An Evolutionarily Conserved Motif in the TAB1 C-terminal Region Is Necessary for Interaction with and Activation of TAK1 MAPKKK*

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TAK1, a member of the MAPKKK family, is involved in the intracellular signaling pathways mediated by transforming growth factor β, interleukin 1, and Wnt. TAK1 kinase activity is specifically activated by the TAK1-binding protein TAB1. The C-terminal 68-amino acid sequence of TAB1 (TAB1-C68) is sufficient for TAK1 interaction and activation. Analysis of various truncated versions of TAB1-C68 defined a C-terminal 30-amino acid sequence (TAB1-C30) necessary for TAK1 binding and activation. NMR studies revealed that the TAB1-C30 region has a unique α-helical structure. We identified a conserved sequence motif, PYDXA/TXF, in the C-terminal domain of mammalian TAB1, Xenopus TAB1, and its Caenorhabditis elegans homolog TAP-1, suggesting that this motif constitutes a specific TAK1 docking site. Alanine substitution mutagenesis showed that TAB1 Phe-484, located in the conserved motif, is crucial for TAK1 binding and activation. The C. elegans homolog of TAB1, TAP-1, was able to interact with and activate the C. elegans homolog of TAK1, MOM-4. However, the site in TAP-1 corresponding to Phe-484 of TAB1 is an alanine residue (Ala-364), and changing this residue to Phe abrogates the ability of TAP-1 to interact with and activate MOM-4. These results suggest that the Phe or Ala residue within the conserved motif of the TAB1-related proteins is important for interaction with and activation of specific TAK1 MAPKKK family members in vivo.

The mitogen-activated protein kinases (MAPKs)1 are a family of serine-threonine kinases that function in a wide variety of biological processes (1). MAPKs are activated by phosphorylation on specific tyrosine and threonine residues by a family of growth factor receptors (2). MAPKs include the Raf kinase group of proteins (4–7). TRAF2, an adaptor protein that interacts with the MEKK and mixed lineage kinase group of proteins (1, 2). TAK1, Tpl2, apoptosis signal-regulating kinase 1 (ASK1), and the MEKK and mixed lineage kinase group of proteins (1, 2). Several components that function upstream of the MAPKKK family have been identified. Ras GTPase functions upstream of the Raf MAPKKK and is itself activated by growth factors that signal through receptor protein-tyrosine kinases (3). Rac and Cdc42 GTPases can interact with the MEKK and mixed lineage kinase group of proteins (4–7). TRAF2, an adaptor protein that functions in the tumor necrosis factor α signaling pathway, has been reported to bind MEKK1 and ASK1 (8, 9). It has been demonstrated that TRAF2 acts on ASK1 after dissociation of ASK1 from its inhibitor thioredoxin (10). This interaction is likely to involve the regulation of ASK1 dimerization by reactive oxygen species (11). In some signaling pathways, Ste20-like kinases have been implicated in the activation of MAPKKKs. For example, Raf-1 is phosphorylated and activated by p21 (Rac/Cdc42)-activated kinase (12). Germinal center kinase functions upstream of MEKK1 in the tumor necrosis factor α signaling pathway, leading to Jun N-terminal kinase activation (8), and hematopoietic progenitor kinase and hematopoietic progenitor kinase/germinal center kinase-like kinase are involved in the activation of TAK1 (13). Thus, different mechanisms are involved in the activation of MAPKKKs in response to a variety of extracellular stimuli. To understand the mechanisms by which extracellular signals regulate the MAPK pathway, it is essential to characterize potential factors involved in MAPK activation.

TAK1 is a member of the MAPKKK family and is activated by various cytokines, including transforming growth factor-β family ligands and interleukin 1 (14, 15). We have previously demonstrated that TAK1 functions in the transforming growth factor β signaling pathways in mammalian cells (15). In early embryos of the amphibian Xenopus, TAK1 also participates in mesoderm induction and patterning mediated by bone morphogenetic protein, another transforming growth factor β family ligand (16). Furthermore, we have recently demonstrated that TAK1 is involved in the interleukin 1 signaling pathway via its activation of two kinase cascades (14), an MAP kinase cascade leading to Jun N-terminal kinase activation and an IκB kinase cascade that ultimately leads to nuclear factor κB activation.

The TAB1 protein was isolated in a yeast two-hybrid screen as a specific partner of TAK1 (17). When ectopically expressed together with TAB1, TAK1 phosphorylation and kinase activity are increased (18). A kinase-deficient mutant of TAK1 is not phosphorylated when co-expressed with TAB1, indicating that TAB1 promotes TAK1 autophosphorylation. Furthermore, mu-
tation of a conserved serine residue (Ser-192) to alanine in the activation loop between kinase domains VII and VIII of TAK1 abolishes both phosphorylation and kinase activation by TAB1. These results suggest that ectopic expression of TAB1 activates TAK1 by promoting TAK1 autophosphorylation on Ser-192. However, the molecular mechanism for this activation remains to be elucidated.

Homologs of TAK1 and TAB1, encoded by mom-4 and tap-1 genes, respectively, have been identified in Caenorhabditis elegans (19). Biochemical analysis has revealed that MOM-4 can phosphorylate MKK6 MAPK kinase in vitro, and that MOM-4 kinase activity is promoted by binding of TAP-1 (19). Thus, biochemical characterization of MOM-4 and TAP-1 suggests that these factors are similar to TAK1 and TAB1. Genetic analysis has shown that TAP-1 and MOM-4 regulate Wnt signaling in C. elegans via activation of the MAPK-like LIT-1. This is consistent with the observation that co-expression of TAK1 and TAB1 in mammalian cells can activate NEMO-like kinase, a mammalian homolog of LIT-1(20), and that the TAK1-NEMO-like kinase MAPK pathway negatively regulates Wnt signaling in mammalian cells. Thus, TAB1-TAK1 and TAP-1-MOM-4 appear to function analogously to regulate Wnt signaling in mammalian cells and C. elegans, respectively.

The C-terminal 68-amino acid portion of TAB1 was previously shown to be sufficient for TAK1 binding and activation (17). To understand the mechanism for TAB1-induced activation of TAK1, we undertook a structural and functional analysis of this domain. We report here that the C-terminal region of TAB1 contains a unique α-helix structure. By analyzing a number of TAB1-related proteins, we identified a conserved motif, PYVDXAAXTF, that is present in the C-terminal domains. Our results suggest that this conserved region functions as a specific docking site for TAK1.

**EXPERIMENTAL PROCEDURES**

**Yeast Assays**—Yeast two-hybrid analysis was performed as described previously (17). For quantitative β-galactosidase assays, a liquid culture of yeast cells expressing the -nitroenylphospho-β-galactosidase substrate was carried out as described previously (21), and activity was expressed in Miller units (22). For the ste11 complementation assay, SY1984-P (his3Δ ste11Δ fus1::his3 ste7::p536) was co-transformed with pNVI1-TAK1 and the pGAD10 vector containing various TAB1 deletion mutants, and the transformants were streaked onto SC-His plates and incubated at 30 °C.

**Identification of the Minimum Site within TAB1 Required for TAK1 Binding and Activation**—We have previously shown that the C-terminal 68-amino acid sequence of TAB1 (TAB1-C68) is sufficient for TAK1 interaction and activation (17). To determine the region of TAB1-C68 responsible for its interaction with TAK1, we examined a series of N- and C-terminal TAB1-C68 truncation mutants in a yeast two-hybrid system. Various deletion mutants of TAB1-C68 were fused to the Gal4 transcription activation domain (GAD) and co-transformed with a plasmid encoding a LexA DNA binding domain-TAK1 chimeric protein. Interaction was detected by expression of a β-galactosidase reporter containing LexA binding sites in its promoter (Fig. 1). Deletion of a serine-rich region within TAB1 (residues 457–469, TAB1-C45) had no effect on the binding of a TAB1 with TAK1. Thus, the serine-rich region of TAB1 is not required for interaction with TAK1, and the minimum TAB1 segment required for TAB1 binding includes residues 480–495.

We have previously shown that a constitutively activated form of mammalian TAK1 can complement the yeast Ste11 MAPKKK in the pheromone-induced MAPK pathway (15). However, expression of normal, full-length TAK1 fails to rescue the ste11Δ mutation. This suggests that yeast cells lack an endogenous activator of TAK1 (15). Using this complementation system, various GAD-TAB1 constructs were tested for their ability to complement the ste11Δ mutation in the pres-
ence of TAK1. Co-expression of GAD-TAB1-C30 with TAK1 was found to rescue the Ste11 deficiency, whereas GAD-TAB1-C25 had no effect (Fig. 1). These results indicate that the serine-rich region of TAB1 is not required for either TAK1 binding or activation, whereas TAB1-C30 contains a minimal region sufficient for both TAK1 binding and activation.

Structure of the TAB1-C30 Region—To determine the three-dimensional structure of the TAB1-C30 region, a peptide comprising residues 475–504 was synthesized and analyzed by NMR (Fig. 2A). For sequence-specific assignments and the identification of secondary structure elements in TAB1-C30, we used sequential nuclear Overhauser effect connectivities and the secondary Hα chemical shifts observed for TAB1-C30 in 40% trifluoroethanol, 55% H2O, and 5% D2O, pH 2.75. On the basis of the superposition of the 10 best-calculated structures, an α-helical region was identified between Tyr-481 and His-495. This region overlaps with the minimum region in TAB1 required for TAK1 binding (Fig. 1). However, the secondary structures of both the N-terminal (residues from Asp-475 to Phe-480) and C-terminal (residues from Asp-496 to Phe-504) regions were not well defined (Fig. 2B). In a wheel projection of the α-helix region, we noticed the alignment of amino acids carrying large hydrophobic side chains with aromatic character on the same side of the wheel (Fig. 2C). This structure may provide local apolar surfaces that facilitate the interaction of TAB1 with TAK1.

The Phe-484 Residue in the TAB1 Protein Is Important for TAK1 Binding—To determine whether individual aromatic amino acids in the α-helix region between Tyr-481 and His-495 are essential for TAB1 interaction with TAK1, each aromatic amino acid in the TAB1-C68 region was replaced with alanine. TAK1 binding activity of wild-type and mutant TAB1-C68 was determined by a yeast two-hybrid protein assay (Fig. 3). Alanine substitution of Phe-484 markedly reduced TAK1 binding. In contrast, substitutions of Tyr-481, Phe-487, Trp-491, or His-495 with alanine had little effect on TAK1 binding. Alanine substitution of Tyr-488 partially reduced interaction with TAK1. Thus, this analysis implicates the Phe-484 residue...
within the α-helix as essential for TAK1 binding.

We next examined whether the F484A mutation would affect the ability of full-length TAB1 to bind to TAK1. We constructed TAB1(F484A), replacing Phe-484 with Ala, and performed co-immunoprecipitation assays in mammalian cells. Expression vectors encoding Flag epitope-tagged TAB1 (Flag-TAB1) or TAB1(F484A) (Flag-TAB1(F484A)) were co-transfected into human embryonic kidney 293 cells along with an expression vector encoding HA epitope-tagged TAK1 (HA-TAK1). Cell lysates were immunoprecipitated with the anti-HA antibody, and co-precipitated TAB1 or TAB1(F484A) was detected by immunoblot analysis with the anti-Flag antibody. We found that the TAB1(F484A) mutation caused a marked reduction in TAK1 binding relative to wild-type TAB1 (Fig. 4). Thus, the F484A mutation disrupts the association of full-length TAB1 with TAK1.

We have previously shown that TAK1 has no kinase activity when ectopically expressed in mammalian cells alone but is activated when co-expressed with TAB1 (17, 18). We therefore examined the effect of the TAB1(F484A) mutation on TAK1 activation. TAK1 immunoprecipitates were subjected to in vitro kinase assay using bacterially expressed MKK6 as an exogenous substrate (Fig. 4). As observed previously (17, 18), TAK1 activity was not detected when TAK1 alone was ectopically expressed but was detected when TAK1 and TAB1 were expressed together. In contrast, TAK1 activity was significantly lower when the TAB1(F484A) mutant was co-expressed with TAK1. These results indicate that the Phe-484 residue in the TAB1 protein is important for both TAK1 binding and activation.

**Evolutionary Conservation of the TAK1 Binding Motif**—The *C. elegans* MOM-4 and TAP-1 proteins are structurally and functionally similar to the vertebrate TAK1 and TAB1 proteins, respectively (19). For example, MOM-4 functions as an MAPKKK that can phosphorylate the MKK6 MAPK kinase in vitro, and TAP-1 can interact with and activate MOM-4. The present studies delineated an essential region required for TAK1 binding within the TAB1-C30 domain. This C-terminal region is homologous between the vertebrate TAB1 and the *C. elegans* TAP-1; in particular, a similar motif PYVDX·F is conserved in TAB1 from human, *Xenopus*, and *C. elegans* (TAP-1) (Fig. 5). The identical and conserved amino acids with those of TAP-1 are indicated by asterisks and dots, respectively. The PYVDX/TFX motif is boxed.

![Figure 3](image-url) **Effects of TAB1 mutants on TAK1 binding activity.** Each aromatic amino acid in the α-helix domain of TAB1-C68 was substituted with alanine. Quantitative analysis of TAK1 binding in GAD-TAB1 mutants was done using the yeast two-hybrid system. The binding ability of LexA DNA binding domain-TAK1 and GAD-TAB1-C68 is shown as 1.0. Data are means ± S.D. of the determinations of three independent transformants from a representative experiment.

![Figure 4](image-url) **Biochemical relationship between TAK1/MOM-4 and TAB1/TAP-1.** 293 cells were transiently transfected with expression vectors for HA-TAK1, HA-MOM-4, Flag-TAB1 (F), Flag-TAB1(F484A) (A), Flag-TAP-1 (A), or Flag-TAP-1(A364F) (F) as indicated. Cell extracts were immunoprecipitated (IP) with anti-HA. The immunoprecipitates were subjected to an in vitro phosphorylation assay using bacterially expressed MKK6 as an exogenous substrate (top panel) and autophosphorylation of TAK1 (second panel). Co-precipitated Flag-TAB1 or Flag-TAP-1 derivatives were detected by immunoblotting (IB) with anti-Flag (third panel). Whole-cell extracts were immunoblotted with anti-HA and anti-Flag to determine total amounts of HA-TAK1 and HA-MOM-4 (fourth panel) and Flag-TAB1 and Flag-TAP-1 derivatives (bottom panel).

![Figure 5](image-url) **Sequence comparison of the TAB1 C-terminal domain from different species.** Sequences of the TAB1 C-terminal domain from human, *Xenopus*, and *C. elegans* (TAP-1) are depicted. The identical and conserved amino acids with those of TAP-1 are indicated by asterisks and dots, respectively. The PYVDX/TFX motif is boxed.
present in both (Fig. 5). Therefore, it is likely that the TAK1/MOM-4 binding function of TAB1/TAP-1 is evolutionarily conserved. However, although mutation of Phe-484 to alanine abrogates the ability of mammalian TAB1 to associate with and activate TAK1, the corresponding site in the C. elegans TAB-1 protein is itself an alanine residue, Ala-364. To determine whether this alanine plays a role in MOM-4 binding and activation, we mutated Ala-364 to Phe. We transiently co-expressed HA epitope-tagged MOM-4 (HA-MOM-4) together with Flag epitope-tagged TAP-1 (Flag-TAP-1) or TAP-1(A364F) (Flag-TAP-1(A364F)) in 293 cells. Cell extracts were subjected to immunoprecipitation with anti-HA antibody, immunoblotting, and then in vitro kinase assay (Fig. 4). Immunoblot analysis revealed that wild-type TAP-1 associated with MOM-4 but that the TAP-1(A364F) mutation abolished MOM-4 binding activity. When expressed alone, HA-MOM-4 had no kinase activity. However, co-expression with TAP-1 stimulated MOM-4 activity, whereas co-expression with TAP-1(A364F) did not. These results indicate that the Ala-364 residue in the C. elegans TAP-1 is important for both MOM-4 binding and activation.

We next examined the species specificity between TAB1/TAP-1 and TAK1/MOM-4 by co-expression in 293 cells (Fig. 4). We found that neither mammalian Flag-TAB1 nor Flag-TAB1(F484A) associated with or activated C. elegans HA-MOM-4 kinase. In contrast, both C. elegans TAP-1 and TAP-1(A364F) interacted with mammalian TAK1 kinase. Interestingly, although TAP-1 did not activate TAK1 kinase activity, TAP-1(A364F) did show weak stimulation of TAK1 autophosphorylation. Thus, activation of mammalian TAK1 requires a Phe residue in the C-terminal region of either TAB1 or TAP-1.

Conclusion—The majority of protein kinases contain a number of conserved sequence motifs, in addition to the canonical ~260-amino acid catalytic core, which have been suggested to be involved in substrate selection, regulation of catalytic activity, and cellular localization (35). In general, our understanding of the functions of these noncatalytic domains is poor, perhaps because there are few studies using purified, well-characterized proteins. In the present study, we examined TAK1, a member of the MAPKKK family. TAK1 is inactive until activated by TAB1. The activation of TAK1 is regulated by protein-protein interactions and protein modifications such as phosphorylation. Recently, we have shown that TAB1-induced activation of TAK1 requires autophosphorylation of TAK1 at Ser-192 in the kinase activation loop. Furthermore, it has been reported that the C-terminal region of TAB1, consisting of residues 480–504, is sufficient for association with and activation of TAK1 (36). In the present study, we characterized in detail the region in TAB1 responsible for binding to TAK1. First, a structural analysis of the TAB1-C30 peptide by NMR study reveals a unique a-helix structure, characterized by the alignment of aromatic residues on one side of the helix. Yeast two-hybrid analyses of wild-type, alanine-substituted, and deletion mutants of TAB1 show that the aromatic Phe-484 residue in the a-helical domain is critical for TAK1 binding. An evolutionarily conserved consensus core sequence required for TAK1 binding, PYD/DEAT/TFX, was identified within the helix domain of TAB1. High-resolution structural studies will be required to define the specific functional role of each amino acid in the core motif important for TAK1 binding. As shown here, TAK1 binding activity can be localized to a small region in the TAB1 C-terminal domain and can be ablated by a single point mutation. These features of the TAB1-TAK1 interaction suggest that small peptides or peptidomimetics that disrupt the interaction could be used to inhibit TAK1-dependent cellular functions.

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