsuji *et al*, 1999; Wang *et al*, 2001; Cheung *et al*, 2003; Huangfu *et al*, 2006), did not raise global *O*-GlcNAc levels to the same extent but still increased *O*-GlcNAcylation of WT TAB1 (Figure 3B and C). This suggested that the *O*-GlcNAcylation levels on TAB1 are modulated by stimuli that are known to activate TAK1 signalling, and could perhaps also affect signalling downstream of TAK1.

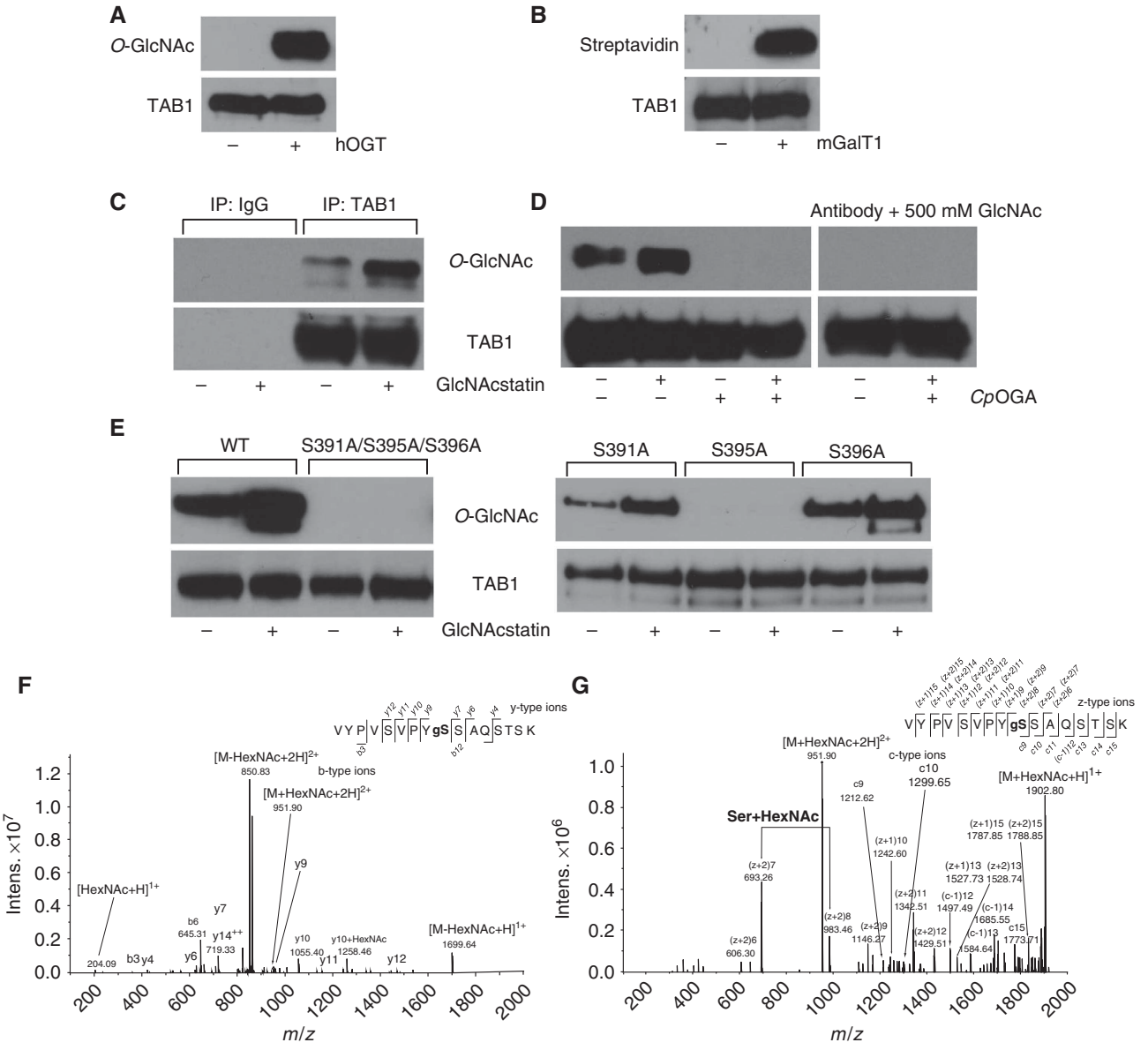

Figure 2 TAB1 is *O*-GlcNAcylated on Ser395. (**A**) Recombinant TAB1 was *O*-GlcNAcylated *in vitro* with recombinant hOGT. The samples were denatured in LDS (lithium dodecyl sulphate), subjected to SDS–PAGE and immunoblotted with a generic *O*-GlcNAc antibody CTD110.6. Total protein was detected with a total TAB1 antibody (TAB1) as a loading control. (**B**) *In vitro O*-GlcNAcylated TAB1 was subjected to enzymatic labelling using galactosyltransferase (mGalT1) labelling and UDP-GalNAz, before reacting with biotin alkyne for detection with HRP. The samples were denatured in LDS, subjected to SDS–PAGE and probed with horseradish peroxidase-conjugated streptavidin (Extravidin-HRP). (**C**) *In vivo O*-GlcNAcylation of TAB1 was detected by immunoprecipitating the endogenous TAB1 from IL-1R cells (treated with or without GlcNAcstatin—1 μM) using an antibody directed against TAB1. A non-specific IgG was included as a control. Immunoprecipitates were denatured in LDS, subjected to SDS–PAGE and immunoblotted with generic *O*-GlcNAc antibody CTD110.6 and then with the TAB1 antibody for loading controls. Treating the cells with the potent and selective OGA inhibitor GlcNAcstatin further increased TAB1 *O*-GlcNAcylation at a concentration of 1 μM. (**D**) TAB1 was immunoprecipitated, from cells treated with or without GlcNAcstatin (1 μM), using the TAB1 antibody. One fraction of the reaction was treated with *Cp*OGA for 30 min at room temperature, denatured in LDS, followed by immunoblotting with the generic *O*-GlcNAc antibody CTD110.6. A parallel set of immunoprecipitated samples were denatured in LDS and subjected to western blotting with antibody pre-incubated with *N*-acetylglucosamine, which blocks the *O*-GlcNAc signal on TAB1. Lower panel shows the corresponding Ponceau-stained membrane before western blotting. (**E**) WT TAB1, the S391A/S395A/S396A TAB1 triple mutant and the S391A, S395A and S396A TAB1 single mutants were transfected into IL-1R cells. After 24 h, the cells were treated with 1 μM of GlcNAcstatin for 16 h and the GSt–TAB1 was pulled out using glutathione-sepharose beads. The samples were denatured in LDS, subjected to SDS–PAGE and immunoblotted with the generic *O*-GlcNAc antibody CTD110.6 and then with a total TAB1 antibody (TAB1) as a loading control. (**F**, **G**) LC-MS/MS CID (**F**) and ETD (**G**) site mapping of the TAB1 *O*-GlcNAc modification site. *In vitro* O-GlcNAcylated TAB1 was digested with trypsin and subjected to LC–MS. The tryptic peptide VYPVSVPYSSAQSTSK ($Mw_{calc} = 1901.9$ Da) containing a HexNAc ($+203.1$ Da) was detected after 25.5 min as $[M + HexNAc + 2H]^{2+}$ m/z 951.904. The observed fragment ions are indicated, in case of the ETD experiment allowing definition of S395 as the site of *O*-GlcNAc modification. Figure source data can be found in Supplementary data.

O-GlcNAc modification of TAB1 modulates TAK1 activation

To investigate the effects of *O*-GlcNAcylation of TAB1 on activation of the TAK1 kinase and downstream signalling, we reintroduced WT TAB1 and the O-GlcNAc-deficient S395A mutant into *Tab1*$^{-/-}$ MEFs. In untransfected *Tab1*$^{-/-}$ MEFs, or the cells transfected with empty plasmid, there was no detectable IL-1α-induced TAK1 activity in agreement with

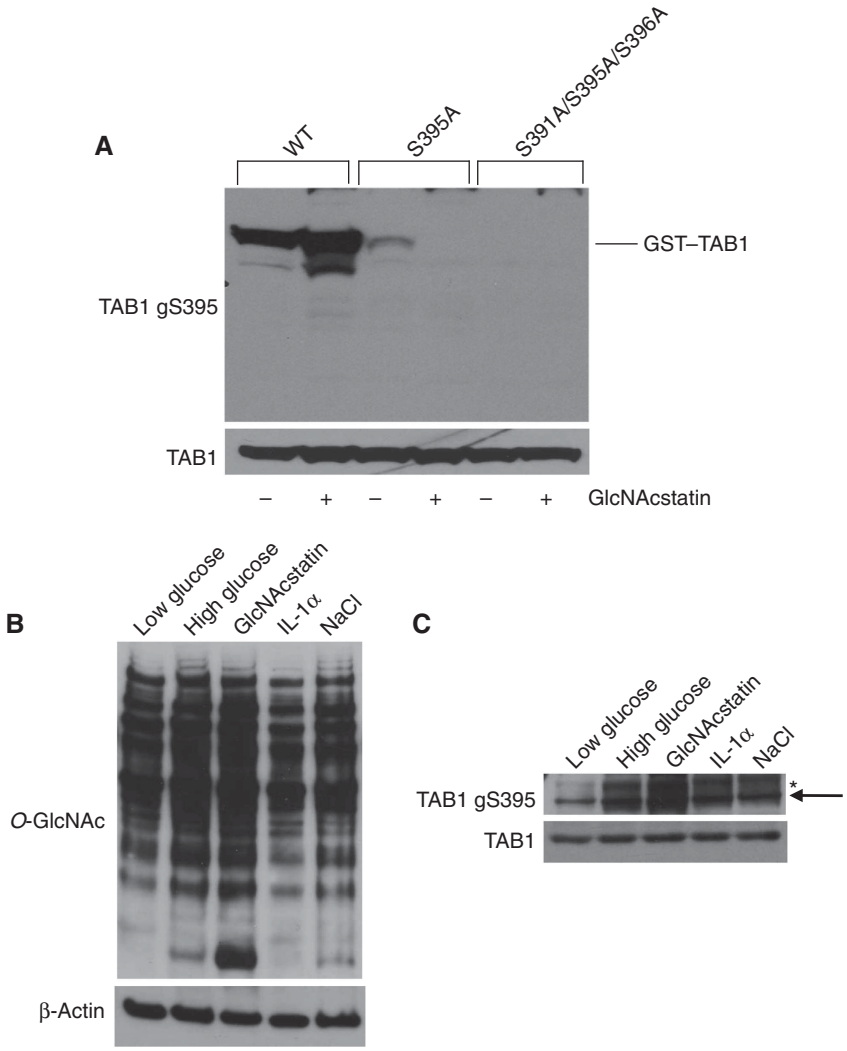

Figure 3 *O*-GlcNAcylation of TAB1 is modulated in response to IL-1 or NaCl stimulation. (**A**) The TAB1-derived glycopeptide CVSVPYS(*O*-GlcNAc)SAQSTSKTS, was used to generate an *O*-GlcNAc site-specific polyclonal antibody against *O*-GlcNAc S395. The specificity of the antibody was determined using cell lysates prepared from IL-1R cells transfected with WT TAB1, S395A TAB1 and the S391A/S395A/S396A TAB1 triple mutant and treated with or without GlcNAcstatin (1 μM). The cell lysates were denatured in LDS, subjected to SDS–PAGE and immunoblotted with the site-specific polyclonal antibody against *O*-GlcNAc S395. The antibody recognized the signal in WT samples only with increased signal intensity when *O*-GlcNAcylation of TAB1 is increased by GlcNAcstatin treatment. (**B**) *Tab1*$^{+/+}$ MEFs cells were (i) maintained in hyperglycaemic conditions (25 mM glucose) or (ii) treated with GlcNAcstatin (1 μM) or (iii) subjected to stress by treatment with IL-1α (10 ng/ml) or 100 mM of NaCl for 6 h under low glucose conditions (5 mM). Cell extracts (30 μg protein) were denatured in LDS, subjected to SDS–PAGE and immunoblotted with *O*-GlcNAc (CTD110.6) antibody and β-tubulin antibody for loading control. (**C**) The samples from (**B**) were probed simultaneously using the site-specific TAB1 *O*-GlcNAc S395 antibody (gS395). Figure source data can be found in Supplementary data.

earlier studies (Mendoza *et al*, 2008), whereas the cells transfected with WT TAB1 showed significant recovery of TAK1 autophosphorylation and activation (Supplementary Figure S1A and B). To establish an optimal reconstituted TAK1–TAB1 system, the *Tab1*$^{-/-}$ MEFs were transfected with varying amounts of TAB1 plasmid (pEBG GST–TAB1) (Supplementary Figure S1C and D). Reconstituting *Tab1*$^{-/-}$ MEFs with 5 μg of the TAB1 plasmid resulted in restoration of TAK1 activity similar to that of *Tab1*$^{+/+}$ MEFs, with TAB1 protein levels similar to endogenous TAB1 levels (Supplementary Figure S1E and F).

To investigate the effect of TAB1 *O*-GlcNAcylation on TAK1 activity, reconstituted *Tab1*$^{-/-}$ MEFs were stimulated with IL-1α for 5 or 15 min, the TAK1 complexes pulled down and analysed for kinase activity and activatory autophosphorylation. When transfected with WT TAB1, IL-1α treatment increased TAB1 *O*-GlcNAcylation and stimulated TAK1 activity, as evidenced by phosphorylation of T187 in the activation loop of TAK1 (Figure 4A and B). Strikingly, both TAK1 kinase activity and T187 autophosphorylation were reduced with the *O*-GlcNAc-deficient TAB1 S395A mutant (Figure 4A and B). To rule out the possibility TAK1 is itself *O*-GlcNAcylated, thus regulating kinase activity, TAK1 *O*-GlcNAcylation was investigated. GST–TAK1 and GST–TAB1 were co-expressed in IL-1R cells maintained in 1 g/l glucose DMEM (low glucose DMEM). Cells were either treated with GlcNAcstatin or stimulated with IL-1β or NaCl before GST pulldowns and probing for *O*-GlcNAcylation. As expected, TAB1 was *O*-GlcNAcylated in the presence of higher glucose conditions or on stimulation with IL-1 or NaCl, whereas TAK1 was not, suggesting that under physiological conditions as well as in stimulated conditions TAK1 is not *O*-GlcNAc modified (Supplementary Figure S2). Furthermore, the AK1–TAB1 complex remains intact in these experiments (Supplementary Figure S2).

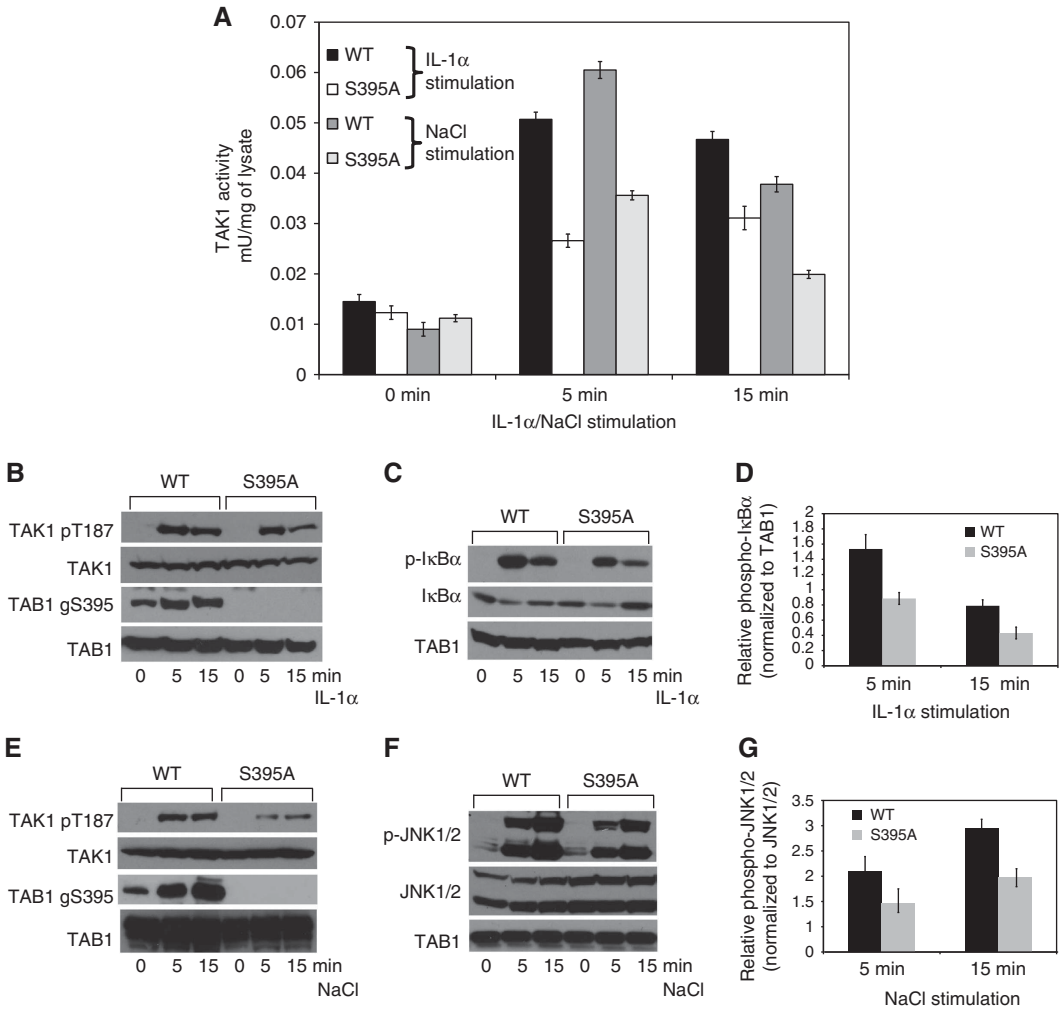

Figure 4 *O*-GlcNAcylation of TAB1 affects activation of TAK1 and phosphorylation of its downstream targets IκBα and JNK1/2. (**A**) IL-1α or NaCl-induced activation of TAK1 in WT and S395A TAB1 transfected *Tab1*$^{-/-}$ MEFs. At 36 h post-transfection, MEFs were serum starved for 6 h, and then stimulated for 5 and 15 min with 10 ng/ml IL-1α or 0.5 M NaCl. The TAK1 complexes were pulled down from the cell extracts (1 mg of protein extract) using glutathione-sepharose beads, and TAK1 activity assays were performed (as described in the Materials and methods section) in addition to immunoblotting as described below in (**B**). The data are expressed as the relative increase in TAK1 activity of the IL-1/NaCl-stimulated samples compared with the basal activity of the unstimulated control samples. Error bars denote standard deviation, determined from three independent experiments. (**B**) In parallel to the experiments in (**A**), TAK1 complexes were denatured in LDS, subjected to SDS–PAGE and immunoblotted with a phospho-specific antibody that recognizes TAK1 autophosphorylation at Thr187 (pT187) and with a further antibody that recognizes all forms of TAK1. *O*-GlcNAcylation of TAB1 was detected with the site-specific *O*-GlcNAc antibody (gS395) versus a total TAB1 antibody control (TAB1). (**C**) In all, 30 μg of the cell lysates from the samples obtained as in (**A**) was immunoblotted for phosphorylated Iκβα p-Iκβα and total Iκβα. (**D**) Densitometry for IκBα phosphorylation after normalization against total TAB1 levels. The data shown are the average of three independent experiments with error bars denoting standard deviation. (**E**) WT and S395A TAB1 were transfected in *Tab1*$^{-/-}$ MEFs. At 36 h post-transfection, MEFs were serum starved for 6 h, and then stimulated for 5 or 15 min with 0.5 M NaCl. The TAK1 complexes were pulled down from the cell extracts (1 mg of protein extract) using glutathione-sepharose beads and taken for kinase assays (**A**) in addition to immunoblotting. For immunoblotting, the samples were denatured in LDS, subjected to SDS–PAGE and immunoblotted with a phospho-specific antibody that recognizes TAK1 autophosphorylation at Thr187 (pT187) and with a further antibody that recognizes all forms of TAK1. *O*-GlcNAcylation of TAB1 was detected with the site-specific *O*-GlcNAc antibody (gS395) versus a total TAB1 antibody control (TAB1). (**F**) In all, 30 μg of the cell lysates from the samples obtained as in (**E**) was immunoblotted for phosphorylated JNK1/2 (p-JNK1/2) and total JNK1/2. (**G**) Densitometry for JNK1/2 phosphorylation after normalization for total JNK1/2. The data shown are the average of minimum of three independent experiments with error bars denoting standard deviation. Figure source data can be found in Supplementary data.

Once activated, TAK1 translocates from the membrane to the cytosol along with TRAF6 and its binding partners, TAB1 and TAB2/3. TAK1 activation subsequently leads to activation of IKK and c-Jun NH2-terminal kinase (JNK) as well as p38α MAPK. Activated IKK phosphorylates IκB proteins, and phosphorylated IκB proteins are degraded by the ubiquitin-mediated proteasome pathway (Karin and Ben-Neriah, 2000). To investigate the effects of TAB1 *O*-GlcNAcylation on these downstream events, we investigated Iκβα phosphorylation. In line with reduced TAK1 activation, IL-1α-stimulated phosphorylation of Iκβα Ser32 and Ser36 was reduced by up to 50% in the *Tab1*$^{-/-}$ MEFs expressing S395A TAB1 as compared with WT TAB1 (Figure 4C and D). Phosphorylation of p38α MAPK or ERK1 was not affected with the S395A mutant (Supplementary Figure S3). Previous work has shown that TAK1 activation can also be robustly induced by osmotic stress, independent of stimulation with cytokines/LPS, leading to downstream activation of JNK1/2 (Inagaki *et al*, 2008). Indeed,

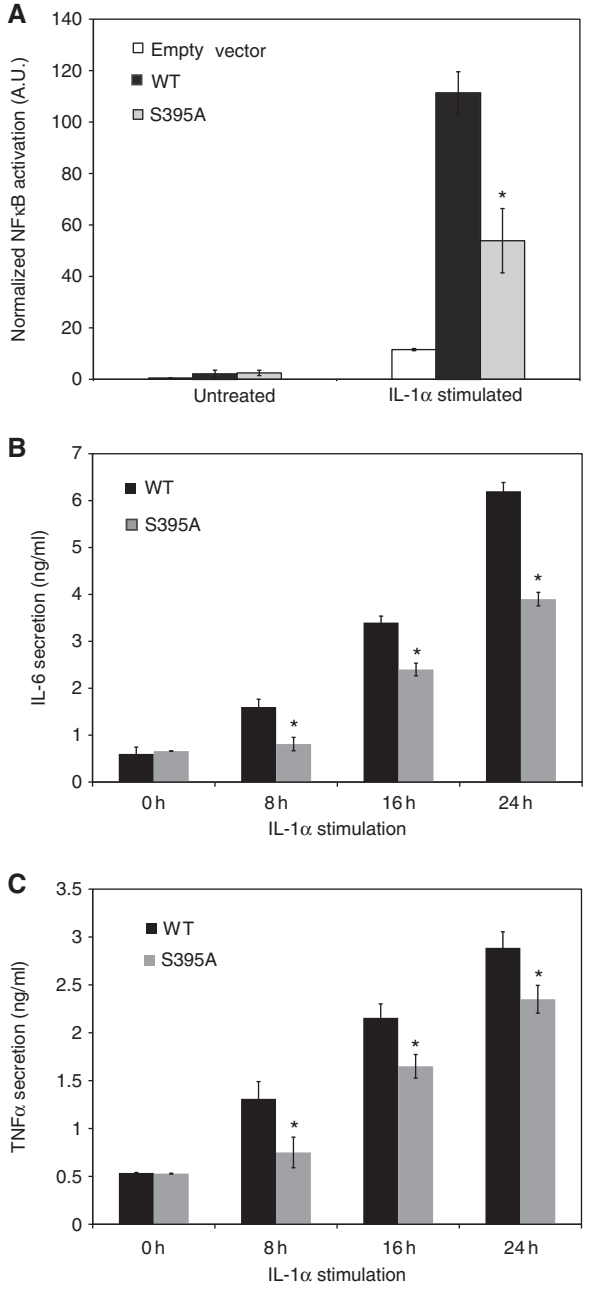

Figure 5 TAB1 *O*-GlcNAcylation affects NFκB-dependent gene transcription and cytokine production. (**A**) *Tab1*$^{-/-}$ MEFs were co-transfected with either WT TAB1, S395A TAB1 or empty plasmid and plasmids encoding the 3 × κB luciferase reporter construct; and pRL-TK. After 24 h of transfection, the cells were stimulated with 10 ng/ml of IL-1α for 24 h before lysing with Passive Lysis Buffer (Promega). NFκB-dependent luciferase reporter gene expression was measured and normalized for transfection efficiency using Renilla luciferase as the internal control. The data are presented as background-corrected mean values ± s.e.m. from three to four independent experiments. (*) denotes $P<0.05$ for significant differences between mutant and WT TAB1. (**B**, **C**) *Tab1*$^{-/-}$ MEFs were transfected with WT or S395A TAB1 plasmid and 24 h post-transfection stimulated with 10 ng/ml IL-1α for 8–24 h. The media was collected and the quantification of (**B**) IL-6 and (**C**) TNFα was performed by ELISA. The data are presented as background-corrected mean values ± s.e.m. from three to four independent experiments. (*) denotes $P<0.05$ for significant differences between mutant and WT TAB1.

osmotic stress (induced with 0.5 M NaCl) also induces phosphorylation of TAK1 T187, and in parallel *O*-GlcNAcylation of TAB1 S395, in the *Tab1*$^{-/-}$ MEFs complemented with WT TAB1 (Figure 4A and E). This effect was reduced with the *O*-GlcNAc-deficient TAB1 S395A mutant (Figure 4A and E), which also translated into reduced phosphorylation of JNK1/2 on Thr183 and Tyr185 (Figure 4F and G).

O-GlcNAcylation of TAB1 modulates NFκB activation and IL-1α-stimulated cytokine release

In the canonical NFκB activation pathway, upon phosphorylation by IKKβ, Iκβα and IκBβ, the cytoplasmic inhibitors of NFκB, are marked for ubiquitination and subsequent proteosomal degradation (DiDonato *et al*, 1996; Ghosh and Karin, 2002). The degradation of IκBα and IκBβ allows translocation of NFκB to the nucleus, leading to transcription of a plethora of genes including those encoding various cytokines (Li and Verma, 2002; Hayden and Ghosh, 2008). Using a reporter assay, we studied the role of *O*-GlcNAc on TAK1/TAB1-mediated activation of NFκB in response to IL-1α stimulation in *Tab1*$^{-/-}$ MEFs overexpressing WT or S395A TAB1 (Figure 5). While a robust level of NFκB activation was observed with WT TAB1, S395A TAB1 showed a significant reduction in NFκB activation (Figure 5A), in line with the observed reduction in phosphorylation of IκBα (Figure 4C). To evaluate whether the observed reduction in NFκB activity of the TAB1 S395A mutant resulted in effects on cytokine production, levels of IL-6 and TNFα cytokines were measured in the cell culture medium at different time points after IL-1α stimulation. IL-6 secretion was reduced by 50% after 8 h and 40% after 24 h in samples from the cells transfected with the S395A TAB1 mutant, as compared with the WT TAB1 control (Figure 5B). Similarly, TNFα production, 8 and 16 h after IL-1α stimulation, was significantly reduced with the *O*-GlcNAc-deficient TAB1 mutant (Figure 5C).

O-GlcNAcylation of TAB1 modulates regulatory phosphorylation

TAB1 possesses three characterized phosphorylation sites (S423, T431 and S438) that are modified under various stimuli and are involved in TAK1 activation (Mendoza *et al*, 2008) (Figure 1A and B). p38α MAPK phosphorylates S423 and T431 whereas ERK1/2 and JNK1/2 phosphorylate S438 (Mendoza *et al*, 2008), although the specific roles of the individual phosphorylation sites have not yet been defined. The regulatory TAB1 *O*-GlcNAc site described here does not correspond to a known phosphorylation site; however, it is in proximity to the phosphorylation sites and the C-terminal region of TAB1 that is required for interaction with TAK1 (Ono *et al*, 2001) (Figure 1B). To investigate possible effects of *O*-GlcNAcylation of TAB1 on phosphorylation at S423, T431 and S438, *Tab1*$^{-/-}$ MEFs transiently transfected with TAB1 (WT and S395A) were stimulated with sodium chloride in the presence or absence of GlcNAcstatin. When stimulated with NaCl, phosphorylation of S438 was observed to increase in both WT and S395A TAB1 mutant when compared with control. However, in the presence of both NaCl and GlcNAstatin, there is a further increase in phosphorylation of S438 in WT, but this further increase was not seen in the S395A mutant. This suggests that O-GlcNAc at S395 may regulate TAB1 phosphorylation at S438.

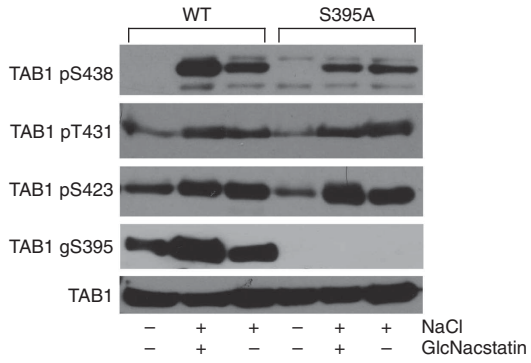

Figure 6 Interplay between TAB1 *O*-GlcNAcylation and phosphorylation. *Tab1*$^{-/-}$ MEFs were transfected with WT or S395A TAB1 plasmids. At 36 h post-transfection, MEFs were serum starved for 6 h with or without GlcNAcstatin (1 μM) and then stimulated for 15 min with 0.5 M NaCl. Aliquots of cell extract (30 μg protein) were denatured in LDS, subjected to SDS–PAGE and immunoblotted with antibodies that recognize TAB1 phosphorylated at Ser423 (pS423), Thr431 (pT431) and Ser438 (pS438) and for *O*-GlcNAcylation at Ser395 (gS395). Data presented are representative of results obtained from three independent experiments. Figure source data can be found in Supplementary data.

WT TAB1 showed increased phosphorylation of S438 in the presence of both GlcNAcstatin and NaCl as compared with the S395A mutant, while there was no change on S423 or T431 phosphorylation (Figure 6). S438 is phosphorylated by ERK1/2 and JNK1/2, and TAB1 has a role in stress response and activation of JNK1/2 pathway (Inagaki *et al*, 2008). The reduced phosphorylation at S438 on S395A TAB1 as compared with that of WT TAB1 could be related to the reduced activation of JNK1/2 as observed earlier, although its not yet clear how TAB1 *O*-GlcNAcylation may regulate JNK1/2 activity towards the TAB1 S438 phosphorylation site.

Discussion

TAK1 is a key regulator of NFκB activity and cytokine production in response to stimulation with LPS and cytokines. Over the past decade, a large number of studies have shown that TAK1 activity is extensively regulated by phosphorylation, ubiquitination, and binding to the regulatory binding partners TAB1–3 (Sakurai *et al*, 2000; Wang *et al*, 2001; Ishitani *et al*, 2003; Ea *et al*, 2004; Singhirunnusorn *et al*, 2005). The data presented here show that TAB1 possesses a single, inducible *O*-GlcNAc site, Ser395 that is responsive to IL-1 and NaCl, known activators of the TAK1 pathway. By reintroducing WT TAB1 or an *O*-GlcNAc-deficient mutant in *Tab1*$^{-/-}$ MEFs, we were able to delineate the role of *O*-GlcNAcylation of TAB1. With IL-1 stimulation, TAB1 *O*-GlcNAcylation enhances Iκβα phosphorylation, which in turn regulates NFκB activation. We show here that *O*-GlcNAcylation of TAB1 is required for full activation of TAK1 on Thr187 and that there is a direct correlation between *O*-GlcNAcylation of TAB1, autophosphorylation of TAK1 Thr187, activation of NFκB and, ultimately, production of IL-6 and TNFα.

Recent studies have shown effects of hyperglycaemia on the transcriptional activity of NFκB and also on IKκβ, key regulators of innate immunity pathways. Both IKKβ and NFκB are also *O*-GlcNAc modified proteins (Yang *et al*, 2008; Kawauchi *et al*, 2009). Taken together with the data presented here, this suggests that the innate immune response is not only regulated by phosphorylation and ubiquitination but also by *O*-GlcNAcylation. Further work will be required to understand how *O*-GlcNAcylation of TAB1 is induced/regulated, and also to unravel the molecular details of *O*-GlcNAc-dependent TAK1 activation by TAB1.

Materials and methods

Materials

GlcNAcstatin was obtained from GlycoBioChem (Dundee, UK). Mouse IL-6 and TNFα Elisa kits were purchased from Peprotech, UK. Human IL-1β was from Sigma, murine IL-1α was from Peprotech and glutathione-sepharose was from GE Healthcare. Luciferase reporter assay kit was from Promega. Click-iT™ *O*-GlcNAc Enzymatic Labeling System and Click-iT Biotin Glycoprotein Detection Kit were from Invitrogen. Dynabeads® Protein G was from Invitrogen.

DNA cloning

Full-length TAB1 was cloned and inserted into pGEX6P1 for recombinant protein production in *E. coli* and pEBG6P for transfection in mammalian cells, as described previously (Cheung *et al*, 2004). Mutations for putative *O*-GlcNAc sites on TAB1 (S391A, S395A and S396A) were created following the Quick Change method (Stratagene) using KOD Hot start Polymerase (Novagen). Recombinant OGT was produced as described previously (Clarke *et al*, 2008). GST–TAK1 was obtained from the Division of Signal Transduction Therapy DSTT in Dundee. *Cp*OGA was produced as described previously (Rao *et al*, 2006). For NFκB reporter assays, DNA encoding a ConA basal promoter incorporating three copies of the NFκB DNA response element (termed '3 × κB luciferase reporter construct') was provided by Professor Ron Hay, College of Life Sciences, University of Dundee (Rodriguez *et al*, 1999). The pRL-TK vector driving *Renilla* luciferase expression was from Promega.

Antibodies

The antibodies that recognize TAK1 phosphorylated at Thr187, total TAB1, TAB1 phosphorylated at Ser423 and TAB1 phosphorylated at Ser438 were used as described previously (Cheung *et al*, 2003). Antibodies recognizing the active phosphorylated forms of ERK1/2, JNK1/2, p38α MAPK and total ERK1/2, JNK1/2 were from Cell Signalling Technologies. ExtrAvidin®-Peroxidase was from Sigma. For immunoblotting with the phospho-specific antibodies for TAK1 and TAB1, the antibodies were incubated at 3 μg/ml in the presence of 30 μg/ml of the unphosphorylated peptide immunogen to neutralize any antibodies that recognize the unphosphorylated form of the protein. The anti-*O*-GlcNAc antibody CTD110.6 was purchased from Abcam. Secondary antibodies conjugated to horseradish peroxidase were from Pierce.

Generation of O-GlcNAc-specific antibody against S395 on TAB1

The *O*-GlcNAc peptide CVSVPYS(O-GlcNAc)SAQSTSKTS, corresponding to residues 389–403 of TAB1, was synthesized on a Liberty microwave-assisted peptide synthesizer (CEM) using MBHA Rink-amide low load resin (Novabiochem) with standard protocols of Fmoc SPS chemistry. The QS dipeptide was introduced as pseudoproline to suppress formation of truncated sequences detected in pilot experiments. 4Ac-GlcNAcSerFmoc was synthesized in-house following a published procedure (Saha and Schmidt, 1997). After high peformance liquid chromatograpy (HPLC) purification of the peptide, it was conjugated to keyhole limpet haemocyanin and injected into rabbits. Antibodies from the serum were first precipitated with ammonium sulphate followed by a one-step purification by passing the resuspended antibody over a non-GlcNAcylated peptide column. The flowthrough from the column was collected and used for immunoblotting.

In vitro O-GlcNAc assay

In vitro o-GlcNAcylation of TAB1 (1 μM) was performed in 20 μl assay volumes containing 100 nM of OGT, reaction buffer (50 mM Tris–HCl, pH 7.5, 1 mM DTT, 12.5 mM $MgCl_2$), and 1 mM UDP-GlcNAc. The reactions were incubated for 90 min at 37°C, stopped by adding 4 × SDS–PAGE sample buffer, resolved on SDS–PAGE, transferred to PVDF and probed with appropriate antibodies.

Enzymatic labelling of O-GlcNAc sites

GST–TAB1 was bound to glutathione-sepharose beads and was *O*-GlcNAcylated *in vitro*. The beads were washed with 10 mM HEPES (pH 7.9) and resuspended in reaction buffer (1% SDS, 20 mM HEPES) then 2 μl of GalT1 Y289L (Invitrogen) and 2 μl of 0.5 mM UDP-GalNAz (Invitrogen) were added in a final reaction volume of 50 μl. The reaction was performed overnight at 4°C. The beads were washed twice with reaction buffer to remove excess UDP-GalNAz. The samples were then reacted with biotin alkyne (Invitrogen) according to the manufacturer's instructions. The proteins were resolved by SDS–PAGE and transferred to PVDF. The blot was incubated with ExtrAvidin-Peroxidase and biotinylated TAB1 was visualized by ECL reaction.

O-GlcNAc site mapping of TAB1

For site mapping analysis of digested TAB1 protein, an Ultimate 3000RSLC nano-HPLC system (Dionex) with a 3D high capacity ion trap mass spectrometer with ETD capability (amaZon ETD; Bruker Daltonics) were used to perform HPLC electrospray MS/MS (ESI-MS/MS). Digested TAB1 samples were reconstituted in 0.1% formic acid, injected and concentrated onto a Dionex PepMap C18 nano-trap column, after a wash step with (2% acetonitrile, 0.1% formic acid (v/v)) peptides were resolved by a Dionex Acclaim PepMap C18 reverse phase column (75 μm i.d. $\times$ 15 cm $\times$ 2 μm) over a 25-min linear gradient from 4% to 50% buffer B (80% acetonitrile and 0.08% formic acid in Milli-Q water (v/v)), followed by another 2 min to 90% buffer B. The column was then washed by holding the 90% buffer B for 10 min before returning to initial conditions of 96% buffer A (0.1% formic acid in Milli-Q water (v/v)). A typical tandem mass spectrometric (MS/MS) cycle (Alternating CID/ETD) in amaZon ETD happens in the following order: (A) 1 MS full-range scan and precursor ion selection. (B) Accumulation of precursor ion ($\sim$10 ms) and fragmentation by CID. (C) (CID-MS/MS spectrum recorded) accumulation of the same precursor ion ($\sim$5 ms), which is allowed to mix and react with fluoranthene (50–100 ms; variable) for ETD fragmentation, and ETD-MS/MS spectrum acquired. Steps (B) and (C) are repeated automatically for each precursor ion. In this study, precursor ion selection was set up to five ions per cycle, excluding singly charged ions, with a dynamic exclusion time of 0.5 min for both CID and ETD. Helium gas was used as collision gas (60–200%), collision energy sweep with amplitude 1.0 parameters was used for CID fragmentation. For ETD experiments, the maximum output of ETD reagent ion (202 m/z) was achieved with the following nCI source tuned parameters: reagent ion charge control target (ICC) 500 000, maximum emission current 4 μA and ionization energy of 75 eV, reactant remove cutoff $\leqslant$210 m/z without supplemental energy activation.

Raw MS data were processed using software packages BioTool 3.2 SR1 and DataAnalysis 4.0 (Bruker Daltonic GMBH). In parallel, two database searches were performed for CID and ETD using Mascot v2.3 (Matrix Science Ltd), database used IPI-human 20110731 (91 522 sequences; 36 630 302 residues) with the following Mascot MS/MS ion search parameters: peptide charges considered 2+, 3+ and 4+ ions, peptide tolerance and fragment tolerance was set to $\pm$0.5 Da, # of $^{13}C = 1$, two missed cleavages allowed, trypsin as proteolysis enzyme, ESI-TRAP (for CID) and ETD-TRAP (for ETD) for instrument type. Carbamidomethyl (C) was used as fixed modification, where Deamidated (NQ), Oxidation (M), Phospho (ST) and HexNAc (ST) (+203.0794 Da) were set as variable modifications.

Cell culture, stimulation and lysis

IL-1R cells HEK293 (cells stably expressing the IL-1 receptor) and immortalized *Tab1*-deficient MEFs (*Tab1*$^{-/-}$ MEFs) were provided by Professor Philip Cohen, MRC Unit, University of Dundee. Cells were cultured in DMEM with 1 or 4.5 mg/ml glucose, containing 10% (v/v) heat inactivated fetal calf serum (FCS), and 2 mM L-glutamine. Prior to stimulation with human IL-1β in IL-1R cells or murine IL-1α in mouse cells, the medium was removed and replaced with DMEM from which FCS had been omitted. IL-1R cells were serum deprived for 16 h and MEFs for 6 h. GlcNAcstatin (1 μM) was added to cells during serum starvation, if required. For osmotic shock, cells were treated with either 0.25 or 0.5 M of NaCl by adding it into DMEM for 15 min before harvesting the cells.

Cells were lysed in ice-cold lysis buffer (50 mM Tris–HCl pH 7.5, 1 mM sodium orthovanadate 1 mM EDTA, 10 mM sodium β-glycerophosphate, 1 mM EGTA, 50 mM sodium fluoride, 5 mM sodium pyrophosphate, 1% (v/v) Triton X-100, 0.27 M sucrose, 0.1% (v/v) 2-mercaptoethanol, 0.1 mM PMSF, 1 mM benzamidine and 5 μM leupeptin). Lysates were centrifuged at 13 000 *g* for 15 min at 4°C and the supernatants were used immediately or snap frozen in liquid nitrogen and stored in aliquots at −80°C until use. Protein concentrations were determined using the Bradford assay.

Immunoprecipitation and OGA treatment of TAB1

To immunoprecipitate endogenous TAB1, 1 mg of cell lysate was incubated for 2 h at 4°C with 10 μg of anti-TAB1 antibody coupled with 10 μl of Dynabeads Protein G. The immunoprecipitates were washed twice with 1 ml of lysis buffer containing 0.25 M NaCl, followed by two washes with 1 ml of 50 mM Tris/HCl, pH 7.5, 50 mM NaCl and 0.1 % (v/v) 2-mercaptoethanol and subjected to SDS–PAGE followed by western blotting.

For OGA treatment on immunoprecipitated *O*-GlcNAcylated TAB1, 3 mg of lysate was used for immunoprecipitation. The samples were divided in three equal volumes. One set of samples was treated with *Cp*OGA (1 μM) for 30 min at room temperature, while the other two sets were left untreated at room temperature. The samples were subjected to SDS–PAGE and western blotting with CTD110.6 antibody. The third set of samples was incubated with CTD110.6 antibody, which was pre-incubated with 500 mM *N*-acetylglucosamine for 1 h.

Cell transfections

IL-1R cells were transfected at 40–50% confluence using polyethyleneimine using DNA encoding GST (glutathione transferase)–TAB1, whereas MEFs were transfected at a density of 3×10^6 cells, with the Amaxa MEF2 kit according to the manufacturer's instructions.

NFκB reporter gene assay

For the measurement of NFκB-dependent luciferase gene expression, *Tab1*$^{-/-}$ MEFs (3×10^6) were co-transfected with either 3 μg of WT TAB1, S395A TAB1 or empty pEBG6 plasmid; 0.5 μg of DNA encoding the 3 $\times$ κB luciferase reporter construct; and 0.5 μg pRL-TK and plated 3×10^5 cells per well. After 24 h, the cells were stimulated with 10 ng/ml of IL-1α for 24 h and then the cells were lysed in Passive Lysis Buffer (Promega). The luciferase activity was then measured using a Dual-Luciferase Reporter Assay System (Promega) as per the manufacturer's instructions. Firefly luciferase activity was normalized by *Renilla* luciferase activity for each transfection.

TAK1 activity assays

TAK1 present in TAB1 immunoprecipitates was assayed by its ability to activate MKK6, as judged by the activation of SAPK2α/p38α. The active SAPK2α/p38α generated in this first stage of assay was then quantitated in a second assay by measuring phosphorylation of myelin basic protein (Cheung *et al*, 2003). The TAK1 complexes were pulled down from 1 mg of cell lysate obtained from TAB1 reconstituted MEFs. The cell lysates were incubated for 2 h at 4°C with 20 μl of glutathione-sepharose beads per sample. The beads were washed twice with 1 ml of lysis buffer containing 0.25 M NaCl, followed by two washes with high salt wash buffer (1 ml of 50 mM Tris–HCl pH 7.5, 0.5 M NaCl and 0.1% (v/v) 2-mercaptoethanol). In all, 25% of the TAK1 complex bound to the beads was used to measure TAK1 activity. The remaining 75% of the TAK1 complex was taken for immunoblotting using appropriate antibodies as described earlier.

Cytokine secretion assay

For measuring TNFα and IL-6 secretion into the medium, 3×10^6 MEFs were transfected with the WT or S395A TAB1 and were seeded in 24-well plates at density of 3×10^5 cells/well. At 24 h after transfection, the cells were stimulated with IL-1α for different lengths of time. The media were collected and after brief centrifugation, 100 μl of clear media was used for cytokine ELISA as per the protocol from Peprotech.

Supplementary data

Supplementary data are available at *The EMBO Journal* Online (http://www.embojournal.org).

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Author contributions: DMFA and SP designed the experiments. SP performed the experiments and analysed the data. VSB synthesized the peptides. OA and DGC performed mass spectrometry. AI cloned the constructs. SP and DMFA wrote the manuscript.

Conflict of interest

The authors declare that they have no conflict of interest.

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