THE JOURNAL OF BIOLOGICAL CHEMISTRY
Vol. 277, No. 20, Issue of May 17, pp. 18029–18036, 2002
© 2002 by The American Society for Biochemistry and Molecular Biology, Inc.
Printed in U.S.A.

Cytoplasmic Localization of Tristetraprolin Involves 14-3-3-dependent and -independent Mechanisms*

Received for publication, October 31, 2001, and in revised form, January 30, 2002
Published, JBC Papers in Press, March 8, 2002, DOI 10.1074/jbc.M110465200

Barbra A. Johnson‡, Justine R. Stehn§, Michael B. Yaffe§, and T. Keith Blackwell‡¶

From the ‡Center for Blood Research and Department of Pathology, Harvard Medical School, Boston, Massachusetts 02115 and the §Center for Cancer Research, Department of Biology, Massachusetts Institute of Technology, Cambridge, Massachusetts 02139

The immediate early gene *tristetraprolin* (*TTP*) is induced transiently in many cell types by numerous extracellular stimuli. *TTP* encodes a zinc finger protein that can bind and destabilize mRNAs that encode tumor necrosis factor-alpha (TNFα) and other cytokines. We hypothesize that TTP also has a broader role in growth factor-responsive pathways. In support of this model, we have previously determined that TTP induces apoptosis through the mitochondrial pathway, analogously to certain oncogenes and other immediate-early genes, and that TTP sensitizes cells to the pro-apoptotic signals of TNFα. In this study, we show that TTP and the related proteins TIS11b and TIS11d bind specifically to 14-3-3 proteins and that individual 14-3-3 isoforms preferentially bind to different phosphorylated TTP species. 14-3-3 binding does not appear to inhibit or promote induction of apoptosis by TTP but is one of multiple mechanisms that localize TTP to the cytoplasm. Our results provide the first example of 14-3-3 interacting functionally with an RNA binding protein and binding *in vivo* to a Type II 14-3-3 binding site. They also suggest that 14-3-3 binding is part of a complex network of stimuli and interactions that regulate TTP function.

The immediate-early protein tristetraprolin (TTP[1]; also Nup475 and TIS11) is expressed transiently during responses to many extracellular stimuli, including TNFα (1). TTP and the related proteins TIS11b and TIS11d (TTP/TIS11 proteins) consist of two conserved Cys-X_8-Cys-X_5-Cys-X_3-His (CCCH) zinc fingers, along with similarly sized but divergent N- and C-terminal regions. Several lines of evidence indicate that TTP binds and destabilizes cytokine mRNAs, through binding to an AU-rich element (ARE) located within their 3′-untranslated regions. This ARE is targeted by conserved signaling pathways, which regulate the localization, stability, and translation of these mRNAs (2–9). *TTP*−/− mice develop a widespread inflammatory syndrome that is mediated largely by TNFα and is associated with elevated levels and half-life of the TNFα mRNA (10–13). A protein complex that contains TTP binds to the TNFα ARE (14) and in transfection assays each TTP/TIS11 protein can bind and destabilize cytokine mRNAs that have related AREs (1, 12, 15–17). These findings suggest that TTP limits expression of TNFα and other cytokines through a feedback mechanism, by destabilizing their mRNAs, and that this is a shared function of TTP/TIS11 proteins. Some other CCCH zinc finger proteins appear to regulate translation of their target genes (18–21), suggesting that many members of this protein family may regulate specific genes post-transcriptionally.

It appears likely that TTP has additional functions and may play a broader role during responses to extracellular stimuli. *TTP* expression is induced rapidly and directly in numerous cultured cell types, by a wide variety of growth factors and mitogens (22–24). In mice, *TTP* is expressed in developing oocytes and regenerating small intestine and liver, in addition to hematopoietic tissues (10, 24–26). Like some other immediate early proteins, in certain contexts TTP is also expressed during induction of apoptosis (27–30). Consistent with the model that TTP/TIS11 proteins influence growth, survival, or apoptotic signals, we have determined that their constitutive expression at modest levels induces apoptosis through the mitochondrial pathway (31). We have also shown that TTP has diverged functionally from the other two TTP/TIS11 proteins, in that TTP alone dramatically sensitizes cells to the apoptotic stimulus of TNFα (31, 32). This last finding suggests that TTP could contribute to the cellular decision between activation or apoptosis in response to TNFα.

Although the isolated TTP zinc finger region can mediate its effects on TNFα mRNA stability in transfection assays (16), we have observed that the TTP zinc finger region is incapable of inducing apoptosis or of sensitizing cells to TNFα-induced apoptosis (32). Together with the zinc fingers, the TTP N- and C-terminal regions each contribute to induction of apoptosis, and the N-terminal region is specifically required to sensitize cells to TNFα (32). In addition, although the isolated TTP zinc finger region is localized predominantly to the nucleus, both the N- and C-terminal regions of TTP promote its localization to the cytoplasm (32). The importance of the TTP N- and C-terminal regions for these TTP activities makes it critical to identify proteins with which these TTP regions interact functionally.

In this study, we have determined that 14-3-3 proteins bind to the TTP C-terminal region sequence-specifically and in a phosphorylation-dependent manner. This interaction appears

* This work was supported by National Institutes of Health Grants CA84418 (to T. K. B.) and GM60594 (to M. B. Y.), by an Arthritis Foundation fellowship (to B. A. J.), and by a C. J. Martin fellowship from the National Health and Medical Research Council of Australia (to J. R. S.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

¶ To whom correspondence should be addressed: Center for Blood Research and Dept. of Pathology, Harvard Medical School, 200 Longwood Ave., Boston, MA 02115. Tel.: 617-278-3150; Fax: 617-278-3153; E-mail: blackwell@cbr.med.harvard.edu.

[1] The abbreviations used are: TTP, tristetraprolin; TNFα, tumor necrosis factor-alpha; ARE, AU-rich element; GFP, green fluorescence protein; GST, glutathione *S*-transferase; MAPK, mitogen-activated protein kinase; NES, nuclear export signal; NLS, nuclear localization sequence; X-gal, 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside; HA, hemagglutinin; nTTP, antiserum raised against the N terminus of TTP; cTTP, antiserum raised against the C terminus of TTP; DMEM, Dulbecco's modified Eagle's medium.

to be conserved among all three TTP/TIS11 proteins. Mutagenesis analysis has identified a specific site in the TTP C terminus that is important for 14-3-3 binding. Binding to 14-3-3 through this site does not appear to be required for the apoptotic effects of TTP but is critical for one of at least three mechanisms that localize TTP to the cytoplasm. Our findings suggest that interactions with 14-3-3 are involved in phosphorylation-mediated signals that may regulate TTP functions *in vivo*.

EXPERIMENTAL PROCEDURES

DNA Constructs—TTP and site-directed *TTP* mutants were introduced into the cytomegalovirus-based expression vector CS2+ (33) by PCR (*Pfu*, Stratagene), with a Kozak consensus and ATG added where appropriate. For two-hybrid analysis, *TTP*, and mutants indicated in Fig. 3*A* were cloned by PCR using *Pfu* (Stratagene) into pC98, a pC97 derivative (34). To remove its 5′-untranslated region, the 14-3-3η prey coding sequence was re-cloned by PCR. TTP and TTP deletion mutants each were fused to green fluorescence protein (GFP) at their N terminus by restriction cloning into C2eGFP (CLONTECH). Analogous GFP fusions of TIS11b and TIS11d were made by PCR cloning in C1eGFP and C2eGFP, respectively.

Cell Culture and Transfections—Transfections were carried out as described (31) using LipofectAMINE (Invitrogen), and 35-mm plates unless otherwise stated. DNA amounts were supplemented to 2 μg by addition of pBluescript. LipofectAMINE Plus and 1 μg of total DNA were used when higher efficiencies were desired. DNA amounts used for 10-cm plates for these two transfection methods were 10 and 5 μg, respectively. FuGENE (Roche Molecular Biochemicals) and 8 μg of total DNA were used to transfect 10-cm plates for Fig. 3*C*. Cell death was assayed by co-transfection of a β-galactosidase reporter and examination of cell morphology after X-gal staining 24 h later (31). Under a variety of conditions, numbers of apoptotic cells identified by this method were reproducibly comparable to those detected by scoring Hoechst-stained pyknotic nuclei (not shown) (31). For serum-dependent relocalization assays, cells were washed twice in Dulbecco's modified Eagle's medium (DMEM) prior to transfection, and DMEM without serum was added 3 h later. 26–28 h after transfection, cells were stimulated with 20% serum for the times stated before fixation and antibody staining.

Western Blotting, Antibody Production, and Immunofluorescence—Cells were lysed in 1% Triton X-100 (or Nonidet P-40), 50 mM Tris, pH 8, 150 mM NaCl, 1 mM $MgCl_2$, 1 mM dithiothreitol, 10% glycerol, Complete protease inhibitors (Roche Pharmaceuticals), 1 mM sodium vanadate, and 50 mM NaF (cell lysis buffer). Electrophoresis was performed on a minigel apparatus unless otherwise indicated. For Western blotting, 100 μg of protein was generally used per lane, unless otherwise stated. The following commercial monoclonal antibodies were used: Mouse anti-GFP (Zymed Laboratories Inc.), anti-14-3-3 (H-8, Santa Cruz Biotechnology), and anti-HA (12CA5, Roche Molecular Biochemicals). A polyclonal GFP antiserum was purchased from CLONTECH. The TTP peptide antibodies nTTP and cTTP (31, 32) were typically used at 1/5000 dilution. Immunofluorescence was carried out as described previously (31), using Cy3- or fluorescein isothiocyanate-conjugated secondaries (Jackson). 200–300 cells per slide were typically counted to determine TTP localization.

Protein-Protein Interaction Assays—Yeast two-hybrid screening and interaction assays were performed as described previously (34). For analysis of binding *in vitro* to glutathione *S*-transferase (GST)-14-3-3 fusion proteins, each 10 cm plate of cells was lysed in 100 μl of lysis buffer. 100 μg of protein was saved as an input control. 0.5–1 mg of lysate protein was incubated with 50 μl of a 1:1 bead slurry of GST-14-3-3 isoforms that had been synthesized as described (35, 36), in 1 ml of lysis buffer at 4 °C with rocking for 1 h, or overnight (Fig. 3*C*). Beads were washed three times with phosphate-buffered saline containing 1% Triton X-100, then boiled in SDS loading buffer for 10 min before electrophoresis and Western blotting. Incubations with calf intestinal phosphatase were performed at 1 unit/10 μg of total protein in lysis buffer (without phosphatase inhibitors) for 30 min at 30 °C. For immunoprecipitation, cells were lysed in 150 μl of lysis buffer, with 3 μl of cleared lysate saved as input. After addition of either 2 μg of rabbit HA antibody (Y-11, Santa Cruz Biotechnology) or 5 μl of cTTP, the remaining cleared lysate was rocked at 4 °C for 1 h. Samples were spun for 5 min to remove precipitates, then incubated for 1 h with 20 μl of protein A beads (Santa Cruz Biotechnology) that had been preincubated in bovine serum albumin. For monoclonal anti-HA immunoprecipitations, 10 μl of antibody-conjugated beads (F-7, Santa Cruz Biotechnology) was added directly to the lysate for 2 h. Beads were washed three times in lysis buffer and boiled in SDS loading buffer for 10 min before electrophoresis and Western blotting.

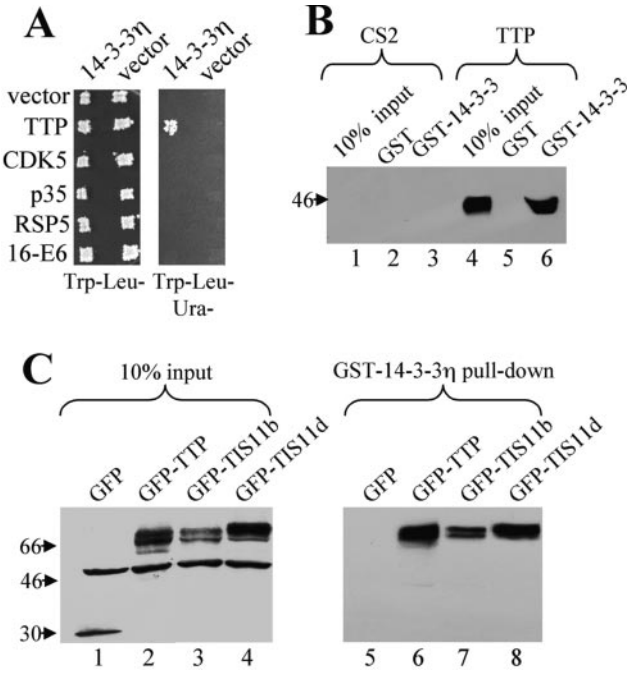


FIG. 1. **Interaction between TTP/TIS11 proteins and 14-3-3.** *A*, TTP interacts with 14-3-3η in the yeast two-hybrid assay, as indicated by growth on Ura$^-$ medium. The 14-3-3η prey binds to TTP but not to the unrelated control baits indicated to the *left*. *B*, TTP from 3T3 cell lysates binds specifically to GST-14-3-3. 3T3 cells were transfected with either 10 μg of CS2 vector control, or CS2TTP. Interaction assays were performed using 1 mg of cell lysate and either GST alone, or a mixture of different GST-14-3-3 isoforms (β, γ, σ, η, and ζ), then analyzed by Western blotting with the nTTP antiserum. The 46-kDa molecular mass marker is indicated to the *left*. *C*, Each TTP/TIS11 protein binds to GST-14-3-3η. Cells were transfected as in *B* with either GFP-expressing control plasmid or plasmids expressing TTP/TIS11 proteins to which GFP was fused at the N terminus. Proteins that bound to GST-14-3-3η (pull-down) were detected by Western blotting with a GFP antibody. GFP and GFP-TTP/TIS11 proteins migrate at roughly 30 and 66 kDa, respectively. A background band running above 46 kDa was present in all *input lanes*.

RESULTS

Specific, Phosphorylation-dependent Binding between TTP/TIS11 and 14-3-3 Proteins—To identify proteins that interact with TTP, we performed a yeast two-hybrid screen of a mouse mixed-stage embryonic cDNA library using a full-length TTP bait. After isolating a full-length 14-3-3η cDNA from this screen, we determined that 14-3-3η bound strongly to TTP but not to various control baits, suggesting that this interaction was specific (Fig. 1*A*). Mammals encode seven closely related 14-3-3 isoforms, each of ~31 kDa (37, 38). 14-3-3 proteins bind to phosphorylated proteins, generally as dimers, and often bind to more than one site in the same protein (35, 37–40). 14-3-3 binding influences the activity of several proteins, and localizes others to the cytoplasm in response to signals (37, 38). 14-3-3 proteins also act as signal-responsive anti-apoptotic factors by binding to phosphorylated forms of regulators such as the A20 protein, Forkhead-related transcription factors, the apoptosis-inducing kinase ASK1, and the BH3-only protein BAD. Because TTP is a phosphoprotein that induces apoptosis, and because its localization within the cell is influenced by extracellular stimuli and growth conditions (14, 31, 41, 42), we chose to investigate the specificity and functional significance of TTP-14-3-3 binding.

To test further the specificity of TTP-14-3-3 binding, we

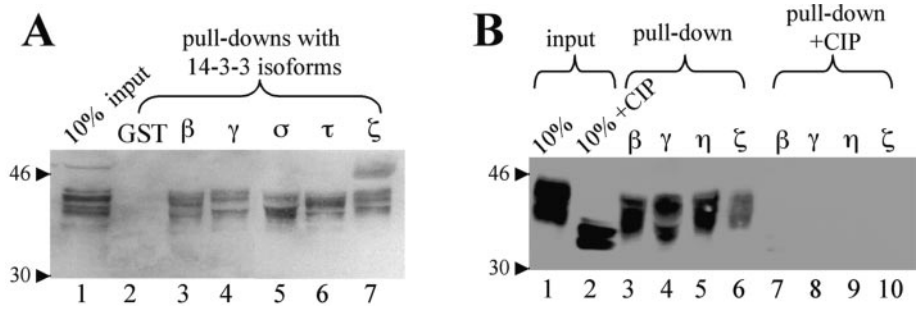


FIG. 2. **Phosphorylation-dependent interaction between TTP and 14-3-3 proteins.** *A*, TTP expressed in 3T3 cells binds specifically to GST-14-3-3 proteins. Lysates were prepared from cells transfected with CS2TTP as in Fig. 1*B*. Binding was assayed by incubation of 500 μg of protein lysate with GST alone, or with the indicated individual GST-fused 14-3-3 isoforms. After electrophoresis on a large-format gel to allow separation of TTP forms, Western blots were probed with the nTTP antiserum. *B*, phosphorylation dependence of TTP-14-3-3 binding. Parallel cell lysate samples (1 mg) were either treated with calf intestinal phosphatase, or incubated without enzyme, then assayed for TTP/14-3-3 binding as in *A*.

expressed TTP in 3T3 cells by transfection, and assayed whether it bound *in vitro* to a mixture of bacterially expressed 14-3-3 isoforms that were fused to GST. TTP bound robustly to the GST-14-3-3 protein mix but not to GST alone (Fig. 1*B*, *lanes 4–6*). Similarly, fusion proteins in which TTP, TIS11b, and TIS11d were each linked at their N terminus to GFP bound comparably well to a GST-14-3-3η protein *in vitro*, whereas GFP alone did not bind, suggesting that binding to 14-3-3 is characteristic of all three TTP/TIS11 proteins (Fig. 1*C*). TTP that was expressed in mammalian cells bound comparably well to fusion proteins that corresponded to each of the seven closely related mammalian 14-3-3 isoforms (Fig. 2, *A* and *B*; GST-14-3-3ϵ is not shown). These GST-linked 14-3-3 isoforms bound preferentially to distinct but overlapping sets of TTP species that appeared larger than the predicted TTP molecular mass of 34 kDa (Fig. 2*A*, *lanes 3–7*). As reported previously (14, 31, 43), treatment of transfected cell lysates with phosphatase converted these TTP species to a less heterogeneous group of faster-migrating forms, indicating that they represented different phosphorylated forms of TTP (Fig. 2*B*, *lanes 1* and *2*). Interaction with 14-3-3 proteins was completely abrogated by dephosphorylation of TTP in these cell lysates (Fig. 2*B*, *lanes 3–10*), indicating that binding of 14-3-3 proteins to TTP is phosphorylation-dependent.

Serine 178 of TTP Is Critical for High Affinity TTP-14-3-3 Binding—Phosphorylation-dependent 14-3-3 binding generally involves a conserved phosphoserine or phosphothreonine residue flanked by basic, aromatic, and aliphatic amino acids, along with additional serine and threonine residues (44). Combinatorial screening using phosphoserine-oriented peptide libraries have identified two optimal classes of 14-3-3 binding consensus motifs, in which an arginine or lysine residue is preferred at either the −3 (Type I) or −4 (Type II) position relative to the phosphorylated residue, with nearby amino acids also being important (35). Of these two consensus motifs, only Type I sites have been definitively identified previously as 14-3-3 targets *in vivo*. These screens have also indicated that individual 14-3-3 isoforms differ only subtly in their binding specificities.

To identify TTP sequences that are required for 14-3-3 binding, we first analyzed a series of TTP deletion mutants (Fig. 3*A*). In the yeast two-hybrid assay, the region of TTP located C-terminal to the zinc fingers was both necessary and sufficient for 14-3-3 binding (Fig. 3*B*). This conclusion was supported by analysis of binding *in vitro* between a mixture of GST-fused 14-3-3 proteins, and GFP-tagged TTP mutants that had been expressed in 293T cells (Fig. 3*C*). GFP-TTP and GFP-TTP(Zn-C) bound robustly to the GST-14-3-3 mix in this *in vitro* assay, even though they were expressed at the lowest relative levels (Fig. 3*C*, *lanes 2* and *6*). GFP-TTP(C) also bound significantly to the GST-14-3-3 mix, but GFP and the other TTP mutants did not (Fig. 3*C*, *lanes 1*, *3*, *4*, and *5*).

Within the TTP C-terminal region, we identified four sequence elements that match the previously identified 14-3-3 binding consensus motifs with varying degrees of success (Fig. 3*D*). Two of these elements are loosely conserved among all three TTP/TIS11 proteins (at TTP Ser-178 and Ser-206), and two are present only in TTP. To investigate whether these TTP elements are required for 14-3-3 binding, within each one we substituted alanine for the amino acid which 14-3-3 binding consensuses predict should be phosphorylated (Fig. 3*D*). Binding of TTP to a 14-3-3 protein mixture was significantly reduced only by the TTP S178A mutation (Fig. 3*E*, *lane 6*), which disrupts a predicted type II 14-3-3 binding site (35). Because 14-3-3 often binds to pairs of sites in the same protein (38), we similarly analyzed pairs of these Ala substitutions in all possible combinations, but none diminished binding compared with the corresponding single amino acid mutations (not shown).

To determine whether TTP and 14-3-3 proteins interact specifically *in vivo*, we assayed for binding between TTP mutants and 14-3-3 proteins that were co-expressed in the cell line 293T, which produces anti-apoptotic adenovirus products and is relatively resistant to TTP-induced apoptosis (31). Supporting the findings shown in Fig. 3 (*B* and *C*), in this assay both TTP and the TTP(Zn-C) mutant could be specifically co-immunoprecipitated along with HA-tagged 14-3-3β by the HA antibody (Fig. 4*A*, *lanes 1–4* and *7*). The S178A substitution significantly reduced co-immunoprecipitation of 14-3-3β and TTP, and eliminated detectable binding of HA-14-3-3β to TTP(Zn-C) (Fig. 4*A*, *lanes 4*, *5*, *7*, and *8*). In parallel transfections, TTP was comparably co-immunoprecipitated by other HA-tagged 14-3-3 isoforms (not shown). To assay for *in vivo* binding between 14-3-3 and other TTP/TIS11 proteins, we co-expressed HA-14-3-3β along with GFP-tagged TTP, TIS11b, and TIS11d. Like GFP-TTP, GFP-TIS11b and GFP-TIS11d each co-immunoprecipitated with HA-14-3-3β (Fig. 4*B*, *lanes 6–9*). Specific co-immunoprecipitation of GFP-TTP and HA-14-3-3β was reduced by the S178A mutation and eliminated by deletion of the TTP C-terminal region (Fig. 4*B*, *lanes 1–5*). We assayed for binding between transfected TTP and endogenous 14-3-3 by using a TTP antibody to co-immunoprecipitate 14-3-3 (Fig. 4*C*). When TTP was present, co-immunoprecipitation of endogenous 14-3-3 was elevated significantly over background (Fig. 4*C*, *lanes 1–3*). This binding was abrogated by the S178A mutation (Fig. 4*C*, *lane 4*), indicating that Ser-178 is important for binding to endogenous 14-3-3 proteins.

TTP Does Not Cause Apoptosis through 14-3-3 Sequestration—14-3-3 proteins inhibit apoptosis by binding to various

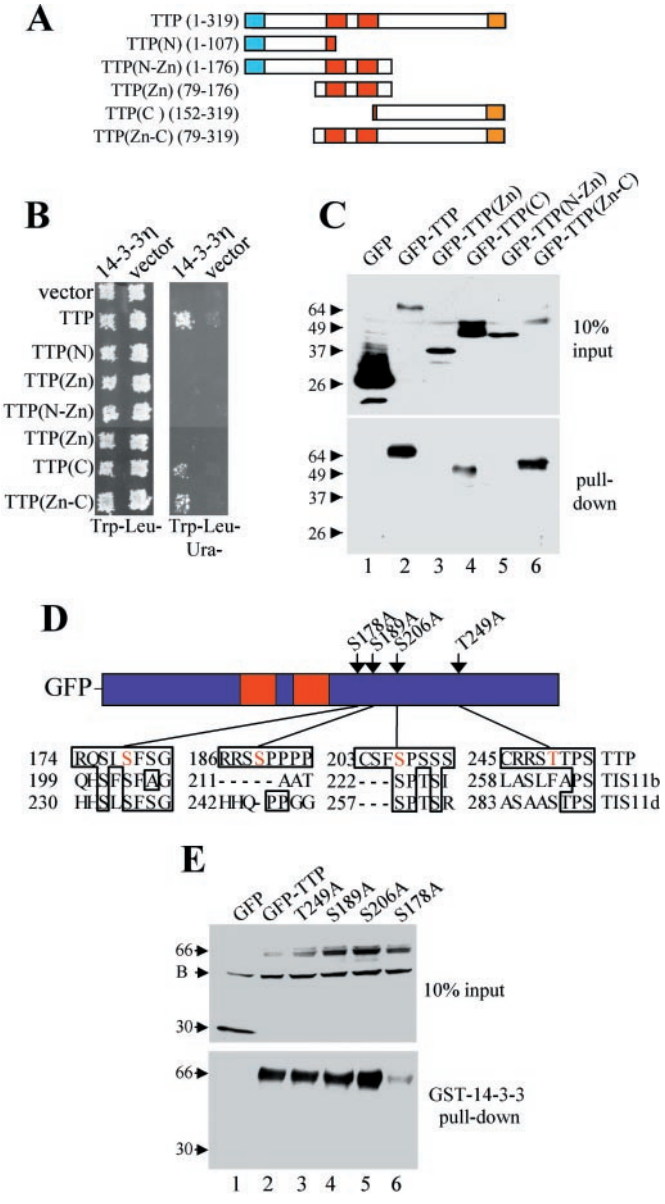


FIG. 3. **Sequence specificity of TTP-14-3-3 binding.** *A*, TTP deletion mutants. *Red boxes* indicate the CCCH zinc fingers, *blue* and *orange boxes* represent the 23 and 21 amino acids at the TTP N and C termini, respectively, against which peptide antibodies were raised (nTTP and cTTP). Amino acids present in each construct are indicated in *parentheses*. *B*, yeast two-hybrid analysis of binding between 14-3-3η and TTP deletion mutants (in *A*), performed as in Fig. 1*A*. *C*, binding of 14-3-3 proteins to the TTP C-terminal region. GFP-fused TTP deletion mutants were expressed in 293T cells by transfection, then assayed for binding to a mixture of GST-fused 14-3-3 isoforms (β, γ, σ, and ζ) as in Fig. 1*B*. Western blots were probed with a polyclonal GFP antiserum. In the input gel, the mobility of the predominant GFP-TTP(Zn-C) species is similar to that of a weaker background band. The relative expression levels of these GFP-fused proteins in transfected cells were confirmed by fluorescence microscopy (not shown). *D*, diagram of serine- or threonine-to-alanine point mutants generated in the C-terminal region of TTP. Predicted possible 14-3-3 binding sites in TTP are indicated, with mutated amino acids shown in *red*. Conserved residues identified by Clustal alignments of TTP, TIS11b, and TIS11d are *boxed*. *E*, reduced binding of TTP S178A to 14-3-3. Binding of GFP-TTP point mutants to a mixture of GST-14-3-3 isoforms (β, γ, σ, and η) was assayed as in Fig. 1*B*, using 1 mg of protein from transfected cells expressing either GFP or GFP-TTP fusions containing the specified point mutations.

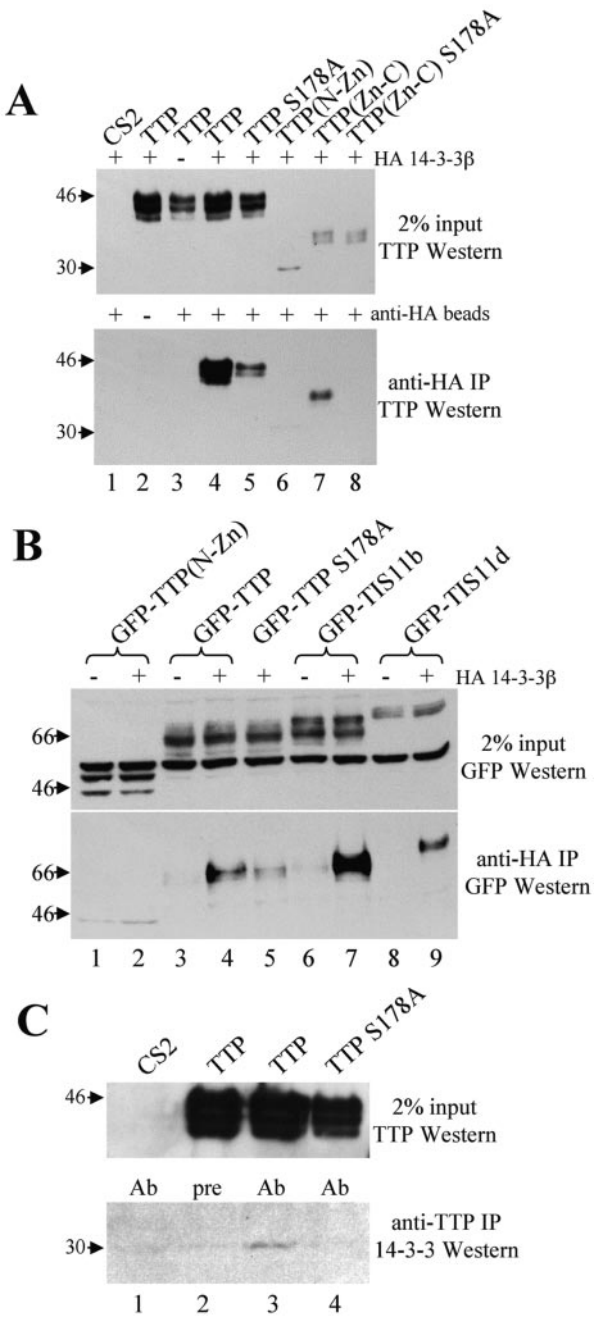


FIG. 4. **Co-immunoprecipitation of TTP/TIS11 and 14-3-3 proteins from mammalian cell lysates.** *A*, interaction between TTP and HA-tagged 14-3-3β. 293T cells were transfected with 3.5 μg of the indicated CS2TTP construct or CS2 vector, 1 μg of HA vector, or HA14-3-3β expression construct, and 0.5 μg of Bcl-2 expression vector to minimize apoptosis. Immunoprecipitation was performed using an anti-HA antibody. TTP was detected in input and co-immunoprecipitated samples by Western blotting with a mixture of nTTP and cTTP antisera. *B*, co-immunoprecipitation of GFP-tagged TTP/TIS11 proteins and HA-tagged 14-3-3. Transfections were carried out in as in *A*. Lysates were immunoprecipitated with anti-HA rabbit polyclonal antibody, then TTP/TIS11 proteins were detected by Western blotting with a GFP antibody. A background band is labeled *B*. *C*, co-immunoprecipitation of transfected TTP and endogenous 14-3-3. 293T cells were transfected as above with 4.5 μg of CS2 empty vector or TTP expression constructs and 0.5 μg of Bcl-2 expression vector. Lysates were immunoprecipitated with cTTP antiserum. Input 14-3-3 (not shown), input TTP, and co-immunoprecipitated 14-3-3 were detected by Western blotting with cTTP or an antibody that detects multiple 14-3-3 isoforms.

pro-apoptotic proteins (37, 38), raising the question of whether TTP might cause apoptosis, in part, by titrating 14-3-3 proteins away from these anti-apoptotic interactions. To address this question, we first investigated whether the S178A substitution influenced the ability of TTP to induce apoptosis. TTP S178A induced apoptosis comparably to TTP over a range of input DNA concentrations (Fig. 5*B*), despite its significantly reduced

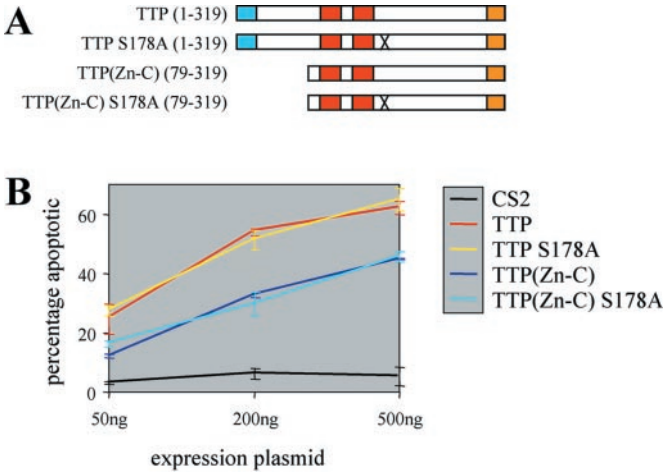


FIG. 5. **Effects of 14-3-3 binding on TTP-induced apoptosis.** *A*, TTP mutants analyzed, described as in Fig. 1*A*. *Crosses* denote the S178A substitution. *B*, binding of 14-3-3 to TTP at Ser-178 is not necessary for induction of apoptosis. 3T3 cells were transfected with the indicated amounts of TTP vector and 100 ng of β-gal plasmid. After 24 h, cells were X-gal-stained and the percentage of apoptotic blue cells was determined. *Bars* represent the mean of four wells; *error bars* represent the standard deviation.

binding to 14-3-3 proteins (Figs. 3*E* and 4). TTP and TTP S178A were also similarly capable of sensitizing cells to the apoptotic stimulus of TNFα (not shown). In addition, although TTP(Zn-C) S178A did not bind detectably to 14-3-3 (Fig. 4*A*), it induced apoptosis comparably to TTP(Zn-C) (Fig. 5, *A* and *B*). The S178A substitution did not influence the levels in which either TTP or TTP(Zn-C) was expressed in these transfections (Fig. 4*A*). Finally, simultaneous overexpression of 14-3-3 proteins did not attenuate TTP-induced apoptosis or differentially affect induction of apoptosis by either TTP or TTP S178A (not shown). These findings suggest that induction of apoptosis by TTP neither requires 14-3-3 binding at Ser-178 nor is mediated by TTP sequestering cellular pools of 14-3-3.

14-3-3 Binding Promotes Cytoplasmic Localization of TTP—Precedents set by various proteins suggest that 14-3-3 binding might influence how TTP is localized within the cell (37, 38). To test this model, we first investigated how 14-3-3 co-expression influences localization of TTP in HeLa cells. We used immunofluorescence to examine the subcellular localization of TTP that was expressed by transfection and scored cells according to whether TTP was predominantly cytoplasmic, generalized, or predominantly nuclear (Fig. 6*A*). TTP was present primarily in the cytoplasm only infrequently in HeLa cells (Fig. 6, *B* and *C*), making nuclear-to-cytoplasmic changes in its localization readily detectable. Co-expression of HA-14-3-3β increased the proportion of HeLa cells in which TTP was predominantly cytoplasmic 6-fold (to 18%, Fig. 6, *B* and *C*). In contrast, 14-3-3β expression did not significantly influence the localization of TTP S178A (Fig. 6, *B* and *C*), suggesting that direct binding was required for 14-3-3β to promote cytoplasmic localization of TTP.

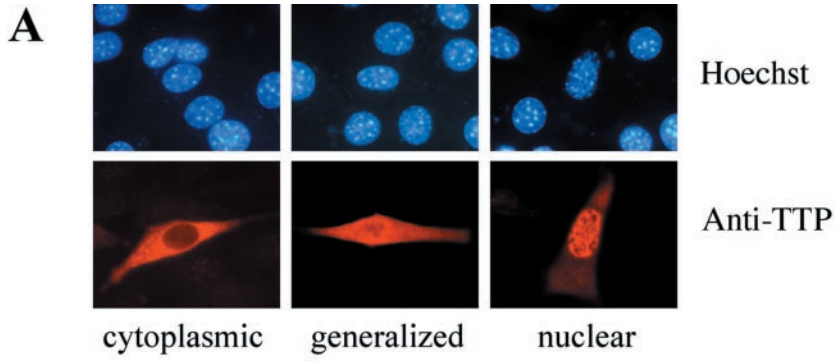
14-3-3 binding also enhanced localization of TTP to the cytoplasm in 3T3 cells. The proportion of transfected 3T3 cells in which TTP was predominantly cytoplasmic was increased significantly by co-expression of HA-14-3-3β (16–45%, Fig. 6*D*). In contrast, HA-14-3-3β did not increase cytoplasmic localization of TTP S178A (Fig. 6*D*). Significantly, TTP S178A was also less likely than TTP to be present primarily in the cytoplasm in these cells (1% *versus* 16% cytoplasmic, Fig. 6*D*), implying that binding by endogenous 14-3-3 proteins promotes cytoplasmic localization of TTP. As reported elsewhere, deletion of the TTP N-terminal region alone (TTP(Zn-C)) also significantly decreased the proportion of cells in which TTP was detected in the cytoplasm (to 15% generalized, Fig. 6*D*). The simultaneous presence of the S178A mutation (TTP(Zn-C) S178A) resulted in TTP being almost completely excluded from the cytoplasm (95% nuclear, Fig. 6*D*). Finally, 14-3-3 co-expression enhanced the cytoplasmic localization of TTP(Zn-C) (to 32% generalized) but not of TTP(Zn-C) S178A (Fig. 6*D*). Together, our findings suggest that direct and specific binding by 14-3-3 proteins at Ser-178 promotes localization of TTP to the cytoplasm.

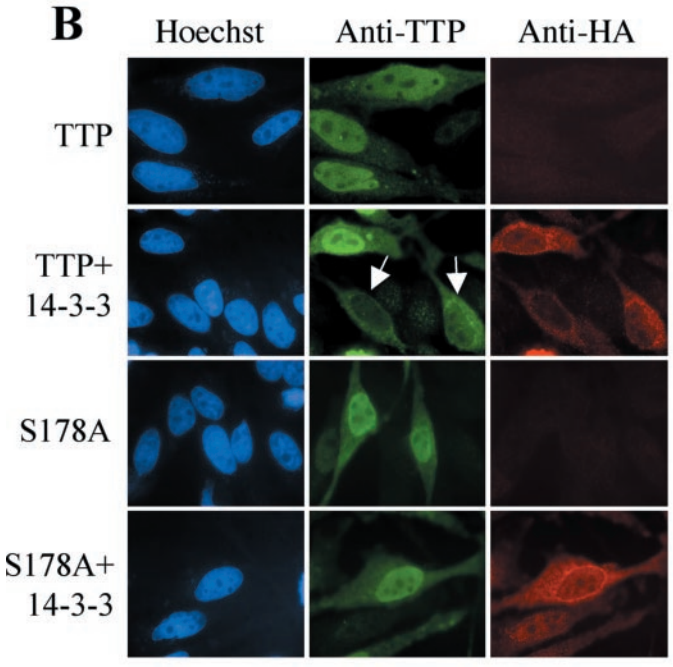
In quiescent cells, a significant fraction of the total TTP protein is present in the nucleus (41). This TTP fraction is largely relocalized to the cytoplasm after serum stimulation, which also increases TTP phosphorylation (41, 42). We investigated whether 14-3-3 binding through Ser-178 is required for this serum-dependent relocalization of TTP. TTP was present in both the cytoplasm and nucleus in serum-starved 3T3 cells (generalized, Fig. 7*A*). The percentage of cells in which TTP was present exclusively in the cytoplasm increased rapidly in response to serum stimulation (Fig. 7*A*), in general agreement with published findings (41). The distribution of TTP later returned to that characteristic of cycling cells (Fig. 6*D*, not shown). The TTP(Zn-C) mutant was significantly more nuclear in its overall distribution but still relocalized to the cytoplasm in response to serum (Figs. 6*D* and 7*B*). In contrast, the predominantly nuclear localization of the TTP(Zn) mutant was not affected by serum stimulation (Fig. 7*C*), indicating that the TTP C-terminal region is important for this response. Remarkably, although the S178A substitution decreased the proportion of both TTP and TTP(Zn-C) that was present in the cytoplasm, it did not detectably impair serum-dependent relocalization of these proteins (Fig. 7, *D* and *E*). The data suggest that 14-3-3 binding to Ser-178 is not essential for TTP to be relocalized in response to serum and that at least two distinct mechanisms act through the C-terminal region of TTP to promote its localization to the cytoplasm.

DISCUSSION

In this study, we have determined that 14-3-3 proteins bind to TTP and that this binding is largely dependent upon a specific site in the TTP C terminus. The following lines of evidence suggest that the interaction between TTP and 14-3-3 proteins is biologically significant. This interaction was readily detectable and specific in yeast two-hybrid and *in vitro* assays and was abrogated by dephosphorylation of TTP (Figs. 1–3). TTP that was expressed by transfection in 293T cells interacted with both co-expressed and endogenous 14-3-3 proteins (Fig. 4). Binding of TTP to 14-3-3 was significantly reduced by Ala substitution of TTP Ser-178, which is located within a predicted 14-3-3 binding site consensus (Figs. 3 and 4). Finally, our findings indicated that 14-3-3 binding through Ser-178 is one of multiple mechanisms that normally promote localization of TTP to the cytoplasm (Fig. 6).

In vitro binding and co-immunoprecipitation assays indicate that substitution of Ser-178 with Ala significantly reduces binding of TTP to over-expressed and endogenous 14-3-3 proteins (Figs. 3*E* and 4). TTP Ser-178 is located within a Type II 14-3-3 binding site (Fig. 3*D*) (35), the first of its kind to be implicated in an interaction *in vivo*. This finding confirms the value of peptide library site selections for predicting 14-3-3 binding sites. TTP phosphorylation is required for 14-3-3 binding (Fig. 2*B*) predicting that Ser-178 is phosphorylated. The electrophoretic profiles of TTP and TTP S178A were not reproducibly distinguishable however (Fig. 4, not shown), supporting the idea that TTP is phosphorylated at multiple additional positions (Fig. 2). It has been reported that TTP is phosphorylated by the mitogen-activated protein kinase (MAPK) p42, the p38 MAPK, and MAPK-activated protein kinase 2 (14, 42, 43).





C

	percentage		
	cytoplasmic	generalized	nuclear
TTP	3±1	8±6	89±6
TTP+14-3-3	18±6	2±0	80±5
S178A	0±0	1±1	99±1
S178A+14-3-3	2±2	3±1	95±1

D

	percentage		
	cytoplasmic	generalized	nuclear
TTP	16±5	84±5	0±0
TTP+14-3-3	45±8	55±8	0±0
S178A	1±1	98±2	1±1
S178A+14-3-3	3±2	96±1	1±1
Zn-C	0±0	15±3	85±3
Zn-C+14-3-3	0±0	32±8	68±8
Zn-C S178A	0±0	5±2	95±2
Zn-C S178A+14-3-3	0±0	5±2	95±2

FIG. 6. **14-3-3 binding promotes cytoplasmic localization of TTP.** *A*, categories of TTP subcellular localization at 80× magnification. 3T3 cells transfected with 100 ng of CS2TTP or TTP mutant plasmids along with 100 ng of Bcl-2 vector were stained with TTP antibodies (nTTP or cTTP). Distributions classified as cytoplasmic, generalized, and nuclear are shown. *B*, localization of exogenous TTP and 14-3-3 proteins in transfected HeLa cells, shown at 100×. Cells were transfected with 100 ng of CS2TTP or CS2TTP S178A plasmid, 100 ng of HA14-3-3β or HA vector, and 200 ng of Bcl-2 plasmid. After fixation, TTP and HA-14-3-3β were detected by staining with cTTP and HA antibodies, respectively. *Arrows* indicate TTP- and HA-14-3-3β-expressing cells in which TTP is predominantly cytoplasmic. *C*, localization of TTP in a typical experiment performed as in *B*. *Numbers* indicate the mean percentage in each category (as in *A*), ± the standard deviation among four samples. *D*, effects of 14-3-3 binding on localization of TTP in 3T3 cells. A representative experiment in which 3T3 cells were transfected with 100 ng of the indicated CS2TTP constructs, 25 ng of HA14-3-3β or HA vector, and 100 ng of Bcl-2 expression plasmid, then stained for TTP and HA14-3-3β expression and classified as in *C*.

p42 MAPK phosphorylates TTP at Ser-220 *in vitro*, however (42), and the amino acids surrounding Ser-178 do not conform well to the known binding preferences of these or other kinases (45). Ser-178 and some nearby residues appear to be conserved among the three TTP/TIS11 proteins (Fig. 3*D*), each of which binds to 14-3-3 proteins *in vitro* and in co-immunoprecipitation assays (Figs. 2*C* and 4*B*). Interaction between human TIS11b and 14-3-3 proteins in a yeast two-hybrid assay has also been reported (46). These findings suggest that binding to 14-3-3 proteins may be a conserved means of regulation of TTP/TIS11 proteins. Consistent with this idea, outside of their respective zinc finger regions, each TTP/TIS11 protein contains multiple potential sites for proline-directed Ser or Thr phosphorylation, some of which are conserved among all three proteins (not shown). Our finding that 14-3-3 interacts functionally with TTP suggests that the range of biological processes influenced by 14-3-3 includes gene regulation at a post-transcriptional level.

14-3-3 proteins commonly bind to other proteins as dimers (37, 38), predicting that TTP may contain additional 14-3-3 binding sites. Supporting this model, different 14-3-3 isoforms each bind preferentially to distinct sets of phosphorylated forms of TTP (Fig. 2*A*). Mutation of other predicted 14-3-3 binding sites in the TTP C-terminal region, even in pairs, did not reduce binding to 14-3-3, however (Fig. 3, *D* and *E*, not shown). These and perhaps other unidentified 14-3-3 binding sites could nevertheless be partially redundant, as has been observed in yeast Cdc25 (40). Assuming that 14-3-3 binds to TTP as a dimer, the broad importance of TTP Ser-178 for 14-3-3 binding suggests that for many 14-3-3 isoforms, binding of Ser-178 to one monomeric subunit of the 14-3-3 dimer may be a critical event. Different isoforms could then each preferentially recognize a distinct subset of second sites. The existence of TTP in numerous phosphorylated forms suggests that TTP may be targeted by multiple signaling pathways (Fig. 2*A*). The preferential binding of individual 14-3-3 isoforms to different phosphorylated forms of TTP may facilitate integration of signals from these pathways. Although peptide library screens

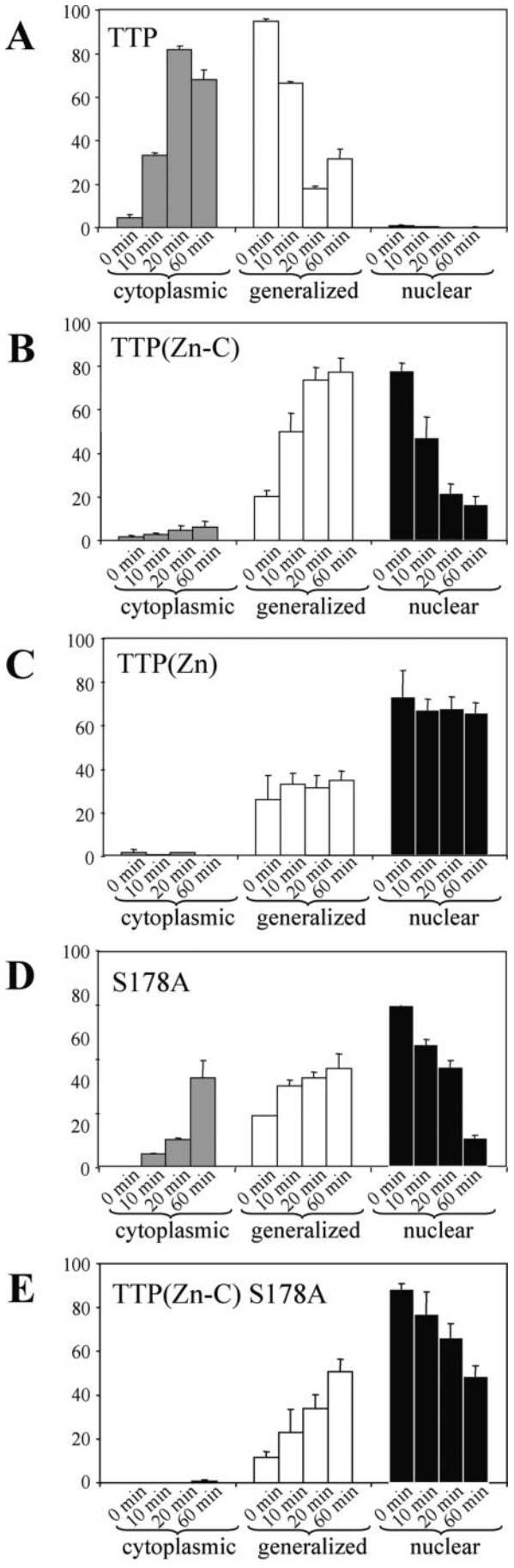


FIG. 7. **14-3-3 binding at Ser-178 is not required for serum-dependent relocalization of TTP.** 100 ng of expression vectors for TTP (*A*), TTP(Zn-C) (*B*), TTP(Zn) (*C*), TTP S178A (*D*), or TTP(Zn-C) S178A (*E*), were transfected into 3T3 cells that were subsequently serum-starved, then stimulated with 20% serum. After staining with cTTP, transfected cells were scored for TTP subcellular localization as in Fig. 6*A*. Times indicated represent the duration of serum stimulation. *0 min* indicates unstimulated controls. A typical experiment is shown for each, with *bars* representing the mean of two wells and *error bars* representing the standard deviation.

indicate that the binding preferences of 14-3-3 isoforms are generally similar (35), our findings suggest that seemingly small differences among them may be biologically significant *in vivo*. The existence of multiple different 14-3-3 isoforms may therefore expand the range of phosphorylated protein sequences that can be recognized by 14-3-3 proteins.

14-3-3 co-expression and TTP mutagenesis studies together indicate that binding of 14-3-3 at Ser-178 promotes localization of TTP to the cytoplasm. Expression of 14-3-3 enhances cytoplasmic localization of both TTP and TTP(Zn-C), an effect that is abrogated by the S178A substitution (Fig. 6, *B–D*). The S178A mutation also decreases cytoplasmic localization of these proteins in the absence of exogenous 14-3-3, suggesting that endogenous 14-3-3 proteins normally contribute to subcellular localization of TTP (Fig. 6, *C* and *D*). It has been proposed that 14-3-3 proteins promote cytoplasmic localization of many proteins through masking of nuclear localization sequences (NLS) or unmasking of nuclear export signals (NES) (38). Accessibility of nuclear import or export elements within TTP could be influenced by 14-3-3 binding, even if along the TTP amino acid sequence these elements are distant from the 14-3-3 binding site at Ser-178. Predicted weak NLS and NES sequences are conserved in TTP/TIS11 proteins immediately upstream of the zinc fingers, and at the C terminus, respectively (not shown) (47, 48). Since submission of this report, it has been reported that a functional NES is present at the TTP N terminus but also that the entire C-terminal region of TTP is dispensable for its localization to the cytoplasm (49). One possible explanation for why the latter observation disagrees with our findings is that it was obtained using GFP-TTP fusion proteins. We have determined that, in general, fusion to GFP significantly enhances the cytoplasmic localization of the various TTP mutants we have analyzed and can thereby mask effects of important localization elements (not shown).

Our analysis of TTP mutants also indicated that relocalization of TTP from the nucleus to the cytoplasm in response to serum stimulation involves the TTP C-terminal region and occurs independently of the N-terminal region (Fig. 7, *A–C*). This relocalization of TTP does not require Ser-178 (Fig. 7, *D* and *E*), suggesting that it may not depend upon 14-3-3 binding. It remains possible, however, that unidentified 14-3-3 recognition sites are involved, or that 14-3-3 binding affects the kinetics of TTP import and export without affecting its final subcellular distribution. Binding of 14-3-3 at Ser-178 presumably represents a response to signals that are constitutive in cultured cells but may regulate TTP activity *in vivo*. Because the TTP N-terminal region also promotes cytoplasmic localization of TTP (Fig. 6*C*) (32, 49), our results indicate that at least three different processes contribute to exclusion of TTP from the nucleus.

In a separate study, we have determined that regions of TTP outside of the zinc fingers are required to induce apoptosis, for its unique effects on TNFα-induced apoptosis, and for localization of TTP to the cytoplasm, but we have not observed a precise correlation between presence of TTP in a particular cellular compartment and its activities in these functional assays (32). Similarly, in this study we found that neither the S178A substitution nor 14-3-3 co-expression appeared to influ-

ence the pro-apoptotic effects of TTP significantly (Fig. 5, not shown). These observations suggest that the TTP N- and C-terminal regions may be required for its biological activities independently of their effects on its localization within the cell. At the same time, it also appears likely that 14-3-3 binding and other mechanisms that modulate localization of TTP may regulate TTP functions in ways that are not detectable in our current functional assays. By implicating 14-3-3 binding in the multiple processes that localize TTP within the cell, our findings represent an important step toward elucidating how TTP functions are regulated and how interactions with the TTP N- and C-terminal regions contribute to its biological functions.

Acknowledgments—We thank Dilara Sarbassova for preparing GST-14-3-3 fusion proteins and Mark Vidal for the gift of the two-hybrid library. We also thank Karl Munger, Grace Gill, Azad Bonni, and members of the Blackwell laboratory for many helpful discussions and critical reading of the manuscript, and Jiping Zha for advice on 14-3-3 co-immunoprecipitations.

REFERENCES

1. Carballo, E., Lai, W. S., and Blackshear, P. J. (1998) *Science* **281,** 1001–1005
2. Chen, C. Y., and Shyu, A. B. (1995) *Trends Biochem. Sci* **20,** 465–470
3. Kotlyarov, A., Neininger, A., Schubert, C., Eckert, R., Birchmeier, C., Volk, H. D., and Gaestel, M. (1999) *Nat. Cell Biol.* **1,** 94–97
4. Kontoyiannis, D., Pasparakis, M., Pizarro, T. T., Cominelli, F., and Kollias, G. (1999) *Immunity* **10,** 387–398
5. Winzen, R., Kracht, M., Ritter, B., Wilhelm, A., Chen, C. Y., Shyu, A. B., Muller, M., Gaestel, M., Resch, K., and Holtmann, H. (1999) *EMBO J.* **18,** 4969–4980
6. Dumitru, C. D., Ceci, J. D., Tsatsanis, C., Kontoyiannis, D., Stamatakis, K., Lin, J. H., Patriotis, C., Jenkins, N. A., Copeland, N. G., Kollias, G., and Tsichlis, P. N. (2000) *Cell* **103,** 1071–1083
7. Rutault, K., Hazzalin, C. A., and Mahadevan, L. C. (2001) *J. Biol. Chem.* **276,** 6666–6674
8. Vasudevan, S., and Peltz, S. W. (2001) *Mol Cell* **7,** 1191–1200
9. Kontoyiannis, D., Kotlyarov, A., Carballo, E., Alexopoulou, L., Blackshear, P. J., Gaestel, M., Davis, R., Flavell, R., and Kollias, G. (2001) *EMBO J.* **20,** 3760–3770
10. Taylor, G. A., Carballo, E., Lee, D. M., Lai, W. S., Thompson, M. J., Patel, D. D., Schenkman, D. I., Gilkeson, G. S., Broxmeyer, H. E., Haynes, B. F., and Blackshear, P. J. (1996) *Immunity* **4,** 445–454
11. Carballo, E., Gilkeson, G. S., and Blackshear, P. J. (1997) *J. Clin. Invest.* **100,** 986–995
12. Carballo, E., Lai, W. S., and Blackshear, P. J. (2000) *Blood* **95,** 1891–1899
13. Carballo, E., and Blackshear, P. J. (2001) *Blood* **98,** 2389–2395
14. Mahtani, K. R., Brook, M., Dean, J. L., Sully, G., Saklatvala, J., and Clark, A. R. (2001) *Mol. Cell. Biol.* **21,** 6461–6469
15. Lai, W. S., Carballo, E., Strum, J. R., Kennington, E. A., Phillips, R. S., and Blackshear, P. J. (1999) *Mol. Cell. Biol.* **19,** 4311–4323
16. Lai, W. S., Carballo, E., Thorn, J. M., Kennington, E. A., and Blackshear, P. J. (2000) *J. Biol. Chem.* **275,** 17827–17837
17. Ming, X. F., Stoecklin, G., Lu, M., Looser, R., and Moroni, C. (2001) *Mol. Cell. Biol.* **21,** 5778–5789
18. Guedes, S., and Priess, J. (1997) *Development* **124,** 731–739
19. Tabara, H., Hill, R. J., Mello, C. C., Priess, J. R., and Kohara, Y. (1999) *Development* **126,** 1–11
20. Schubert, C. M., Lin, R., de Vries, C. J., Plasterk, R. H., and Priess, J. R. (2000) *Mol Cell* **5,** 671–682
21. Tenenhaus, C., Subramaniam, K., Dunn, M. A., and Seydoux, G. (2001) *Genes Dev.* **15,** 1031–1040
22. Lai, W. S., Stumpo, D. J., and Blackshear, P. J. (1990) *J. Biol. Chem.* **265,** 16556–16563
23. Varnum, B. C., Lim, R. W., Sukhatme, V. P., and Herschman, H. R. (1989) *Oncogene* **4,** 119–120
24. DuBois, R. N., Bishop, P. R., Graves-Deal, R., and Coffey, R. J. (1995) *Cell Growth Differ* **6,** 523–529
25. Heximer, S. P., and Forsdyke, D. R. (1993) *DNA Cell Biol.* **12,** 73–88
26. te Kronnie, G., Samallo, J., Schipper, H., and Stroband, H. W. (2001) *Dev. Genes Evol.* **211,** 261–262
27. Haas, C. A., Donath, C., and Kreutzberg, G. W. (1993) *Neuroscience* **53,** 91–99
28. Mesner, P. W., Epting, C. L., Hegarty, J. L., and Green, S. H. (1995) *J. Neurosci.* **15,** 7357–7366
29. Mittelstadt, P. R., and DeFranco, A. L. (1993) *J. Immunol.* **150,** 4822–4832
30. Harkin, D. P., Bean, J. M., Miklos, D., Song, Y. H., Truong, V. B., Englert, C., Christians, F. C., Ellisen, L. W., Maheswaran, S., Oliner, J. D., and Haber, D. A. (1999) *Cell* **97,** 575–586
31. Johnson, B. A., Geha, M., and Blackwell, T. K. (2000) *Oncogene* **19,** 1657–1664
32. Johnson, B. A., and Blackwell, T. K. (2002) *Oncogene*, in press
33. Turner, R., and Tjian, R. (1989) *Science* **243,** 1689–1694
34. Vidal, M. (1997) in *The Yeast Two-hybrid System* (Bartel, P. L., and Fields, S., eds) pp. 109–147, Oxford University Press, Oxford
35. Yaffe, M. B., Rittinger, K., Volinia, S., Caron, P. R., Aitken, A., Leffers, H., Gamblin, S. J., Smerdon, S. J., and Cantley, L. C. (1997) *Cell* **91,** 961–971
36. Rittinger, K., Budman, J., Xu, J., Volinia, S., Cantley, L. C., Smerdon, S. J., Gamblin, S. J., and Yaffe, M. B. (1999) *Mol Cell* **4,** 153–166
37. Yaffe, M. B., and Elia, A. E. (2001) *Curr. Opin. Cell Biol.* **13,** 131–138
38. Muslin, A. J., and Xing, H. (2000) *Cell. Signal.* **12,** 703–709
39. Muslin, A. J., Tanner, J. W., Allen, P. M., and Shaw, A. S. (1996) *Cell* **84,** 889–897
40. Zeng, Y., and Piwnica-Worms, H. (1999) *Mol. Cell. Biol.* **19,** 7410–7419
41. Taylor, G. A., Thompson, M. J., Lai, W. S., and Blackshear, P. J. (1996) *Mol. Endocrinol.* **10,** 140–146
42. Taylor, G. A., Thompson, M. J., Lai, W. S., and Blackshear, P. J. (1995) *J. Biol. Chem.* **270,** 13341–13347
43. Carballo, E., Cao, H., Lai, W. S., Kennington, E. A., Campbell, D., and Blackshear, P. J. (2001) *J. Biol. Chem.* **276,** 42580–42587
44. van Hemert, M. J., Steensma, H. Y., and van Heusden, G. P. (2001) *Bioessays* **23,** 936–946
45. Pearson, R. B., and Kemp, B. E. (1991) *Methods Enzymol.* **200,** 62–81
46. Bustin, S. A., and McKay, I. A. (1999) *DNA Cell Biol* **18,** 653–661
47. Gorlich, D., and Mattaj, I. W. (1996) *Science* **271,** 1513–1518
48. Nigg, E. A. (1997) *Nature* **386,** 779–787
49. Phillips, R., Ramos, S., and Blackshear, P. J. (2002) *J. Biol. Chem.* **277,** 11606–11613