Identification of the Anti-proliferative Protein Tob as a MAPK Substrate*

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Mitogen-activated protein kinases (MAPKs) regulate a wide variety of cellular functions by phosphorylating their specific substrates. Here we have identified Tob as a novel substrate of MAPK. Tob, a member of the Tob and B-cell translocation gene anti-proliferative protein family, is shown to negatively regulate the proliferation of osteoblasts and T cells. In this study, our two-hybrid screening has identified Tob as an ERK2-interacting protein. Biochemical analyses have then shown that ERK MAPK (ERK2) and JNK/SAPK (JNK2) bind to and phosphorylate Tob in vitro. ERK catalyzes the phosphorylation more efficiently than JNK. When the ERK pathway is activated in cells, phosphorylation of Tob is induced. An ERK-binding or -docking site locates in the N-terminal portion of Tob, and phosphorylation sites reside in the C-terminal stretch region. The docking is crucial for efficient phosphorylation. Mutant forms of Tob, in which serines are replaced by glutamic acids to mimic phosphorylation, show a much reduced ability to inhibit the cell cycle progression to S phase from G0/G1 phase, as compared with wild-type Tob, indicating that ERK phosphorylation negatively regulates the anti-proliferative function of Tob.

The mitogen-activated protein kinase (MAPK)1 cascades play pivotal roles in diverse cellular functions. To date, at least four independent MAPK pathways have been identified. They include the extracellular signal-regulated protein kinase (ERK) pathway, the c-Jun N-terminal kinase (JNK)/stress-activated protein kinase (SAPK) pathway, the p38 pathway, and the ERK5/Big MAPK1 (BMK1) pathway. Activation of these pathways leads to variable responses, such as cytokine production, cell proliferation, differentiation, cell cycle arrest, apoptosis, etc., depending on the cell type (1–10).

MAPKs fulfill their specific roles by phosphorylating several substrates, such as transcription factors and MAPKAPKs (MAPK-activated protein kinases). The phosphorylation often causes a conformational change of a substrate and regulates its association with other molecules. Despite a wide variety of cell responses in which MAPKs play roles, substrates of MAPKs have not been fully elucidated. To gain further insights into the function of MAPKs, we performed a yeast two-hybrid screening using ERK2 as bait to identify novel substrates. We then identified Tob as a novel ERK2-interacting protein.

Tob is a member of the Tob and BTG anti-proliferative protein family. The family includes B cell translocation gene (BTG1), BTG2, Tob, abundant in neuroepithelial area (ANA), and Tob2 (11–16). The family can be divided into two subgroups, the BTG family and the Tob family. Both families have been reported to suppress cell proliferation when expressed exogenously in cultured cells. They commonly share an N-terminal BTG domain, which is responsible for their anti-proliferative function. Tob proteins have a C-terminal stretch whose function has not been elucidated. Members of the Tob family are expressed in a wide variety of organs and conserved across species, from invertebrates to vertebrates (17, 18). However, molecular mechanisms of their actions have not been fully understood. Recently, two reports appeared. One report showed that mice carrying a targeted deletion of the Tob gene have a greater bone mass resulting from increased numbers of osteoblasts, indicating that Tob negatively regulates the proliferation of osteoblasts (19). The other report showed that Tob functions as a negative regulator of T cell proliferation and cytokine transcription (20).

In this study, our two-hybrid screening has suggested Tob as a possible target of MAPKs. Our results have shown that among the members of the MAPK family, ERK2 and JNK2 bind to and phosphorylate Tob. ERK2 catalyzes the phosphorylation more efficiently than JNK2. We have identified an N-terminal portion of Tob as a MAPK-docking site, which is required for optimal phosphorylation. The phosphorylation sites locate in the C-terminal stretch region of Tob, a region unique to the Tob family. Finally, we have shown that mutant forms of Tob, in which phosphorylatable serines are mutated to glutamic acids, show a much reduced ability to inhibit the cell cycle progression, as compared with wild-type Tob. These results suggest that Tob is a substrate of ERK MAPK.

EXPERIMENTAL PROCEDURES

Plasmids—Human Tob was cloned by PCR from a fetal human brain cDNA library. The primers used were 5’-agatctatagctgatatc-3’ and 5’-gaattcttacagctatcagcga-3’. The expression vector used for human Tob is pDL-SRe-3XMyc. For ERK2 (Xenopus), JNK2 (rat), and p38a (human), pDL-SRe-HA was used.

Two-hybrid Screening—Yeast two-hybrid screening of the Gal4 system was performed with catalytically inactive ERK2 (Xenopus) as bait. Full-length catalytically inactive ERK2 was cloned into pBridge Gal4.
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**Materials and Methods**

**Cell Cultures and Transfection**
C2C12 cells were cultured in Dulbecco’s modified Eagle’s medium containing 10% fetal calf serum (COST7 and NIH3T3) or 15% fetal calf serum (C2C12). B-Raf:ER cells were cultured in Dulbecco’s modified Eagle’s medium containing 10% fetal bovine serum and 25 mM Hepes, pH 7.4, without phenol red. The cells were maintained in 5% CO2 at 37 °C. Cells were split on a 35-mm dish or a 60-mm dish at 2 × 10² or 5 × 10² cell number per dish, respectively. After 19 h, cells were transfected using LipofectAMINE Plus reagent (Invitrogen) according to the manufacturer’s protocol.

**GST Pull-down Assay**
Cells were lysed in 50 mM Hepes, pH 7.4, 2 mM EGTA, 2 mM MgCl₂, 10% glyceral, 1% Nonidet P-40, 1 mM phenylmethylsulfonyl fluoride, and 20 μM/ml aprotinin. Following homogenization, the cell lysates were centrifuged at 15,000 × g for 30 min. Tagged proteins were immunoprecipitated from the supernatant of the cell lysates (about 3 × 10⁵ cells in each sample) by incubation with 5 μg of anti-c-Myc antibody (9E10) (Santa Cruz) or 5 μg of anti-HA antibody (12CA5) and protein A-Sepharose beads (25 μl) (Amersham Biosciences) for 2 h at 4 °C. The precipitates were then washed twice with a lysis buffer. The immunoprecipitant was eluted by incubation with an elution buffer (100 mM Tris-HCl, pH 2.2). The precipitates were then washed twice with a lysis buffer. The proteins were separated by SDS-PAGE and analyzed by immunoblotting.

**Kinase Assay**
Cells were lysed in a buffer (20 mM Tris-HCl, pH 7.5, 1M glycerophosphate, 150 mM NaCl, 1.5 mM MgCl₂, 2 mM EGTA, 10 mM NaF, 1% Triton X-100, 1 mM phenylmethylsulfonyl fluoride, 1 mM sodium vanadate, and 20 μg/ml aprotinin). Tagged proteins were immunoprecipitated from cell lysates (about 1 × 10⁶ cells in each sample) by incubation with 2 μg of appropriate antibody and protein A-Sepharose beads (15 μl) (Amersham Biosciences) for 2 h at 4 °C. Each precipitate was washed twice with TBS (20 mM Tris-HCl, pH 7.5, 0.5 mM NaCl, 1 mM PMSE, 1% dithiothreitol, 1 mM sodium vanadate, and 20 μg/ml aprotinin), and then washed with Tris buffer (20 mM Tris-HCl, pH 7.5). The washed beads were mixed with substrates in a kinase reaction buffer (20 mM Tris-HCl, pH 7.5, 10 mM MgCl₂, and 100 μM ATP) (2 μCi [γ-32P]ATP), and incubated for 10 min at 30 °C. The reaction was stopped by addition of Laemmli’s sample buffer. Substrate phosphorylation was detected by autoradiography and BAS 2500 (Fuji Film) after SDS-PAGE.

**Results and Discussion**

**MAPKs Bind to Tob and Phosphorylate it in Vitro**
We screened the human liver cDNA library by the yeast two-hybrid method with catalytically inactive ERK2 as bait. Among several positive clones, a full-length Tob was obtained. To determine whether the three major members of the MAPK family (ERK2, JNK2, and p38α) bind to Tob, we performed a co-immunoprecipitation assay. ERK2 and JNK2 co-immunoprecipitated well with Tob, whereas p38α did not (Fig. 1A). To further confirm the binding between ERK2 or JNK2 and Tob, we performed a GST pull-down assay. As shown in Fig. 1B, HA-ERK2 or HA-JNK2, but not p38α, was efficiently co-precipitated with GST-Tob protein but not with GST protein (Fig. 1B). As Tob has seven putative MAPK-phosphorylation sites in its C-terminal stretch (see Fig. 2A), we examined whether or not Tob is directly phosphorylated in vitro by MAPK. Recombinant Tob protein (as a GST fusion protein) was expressed in E. coli, purified, and assayed. As shown in Fig. 1C, ERK2 and JNK2 efficiently phosphorylated GST-Tob protein, but not GST protein (data not shown), in vitro. ERK2 catalyzed the phosphorylation much more efficiently than JNK2 (Fig. 1C). p38α could not phosphorylate Tob efficiently. Thus, among the members of the MAPK family, ERK2 and JNK2 bind to Tob and phosphorylate it in vitro. ERK2 phosphorylates Tob much more efficiently than JNK2 does.

**FIG. 1.** ERK2 and JNK2 bind to and phosphorylate Tob. A, binding between MAPKs and Tob was examined by a co-immunoprecipitation assay. Lysates of COS7 cells (60-mm dish) co-transfected with indicated combinations of constructs were immunoprecipitated with anti-Myc antibody. Co-immunoprecipitated HA-MAPK (ERK2, JNK2, or p38) was detected (upper panel, αHA (IP)). The expression levels of HA-MAPK in each sample were similar (middle panel, αHA (whole)). Comparable amounts of Myc-Tob were immunoprecipitated in each lane (lower panel, αMyc (IP)). Similar results were obtained in three different experiments. B, ERK2 and JNK2 were co-precipitated with a GST-Tob protein in a GST pull-down assay. A GST fusion of Tob was expressed in bacteria and purified by the method described previously (21). An equal amount (20 μg) of GST or GST-Tob was incubated with each lysate from COS7 cells expressing HA-ERK2, JNK2, or p38α. Co-precipitated HA-MAPK was detected (upper panel, αHA (pull-down)). Comparable amounts of MAPK were expressed (lower panel, αHA (whole)). Similar results were obtained in three different experiments. C, Tob is phosphorylated by MAPKs in vitro. HA-JNK2 or HA-p38α was expressed in COS7 cells and activated by 0.5 mM NaCl. 5 μg of anti-HA antibody (12CA5) was co-expressed with constitutively active MEK1 (MEK1 S556D) in COS7 cells. Activated HA-MAPKs were immunoprecipitated using anti-HA antibody (12CA5). After extensive wash with TBS (0.5 mM NaCl), HA-MAPK was examined for the ability to phosphorylate GST-Tob in vitro. Phosphorylated GST-Tob was detected by autoradiography (upper panel, p-GST-Tob). Immunoprecipitated HA-MAPKs were detected by Western blotting (lower panels).
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Fig. 2. Tob is phosphorylated on its C-terminal portion by ERK2. A, schematic representation of human Tob. In the C-terminal portion of Tob, seven putative MAPK-phosphorylation sites (SP site) exist. Mutant forms of Tob were created in which the putative phosphorylation site was replaced by alanine or glutamic acid. B, AB-Raf:ER cells transfected with SR-Myc-Tob were left untreated or stimulated by estrogen for 30 min. Myc-Tob immunoprecipitated with anti-Myc antibody was treated with calf intestine alkaline phosphatase (0.5 unit/μl, 37 °C, 2 h) or a control buffer and then analyzed by SDS-PAGE followed by immunoblotting. C, AB-Raf:ER cells transfected with various mutant forms of Myc-Tob were left untreated or stimulated by estrogen for 30 min. The immunoprecipitated Myc-Tob was analyzed by immunoblotting with anti-Myc antibody. D, the mobility of Tob S154E and Tob EEE (S152E, S154E, and S164E) in SDS-PAGE was examined. AB-Raf:ER cells transfected with various mutant forms of Myc-Tob were left untreated or stimulated by estrogen for 40 min. Myc-Tob was analyzed by immunoblotting with anti-Myc antibody. E, phosphorylation of mutant forms of Tob in vitro was examined. Myc-tagged wild-type, S154A or S152A, S154A, S164A Tob was expressed in COS7 cells and immunoprecipitated. After washing twice with TBS (0.5 μM NaCl), the immunoprecipitates were subjected to the phosphatase treatment and then washed with TBS (0.5 μM NaCl) for a further three times and once with 20 mM Tris-HCl, pH 7.4. GST-ERK2 was co-expressed with constitutively active MEK1 (MEK1 S36D) in COS7 cells. Activated GST-ERK2 was then precipitated using GSH-beads. The comparable amounts of various forms of Tob were assayed for their ability to be phosphorylated by ERK2. The rate of Tob phosphorylation was quantified, and the value relative to the wild-type Tob is shown. Essentially the same results were obtained in three independent experiments. F, NIH3T3 cells transfected with various mutant forms of Myc-Tob were left untreated or stimulated by platelet-derived growth factor (20 ng/ml) for 10 min after incubation in serum-free medium for 24 h. The cells were lysed in a buffer containing MgCl2 (50 mM). The immunoprecipitated Myc-Tob was analyzed by immunoblotting with anti-Myc antibody. G, CO2 cells transfected with Myc-Tob were left untreated or stimulated by EGF (20 ng/ml) or FGF (50 ng/ml) in the presence or absence of U0126 (20 μM). The immunoprecipitated Myc-Tob was analyzed by immunoblotting with anti-Myc antibody. H, endogenous Tob was detected in C2C12 cells using anti-Tob antibody (IBL). The cells were treated with 10% fresh fetal calf serum in the presence or absence of U0126 (20 μM). U0126 was added 1 h before the stimulation. MG132 was added to the medium 2 h before the stimulation to avoid degradation of Tob protein. Tob was analyzed by immunoblotting. Activation of ERK MAPKs was examined using an anti-phospho-specific ERK antibody (Promega).

appeared after the stimulation (Fig. 2B). As the shifted band disappeared after in vitro treatment of immunoprecipitated Myc-Tob with calf intestine alkaline phosphatase (Fig. 2B), the shifted band of Tob after the stimulation may be caused by phosphorylation by ERK (Fig. 2B). Tob has seven putative MAPK-phosphorylation sites (SP site) in its C-terminal stretch region (Fig. 2A). To determine a phosphorylation site responsible for the mobility shift, we created seven mutant forms of Tob in which each of the putative MAPK-phosphorylation sites was replaced by alanine. As shown in Figs. 2C, when Ser154 was replaced by alanine the mobility shift was completely inhibited. To mimic the phosphorylation state of Tob, we created a mutant form of Tob (Tob S154E), in which Ser154 was replaced by glutamic acid. A migrating position of this mutant was almost the same as that of the shifted band of wild-type Tob in SDS-PAGE, and no further band-shift was observed after stimulation with estrogen (Fig. 2D). Furthermore, even when the other two serine residues, Ser152 and Ser154, along with Ser154 were simultaneously replaced by glutamic acids (Tob EEE), no further mobility shift was observed (Fig. 2D). These results indicate that Ser154 is a major phosphorylation site responsible for the mobility shift. A Myc-tagged S154A Tob was then expressed in COS7 cells, purified, and assayed for its ability to be phosphorylated by ERK2 in vitro. This mutant Tob was less efficiently phosphorylated by ERK2 than wild-type Tob (Fig. 2E). Because it appeared possible that other sites might also be phosphorylated, we created a mutant form of Tob (Tob AAA), in which Ser152, Ser154, and Ser164 were replaced by alanines. This mutant was more defective than Tob S154A in the ability to be phosphorylated by ERK2 (Fig. 2E). These results suggest that these serine residues constitute at least part of the phosphorylation sites of Tob, which may be catalyzed by ERK MAPK. Next, we examined whether Tob is phosphorylated on Ser154 in cells in response to physiological stimuli. NIH3T3 cells were transfected with wild-type or mutant forms of Tob, and stimulated with platelet-derived growth factor, which is a well known activator of the ERK MAPK pathway. Although wild-type Tob showed a mobility-shifted band, neither Tob S154A nor Tob AAA showed its mobility shift (Fig. 2F). In COS7 cells also, exogenously expressed wild-type Tob showed its mobility shift in response to EGF or FGF (Fig. 2G). Furthermore, in the presence of the MEK-specific inhibitor U0126, which inhibited the activation of ERK by EGF or FGF (Fig. 2G, lower panel), the mobility-shift was suppressed (Fig. 2G, upper panel). We could detect endogenous Tob only in C2C12 cells among several cultured cell lines tested. When C2C12 cells were stimulated by fresh serum, a mobility-shifted band of endogenous Tob appeared. The appearance of the shifted band was markedly inhibited in the presence of U0126 (Fig. 2F). These results taken together suggest that Tob is phosphorylated, at least on Ser154, in cells in response to growth factor stimulation, and the phosphorylation may be mediated by ERK.

Tob Has a MAPK-docking Site in its N-terminal Portion—It is known that several MAPK-interacting molecules have a MAPK-docking site (22–25). The MAPK-docking site is featured by a cluster of positively charged amino acids and surrounding hydrophobic amino acids. We found that Tob has a putative MAPK-docking site in its N-terminal portion (Fig. 3A). To determine whether this site is needed for binding to ERK MAPK, we created a mutant form of Tob (RRMM) in which two positively charged amino acid residues (Arg22 and Arg28) in a putative MAPK-docking site were replaced by methionines (Fig. 3A). Although wild-type Tob bound to ERK2 efficiently in a co-immunoprecipitation assay, the mutant form of Tob (RRMM) did not (Fig. 3B). Furthermore, the mutant form of
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Tob was less efficiently phosphorylated by ERK2 than wild-type Tob was (Fig. 3C). These results clearly show that the N-terminal portion of Tob serves as a MAPK-docking site. The MAPK-docking site in the N-terminal portion is well conserved among the members of the Tob family, Tob, Tob2, and ANA, but not in the BTG family. Moreover, Xenopus Tob, Drosophila Tob, and Caenorhabditis elegans Tob also possess the conserved MAPK-docking site in their N-terminal portion (see Fig. 3D), suggesting that docking to ERK MAPK is evolutionarily conserved.

Several substrates of ERK have a specific motif (the FXFP site) C-terminal to the phosphorylation site (25). The FXFP motif facilitates the efficiency of phosphorylation by MAPKs. Tob also has an FXFP motif C-terminal to the phosphorylation sites (residues 274–277). This fact may be consistent with the idea that Tob is a physiological substrate of ERK. There are several common functions in the Tob family and the BTG family. For example, both can inhibit cell proliferation when expressed in cells, and both can bind to Caf1 and Pop2, components of the CCR4-NOT transcription complex. Because the MAPK-docking site of Tob is not conserved in the BTG family, and because the ERK-phosphorylation sites exist in the C-terminal stretch, a region unique to the Tob family, regulation of the activity by ERK MAPK might be specific to the Tob family member (see below).

Phosphorylation by ERK2 Negatively Regulates the Anti-proliferative Function of Tob—To address the role of ERK phos-
phorylation of Tob, we examined the effect of expression of mutant forms of Tob on cell proliferation. When exogenously expressed in cells, Tob is known to inhibit cell proliferation (12, 14, 15). On the other hand, it is known that ERK activation is necessary for serum stimulation-induced cell cycle progression into S phase from the quiescent state (6, 7, 26). Then, we examined the effect on the serum-induced S phase entry of the cells. Serum-starved NIH3T3 cells were microinjected with S-Ras-Myc-wild-type Tob or each of S-Ras-Myc-mutant forms of Tob and stimulated with 20% fetal calf serum. Cells expressing Tob were detected by immunostaining with anti-Myc antibody, and the cells that entered the S phase were identified by BrdUrd incorporation into nucleus. Expression of an empty vector did not affect the 20% serum-induced S phase entry (Fig. 4B). Expression of wild-type Tob blocked the serum-induced S phase entry (Fig. 4, A and B). Tob S154A and Tob AAA, in which Ser152, Ser154, and Ser164 were replaced by alanines, also blocked the S phase entry. However, Tob S154E or Tob EEE did not block the S phase entry (Fig. 4, A and B). These results strongly suggest that phosphorylation of Tob by ERK negatively regulates its anti-proliferative activity. When the expression level of wild-type Tob was decreased, the anti-proliferative effect was decreased reproducibly (Fig. 4, C and D). In this case, the amount of the unshifted band of wild-type Tob after serum stimulation was significantly decreased (Fig. 4D). When the expression level of Tob S154A or Tob AAA was decreased, their anti-proliferative effect was not significantly decreased under the conditions used (Fig. 4D). These data suggest that although the maximal anti-proliferative activity of the alanine mutants of Tob (Tob S154A and Tob AAA) is weaker than that of wild-type Tob, the concentration of the mutants required for the half-maximal effect is lower than that of wild-type Tob, because the activity of the mutants is not subject to regulation by ERK-mediated phosphorylation. It is likely that negative regulation of the anti-proliferative activity of Tob by ERK-mediated phosphorylation is important for growth factor stimulation-induced S phase entry of the cells.

Upon mitogenic stimuli, ERK positively regulates the expression of immediate early genes, which are necessary for cell proliferation. To regulate the expression of immediate early genes, ERK phosphorylates several transcription factors, including Elk-1 and SAP1. Here we have identified Tob as a candidate of a novel substrate of ERK during G1-S transition. Although the precise molecular mechanism of the action of Tob has not yet been elucidated, Tob was reported to associate with CAF1 and POP2, components of the CCR4-NOT protein complex. The genetic analyses of CCR4-NOT functions in yeast demonstrated that these proteins are involved in both the diverse transcriptional responses and the control of mRNA metabolism (27–30). Whether Tob plays a role in the regulation of transcription or the control of mRNA stabilization via the CCR4-NOT complex is currently unknown. In our preliminary experiment, Tob was stabilized upon mitogenic stimuli. This observation seems contradictory to the negative role of Tob in cell proliferation. However, as the CCR4-NOT complex plays both positive and negative roles in the regulation of expression of diverse genes, Tob might regulate transcription both positively and negatively depending on the target. Or, stabilization of Tob might have some negative feedback role. In any case, further studies are needed to fully elucidate the molecular mechanisms of the action of Tob and its regulation by ERK MAPK. This study provides us with the first cue to address these problems. After submission of this work, Yamamoto et al. (31) reported that Tob is phosphorylated and negatively regulated by ERK MAPK.

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