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Identification and characterization of *Xenopus laevis* homologs of mammalian TRAF6 and its binding protein TIFA

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Abstract

Tumor necrosis factor receptor (TNFR)-associated factor 6 (TRAF6) transduces signals from members of the TNFR superfamily and the Toll/IL-1R family, leading to activation of transcription factors such as NFκB and AP-1. Genetic disruption of the *TRAF6* gene in mice results in various developmental abnormalities during embryogenesis, including osteopetrosis, failure of neural tube closure, defective formation of skin appendices, absence of lymph nodes, and absence of mature thymic epithelial cells. To clarify the effect of TRAF6 in development, we previously identified a TRAF-interacting protein with a forkhead-associated domain (TIFA), which binds and activates TRAF6 upon extracellular stimulation. To understand the physiological roles of TRAF6 and TIFA in early development, we studied these genes in *Xenopus laevis*. Here, we describe identification of *X. laevis* homologs of mammalian TRAF6 (XTRAF6) and TIFA (XTIFA). As was the case for the mammalian homologs, overexpression of XTRAF6 or XTIFA activated NFκB, whereas XTIFA carrying a mutation that abolishes XTRAF6 binding failed to activate NFκB, suggesting that XTIFA activates NFκB by binding to XTRAF6. *XTIFA* and *XTRAF6* mRNAs were expressed at similar levels in zygotes from the neurula stage and then increased. Whole-mount in situ hybridization revealed that *XTRAF6* mRNA was expressed in the head region and neural tube during the neurula stage, and the expression expanded to the pharyngeal apparatus during the tailbud stage. This localization is consistent with the defective neural tube closure and abnormal thymus organogenesis observed in TRAF6-deficient mice. Our results suggest possible cooperation between XTRAF6 and XTIFA during embryogenesis.

Keywords: NFκB; Forkhead-associated domain; Whole-mount in situ hybridization; Neural tube; Pharyngeal apparatus

1. Introduction

The tumor necrosis factor receptor (TNFR)-associated factor (TRAF) family proteins are cytoplasmic proteins that mediate cytokine signaling emanating from members of the TNFR superfamily and the Toll/interleukin-1 receptor (IL-1R) family (Arch et al., 1998; Inoue et al., 2000). To date, seven members of the TRAF family have been described. TRAF2, TRAF5, TRAF6, and TRAF7 are involved in the activation of transcription factors NFκB through IκB kinase (IKK) and AP-1 through mitogen-activated protein kinases such as Jun N-terminal kinase (JNK), p38, and extracellular signal-regulated kinase (Inoue et al., 2000; Bouwmeester et al., 2004). TRAF6 is the only TRAF that is essential for the MyD88-dependent signaling pathways originating from the Toll/IL-1R family (Cao et al., 1996; Ishida et al., 1996).

Abbreviations: BLAST, basic local alignment search tool; FHA, forkhead-associated; IKK, IκB kinase; IL-1R, interleukin-1 receptor; IRAK, IL-1R associated kinase; JNK, Jun N-terminal kinase; PCR, polymerase chain reaction; RANK, receptor activator of NFκB; TIFA, TRAF-interacting protein with an FHA domain; TRAF, tumor necrosis factor receptor associated factor; UEA-1, *Ulex europaeus* agglutinin-1.

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To elucidate the molecular mechanism by which TRAF6 is activated upon extracellular stimulation, we previously screened for proteins that bind and activate TRAF6, and we identified a TRAF-interacting protein with a forkhead-associated (FHA) domain (TIFA) (Takatsuna et al., 2003). When overexpressed, TIFA activates NFκB and JNK. Furthermore, introduction of a mutation into TIFA that abolishes binding to TRAF6 abolishes TIFA activation of NFκB and JNK, indicating that interaction with TRAF6 is essential for TIFA activity. FHA domains are conserved sequences of 60–100 amino acids found mainly in eukaryotic nuclear proteins (Hofmann and Bucher, 1995). Some FHA domain-containing proteins bind directly to phosphoserine/phosphothreonine residues via the FHA domain in much the same way that SH2 domains interact with phosphotyrosine residues (Yaffe and Cantley, 1999; Li et al., 2000). TIFA carrying mutations in the FHA domain (*G50ES66A* mutant) that are known to abolish FHA domain binding to phosphopeptide cannot activate NFκB and JNK, suggesting that TIFA may be regulated by an unidentified phosphoprotein. Furthermore, analysis of endogenous proteins indicated that TIFA associates with TRAF6 constitutively, whereas it associates with IL-R-associated kinase-1 (IRAK-1) in an IL-1 stimulation-dependent manner. Therefore, TIFA may be a signal-dependent activator of TRAF6 in vivo (Takatsuna et al., 2003).

We previously reported that TRAF6-deficient (*TRAF6*$^{-/-}$) mice exhibit severe osteopetrosis and lack lymph nodes due to defective signaling from RANK upon binding of RANK ligand (Naito et al., 1999; Kobayashi et al., 2001; Yoshida et al., 2002). *TRAF6*$^{-/-}$ mice also display hypohidrotic ectodermal dysplasia, which is a congenic disorder of the formation of skin structures, including hair follicles and sweat glands (Naito et al., 2002). Furthermore, we recently reported that TRAF6 deficiency results in abnormal development of thymic stroma, which alters immune self-tolerance (Akiyama et al., 2005). The developmental defects described above appear during embryogenesis. In addition, approximately half the *TRAF6*$^{-/-}$ mice die in utero (Lomaga et al., 1999; Naito et al., 1999; Lomaga et al., 2000). These results indicate that TRAF6 plays several important roles in embryogenesis. In the present study, we cloned the cDNAs encoding *X. laevis* homologs of TRAF6 (XTRAF6) and TIFA (XTIFA) and examined their physical and functional interactions. We also examined the expression patterns of *XTRAF6* and *XTIFA* mRNAs during embryogenesis and in adult tissue.

2. Materials and methods

2.1. Cloning

One microgram of total RNA extracted from liver of adult *X. laevis* was converted to cDNA with 50 ng of random hexanucleotides (GIBCO BRL) and 200 U of Superscript II Reverse Transcriptase (GIBCO BRL) at 37 °C for 1 h. cDNAs encoding XTRAF6 and XTIFA were amplified by polymerase chain reaction (PCR) from 1 μl of cDNA mixture as a template with primer pairs. TRAF6-5′, 5′-GCGAATTCATGAGTATCCTGAACCCAAGG-3′ and TRAF6-3′, 5′-GCGGATCCCTATAGGGTCCCCTCTCCGCTG-3′; and TIFA-5′, 5′-GCGAATTCATGGATAAAGAATTAATTGATG-3′ and TIFA-3′, 5′-GGATCCTTAACATTCATTTTCGTCAATTTC-3′.

2.2. Plasmids and antibodies

pME-Myc-XTRAF6, pME-FLAG-XTIFA, and pME-FLAG-XTIFA-E172A were constructed by insertion of a DNA fragment encoding Myc-tagged XTRAF6, FLAG-tagged XTIFA, and FLAG-tagged XTIFA-E172A (Ala substituted for Glu-172), respectively, into pME-18S (Shiio et al., 1992). Anti-Myc (9E10) monoclonal antibody and anti-TRAF6 (H-274) polyclonal antibody were purchased from Santa Cruz Biotechnology. Anti-XTIFA polyclonal antibody was generated by injection of maltose-binding protein (MBP)-XTIFA fusion protein into rabbits and purified from anti-serum by passing follow-through fractions from the MBP affinity column over MBP-XTIFA affinity columns.

2.3. Immunoprecipitation and Western blotting

HEK293T cells (1×10^6) in 90-mm dishes were transfected with pME-Myc-TRAF6 alone or together with pME-FLAG-XTIFA or pME-FLAG-XTIFA-E172A. Thirty-six hours after transfection, cells were lysed in TNE buffer as described previously (Kobayashi et al., 2001) and subjected to immunoprecipitation by addition of anti-Myc monoclonal antibody and protein G-Sepharose (Amersham-Pharmacia Biotech). The resulting immunoprecipitates were separated on 10% or 12.5% polyacrylamide/SDS gels and transferred to PVDF membrane (Millpore Corp.). The membranes were immunoblotted with either anti-TRAF6 (H-274) or anti-XTIFA polyclonal antibody, and each protein was visualized by horseradish peroxidase-conjugated secondary antibody with the ECL Western blotting system (Amersham-Pharmacia Biotech). Cell lysates were also used to analyze the level of expression of each protein. For luciferase reporter assays, amounts of TIFA and TIFA-E172A were determined in cell lysates by Western blotting with anti-XTIFA polyclonal antibody.

2.4. Luciferase reporter assay

HEK293T cells (2×10^5) in 6-well dishes were transfected with 5 ng of 3×κB-luc, 50 ng of β-actin-β-gal, and the indicated amounts of pME-FLAG-XTRAF6, pME-FLAG-XTIFA, or pME-FLAG-XTIFA-E172A. Thirty-six hours after transfection, luciferase activity was measured with the PicaGene Luciferase Assay System (Toyo Ink), and