ACCELERATED PUBLICATION

TAB3, a new binding partner of the protein kinase TAK1

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We have identified a new binding partner of the TGFβ (transforming growth factor-β)-activated protein kinase (TAK1), termed TAB3 (TAK1-binding protein-3), which shares 48 % amino acid sequence identity with TAB2. Our results indicate that two distinct TAK1 complexes are present in cells. One comprises TAK1 complexed with TAB1 and TAB2, and the other TAK1 complexed with TAB1 and TAB3. Both complexes are activated in response to tumour necrosis factor-α or interleukin-1 in human epithelial KB cells or bacterial lipopolysaccharide in RAW264.7 macrophages, and are subject to feedback control by stress-activated protein kinase 2a (SAPK2a; also called p38α). The electrophoretic mobility of TAB2 and TAB3 decreases in response to these agonists or osmotic shock, and is reversed by treatment with protein phosphatase-1. The decrease in mobility of TAB3 is prevented if the cells are incubated with SB 203580 before stimulation, but treatment with SB 203580 produces forms of TAB2 with a mobility intermediate between that observed for TAB2 in unstimulated and stimulated cells. Similar results were obtained in embryonic fibroblasts from mice deficient in SAPK2a/p38α. Our results indicate that TAB3 is phosphorylated via the SAPK2a/p38α pathway, whereas TAB2 is phosphorylated at two or more sites by both an SAPK2a/p38α-dependent and an SB 203580-independent kinase. The SAPK2a/p38α-mediated phosphorylation of TAB2 and TAB3 may contribute to the SAPK2a/p38α-mediated feedback control of TAK1 activity that also involves the phosphorylation of TAB1. We also show that the agonist-induced activation of TAK1 complexes requires the phosphorylation of the TAK1 catalytic subunit at a serine/threonine residue(s).

Key words: inflammation, interleukin-1 (IL-1), lipopolysaccharide (LPS), p38 MAP kinase, stress-activated protein kinase 2a (SAPK2a), tumour necrosis factor (TNF).

INTRODUCTION

The TGFβ (transforming growth factor-β)-activated protein kinase (TAK1) is a widely expressed enzyme which, as implied by its name, was originally identified as a TGFβ-activated enzyme [1]. However, there is growing evidence that one of its major roles is to mediate some of the intracellular actions of pro-inflammatory cytokines, such as interleukin-1 (IL-1) and tumour necrosis factor-α (TNFα), and responses to bacterial lipopolysaccharide (LPS) [2,3]. Once activated, TAK1 is thought to switch on several protein-kinase cascades, including those that lead to the activation of stress-activated protein kinase 2a (SAPK2a; also called p38α), c-Jun N-terminal kinase (JNK) and the transcription factor NF-κB (nuclear factor κB).

TAK1 is bound to a regulatory subunit TAB1 (TAK1-binding protein 1) that seems to be required for activity [4], and a second subunit TAB2 (TAK1-binding protein 2), which is thought to interact with upstream elements of the signalling pathway [5]. We have recently identified a feedback control mechanism in which TAK1 activity is down-regulated, and which involves the direct phosphorylation of TAB1 at two residues (Ser^{423} and Thr^{431}) by SAPK2a/p38α [6]. Thus the activation of TAK1 by IL-1, TNFα or LPS is enhanced by incubating cells with the SAPK2a/p38α inhibitor SB 203580, or in cells that do not express SAPK2a/p38α. An important consequence of blocking this feedback control mechanism is that inhibitors of SAPK2a/p38α also enhance the activation of JNK and NF-κB [6].

In the present study, we identify a new subunit of TAK1, termed TAB3 (TAK1-binding protein 3), that is homologous with TAB2. We find that cells express two distinct forms of TAK1 in which this protein kinase is complexed with TAB1 and either TAB2 or TAB3. We demonstrate that both forms of TAK1 are activated by IL-1, TNFα or LPS, and that this is accompanied by the phosphorylation of TAB2 and TAB3 via the SAPK2a/p38α pathway.

MATERIALS AND METHODS

Materials

Molecular-mass markers were purchased from BioRad (Hemel Hempstead, Herts, U.K.), and NuPAGE precast SDS/polyacrylamide gels were from Invitrogen (Groningen, The Netherlands). IL-1α, TNFα and LPS were from Sigma (Poole, Dorset, U.K.). The SAPK2a/p38α inhibitor SB 203580 was from Calbiochem (La Jolla, CA, U.S.A.). The sources of all other materials have been described previously [6].

cDNA cloning

EST (expressed sequence tag) clones ID 3916085 and ID 1580302 encoding human TAB2, and ID 5502039 encoding human TAB3

Abbreviations used: TGFβ, transforming growth factor-β; TAK1, TGFβ-activated protein kinase; TAB1/TAB2/TAB3, TAK1-binding protein-1, -2 and -3 respectively; SAPK2a, stress-activated protein kinase 2a; IL-1, interleukin-1; TNFα, tumour necrosis factor-α; LPS, lipopolysaccharide; JNK, c-Jun N-terminal kinase; NF-κB, nuclear factor κB; GST, glutathione S-transferase; CUE, coupling of ubiquitin conjugation to endoplasmic reticulum degradation; NZF, Npl4 zinc finger; TRAF, TNF-receptor-associated factor.

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The nucleotide sequence data reported for human TAB3 will appear in DDBJ, EMBL, GenBank® and GSDB Nucleotide Sequence Databases under the accession number AY371491.

were obtained from the IMAGE consortium [Human Genome Mapping Project (HGMP), Hinxton Hall, Cambs, U.K.]. Full-length cDNAs encoding TAB2 or TAB3 were amplified by PCR and subcloned into the pGEX6P-1 vector (Amersham Pharmacia Biotech, Little Chalfont, Bucks, U.K.) using the restriction enzymes *Bam*HI/ *Eco*RI.

Northern blot analysis

A human multiple-tissue Northern blot (Clontech, Palo Alto, CA, U.S.A.) was probed with cDNA encoding residues 445–677 of human TAB3. The 636 bp cDNA fragment was excised from the pGEX-6P1-TAB3 plasmid by restriction digestion with *Xho*I, gel-purified and randomly labelled with [α-^{32}P]ATP. The Northern blot was hybridized overnight at 60 °C with the radiolabelled cDNA probe in ExpressHyb (Clontech). The blot was washed for 40 min at room temperature (21 °C) in 2 × SSC (where 1 × SSC is 0.15 M NaCl/0.015 M sodium citrate)/0.05 % SDS, followed by a 40 min wash at 50 °C in 0.1 × SSC/0.1 % SDS. The blot was autoradiographed for 1 day at − 70 °C.

Expression of TAB2 and TAB3 in *Escherichia coli*

The plasmids pGEX6P-1-TAB2 or pGEX6P-1-TAB3 encoding glutathione S-transferase (GST) fusion proteins of full-length human TAB2 or TAB3 respectively were transformed into BL21 cells. The transformed cells were grown at 37 °C in LB (Luria–Bertani) medium containing 100 μg/ml of ampicillin to an attenuance at 600 nm of 0.8. They were then induced with 30 μM isopropyl β-D-thiogalactoside, grown overnight at 26 °C, and the bacteria were harvested by centrifugation for 30 min at 10 000 ***g***. The bacterial pellet was resuspended in 50 mM Tris/HCl, pH 7.5, containing 150 mM NaCl, 0.1 % (v/v) 2-mercaptoethanol, 0.03 % (w/v) Brij 35, 5 % (v/v) glycerol, 0.1 mM PMSF, 1 mM benzamidine and 1 mM dithiothreitol, subjected to one freeze–thaw cycle and sonicated. Triton X-100 was added to a final concentration of 1 % (v/v) and incubated for 30 min on ice. The lysate was clarified by centrifugation for 30 min at 30 000 ***g*** and the expressed fusion protein was isolated from the supernatant by affinity chromatography on glutathione–Sepharose 4B (Amersham Pharmacia Biotech) at 4 °C. The resin was washed with 20 bed-volumes of 50 mM Tris/HCl, pH 7.5, containing 0.5 M NaCl, 0.1 % (v/v) 2-mercaptoethanol and 0.27 M sucrose, then with 20 bed-volumes of 50 mM Tris/HCl, pH 7.5, 0.1 % (v/v) 2-mercaptoethanol and 0.27 M sucrose. The bound GST fusion proteins were eluted with 20 mM glutathione in 50 mM Tris/HCl, pH 7.5, containing 0.1 % (v/v) 2-mercaptoethanol and 0.27 M sucrose. Finally, the eluted protein was dialysed overnight in 50 mM Tris/HCl, pH 7.5, containing 0.1 % (v/v) 2-mercaptoethanol and 50 % glycerol at 4 °C, and stored at − 20 °C.

Antibodies

The GST–TAB3 fusion protein was injected into a sheep at Diagnostics Scotland (Edinburgh, U.K.) and the antisera were affinity-purified on GST–TAB3 bound to glutathione–Sepharose. The antibody was then passed through a GST–TAB2 column to deplete it of anti-GST antibodies and any antibodies that might cross-react with TAB2. An anti-TAB2-specific antibody (K-20) was purchased from Santa Cruz Biotechnology (Santa Cruz, CA, U.S.A.). As shown in the Results section, this antibody does not recognize TAB3. The immunoprecipitating anti-TAB1 and anti-TAK1 antibodies have been described previously [6].

Cell culture and stimulation

Cells were grown in 10 cm-diameter dishes to confluence in DMEM (Dulbecco's modified Eagle's medium) containing 10 % (v/v) foetal-calf serum and antibiotic/antimycotic (100 units/ml penicillin, 100 μg/ml streptomycin and 25 ng/ml amphotericin B), as described previously [6]. Cells were grown overnight in serum-free medium containing antibiotic/antimycotic prior to stimulation with IL-1α or TNFα. Cells that were stimulated with LPS or exposed to sorbitol were not serum-starved before stimulation.

Immunoprecipitation of proteins

Cells were harvested by lysis in buffer A [50 mM Tris/HCl (pH 7.5)/0.1 mM EGTA/1 mM EDTA/1 % (w/w) Triton X-100/1 mM Na_3VO_4/50 mM NaF/5 mM sodium pyrophosphate/0.27 M sucrose/1 μM microcystin-LR/0.1 % (v/v) 2-mercaptoethanol]. Each 50 ml of buffer A also contained one tablet of Complete™ protease inhibitor cocktail (Roche, Lewes, East Sussex, U.K.). After centrifugation for 5 min at 13 000 ***g***, the supernatant was removed and an aliquot of cell lysate (amounts specified in the Figure legends) was added to 2 μg of antibody coupled with 10 μl of Protein G–Sepharose. Following incubation for 1 h at 4 °C, the suspension was centrifuged, the supernatant was discarded and the Protein G–Sepharose was washed twice with 1 ml of buffer A containing 0.5 M NaCl, followed by two washes with 1 ml of 50 mM Tris/HCl, pH 7.5, containing 0.27 M sucrose and 0.1 % (v/v) 2-mercaptoethanol.

Assay of TAK1

TAK1 activity in TAB2 and TAB3 immunoprecipitates was measured by the activation of MKK6 (MAP kinase kinase 6) and coupled to the activation of SAPK2a/p38α. The active SAPK2a/p38α generated in this reaction was then quantified in a second assay by the phosphorylation of myelin basic protein. This coupled assay is described and validated elsewhere [6].

RESULTS

Structure and tissue distribution of TAB3

We searched the human genome database for proteins related to the three known subunits of the TAK1 heterotrimeric complex. Interrogation with the amino acid sequence of human TAB2 identified a related protein, termed here TAB3. Human TAB3 consists of 712 amino acid residues, and has a predicted molecular mass of 78 kDa (Figure 1A). This compares with human TAB2, which comprises 693 residues and has a predicted molecular mass of 76 kDa (Figure 1A).

Alignment of the two sequences shows that TAB3 and TAB2 share 48 % identity, with especially high sequence conservation near the N- and C-termini (Figure 1A). A 40-amino-acid sequence near the N-terminus of TAB2 is reported to encode a ubiquitin-binding CUE (coupling of ubiquitin conjugation to endoplasmic reticulum degradation) domain (Figure 1B) [7], whereas the C-terminal 30 residues of TAB2 encode an NZF [Npl4 (nuclear protein localization 4) zinc finger] domain (Figure 1C) that has been shown to bind ubiquitin [8]. These domains are also present in TAB3. The putative nuclear-export signal identified previously in TAB2 (residues 549–553) [9] is conserved in TAB3 (residues 531–535). We have also identified murine and *Xenopus* orthologues of both TAB2 and TAB3 (results not shown).

The mRNA encoding TAB3 was detected in all the tissues examined as a single transcript of approx. 7.5 kb (Figure 2A). The

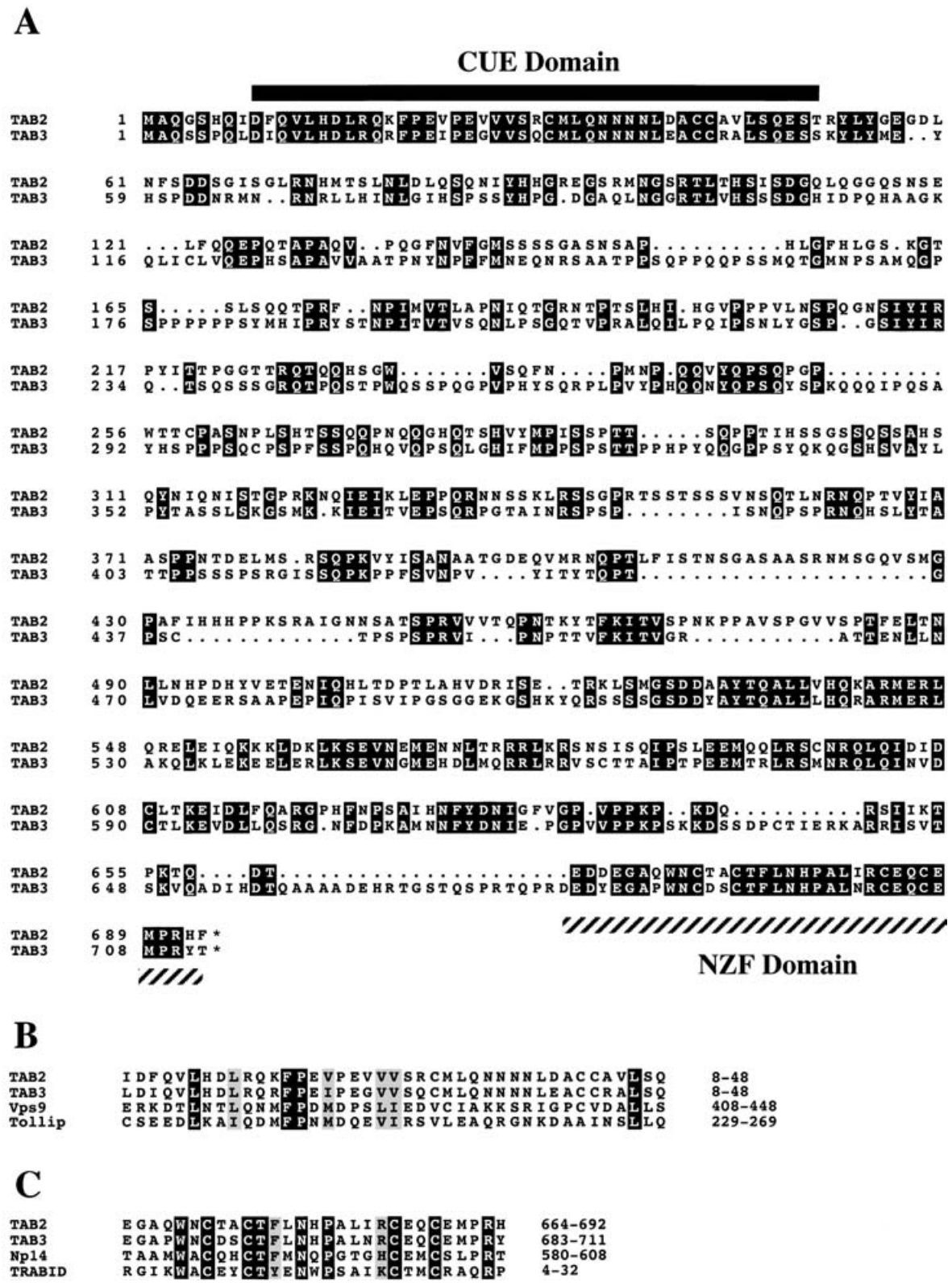

Figure 1 Primary structure of human TAB3

(**A**) Comparison of the amino acid sequences of human TAB2 and TAB3. The sequences were aligned using the Pile Up programme (University of Wisconsin GCG package). Dots indicate gaps introduced to maximize the alignment and identical residues are boxed in black. The positions of the CUE (black bar) and NZF (hatched bar) domains are indicated. (**B**) Alignment of the CUE domains of TAB2 and TAB3 with those of Vps9 (vacuolar protein sorting 9) from *Saccharomyces cerevisiae* and Tollip (Toll-interacting protein) from human cells. Identical and similar residues are boxed in black and grey respectively. (**C**) Alignment of the NZF domains of TAB2 and TAB3 with those of Npl14 and TRABID (TRAF-binding domain) from human cells. Identical and similar residues are enclosed in black and grey boxes respectively.

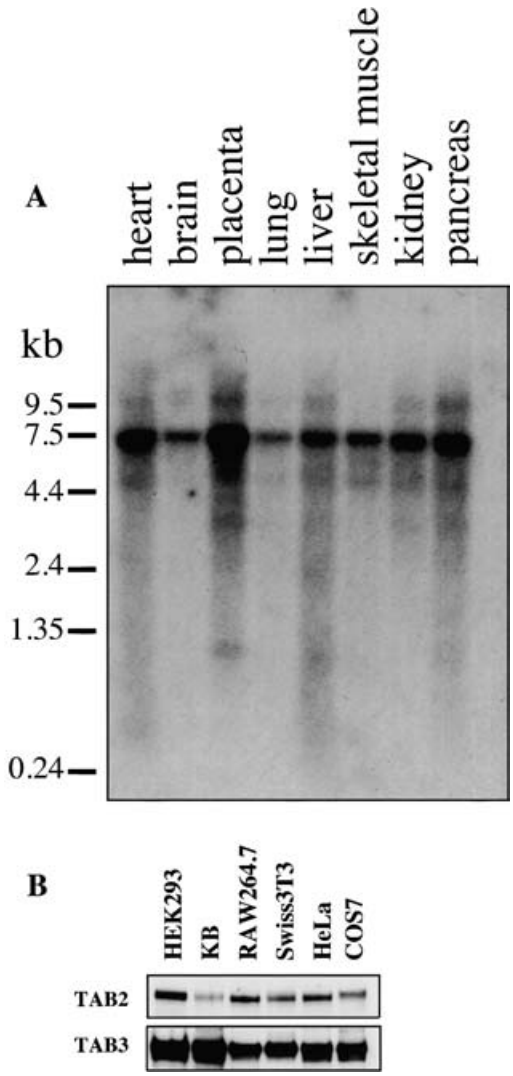

Figure 2 Distribution of TAB3 in human tissues and in mammalian cell lines

(**A**) A human multiple-tissue Northern blot was hybridized at 60 °C overnight with a radiolabelled cDNA probe encoding amino acid residues 446–676 of human TAB3. The blot was washed with 0.1 × SSC/1 % SDS at 50 °C, and autoradiographed at − 70 °C. (**B**) TAB2 or TAB3 were immunoprecipitated from 1 mg of lysate protein from the cell lines indicated, and analysed for the presence of TAB2 or TAB3 protein by immunoblotting (see the Materials and methods section).

TAB3 protein is also expressed in a number of well-characterized cell lines (Figure 2B). Thus, like TAB2, TAB3 is a widely expressed protein.

TAB3 is associated with TAB1 and TAK1 *in vivo*

Consistent with its slightly larger molecular mass, the endogenous TAB3 in HEK-293 cells migrates on SDS/PAGE with a slightly slower electrophoretic mobility than TAB2 (Figure 3A). As TAB2 binds to TAK1 [5], we next examined whether this was also the case for TAB3. TAK1, TAB1, TAB2 or TAB3 were immunoprecipitated from HEK-293 cell lysates using the four different antibodies raised against each protein and a control IgG (Figure 3B). TAK1 or TAB1 immunoprecipitates contained TAB2 and TAB3 (as well as TAB1 and TAK1). However, TAB2 immunoprecipitates contained TAK1, TAB1 and TAB2, but not TAB3.

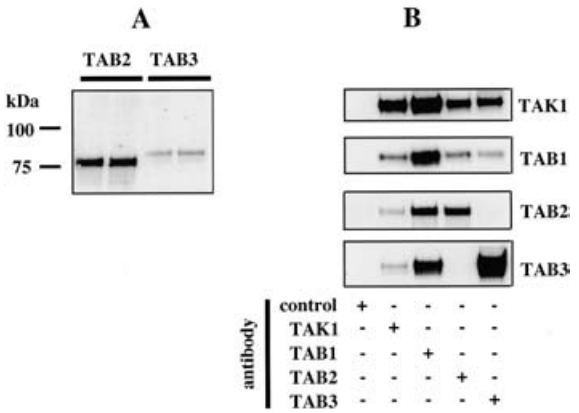

Figure 3 Evidence that TAK1 interacts with TAB1 and either TAB2 or TAB3 in HEK-293 cells

(**A**) TAB3 migrates more slowly than TAB2 on SDS/PAGE. TAB2 and TAB3 were immunoprecipitated from 1.0 mg and 0.1 mg of lysate protein respectively, and immunoblotted with specific antibodies. (**B**) Antibodies specific for TAK1, TAB1, TAB2, TAB3 or control IgG were coupled separately to Protein G–Sepharose, and each was incubated for 1 h at 4 °C with 1 mg of lysate protein. After centrifugation, washing and denaturation in SDS, the immunoprecipitates were analysed for the presence of TAK1, TAB1, TAB2 or TAB3 by immunoblotting.

Conversely, TAB3 immunoprecipitates contained TAK1, TAB1 and TAB3, but not TAB2. This indicates that TAK1 exists as two distinct complexes which contain TAB1 and TAB2 or TAB1 and TAB3 (Figure 3B).

The activation of TAK1 complexes containing TAB2 or TAB3

We investigated whether the two forms of TAK1 were activated in response to IL-1, TNFα or LPS. These experiments showed that both complexes were indeed activated by these agonists (Figure 4). We have reported previously that SAPK2a/p38α is involved in a feedback loop which leads to the inhibition of TAK1, because the agonist-induced activation of TAK1 is enhanced in cells that have been incubated with SB 203580, a relatively specific inhibitor of SAPK2a/p38α, or in SAPK2a/p38α-deficient fibroblasts [6]. In the present study, we showed that this was true for both the TAB1–TAK1–TAB2 complex and the TAB1–TAK1–TAB3 complex (Figure 4). Interestingly, osmotic shock by itself hardly activated either TAK1 complex, but activation was pronounced if the cells were also incubated with SB 203580.

The agonist-induced activation of the TAK1 complexes was also accompanied by a decrease in the electrophoretic mobility of the TAK1 catalytic subunit, which was not prevented by SB 203580. Indeed, incubation of cells with SB 203580 actually enhanced the sorbitol-induced decrease in the electrophoretic mobility of TAK1 in the TAB1–TAK1–TAB3 complex (Figure 4C). The agonist-induced decrease in the mobility of TAK1 was reversed by incubation of the immunoprecipitates with protein phosphatase-1, and this abolished TAK1 activity (results not shown).

TAB2 and TAB3 are phosphorylated by the SAPK2a/p38α pathway in cells

In our earlier paper, we showed that SAPK2a/p38α phosphorylates TAB1 at Ser^{423} and Thr^{431} in response to stimuli that activate this pathway [6]. It has also been reported by others that IL-1 induces a decrease in the electrophoretic mobility of TAB2 [10]. In the present study, using 7 % instead of the 10 %

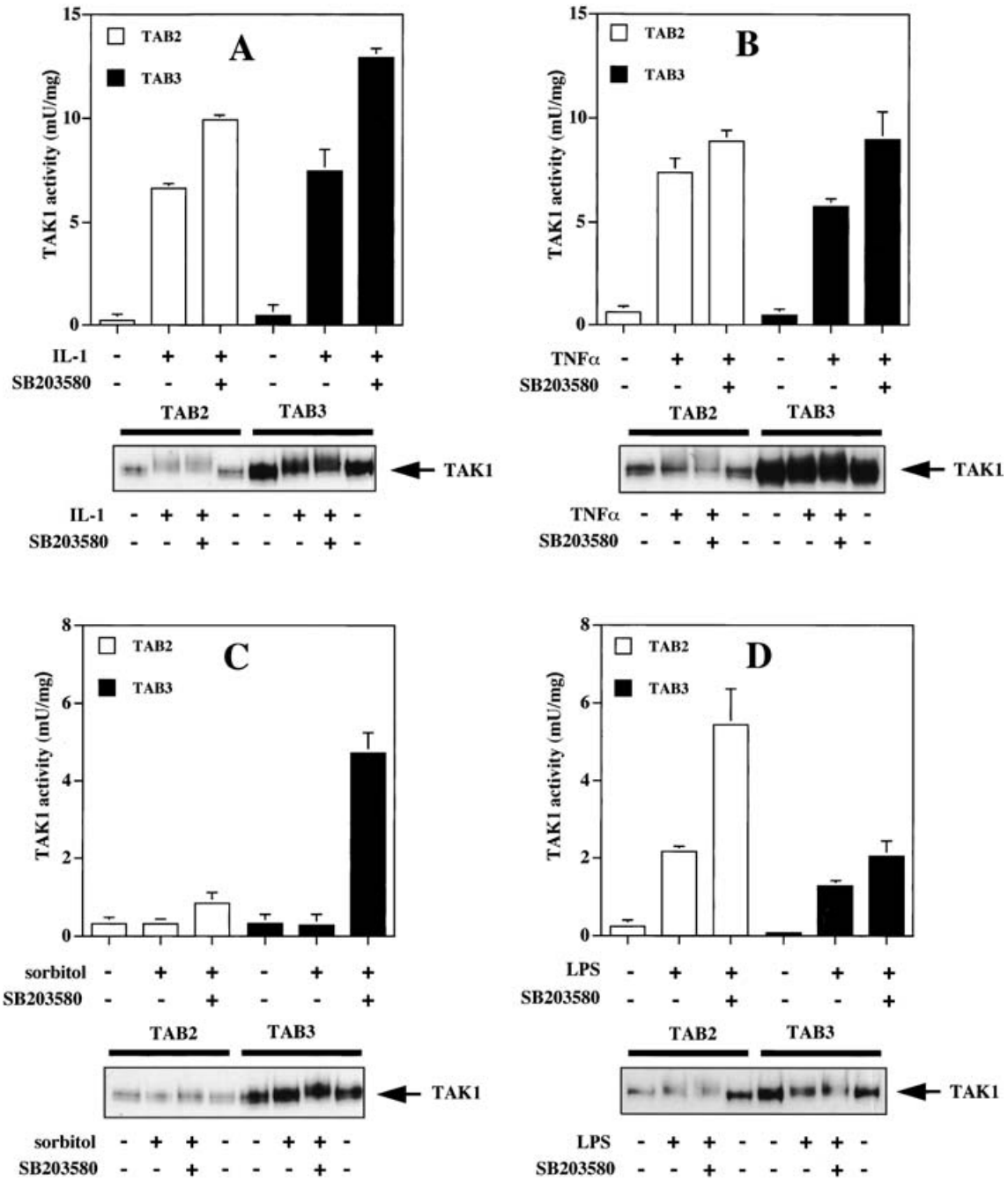

Figure 4 Effect of agonists on the activation of TAK1 complexes containing TAB2 or TAB3

(**A**–**C**) Human epithelial KB cells were incubated for 1 h without (−) or with (+) 10 μM SB 203580, then stimulated with human IL-1 (20 ng/ml for 20 min), human TNFα (50 ng/ml for 5 min) or sorbitol (0.5 M for 30 min). The cells were lysed, and TAB2 or TAB3 was immunoprecipitated from 150 μg of cell lysate protein by incubation for 1 h with 2 μg of anti-TAB2 or anti-TAB3 antibody coupled to Protein G–Sepharose. After centrifugation and washing, an aliquot of each immune complex was assayed for TAK1 activity (upper panels in **A**, **B** and **C**), and the results are presented as $\pm$ S.E.M. for two experiments, each performed in triplicate. A second aliquot was denatured in SDS, subjected to PAGE, transferred on to nitrocellulose and immunoblotted for the presence of TAK1 (lower panels in **A**, **B** and **C**). (**D**) Same as in (**A**–**C**), except that murine RAW 264.7 macrophages were stimulated with 100 ng/ml LPS for 15 min.

polyacrylamide gels we used previously [6], we were also able to demonstrate a marked decrease in the electrophoretic mobility of both TAB2 and TAB3 in response to IL-1, LPS or osmotic shock (Figure 5). The decrease in electrophoretic mobility of TAB2 and TAB3 was reversed by treatment of the immunoprecipitates with protein phosphatase-1, a serine/threonine-specific phosphatase (results not shown), indicating that the decreased electrophoretic mobility of TAB2 and TAB3 results from phosphorylation.

The decrease in electrophoretic mobility of TAB3 did not occur if wild-type cells were incubated with SB 203580 prior to agonist stimulation, or in fibroblasts deficient in SAPK2a/p38α (Figure 5), indicating that this protein is phosphorylated in cells via the SAPK2a/p38α pathway. In contrast, agonist stimulation in the presence of SB 203580 consistently induced a small decrease in the electrophoretic mobility of TAB2, but the mobility did not return to that seen in unstimulated cells. Similarly, IL-1 or

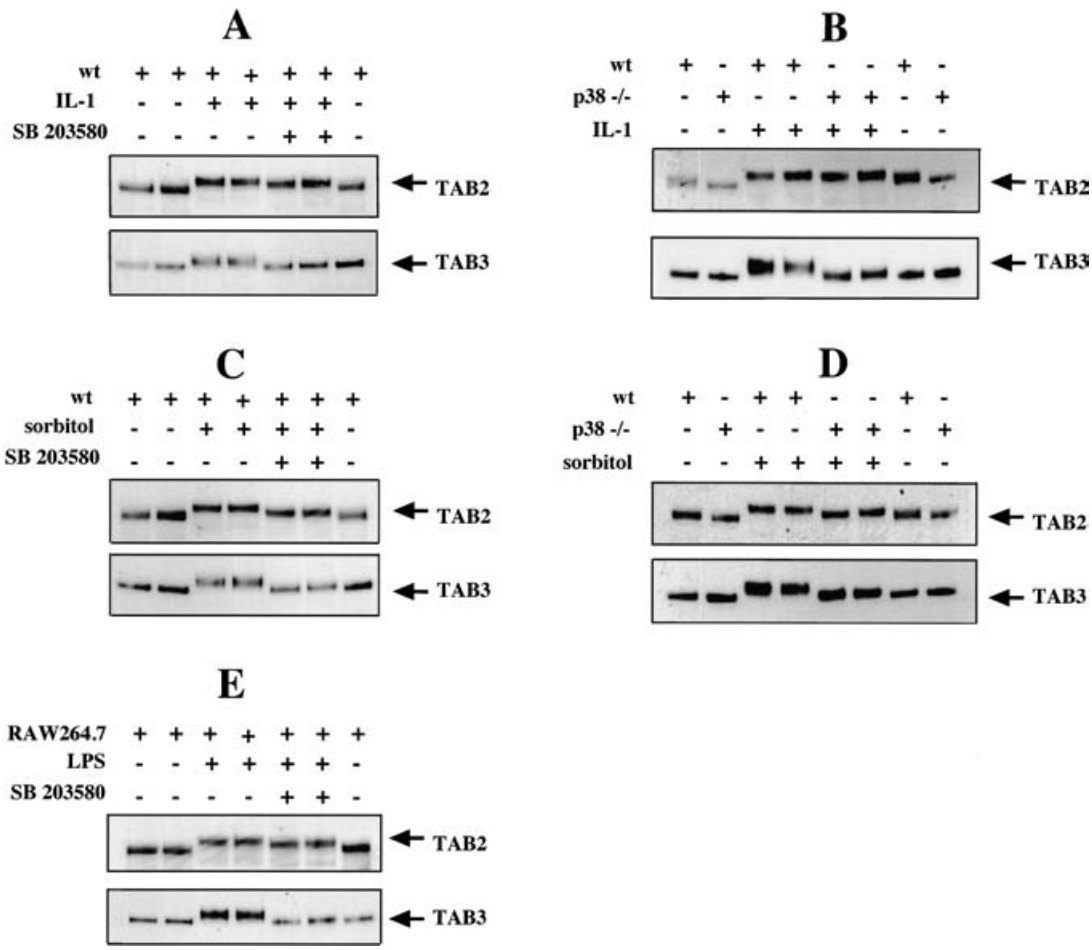

Figure 5 Electrophoretic mobility of TAB2 and TAB3 decreases after stimulation with IL-1, LPS or sorbitol

(**A**–**D**) Wild-type (wt) or SAPK2a/p38α-deficient (p38−/−) immortalized murine embryonic fibroblasts were incubated for 1 h without (−) or with (+) 10 μM SB 203580, and then were stimulated with murine IL-1 (20 ng/ml for 20 min) or exposed to sorbitol (0.5 M for 30 min). Cell lysate (250 μg of protein) was incubated for 1 h with 2 μg of anti-TAB2 or anti-TAB3 antibody coupled to Protein G–Sepharose. After centrifugation and washing, proteins bound to the antibodies were denatured in SDS and analysed for the presence of TAB2 or TAB3 by immunoblotting (see the Materials and methods section). Similar results were obtained in several different experiments. (**E**) Same as in (**A**–**D**), except that murine RAW 264.7 macrophages were stimulated with LPS (100 ng/ml for 15 min).

osmotic shock decreased the electrophoretic mobility of TAB2 in SAPK2a/p38α-deficient fibroblasts, but not to the extent seen in wild-type cells. Similar results were observed when cells were stimulated with TNFα (results not shown).

DISCUSSION

In this paper, we describe a widely expressed protein TAB3 that is structurally related to TAB2, and show that it can replace TAB2 in TAK1 complexes. Thus cells contain two distinct TAK1 complexes in which this protein is bound to TAB1 and either TAB2 or TAB3. While our work was in progress, TAB3 was also identified by others as an mRNA that is up-regulated in response to TGF-β [11], but its function was not addressed in their paper.

Our findings raise the question of the physiological roles of TAB2 and TAB3, and the significance of two distinct TAK1 complexes in cells. The mechanism(s) by which TAK1 is activated is still incompletely understood, but the interaction of pro-inflammatory cytokines or LPS with their receptors at the plasma membrane is thought to trigger the oligomerization of a family of proteins, termed TRAFs (TNF-receptor-associated factors). It has been reported that IL-1 triggers the ubiquitination of TRAF6 through the action of the ubiquitin conjugation factors Ubc13–Uev1a, allowing it to interact with the TAB2 component of the TAK1 complex [5,12]. The presence of ubiquitin-binding CUE and NZF domains in both TAB2 and TAB3 therefore suggests that ubiquitinated TRAFs may interact with the TAK1 complex via the CUE and/or the NZF domains of TAB2 and TAB3. However, how this might trigger the activation of TAK1 is unclear. We have found that both the TAB1–TAK1–TAB2 and TAB1–TAK1–TAB3 complexes can be inactivated by treatment with protein phosphatase-1, demonstrating that phosphorylation of one or more serine/threonine residues is required for TAK1 complexes to be active (results not shown), perhaps a residue in the activation loop of the TAK1 catalytic subunit [13]. Consistent with this idea, the agonist-induced activation of TAK1 is accompanied by a decrease in the electrophoretic mobility of the TAK1 catalytic subunit ([13]; also see Figure 4), which can be reversed by treatment with serine/threonine-specific protein phosphatases, thereby inactivating TAK1 ([13] and our unpublished results). One possible scenario is that the binding of ubiquitinated TRAFs to the TAK1 complex may allow another protein kinase, or even TAK1 itself, to phosphorylate the TAK1 catalytic subunit and so activate the complex.

It has been reported that IL-1 and LPS signalling to JNK and NF-κB is impaired in TRAF6-deficient fibroblasts, but TNFα signalling to NF-κB is not [14]. Conversely, TNFα signalling to JNK is severely impaired in TRAF2-deficient mice, but TNFα signalling to NF-κB is only reduced mildly [15]. TRAF6 has been reported to interact with TAB2 in response to IL-1 [5], whereas TRAF2 has been reported to bind to the TAK1 complex in response to TNFα [16]. These observations raise the questions of whether the TAB1–TAK1–TAB2 complex might be involved in TRAF-6-dependent signalling by IL-1 and LPS, and whether the TAB1–TAK1–TAB3 complex might be involved in TRAF-2-dependent signalling by TNFα. However, this does not seem to be the case, because we have found that both complexes can be activated by TNFα, as well as by IL-1, in human epithelial KB cells, and by LPS in the murine macrophage cell line RAW264.7 (Figure 4). Nevertheless, it would clearly be important to find out whether TAB2 and TAB3 interact preferentially with distinct members of the TRAF family of adaptor proteins. The existence of TAB3 and the presence of two different TAK1 complexes may explain why IL-1 and TNFα signalling is not impaired in TAB2-deficient mice [17].

It is also possible that the two TAK1 complexes are activated differentially by other extracellular agonists. For example, it has been reported that TAK1 is activated in response to activin A and hepatocyte growth factor [18], ephrins [19] and TGFβ [1]. TAB2 and/or TAB3 may therefore interact specifically with protein signalling intermediates in these other pathways, allowing them to couple with TAK1.

Pro-inflammatory cytokines, LPS and cellular stresses are all able to induce a decrease in the electrophoretic mobility of TAB2 and TAB3 (Figure 5). The decrease in mobility of TAB3 did not occur in SAPK2a/p38α-deficient cells or in wild-type cells that had been incubated with SB 203580 before stimulation. This indicates that TAB3 is either phosphorylated directly by SAPK2a/p38α or by another protein kinase that is activated by SAPK2a/p38α. In contrast, agonist stimulation of SB 203580-treated cells produced a form of TAB2 with a mobility intermediate between that observed in unstimulated and stimulated cells, and this species was also formed after stimulation of fibroblasts from SAPK2a/p38α-deficient cells. These observations indicate that TAB2 is not only phosphorylated via the SAPK2a/p38α pathway, but also by an SB 203580-insensitive pathway. Moreover, the results imply that TAB2 must be phosphorylated at two or more sites.

We have recently identified a feedback control mechanism in which TAK1 activity is down-regulated by SAPK2a/p38α [6]. Thus the activation of TAK1 by IL-1, TNFα or LPS is enhanced by incubating cells with SB 203580, or in cells that do not express SAPK2a/p38α. In these earlier studies, TAK1 activity was measured after immunoprecipitation from cell extracts with an anti-TAB1 antibody and would therefore have measured the activity of both TAK1 complexes. In the present study we used anti-TAB2 or anti-TAB3 antibodies to immunoprecipitate and assay each TAK1 complex separately. These studies showed that the TAB1–TAK1–TAB2 and TAB1–TAK1–TAB3 complexes are both inhibited by the SAPK2a/p38α pathway. The findings presented in this paper (Figure 5) raise the possibility that this feedback control mechanism also involves the SAPK2a/p38α-mediated phosphorylation of TAB2 and TAB3, as well as the previously reported phosphorylation of TAB1 at Ser^{423} and Thr^{431} [6]. The feedback inhibition of TAK1 by SAPK2a/p38α does not involve the dissociation of TAB1 or TAB2 from TAκ1 [6]; nor does it involve the dissociation of TAB3, because the amount of TAK1 bound to TAB3 is unaffected by stimulation with agonists that activate SAPK2a/p38α, or by blockade of the pathway with SB 203580 (Figure 4). It is possible that the phosphorylation of TAB2 and TAB3 inhibits the activation of TAK1 by preventing interaction with upstream elements of the signalling pathway, such as TRAFs. It should also be emphasized that the SAPK2a/p38α-mediated phosphorylation of TAB2 or TAB3 is distinct from the phosphorylation event required for TAK1 activation, because the agonist-induced activation of TAK1 is enhanced by SB 203580 or in SAPK2a/p38α-deficient cells.

After submission of the present paper, Matsumoto and co-workers published a paper on the characterization of TAB3 [20]. In agreement with the present study, they showed that IL-1 and TNFα could activate TAK1 complexes containing TAB3, and that these agonists do not affect the interaction of TAB2 and TAB3 with TAK1. They also presented evidence that TAB2 and TAB3 play essential roles in the activation of TAK1, and that TRAF6 and TRAF2 interact with TAB2 and TAB3 in response to IL-1 and TNFα respectively. In disagreement with our work, they also reported that TAB2 and TAB3 interact with one another, and therefore that there is a single TAK1 complex containing TAK1, TAB1, TAB2 and TAB3. In our studies anti-TAB2 antibodies did not immunoprecipitate TAB3, and vice versa, demonstrating that there are two distinct TAK1 complexes (Figure 3B).

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