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Phosphorylation of Recombinant Tristetraprolin *in Vitro*

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Abstract

Tristetraprolin/zinc finger protein 36 (TTP/ZFP36) binds and destabilizes some proinflammatory cytokine mRNAs. TTP-deficient mice develop a profound inflammatory syndrome due to excessive production of proinflammatory cytokines. TTP gene expression is induced by various factors including insulin, cinnamon, and green tea extracts. Previous studies have shown that TTP is highly phosphorylated *in vivo* and multiple phosphorylation sites are identified in human TTP. This study evaluated the potential protein kinases that could phosphorylate recombinant TTP *in vitro*. Motif scanning suggested that TTP was a potential substrate for various kinases. SDS-PAGE showed that *in vitro* phosphorylation of TTP with p42 and p38 MAP kinases resulted in visible electrophoretic mobility shift of TTP to higher molecular masses. Autoradiography showed that TTP was phosphorylated *in vitro* by GSK3b, PKA, PKB, PKC, but not Cdc2, in addition to p42, p38, and JNK. These results demonstrate that TTP is a substrate for a number of protein kinases *in vitro*.

Keywords

Glycogen synthase kinase 3b; Inflammation; Phosphorylation; Protein kinase; Recombinant protein; Tristetraprolin; Zinc finger protein

1. INTRODUCTION

Tristetraprolin (TTP) is the best-understood member of a small family of tandem CCCH zinc finger proteins (ZFP). Similar tandem CCCH zinc finger sequences have been found in many species, ranging from human through yeasts and plants. TTP protein family consist of three known members in mammals (ZFP36 or TTP, ZFP36L1 or TIS11B, and ZFP36L2 or TIS11D) and the fourth member in mouse and rat but not in humans (ZFP36L3) (Blackshear 2002; Blackshear *et al*. 2005). Separate genes encode these four proteins, and their patterns of cell- and tissue-specific expression and agonist-stimulated expression are quite different. However, they share certain sequence characteristics: All four have highly conserved tandem zinc finger domains, in which each C8×C5×C3×H zinc finger is preceded by the sequence (R/K)YKTEL, and the two fingers are separated by 18 amino acids; and all are capable of binding AU-rich elements (ARE) within single stranded RNAs (Blackshear *et al*. 2003; Cao *et al*. 2003; Cao 2004; Carballo *et al*. 1998; Carballo *et al*. 2001; Phillips *et al*. 2004; Worthington *et al*.

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2002) and promoting the deadenylation and subsequent destruction of those transcripts, both in transfection studies and in cell-free experiments (Lai *et al*. 1999; Lai *et al*. 2003). In intact animals, TTP deficiency causes a profound inflammatory syndrome with erosive arthritis, autoimmunity, and myeloid hyperplasia (Phillips *et al*. 2004; Taylor *et al*. 1996). This is apparently due almost entirely to excessive production of tumor necrosis factor α (TNF) and granulocyte-macrophage colony-stimulating factor, whose mRNAs are direct targets of TTP but are stabilized in cells from TTP knockout mice (Carballo *et al*. 1998; Carballo *et al*. 2000; Lai *et al*. 1999). For these reasons, TTP can be thought of as an anti-inflammatory protein and arthritis suppressor.

The cDNAs encoding TTP were originally cloned by three groups based on its very rapid and dramatic transcriptional induction in fibroblasts in response to insulin, phorbol esters, and serum (DuBois *et al*. 1990; Lai *et al*. 1990; Varnum *et al*. 1991). Recently, we have shown that TTP protein is a very low abundance cytosolic protein, whose level is also dramatically induced by lipopolysaccharide (LPS), fetal calf serum, cinnamon polyphenols, and green tea extract (Cao *et al*. 2004; Cao *et al*. 2007a; Cao *et al*. 2007b). The protein is very stable once induced, in contrast to its very labile mRNA (Cao *et al*. 2004). In addition, TTP in normal tissues and in stimulated cells exhibits a much larger molecular mass than the predicted size (Cao *et al*. 2004), probably due to extensive phosphorylation in intact cells and tissues. This conclusion is supported by the fact that dephosphorylation of TTP from transfected human 293 cells and LPS-stimulated mouse RAW 264.7 cells results in TTP close to the size of TTP expressed and purified from *E. coli* (Cao. 2004; Cao *et al*. 2004).

TTP phosphorylation has been studied by several laboratories. TTP could be phosphorylated in intact cells and in cell-free systems by p42 mitogen-activated protein kinase (MAPK) (ERK2) (Cao *et al*. 2003; Cao 2004; Taylor *et al*. 1995), p38 MAP kinase (Cao *et al*. 2003; Cao 2004; Carballo *et al*. 2001; Zhu *et al*. 2001), c-Jun N-terminal kinase (JNK) (Cao *et al*. 2003), and MAP kinase-activated protein kinase 2 (MAPKAP kinase 2 or MK2) (Chrestensen *et al*. 2004; Mahtani *et al*. 2001; Ming *et al*. 2001; Stoecklin *et al*. 2004). Mass spectrometry and site-directed mutagenesis have identified a number of phosphorylation sites in human and mouse TTP (Cao *et al*. 2006; Chrestensen *et al*. 2004; Taylor *et al*. 1995; Cao and Deterding 2007). However, much needs to be learned about the potential kinases that phosphorylate TTP. In this study, we extended our investigation on the identification of potential protein kinases targeting TTP using motif scanning, electrophoretic mobility shift assay, and *in vitro* phosphorylation assay. Our results demonstrate that TTP is a substrate for a number of protein kinases *in vitro*.

2. MATERIALS AND METHODS

2.1. Chemicals and Reagents

The protein kinases used are glycogen synthase kinase b (GSK3β) (recombinant histidine-tagged rabbit protein from *E. coli*, Sigma, St. Louis, MO), protein kinase (PK)A (cAMP-dependent protein kinase, catalytic subunit from bovine heart, Calbiochem, La Jolla, CA), PKBα/Akt1 (histidine-tagged, activated human protein from *S. frugiperda*, Calbiochem), PKCμ (catalytic domain from rat brain, Calbiochem), Cdc2/CDK1/p34^{cdc2} (recombinant human Cdc2 kinase catalytic subunit from Sf9 insect cells, Sigma); p42/ERK2 MAPK (recombinant His-tagged rat protein purified from *E. coli*, Calbiochem,), p38 MAPK (GST-tagged mouse p38 MAP kinase purified from *E. coli*, Calbiochem), and JNK (calmodulin-binding peptide with 24 amino acid residues-tagged rat protein purified from *E. coli*, Calbiochem).

2.2. Protein Concentration Determination

Protein concentrations were determined with modifications using the Protein Assay Dye Reagent Concentrate (Bio-Rad) following NaOH treatment of the samples (Cao 2004). Bovine serum albumin from Bio-Rad was used as the protein standard.

2.3. SDS-PAGE and Immunoblotting

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and immunoblotting were performed (Cao *et al*. 2003). Briefly, proteins were separated by 10% SDS-PAGE and transferred onto nitrocellulose membranes. The membranes were blocked with 5% nonfat dry milk in TTBS buffer, and successively incubated in buffers containing the primary antibodies (1:10000 dilution) overnight and the secondary antibodies (1:10000 dilution) for 4 h. Proteins on the immunoblots were detected using SuperSignal West Pico Chemiluminescent Substrate (Pierce, Rockford, IL). The primary antibodies were anti-MBP-hTTP serum (Cao 2004) and anti-MBP-mTTP serum (Cao *et al.* 2004) raised against the recombinant full-length human or mouse TTP fused to *E. coli* maltose-binding protein. The secondary antibodies were affinity-purified goat anti-rabbit IgG (H+L) horseradish peroxidase conjugate with human IgG absorbed (GAR-HRP, Bio-Rad).

2.4. Recombinant TTP Expression and Purification

Plasmids pMBP-hTTP and pMBP-mTTP were used to express the full-length human TTP (hTTP, GenBank accession no. NP_003398) and mouse TTP (mTTP, GenBank accession no. NP_035886) as recombinant proteins fused to *E. coli* maltose binding protein (MBP) (Cao *et al*. 2003). Recombinant TTP was expressed and purified from *E. coli* (Cao *et al*. 2003). Briefly, plasmids were transformed into *E. coli* BL21(DE3) cells. TTP fusion proteins were induced with 0.4 mM isopropylthio-β-D-galactoside. Cells were homogenized in a buffer containing 20 mM Tris-HCl, pH 7.4, 200 mM NaCl, 10 mM β-mercaptoethanol, 1 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, 2 μM leupeptin, 2 μM pepstatin, 2 μg/ml aprotinin and with 2 mM zinc chloride.

MBP-TTP fusion proteins were purified by fast protein liquid chromatography (Amersham Pharmacia Biotech) from the 10000 ***g*** supernatant with amylose resin affinity chromatography in buffers with 1 mM zinc chloride (Cao *et al*. 2003). TTP fusion proteins were stored at −20 °C in 20% glycerol (v/v), 10 mM maltose, 20 mM Tris-HCl, pH 7.4, 200 mM NaCl, 10 mM β-mercaptoethanol), and 1 mM zinc chloride. MBP was affinity-purified from *E. coli* expressing plasmid pMAL2c (New England Biolabs) by the same amylose resin chromatography and used as a control in these experiments.

2.5. Protein Kinase Activity Assay *in Vitro*

The purified recombinant MBP-TTP or MBP alone was used for phosphorylation reactions with GSK3β, PKA, PKBα/Akt1, PKCμ, Cdc2, p42, p38, and JNK. The general procedures for the *in vitro* protein kinase assays were similar to those previously described (Cao *et al*. 2003). The phosphorylation reactions were initiated by the addition of labeled [γ^{32}-P]-ATP, incubated at 30 °C for various times, and terminated by the addition of 1/5 volume of 5x SDS-PAGE sample buffer (Cao *et al*. 2003). The labeled protein was separated from free ATP by 10% SDS-PAGE. The gel was dried on Whatman paper for 60–90 min at 80 °C before being exposed to X-ray film and/or Phosphorimager (Molecular Dynamics, Sunnyvale, CA).

The GSK3β kinase assay was performed for various times at 30 °C in 20 μl containing ~10 pmol protein, 1 unit enzyme, and 10 μM ATP (200 pmol, ~1 μCi) in GSK3β buffer (25 mM Tris-HCl, pH 7.4, 10 mM $MgCl_2$, 1 mM DTT, 0.1 mM EGTA, and 0.1 mM Na_3VO_4). The PKA kinase assay was performed for various times at 30 °C in 20 μl containing ~10 pmol

protein, 1 μl enzyme, and 10 μM ATP (200 pmol, ~1 μCi) in PKA buffer (40 mM MES, pH 6.0, 1 mM EGTA, 10 mM $MgCl_2$). The PKBα (Akt1) kinase assay was performed for various times at 30 °C in 20 μl containing ~10 pmol protein, 12 unit (1 μl) enzyme, and 10 μM ATP (200 pmol, ~1.5 μCi) in PKBα buffer (20 mM Tris-HCl, pH 7.5, 25 mM β-glycerol phosphate, 5 mM EGTA, 1 mM sodium orthovanadate, 1 mM DTT, 20 mM $MgCl_2$). The PKCμ kinase assay was performed for various times at 30 °C in 20 μl containing ~10 pmol protein, 0.1 μl enzyme, and 10 μM ATP (200 pmol, ~2.5 μCi) in PKCμ buffer (40 mM Hepes, pH 7.4, 1 mM EGTA, 10 mM $MgCl_2$, 1 mM $CaCl_2$). The time course of GSK3β phosphorylation was done for 0–80 min at 30 °C in 150 μl containing ~100 pmol protein, 7.5 unit enzyme, and 10 μM ATP (1500 pmol, ~8 μCi) in GSK3β buffer and the phosphorylation product was analyzed at each time point using 10 μl reaction mixture (~6.5 pmol protein, 0.5 unit enzyme, 100 pmol ATP). The kinetics of GSK3β phosphorylation were done for 15 min at 30 °C in 10 μl containing 0–20 pmol protein, 0.35 unit enzyme, and 10 μM ATP (100 pmol, ~1 μCi) in GSK3β buffer. P42, p38, and JNK phosphorylation assays were previously described (Cao *et al*. 2003). The Cdc2 kinase assay was performed for various times at 30 °C in 20 μl containing ~10 pmol protein, 0.1 μl enzyme, and 100 μM ATP (200 pmol, ~2.5 μCi) in a buffer (50 mM Tris-HCl, pH 7.5, 1 mM EGTA, 10 mM $MgCl_2$, 2 mM DTT).

3. RESULTS

3.1. Prediction of Putative Protein Kinases for the Phosphorylation of TTP

Human TTP protein sequence was imported into a motif scanning program (http://scansite.mit.edu) (Obenauer *et al*. 2003; Yaffe *et al*. 2001) for predicting potential phosphorylation sites and their putative protein kinases. Motif scanning results suggested that human TTP was a potential substrate for a variety of protein kinases (Table 1), including: 1) ERK1 [The consensus sequence motif is $Px_{1-4}(S/T)P$] (Pearson *et al*. 2001) sites at S^{41} [$P_{WSL}SP$ in hTTP], S^{88} [$P_{EL}SP$ in hTTP], S^{218} [$P_{SS}SP$ in hTTP], and S^{228} [$P_{L}SP$ in hTTP]; 2) p38 MAPK [The consensus sequence motif is Px(S/T)P] (Hawkins *et al*. 2000) sites at S^{93} [$P_{T}SP$ in hTTP] and T^{238} [$P_{G}TP$ in hTTP]; 3) a Cdc2 [The consensus sequence motif is x(S/T)Px(R/K)] (Blom *et al*. 2004) site at T^{238} [$_{G}TP_{LA}R$ in hTTP]; 4) a GSK3 [The consensus sequence motif is (S/T)xxx(S/T)] (Frame and Cohen 2001) site at S^{218} [$S_{PSS}S$ in hTTP]; 5) PKA [The consensus sequence motif is $Rx_{1-2}S/Tx$] (Blom *et al*. 2004) sites at S^{197} [$R_{T}SP$ in hTTP] and T^{257} [$R_{A}TP$ in hTTP]; 6) a PKCμ [The consensus sequence motif is LVxxxS] (Nishikawa *et al*. 1997) site at S^{66} [$LV_{EGR}S$ in hTTP]; and 7) a PKC [The consensus sequence motif is x(S/T)x(R/K)] (Blom *et al*. 2004) site at S^{252} [$_{P}S_{C}R$ in hTTP]. Some of the predicted phosphorylation sites are identified by mass spectrometry (Table 1) (Cao *et al*. 2006).

3.2. Scanning Protein Kinases by Electrophoretic Mobility Shift Assay

Non-fusion TTP is difficult to be purified from expressed *E. coli* due to insolubility of the protein (Cao *et al*. 2003). Therefore, recombinant MBP-TTP was selected for our current experiments. MBP-mTTP was incubated with various protein kinases. The results showed that only p42 and p38 MAPK reactions resulted in visible shift of the protein on SDS-PAGE (Fig. 1). The MBP-TTP bands were positively identified by immunoblotting using antibodies against recombinant MBP-TTP proteins (data not shown). No apparent mobility shift of TTP was detected by in vitro phosphorylation with GAS3b, PKA, PKB, PKC, Cdc2, or JNK kinases (Fig. 1).

3.3. Phosphorylation of Recombinant TTP by Glycogen Synthase Kinase-3β *in Vitro*

The increased size of MBP-mTTP on SDS-PAGE suggest that the induced TTP was phosphorylated by p42 and p38 MAPK, but the mobility shift assay is not always definitive and other kinases might phosphorylate TTP but not cause visible changes in gel mobility. We therefore confirmed the test by use of an in vitro phosphorylation assay.

We first investigated if TTP is a substrate for GSK3β *in vitro*. MBP-TTP (both MBP-hTTP and MBP-mTTP) purified from *E. coli* by amylose resin affinity columns was used to investigate the phosphorylation of TTP. MBP-TTP but not MBP was phosphorylated by GSK3β (Fig. 2, lanes 5 and 7), while MBP-TTP was not auto-phosphorylated (lanes 4 and 6). There was, however, apparent auto-phosphorylation of GSK3β protein (lanes 1, 3, 5 and 7). These results suggest that TTP is specifically phosphorylated by GSKβ *in vitro*.

When 1 μM MBP-mTTP was used as a substrate, half-maximal phosphorylation was achieved within 30 min and phosphorylation reached maximal at about 60 min at 30 °C with GSK3β (Fig. 3A). Increasing concentrations of MBP-mTTP showed half-maximal phosphorylation at approximately 0.8 μM in the linear portion of the time course analysis at 15 min (Fig. 3B).

3.4. Phosphorylation of Recombinant TTP by Protein Kinases A, B, and C *in Vitro*

Motif scanning (http://scansite.mit.edu) also predicted that TTP was potentially phosphorylated by PKA and PKCμ (Table 1). MBP-mTTP could be phosphorylated *in vitro* by PKA (Fig. 4A), PKBα (Fig. 4B), and PKCμ (Fig. 4C), while MBP was not phosphorylated by these same protein kinases (data not shown).

4. DISCUSSION

It has been known for many years that TTP is highly phosphorylated in intact cells, and that this modification can affect the electrophoretic mobility of the protein (Blackshear 2002). Mass spectrometry and site-directed mutagenesis have identified a number of phosphorylation sites in human TTP, including S^{66}, S^{88}, T^{92}, S^{169}, S^{186}, S^{197}, S^{218}, S^{228}, S^{276}, and S^{296}, and 29 other potential phosphorylation sites (Cao *et al*. 2006; Cao and Deterding 2007). To better understand TTP phosphorylation reactions, we investigated the protein kinases that may phosphorylate TTP using motif scanning, electrophoretic mobility shift assay, and *in vitro* phosphorylation assay. Our results demonstrate that TTP is a substrate for various protein kinases *in vitro*.

In this report, we have shown by motif scanning program that human TTP is potentially phosphorylated at multiple sites by a variety of protein kinases, including: 1) ERK1; 2) p38 MAPK; 3) Cdc2; 4) GSK3; 5) PKA; 6) PKCμ; and 7) PKC. Some of the predicted phosphorylation sites are experimentally identified by mass spectrometry (Table 1) (Cao *et al*. 2006). Electrophoretic mobility shift assay showed that p42 and p38 MAP kinases caused slower mobility of recombinant TTP on SDS-PAGE after in vitro phosphorylation reactions than those untreated TTP. Previous studies have demonstrated that decreases in the electrophoretic mobility of proteins on SDS-PAGE can serve as an indicator of stoichiometric phosphorylation in some proteins (Rangel-Aldao *et al*. 1979;Rodriguez *et al*. 2003). Similar to these reports, *in vitro* phosphorylation of TTP using protein kinases results in a decrease in mobility on SDS-PAGE (Cao 2004). On the other hand, dephosphorylation of TTP from mammalian cells significantly increases its electrophoretic mobility on SDS-PAGE (Cao 2004;Cao *et al.* 2004;Carballo *et al.* 2001;Taylor *et al.* 1995). These results support the notion that p42 and p38 MAP kinases stoichiometrically phosphorylate recombinant TTP *in vitro* (Cao *et al*. 2003;Cao 2004).

Recombinant TTP was further shown to be phosphorylated by GSK3β, PKA, PKBα (Akt1), and PKCμ under *in vitro* conditions. Recombinant MBP-TTP was used in this study because non-fusion TTP protein is difficult to be purified from expressed *E. coli* due to insolubility and MBP fusion increases the solubility of its fusion partners (Cao *et al.* 2003; Kapust and Waugh 1999). Because MBP forms separate domains from its fusion partner, MBP may not interfere with its fusion partner's conformation (Liu *et al*. 2001). Therefore, recombinant MBP could be an appropriate negative control for the phosphorylation reactions of recombinant MBP-TTP.

Under this assumption and since MBP alone was not phosphorylated by the protein kinases we tested, we conclude that TTP is phosphorylated by GSK3β, PKA, PKBα (Akt1), and PKCμ. These results extend previous observations that recombinant TTP is phosphorylated *in vivo* and *in vitro* by a number of protein kinases, including p42 MAP kinase, p38 MAP kinase, JNK, and MK2 (Chrestensen *et al*. 2004; Mahtani *et al*. 2001; Ming *et al*. 2001; Stoecklin *et al*. 2004). Since GSK3β and PKBα (Akt1) act downstream in the insulin signal transduction pathway (Frame and Cohen 2001), our results suggest that TTP phosphorylation may be similarly affected by insulin induced protein kinases.

It is noted that recombinant MBP-TTP without phosphorylation and those treated with several protein kinases appeared as sharp bands on SDS-PAGE after silver staining (Fig. 1). However, those MBP-TTP molecules treated with GSK3β, PKA, PKBα, and PKCμunder *in vitro* phosphorylation conditions resulted in much broader and more diffuse bands on autoradiograms (Figs. 2–4). The reason for this discrepancy is probably due to the much more sensitive detection method with autoradiography than that with silver staining.

A major task for future studies is to understand the phosphorylation reactions on TTP function (s). A number of investigations have studied the effects of phosphorylation on TTP function (s). MK2 phosphorylation on TTP stability and function is well documented in recent publications. MK2 was shown to be essential for the stabilization of TTP mRNA. MK2 phosphorylation leads to increased TTP protein stability but reduced ARE affinity (Hitti *et al*. 2006). Mouse TTP is phosphorylated by MK2 at S^{52} (corresponding to S^{60} in human TTP) and S^{178} (corresponding to S^{186} in human TTP) *in vivo* and *in vitro* (Chrestensen *et al*. 2004). The regulation of both subcellular localization and protein stability of mouse TTP is dependent on MK2 and on the integrity of S^{52} and S^{178} (Brook *et al*. 2006). Phosphorylation of mouse TTP at S^{178} increases the relative ratio of TTP protein in the cytoplasm (Johnson *et al*. 2002). Recent experiments have shown that mutation of S^{52} to A^{52} in mouse TTP weakly reduces the assembly of TTP-14-3-3, whereas mutation of S^{178} to A^{178} and of $S^{52/178}$ to $A^{52/178}$ substantially reduces the association of mouse TTP with 14-3-3 (Sun *et al*. 2007). Finally, global dephosphorylation of mouse TTP by calf intestine alkaline phosphatase (CIAP) prevents TTP from binding to 14-3-3 proteins (Johnson *et al*. 2002). However, little information is available about the impact of phosphorylation by other protein kinases on TTP functions.

The relationship between TTP phosphorylation and its mRNA binding activity requires more studies in the future. It is well known that phosphorylation of TTP decreases its mRNA binding activity. TTP expressed in human 293 cells and then dephosphorylated by CIAP is able to bind more tightly to an ARE probe than native, phosphorylated TTP (Carballo *et al*. 2001). TTP purified from overexpressed *E. coli* exhibits approximately 2-fold greater affinity for the TNF mRNA ARE than the protein purified from transfected human 293 cells (Cao 2004). However, mRNA binding activity of TTP is not apparently affected by individual phosphorylation with p42/ERK2, p38, or JNK MAP kinases (Cao *et al*. 2003), or by MK2 (Worthington *et al*. 2002) under *in vitro* conditions. Understanding the importance of TTP phosphorylation on its function(s) will require further studies to confirm the types of protein kinases and their TTP targeting sites *in vivo*.

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Abbreviations

TTP tristetraprolin

hTTP human TTP

mTTP mouse TTP

ARE AU-rich element

Cdc2 cyclin-dependent kinase 2

CIAP calf intestine alkaline phosphatase

GSK3b glycogen synthase kinase 3B

JNK c-Jun-N-terminal kinase

LPS lipopolysaccharide

MAPK mitogen-activated protein kinase

MBP maltose binding protein

MK2 MAP kinase-activated protein kinase 2

PK protein kinase

SDS-PAGE sodium dodecyl sulfate-polyacrylamide gel electrophoresis

TNF tumor necrosis factor alpha

ZFP zinc finger protein

ZFP36L ZFP36-like

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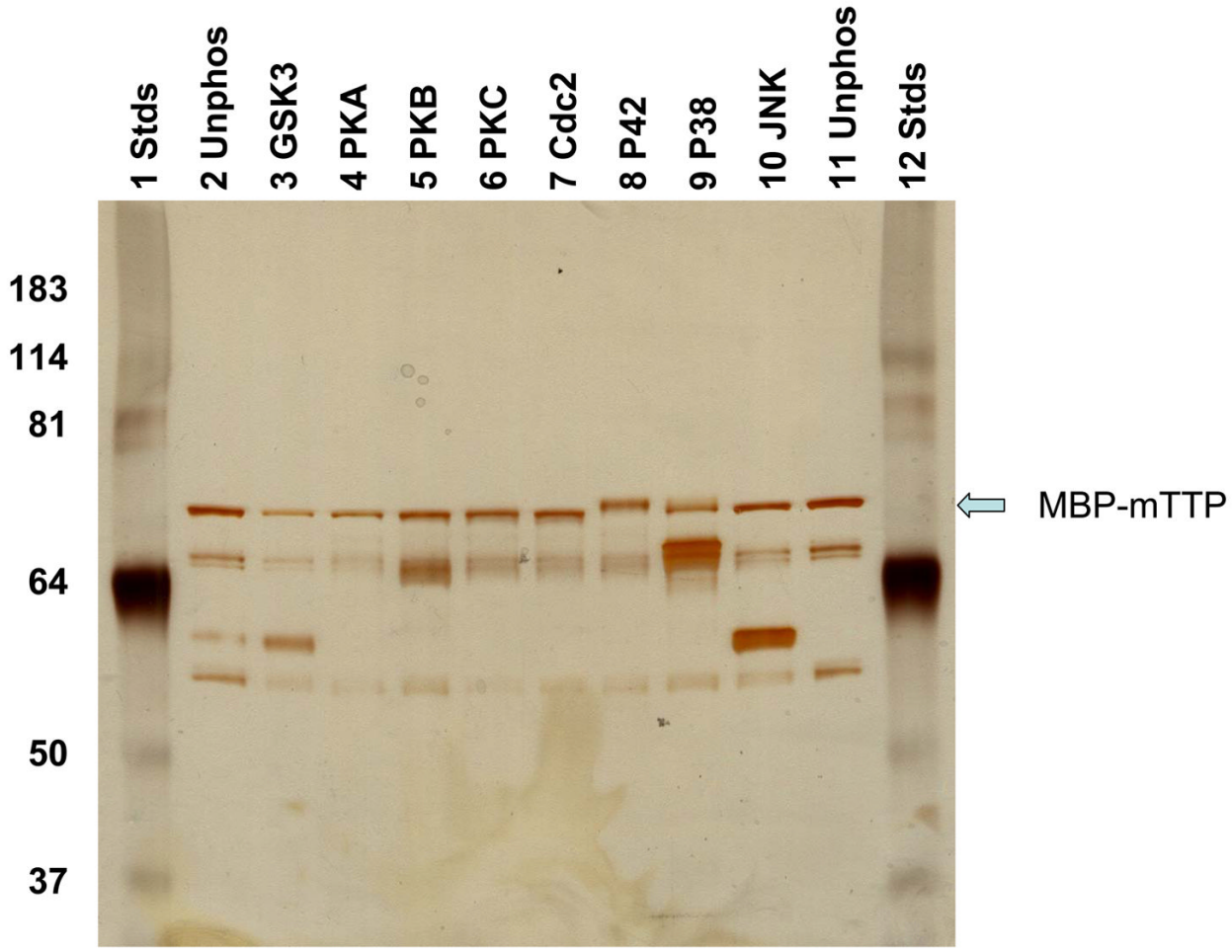

Fig. 1.

Effects of in vitro phosphorylation on the electrophoretic mobility of TTP. MBP-mTTP was phosphorylated in vitro. The reaction mixtures were separated by SDS-PAGE. The proteins were visualized by staining the gel with silver reagent.

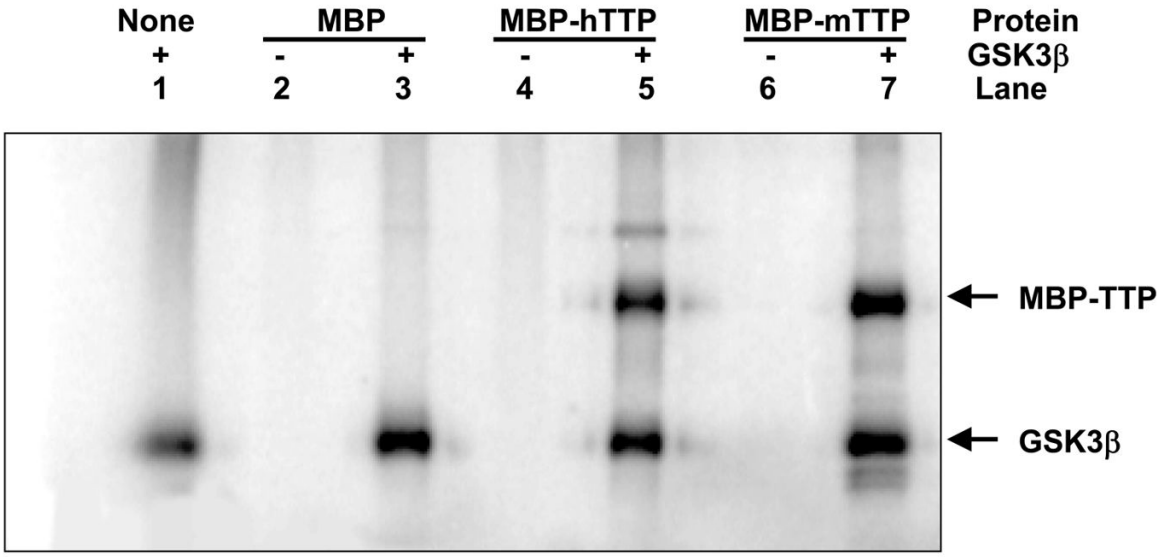

Fig. 2.

Phosphorylation of TTP by GSK3β *in vitro*. MBP-mTTP and MBP-hTTP (1 μg) were purified by amylose resin column and used as a substrate for GSK3β (1 unit). The GSK3β kinase assay was performed for 30 min at 30 °C in 20 μl containing ~10 pmol protein, 1 unit enzyme, and 10 μM ATP (200 pmol, ~1 μCi). The labeled protein was separated from free ATP by 10% SDS-PAGE. The gel was dried and exposed to X-ray film.

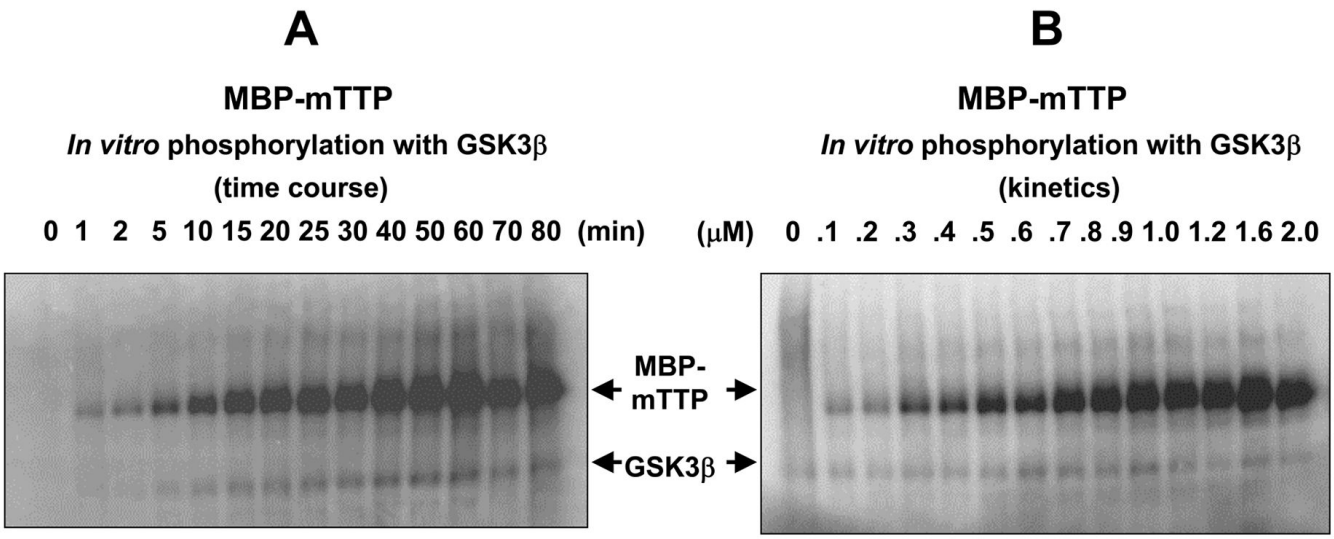

Fig. 3.

Time-course and kinetics of phosphorylation of TTP by GSK3β *in vitro*. (A) Time course of the phosphorylation reactions. The reactions were performed at 30 °C for various times using 1 pmol MBP-mTTP as the substrate in 150 μl (10 μl per time point). (B) Substrate concentration dependence of the phosphorylation reactions. The reactions were performed at 30 °C for 15 min using various amounts of MBP-mTTP in 20 μl. The labeled protein was separated from free ATP by 10% SDS-PAGE. The gel was dried and exposed to Phosphorimager.

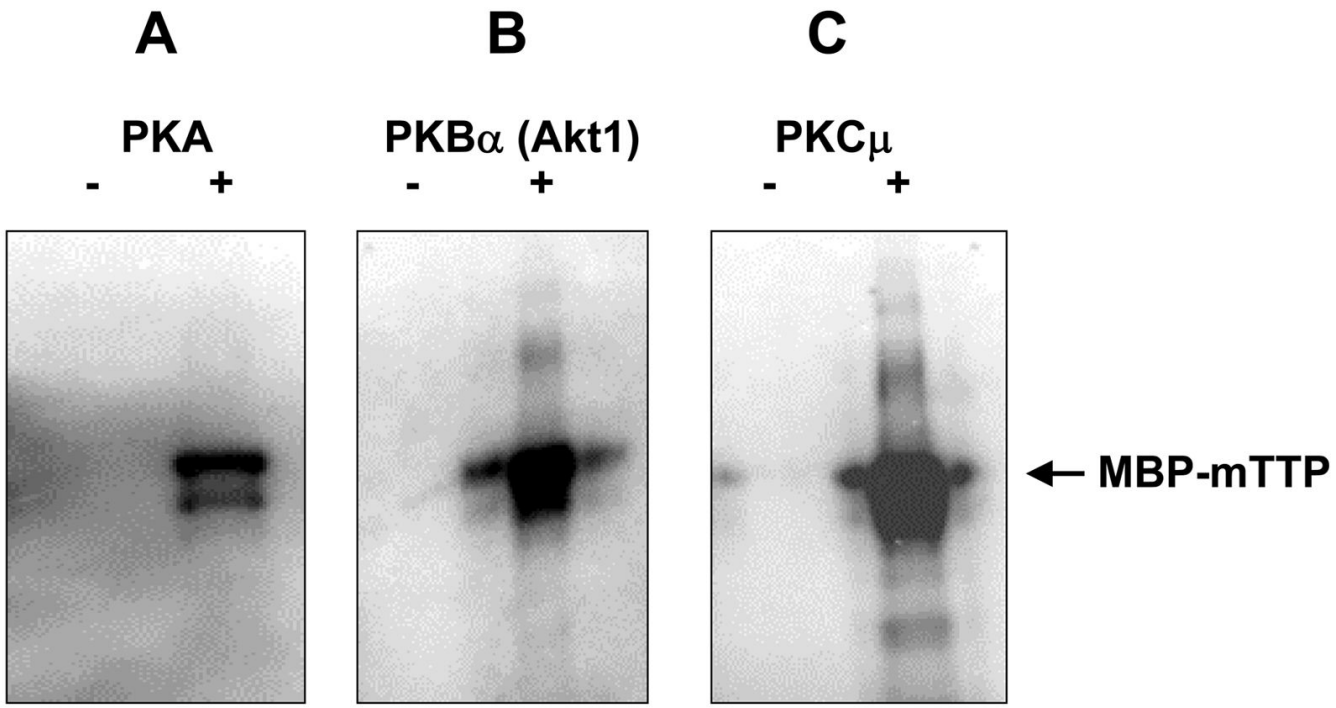

Fig. 4.

Phosphorylation of TTP by PKA, PKBα (Akt1), and PKCμ *in vitro*. MBP-mTTP (1 μg) was purified by amylose resin column and used as a substrate for the protein kinase assays in 20 μl for 30 min at 30 °C. The labeled protein was separated from free ATP by 10% SDS-PAGE. The gel was dried and exposed to X-ray film. (A) The PKA kinase assay contained ~10 pmol protein, 1 μl enzyme, and 10 μM ATP. (B) The PKBα (Akt1) kinase assay contained ~10 pmol protein, 12 unit (1 μl) enzyme, and 10 μM ATP. (C) The PKCμ kinase assay contained ~10 pmol protein, 0.1 μl enzyme, and 10 μM ATP.

Table 1

TTP is predicted to be phosphorylated at multiple sites by several protein kinases

Predicted protein kinase	Predicted phosphorylaton site position	Predicted peptide sequence with phosphorylaton site in bold letter	Phosphorylation sites observed by mass spectrometric analysis (Cao *et al*. 2006)
ERK1	**41**	SSGPWSL**S**PSDSSPS	S41
	88	PRLGPEL**S**PSPTSPT	S88
	214	SLSSSSF**S**PSSSPPP	
	218	SSFSPSS**S**PPPPGDL	S218
	228	PPGDLPL**S**PSAFSAA	S228
P38	**93**	ELSPSPT**S**PTATSTT	S93
	238	AFSAAPG**T**PLARRDP	T238
GSK3	**35**	SSPGWGS**S**GPWSLSP	
	39	WGSSGPW**S**LSPSDSS	
	52	SSPSGVT**S**RLPGRST	
	214	SLSSSSF**S**PSSSPPP	
	218	SSFSPSS**S**PPPPGDL	S218
PKA	**257**	CPSCRRA**T**PISVWGP	T257
	197	LPSGRRT**S**PPPPGLA	S197
PKB/Akt1	**60**	RLPGRST**S**LVEGRSC	
	113	TELCRTF**S**ESGRCRY	
PKC-a/b/g	**252**	PTPVCCP**S**CRRATPI	S252
-epsilon	**111**	YKTELCR**T**FSESGRC	T111
-mu	**66**	TSLVEGR**S**CGWVPPP	S66
-zeta	**144**	NRHPKYK**T**ELCHKFY	
Cdc2/Cdk5	**238**	AFSAAPG**T**PLARRDP	T238
	100	SPTATST**T**PSRYKTE	