Phosphorylation-dependent activation of TAK1 mitogen-activated protein kinase kinase kinase by TAB1

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Abstract TAK1 is a mitogen-activated protein kinase kinase kinase (MAP3K) that is involved in the c-Jun N-terminal kinase/p38 MAPKs and NF-κB signaling pathways. Here, we characterized the molecular mechanisms of TAK1 activation by its specific activator TAB1. Autophosphorylation of two threonine residues in the activation loop of TAK1 was necessary for TAK1 activation. Association with TAK1 and induction of TAK1 autophosphorylation required the C-terminal 24 amino acids of TAB1, but full TAK1 activation required additional C-terminal Ser/Thr rich sequences. These results demonstrated that the association between the kinase domain of TAK1 and the C-terminal TAB1 triggered the phosphorylation-dependent TAK1 activation mechanism.

Key words: TAK1; TAB1; MAP3K; IKK

1. Introduction

Transforming growth factor-β (TGF-β)-activated kinase 1 (TAK1) was originally identified as a mitogen-activated protein kinase kinase kinase (MAP3K) which can be activated by TGF-β and bone morphological protein (BMP) [1]. Association with its specific activator protein, TAK1 binding protein 1 (TAB1), triggers TAK1 kinase activity [2]. The TAK1–TAB1 complex functions in the BMP signaling pathway in early *Xenopus* development, in cooperation with the BMP signaling molecule, Smad1 or Smad5 [3]. In addition, TAK1 is suggested to act as a MAP3K in the c-Jun N-terminal kinase (JNK) and the p38 MAPK cascades, in which TAK1 phosphorylates MAPK kinases MKK4 and MKK3/6, respectively [4,5]. In fact, hematopoietic progenitor kinase 1 (HPK1) [6] and ceramide [5] have been shown to induce the activation of the JNK pathway mediated through TAK1.

We originally reported that TAK1–TAB1 was involved in the NF-κB activation pathway [7], in which TAK1 activated two IκB kinases, IKKα and IKKβ [8]. Ninomiya-Tsuji et al. demonstrated that TAK1 associated with tumor necrosis factor (TNF)-α receptor-associated factor 6 (TRAF6) and activated NF-κB-inducing kinase (NIK), the upstream kinase of IKKs, in the interleukin-1 (IL-1) signaling pathway [9]. TAK1 is also activated by TNF-α [4,8]. However, TAK1 does not associate with TRAF2, which is an adapter protein in the TNF-α receptor complex [9]. These observations suggest that the intracellular signals bifurcate into the MAPK and NF-κB signaling pathways at the TAK1–TAB1 complex.

It has been shown that TAB1 associates with the catalytic domain of TAK1 and induces its kinase activity [2]. However, little is known about the TAK1 activation mechanism by TAB1. In this study, we characterized the molecular mechanism of TAB1-mediated TAK1 activation in transient transfection experiments. The association with TAB1 induced TAK1 autophosphorylation at two threonine residues in the activation loop. The TAK1 activation domain of TAB1 was present within its C-terminal 68 amino acids, which consisted of two functional subdomains for association with TAK1 and activation of TAK1.

2. Materials and methods

2.1. Mammalian expression vectors

The expression vector for Flag-tagged TAK1 was previously described [8]. Point mutations were made by using a QuickChange site-directed mutagenesis kit (Stratagene) and all of the mutations were verified by DNA sequencing analysis. The expression vectors for HA-tagged and EGFP-fused TAB1 were generated by insertion of TAB1 cDNA encoding full length, N-terminal (amino acids 1–436), C-terminal (437–504), C1 (437–460), C2 (459–482) and C3 (480–504) into pcDNA3.1 (Invitrogen) and pEGFP-C1 (Clontech), respectively.

2.2. Cell cultures and transfection

HeLa cells were maintained in Dulbecco's modified Eagle's medium (high glucose) supplemented with 10% fetal calf serum, 100 units/ml penicillin, and 100 μg/ml streptomycin at 37°C in 5% CO_2. Cells were transfected with expression vectors using Lipofectamine reagents (Life Technologies).

2.3. Generation of recombinant TAK1 and TAB1

Full length TAK1 cDNA was inserted into pAcHLT baculovirus expression vector (Pharmingen). Sf21 insect cells were infected with recombinant virus, and TAK1 was expressed as a 6×His-tagged protein. Protein expression was determined by Western blotting with an anti-TAK1 antibody (Santa Cruz, M-579). cDNAs encoding full length, N-terminal and C-terminal TAB1 were inserted into a pGEX-2T bacterial expression vector (Amersham Pharmacia Biotech). GST-fused TAB1 proteins were expressed in *Escherichia coli* and purified with glutathione-Sepharose (Amersham Pharmacia Biotech). The purified proteins were separated on SDS–PAGE and stained with CBB. Recombinant TAK1 was incubated with GST-TAB1 in a kinase buffer and then determined kinase activity as described previously [10].

2.4. In vitro immunocomplex kinase assay

Flag-TAK1 was immunoprecipitated from whole cell lysates with

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Abbreviations: TGF-β, transforming growth factor-β; BMP, bone morphological protein; TAK1, TGF-β-activated kinase 1; TAB1, TAK1 binding protein 1; MAPK, mitogen-activated protein kinase; MAP3K, MAPK kinase kinase; JNK, c-Jun N-terminal kinase; NF-κB, nuclear factor-κB; IKK, IκB kinase; NIK, NF-κB-inducing kinase; GST, glutathione *S*-transferase; EGFP, enhanced green fluorescence protein; TNF-α, tumor necrosis factor-α; TRAF, TNF-α receptor-associated factor; IL-1, interleukin-1

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an M5 anti-Flag antibody (Sigma). The immunoprecipitates were analyzed for TAK1 kinase activity with substrate protein (6×His-MKK6) as described previously [8].

2.5. Coimmunoprecipitation assay

Association between TAK1 and TAB1 was analyzed by Western blotting with anti-HA (Santa Cruz, Y-11) or anti-GFP antibodies (Clontech) of anti-Flag immunoprecipitates. Western blotting was performed as described previously [7].

2.6. IKK and JNK activation assay

The endogenous IKK and JNK were immunoprecipitated with anti-IKKα (Santa Cruz, H-744) and anti-JNK1 antibodies (Santa Cruz, FL). The immunoprecipitates were examined for the IKK and JNK kinase reactions as described previously [8].

3. Results and discussion

3.1. Activation of TAK1 mutants by TAB1

We investigated the molecular mechanism of TAB1-induced TAK1 activation in HeLa cells. Flag-tagged wild-type TAK1 was activated by HA-tagged full length TAB1, where TAK1 associated with TAB1 (Fig. 1). A kinase negative mutant (TAK1-K63W) also associated with TAB1, but no kinase activity was detected (Fig. 1). When expressed alone, TAB1 was detected as doublet bands in Western blotting (Fig. 1). TAB1 further migrated slowly when coexpressed with wild-type TAK1, but not with TAK1-K63W. TAK1, but not TAK1-K63W, also migrated slowly when coexpressed with TAB1 (Fig. 1). We previously reported that the band shift was due to phosphorylations of these proteins [8]. In addition, endogenous TAK1 and TAB1 were phosphorylated upon IL-1 stimulation in human embryonal kidney 293 cells [9,11]. The phosphorylation was required for TAK1 activation as described below. Furthermore, the phosphorylation resulted in the stable expression of these proteins (Fig. 1). The stabilization was dependent on the TAK1 kinase activity, since this was not detected when TAK1-K63W was expressed with TAB1.

A TAK1 mutant that lacks the N-terminal 22 amino acids (TAK1-ΔN) acts as a TAB1-independent constitutively active kinase in the TGF-β signaling in MV1Lu and MC3T3-E1 cells [1,2]. In contrast to the previous reports, the kinase activity of TAK1-ΔN was completely TAB1-dependent in HeLa cells (Fig. 1). The nuclear translocation of NF-κB by TAK1-ΔN, a downstream signaling pathway of TAK1, was also TAB1-dependent (data not shown). In addition, the phosphorylation of TAK1-ΔN was similar in extent to that of wild-type TAK1 (Fig. 1). Therefore, the serine cluster in the N-terminal 22 amino acids is not the site of the TAB1-induced TAK1 phosphorylation. These results demonstrate that TAK1-ΔN activation is completely dependent on TAB1 in HeLa cells. These distinct results might be derived from the cell-type specificity to the TGF-β responsibility of TAK1 activation. In fact, TAK1 is activated by TGF-β in MV1Lu and MC3T3-E1 cells [1,2], but not in HeLa cells [8]. Therefore, it should be noted that the cell-type specificity of the physiological TAK1 functions in the TGF-β/BMP and IL-1/TNF-α signaling pathways.

It has been shown that the serine or threonine residues in the activation loop of protein kinases are important for enzyme activation, and their substitution with acidic amino acids (glutamic or aspartic acid) produces constitutively active mutants [12]. We generated a TAK1 mutant (TAK1-TT/EE), in which two threonine residues (Thr-184 and Thr-187) were

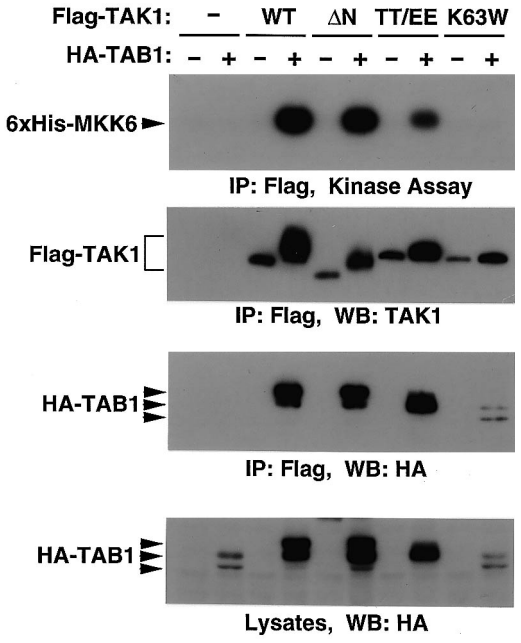

Fig. 1. Activation of TAK1 mutants by TAB1. HeLa cells (1×10^6 cells/60 mm dish) were transfected with expression vectors for wild-type and mutant Flag-TAK1 (1 μg each) and HA-TAB1 (1 μg). The total amount of DNA was adjusted with an empty vector at 2 μg. The TAK1 kinase activity was determined by an anti-Flag immunocomplex kinase assay using 6×His-MKK6 as a substrate (upper panel). Associations between Flag-TAK1 and H-TAB1 were determined by a coimmunoprecipitation assay using anti-Flag and anti-HA antibodies (middle panel). Protein expression levels were determined by Western blotting of the immunoprecipitates (TAK1) or whole cell lysates (TAB1).

substituted with glutamic acids. The association of TAK1-TT/EE with TAB1 was comparable to that of the wild-type TAK1, but the kinase activity was severely reduced. The reduced activity resulted in impaired phosphorylation of both TAK1 and TAB1, strongly suggesting that the phosphorylations were mediated by the TAK1 kinase activity. This result indicates that the autophosphorylation of Thr residues is necessary for maximal TAK1 activation. However, the migration of TAK1-TT/EE on SDS–PAGE remained to be reduced slightly. Kishimoto et al. reported that Ser-192 in the activation loop was autophosphorylated upon IL-1 stimulation [11]. Interestingly, the autophosphorylation of Ser-192 could not be mimicked by replacement with negatively charged amino acid (aspartic acid) [11]. These observations indicate that TAK1 activation requires multiple phosphorylations in the activation loop of TAK1, which can not be mimicked by replacement with negatively charged residues.

3.2. TAK1 activation by TAB1 in a cell free system

To characterize the TAK1 activation domain of TAB1, we generated recombinant TAK1 in the baculovirus system as a 6×His-tagged protein (Fig. 2A) and TAB1 in bacteria as a GST-fusion protein (Fig. 2B). Incubation of TAK1 and full length TAB1 in a cell free system containing ATP induced the TAK1 kinase activity (Fig. 2C). In contrast, TAK1 and TAB1 alone did not induce the phosphorylation activity. Shibuya et al. previously reported that the C-terminal TAB1 was sufficient for association with and activation of TAK1 in the TGF-β signaling [2]. We investigated the ability of N-terminal

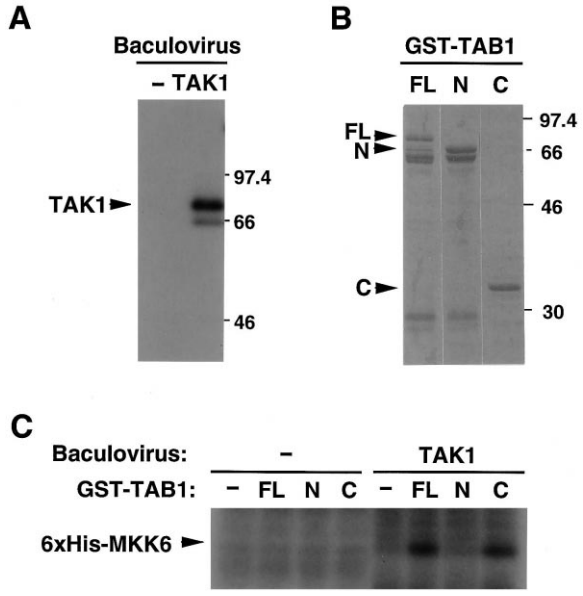

Fig. 2. Activation of TAK1 by TAB1 in a cell free system. A: Lysates prepared from Sf21 cells infected with control or recombinant TAK1 baculoviruses were immunoblotted with an anti-TAK1 antibody. B: Full length, N-terminal and C-terminal TAB1 fused with GST were expressed in *E. coli* and stained with CBB. C: Control cell lysates or TAK1-expressing lysates were incubated with GST-TAB1 at an ATP-containing condition and then TAK1 kinase activity was determined.

(amino acids 1–436) and C-terminal (437–504) TAB1 in the cell free system. GST-TAB1-C, but not GST-TAB1-N, activated TAK1 to a similar extent to GST-TAB1-FL (Fig. 2C). These results indicate that the C-terminal 68 amino acids of TAB1 are sufficient for triggering the TAK1 activation mechanism.

3.3. *TAK1 activation by the C-terminal domain of TAB1 in vivo*

To confirm the activity of the C-terminal domain of TAB1 in vivo, full length, N-terminal and C-terminal TAB1 were expressed as fusion proteins with EGFP in HeLa cells. An anti-Flag immunocomplex kinase assay showed that EGFP-TAB1-C, but not EGFP-TAB1-N, induced the TAK1 kinase activity (Fig. 3). A coimmunoprecipitation assay showed that EGFP-TAB1-C associated with TAK1 (Fig. 3). In addition, EGFP-TAB1-C was phosphorylated at many sites, and was detected as quadruplet on SDS–PAGE (Fig. 3). These results indicate that the C-terminal 68 amino acids of TAB1 are sufficient for association with and activation of TAK1 in vivo. The N-terminal TAB1 may play a role in regulating TAK1, including regulation of the intracellular distribution of TAB1–TAK1. In fact, XIAP was isolated in the yeast two-hybrid system by using the N-terminal TAB1 as bait [13]. XIAP is a member of the cellular inhibitor of apoptosis protein (IAP) family that links the TAB1–TAK1 and BMP type I receptor [13]. However, it has not yet been demonstrated whether XIAP plays a role in the IL-1/TNF-α signaling pathways.

3.4. *Characterization of the C-terminal TAK1 activation domain of TAB1*

We further characterized the TAK1 activation domain in the C-terminal TAB1. The 68 amino acids were divided into three fragments (C1, amino acids 437–460; C2, 459–482; C3, 480–504) and expressed as fusion proteins with EGFP. An immunocomplex kinase assay showed that only the C3 fragment activated TAK1 (Fig. 4). The kinase activity of C3-activated TAK1 was decreased severely compared with that of TAB1-C-activated TAK1, whereas the autophosphorylation of TAK1 was detected normally (Fig. 4). In addition, TAK1 associated with the C3 fragment to a similar extent as TAB1-C (Fig. 4). These results indicate that the C3 fragment was sufficient for the association with TAK1 and the induction of TAK1 autophosphorylation. However, other mechanisms are necessary to induce maximal TAK1 activity, in which the C1 and/or C2 fragments may play an important role. Interestingly, the C3 fragment was detected as a single band in Western blotting (Fig. 4), indicating that it was not phosphorylated in a complex with TAK1. Thus, the phosphorylation sites on TAB1 are present within the C1 and/or C2 fragments. In fact, C1 was Ser/Thr rich with eight serine and five threonine residues. These results raise the possibility that the phosphorylation of the C1 fragment of TAB1 was necessary for the full TAK1 activation.

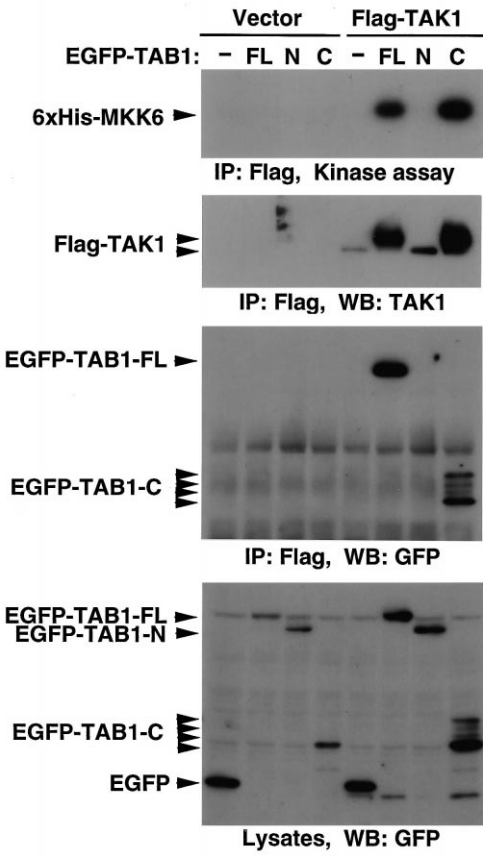

Fig. 3. TAK1 activation by the C-terminal TAB1 in vivo. HeLa cells were transfected with expression vectors for Flag-TAK1 (1 μg) and EGFP-fused TAB1 (1 μg). The total amount of DNA was adjusted with empty vectors at 2 μg. The TAK1 kinase activity was determined by an anti-Flag immunocomplex kinase assay (upper panel). Associations between Flag-TAK1 and H-TAB1 were determined by a coimmunoprecipitation assay using anti-Flag and anti-GFP antibodies (third panel). Protein expression levels of TAK1 (second panel) and TAB1 (bottom panel) were determined by Western blotting with anti-TAK1 or anti-GFP antibodies, respectively.

3.5. IKK and JNK activation by TAK1 and TAB1

It has been reported that TAK1 functions as an upstream kinase of the IKK-NF-κB and JNK/p38 MAPK signaling pathways [4–11]. Here, we examined the effects of TAK1 and TAB1 mutants on IKK and JNK activation. First, wild-type and mutant TAK1 were expressed with full length HA-TAB1 in HeLa cells. Endogenous IKK complex and JNKs were immunoprecipitated with anti-IKKα and anti-JNK antibodies, and the kinase activity was determined by in vitro kinase assays with GST-IκBα (1–54) and GST-c-Jun (1–79) as substrates, respectively (Fig. 5A). Wild-type TAK1 and TAK1-ΔN, but not TAK1-K63W, induced IKK and JNK activities. Interestingly, TAK1-TT/EE, which showed the slight kinase activity as shown in Fig. 1, induced only JNK activation. This result suggests that TAK1-TT/EE could not recognize the substrate for the IKK pathway, strongly supporting the previous observation that the intracellular signals from IL-1 receptor bifurcate into the MAPK pathway and the IKK pathway at the level of TAK1. Indeed, it has been shown that TAK1 activates MKK4 for JNK activation [5] and NIK for IKK activation [9].

Next, we examined the effect of TAB1 mutants on the TAK1-induced IKK and JNK activation (Fig. 5B). In vitro kinase assays showed that full length and C-terminal TAB1, but not N-terminal TAB1, induced the activity of IKK and JNK. This result indicates that the C-terminal TAK1 activation domain of TAB1 is sufficient for the TAK1-induced signal transduction.

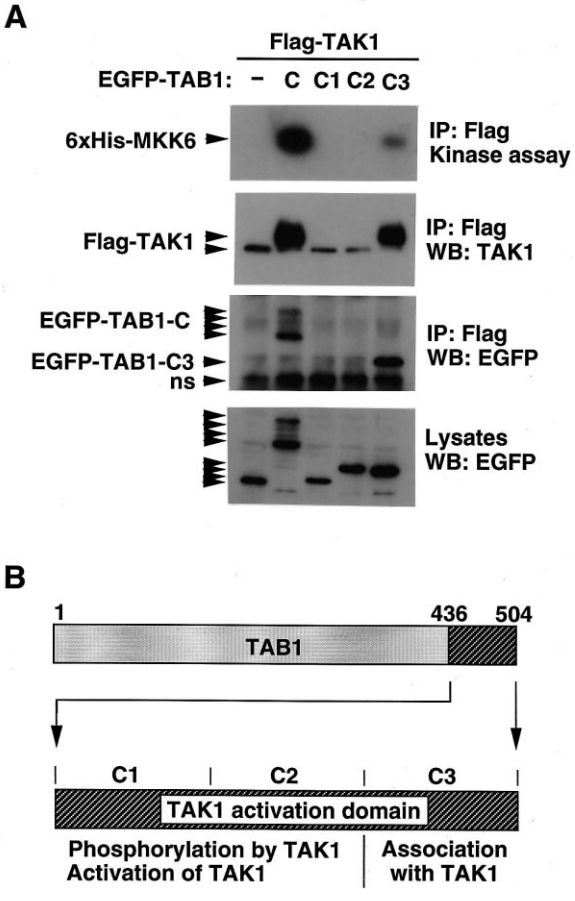

Fig. 4. Characterization of TAK1 activation domain of TAB1. The 68 amino acids of TAB1-C were divided into three fragments (C1, amino acids 437–460; C2, 459–482; C3, 480–504) and expressed as fusion proteins with EGFP. TAK1 kinase activity (upper panel), the association between TAK1 and TAB1 (third panel), and protein expression levels of TAK1 (second panel) and TAB1 (bottom panel) were determined as described in Fig. 3. ns means non-specific band.

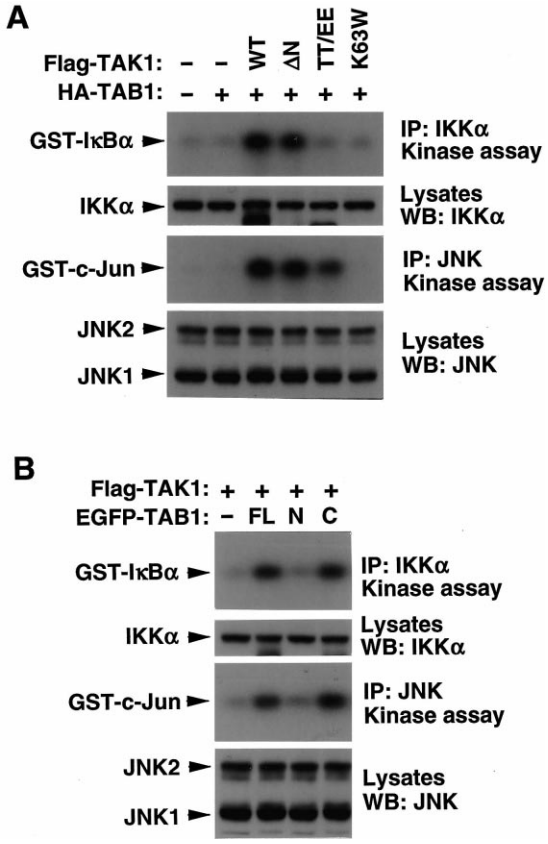

Fig. 5. IKK and JNK activation by TAK1 and TAB1. Effects of TAK1 mutants (A) and TAB1 mutants (B) on IKK and JNK activation were examined. HeLa cells were transfected with expression vectors for TAK1 (1 μg) and TAB1 (1 μg). The total amount of DNA was adjusted with an empty vector at 2 μg. Endogenous IKK complex and JNKs were immunoprecipitated with anti-IKKα and anti-JNK antibodies and examined the kinase activities for GST-IκBα (1–54) and GST-c-Jun (1–79), respectively. Protein expression levels of endogenous IKKα and JNK1/2 were determined by Western blotting with the antibodies.

3.6. A model for TAK1 activation by TAB1

The results presented above provide a possible model for the molecular mechanism of TAK1 activation by TAB1. Association between the catalytic domain of TAK1 and the C-terminal TAB1 (C3 fragment) triggers the TAK1 activation mechanism. The association may cause conformational change of the catalytic domain, inducing a kinase activity of TAK1 for autophosphorylation at Thr-184, Thr-187 and Ser-192 in the activation loop. The autophosphorylated TAK1 further phosphorylates the C-terminal Ser/Thr rich domain of TAB1. The highly phosphorylated TAB1 still associates with TAK1, therefore, the conformation of TAK1 may be further influenced by the negative charges of phosphate groups on TAB1.

Endogenous TAK1 constitutively associates with TAB1 in unstimulated cells, but no TAK1 kinase activity is detected [11,14]. Upon IL-1 stimulation, TAK1 and TAB1 are recruited to TRAF6, and may trigger the TAK1 activation

mechanism [9]. Takaesu et al. recently reported that TAB2, another TAK1 binding protein, is an adapter protein that links between TRAF6 and TAK1 [14]. They suggest a possibility that the binding to an as yet unidentified negative regulator inactivates endogenous TAK1–TAB1 in the absence of IL-1. TRAF-interacting proteins that inhibit NF-κB activation are candidates for this negative regulator. These include A20 [15,16], TRIP [17] and I-TRAF/TANK [18]. Identification of the negative regulator provides the new insights into the molecular mechanism for triggering the TAB1-mediated TAK1 activation upon cytokine stimulation.

In summary, we showed a novel phosphorylation-dependent mechanism for the activation of TAK1 by TAB1. Future investigation of the crystal structure of TAK1 associated with or without TAB1 will provide more information on the TAB1-induced TAK1 activation mechanism. Furthermore, characterization of those residues in the C-terminal TAB1 critical to the TAK1 activation may contribute to drug design for the generation of a specific inhibitor of TAK1 kinase.

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References

[1] Yamaguchi, K., Shirakabe, K., Shibuya, H., Irie, K., Oishi, I., Ueno, N., Taniguchi, T., Nishida, E. and Matsumoto, K. (1995) Science 270, 2008–2011.
[2] Shibuya, H., Yamaguchi, K., Shirakabe, K., Tonegawa, A., Gotoh, Y., Ueno, N., Irie, K., Nishida, E. and Matsumoto, K. (1996) Science 272, 1179–1182.
[3] Shibuya, H., Iwata, H., Masuyama, N., Gotoh, Y., Yamaguchi, K., Irie, K., Matsumoto, K., Nishida, E. and Ueno, N. (1998) EMBO J. 17, 1019–1028.
[4] Moriguchi, T., Kuroyanagi, N., Yamaguchi, K., Gotoh, Y., Irie, K., Kano, T., Shirakabe, K., Muro, Y., Shibuya, H., Matsumoto, K., Nishida, E. and Hagiwara, M. (1996) J. Biol. Chem. 271, 13675–13679.
[5] Shirakabe, K., Yamaguchi, K., Shibuya, H., Irie, K., Matsuda, S., Moriguchi, T., Gotoh, Y., Matsumoto, K. and Nishida, E. (1997) J. Biol. Chem. 272, 8141–8144.
[6] Wang, W., Zhou, G., Hu, M.C.T., Yao, Z. and Tan, T.H. (1997) J. Biol. Chem. 272, 22771–22775.
[7] Sakurai, H., Shigemori, N., Hasegawa, K. and Sugita, T. (1998) Biochem. Biophys. Res. Commun. 243, 545–549.
[8] Sakurai, H., Miyoshi, H., Toriumi, W. and Sugita, T. (1999) J. Biol. Chem. 274, 10641–10648.
[9] Ninomiya-Tsuji, J., Kishimoto, K., Hiyama, A., Inoue, J., Cao, Z. and Matsumoto, K. (1999) Nature 398, 252–256.
[10] Sakurai, H., Chiba, H., Miyoshi, H., Sugita, T. and Toriumi, W. (1999) J. Biol. Chem. 274, 30353–30356.
[11] Kishimoto, K., Matsumoto, K. and Ninomiya-Tsuji, J. (2000) J. Biol. Chem. 275, 7359–7364.
[12] Raingeaud, J., Whitmarsh, A.J., Barrett, T., Derijard, B. and Davis, R.J. (1996) Mol. Cell. Biol. 16, 1247–1255.
[13] Yamaguchi, K., Nagai, S., Ninomiya-Tsuji, J., Nishita, M., Tamai, K., Irie, K., Ueno, N., Nishida, E., Shibuya, H. and Matsumoto, K. (1999) EMBO J. 18, 179–187.
[14] Takaesu, G., Kishida, S., Hiyama, A., Yamaguchi, K., Shibuya, H., Irie, K., Ninomiya-Tsuji, J. and Matsumoto, K. (2000) Mol. Cell 5, 649–658.
[15] Song, H.Y., Rothe, M. and Goeddel, D.V. (1996) Proc. Natl. Acad. Sci. USA 93, 6721–6725.
[16] Heyninck, K. and Beyaert, R. (1999) FEBS Lett. 442, 147–150.
[17] Cheng, G. and Baltimore, D. (1996) Genes Dev. 10, 963–973.
[18] Rothe, M., Xiong, J., Shu, H.B., Williamson, K., Goddard, A. and Goeddel, D.V. (1996) Proc. Natl. Acad. Sci. USA 93, 8241–8246.