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Activation of Mitogen-activated Protein Kinase Cascade Regulates Pituitary Tumor-transforming Gene Transactivation Function*

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Pituitary tumor-transforming gene (PTTG) is a recently characterized oncogene that can act as a transcriptional activator. In this study, we have characterized the transactivation domain of PTTG. Transient transfection of fusion constructs containing GAL4 DNA-binding domain and different parts of PTTG indicated the transactivation domain of PTTG is located between amino acids 119 and 164. Mitogen-activated protein (MAP) kinase cascade is important in the regulation of cell growth, apoptosis, and differentiation. Therefore, we have explored the possibility that this kinase cascade plays a role in regulating PTTG transactivation function. Activation of the MAP kinase cascade by epidermal growth factor or an expression vector for a constitutively active form of the MAP kinase kinase (MEK1) led to stimulation of PTTG transactivation activity. We showed that PTTG is phosphorylated *in vitro* on Ser^{162} by MAP kinase and that this phosphorylation site plays an essential role in PTTG transactivation function. We demonstrated that PTTG interacts directly with MEK1 through a putative SH3 domain-binding site located between amino acids 51 and 54 and that this interaction is crucial for PTTG transactivation function. In addition, we showed that activation of MAP kinase phosphorylation cascade resulted in nuclear translocation of PTTG. Together, our data establish that a growth factor-stimulated MAP kinase plays an important role in modulating PTTG function.

PTTG[1] was originally isolated by its differential expression in pituitary tumor cells (1). Overexpression of PTTG protein in 3T3 fibroblasts resulted in cell transformation *in vitro*, and injection of transfected 3T3 cells into nude mice resulted in tumor formation, indicating PTTG is a transforming gene (1, 2). In addition to pituitary tumors, PTTG mRNA is also expressed in a variety of primary tumors and tumor cell lines including carcinomas of lung, breast, melanoma, leukemia, and lymphoma (3, 4), suggesting that PTTG may be involved in tumorigenesis of other tissues in addition to the pituitary. However, among normal adult tissues, PTTG mRNA is only expressed to high levels in the testis, with lower expression detected in thymus and intestines (1, 2, 5). PTTG mRNA is expressed in spermatocytes and spermatids in a stage-specific manner during the rat spermatogenic cycle (6), and an enhancer element important for PTTG transcriptional activation in germ cells has been identified (7). Yeast two-hybrid screening identified ribosomal protein S10 and a novel DnaJ homologue HSJ2 as binding partners to PTTG in testicular germ cells (7). These findings suggest that PTTG may be involved in regulating male germ cell differentiation. In a recent study, PTTG was identified as a vertebrate sister-chromatid separation inhibitor, and degradation of PTTG was required for sister-chromatid separation (8). The expression level of PTTG was found to be up-regulated in rapidly proliferating cells and was regulated in a cell cycle-dependent manner, peaking in mitosis (9), suggesting that PTTG may play a role in regulatory pathways involved in controlling cell proliferation.

Transcriptional regulation is an essential control point for diverse cellular functions such as cell proliferation, differentiation, and transformation. The involvement of PTTG in transcriptional control was first demonstrated by the finding that the C-terminal region of PTTG could act as a transactivation domain when fused to GAL4 DNA-binding domain (5). One of the target genes for PTTG transactivation function is basic fibroblast growth factor (bFGF). Overexpression of PTTG in transfected cells stimulated expression of bFGF, and point mutations of the putative SH3-binding sites within the C-terminal region of PTTG abrogated the increase in bFGF expression (2). Recently, we demonstrated that PTTG was able to transactivate the bFGF transcription in the presence of a novel PTTG-binding factor (10). This evidence suggests that transcriptional activation is one of the important mechanisms for PTTG actions. However, the mechanism of PTTG transactivation function and the amino acid sequences involved in this function have yet to be characterized. In this study we have performed detailed deletion and point mutation analysis to localize precisely PTTG transactivation domain.

The stimulation of cellular proliferation and differentiation involves the activation of signaling pathways that are initiated by specific receptors at the cell surface. Many of these signaling pathways converge on the mitogen-activated protein (MAP) kinase cascade, a module consisting of MAP kinase kinase (MEK1 and -2, also known as ERK kinase or as MKK), MAP kinase (MAPK 1 and 2, also known as extracellular signal-regulated kinase or ERK), and its downstream targets. These kinases form three successive tiers of a cascade in which MEK phosphorylates and activates MAPK and mitogen-activated protein kinase phosphorylates and activates its downstream target (11–16). An important target of MAP kinase cascade is

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[1] The abbreviations used are: PTTG, pituitary tumor-transforming gene; MAP, mitogen-activated protein; MAPK, mitogen-activated protein kinase; ERK, extracellular signal-regulated kinase; MEK, MAP/ERK kinase; HA, hemagglutinin; PAGE, polyacrylamide gel electrophoresis; EGF, epidermal growth factor; GFP, green fluorescent protein; GST, glutathione *S*-transferase; STATs, signal transducers and activators of transcription; PBS, phosphate-buffered saline; bFGF, basic fibroblast growth factor.

the regulation of gene expression in the nucleus. MAP kinase was shown to phosphorylate and activate several nuclear transcription factors. The proto-oncogene product c-Myc is phosphorylated on Ser^{62} by MAP kinase (17, 18). Phosphorylation at this site is associated with enhanced transactivation function of c-Myc (18–20). Similarly, phosphorylation of NF-IL6 at Thr^{235} by MAP kinase caused an increase in transactivation of gene expression by NF-IL6 (21). MAP kinase phosphorylation of p62TCF/Elk-1, a component of the ternary complex bound to the serum response element, was shown to be responsible for mitogen-stimulated function of serum response element (22, 23).

The possible involvement of PTTG in regulating cellular proliferation as well as the presence of a consensus MAP kinase phosphorylation site (Pro-*X*-Ser/Thr-Pro) (17) within the transactivation domain of PTTG led us to investigate whether activation of MAP kinase signal transduction pathway affects PTTG transactivation function. We show here that transactivation function of PTTG is enhanced by activation of MAP kinase cascade. We demonstrate that PTTG is phosphorylated by MAP kinase at Ser^{162} *in vitro* and that this site plays a critical role in PTTG transactivation function. We explored the possibility that PTTG directly interacts with one or more components of the MAP kinase cascade. We show here that PTTG interacts with MEK1 through an SH3 domain-binding motif located at the N terminus of PTTG and that this interaction is required to mediate the effect of MAPK cascade activation on PTTG transactivation function. Furthermore, we provide evidence that activation of MAPK phosphorylation cascade leads to PTTG nuclear translocation. Together our data establish that MAP kinase phosphorylation cascade has an important functional role in regulating PTTG transactivation activity.

MATERIALS AND METHODS

Plasmids—The reporter plasmid (5×GAL4-E1B-LUC) containing five GAL4-binding sites inserted in front of E1B minimal promoter linked to luciferase (24) was kindly provided by Dr. R. A. Maurer (Oregon Health Science University, Portland, OR). The construction of expression vector containing GAL4 DNA-binding domain (pGAL4DBD) and the GAL4DBD-VP16 fusion constructs were described previously (25) and were obtained from Dr. Bariahmad (Justus-Liebig-Universitat, Germany). The bFGF-luciferase fusion containing 1 kilobase pair of bFGF promoter linked to luciferase was described previously (10). The GAL4DBD-PTTG fusion constructs were constructed as follows. The wild type PTTG and the N-terminal 60-amino acid deletion mutants were constructed by digesting pAS2PTTG and pAS2PTTGm2 (6) with *Nco*I and *Bam*HI. After filling the ends with DNA polymerase I, the blunt ended insert was clone at *Sma*I site of GAL4DBD to generate GAL-PTTG-(1–199) and GAL-PTTG-(61–199). To generate N-terminal 118-amino acid deletion mutant, GST-PTTG (6) was cut with *Xba*I and *Xho*I; the ends were repaired by DNA polymerase I, and the blunt ended insert was cloned at the PuvII site of GAL4DBD, resulting in plasmid GAL-PTTG-(119–199). To generate the deletion mutant containing only the C-terminal 35 amino acids, GST-PTTG (6) was digested with *Pst*I and *Xho*I. The 5′ overhang and the 3′ overhang were repaired by DNA polymerase I and T4 DNA polymerase, respectively. The blunt-ended insert was cloned into *Sma*I site of GAL4DBD, resulting in plasmid GAL-PTTG-(164–199). Deletion mutant GAL-PTTG-(119–164) was generated using ExSite polymerase chain reaction-based mutagenesis kit following the manufacturer's instructions (Stratagene). To generate C-terminal 81-amino acid deletion mutant, GAL-PTTG-(1–199) was digested with *Xba*I. After filling the end with DNA polymerase I, the plasmid was digested with *Sma*I. The religation of the larger fragment resulted in GAL-PTTG-(1–118). Point mutations of PTTG were generated using QickChange site-directed mutagenesis kit following the manufacturer's instructions (Stratagene). All mutations were verified by DNA sequencing.

Fusion protein between PTTG and green fluorescent protein (GFP) was obtained by subcloning the coding region of PTTG into the *Bgl*II and *Hin*dIII sites of pEGFP-C1. Point mutations of PTTG were generated as described above. Histidine-tagged PTTG was generated by inserting the coding region of PTTG at *Eco*RI site of pcDNA/His expression vector (Invitrogen). GST-PTTG fusion construct was described previously (6). Deletions and point mutations of the fusion protein were made similar to GAL-PTTG fusion plasmids.

HA-tagged, constitutively active and inactive mutants of MEK1 were provided by Dr. N. G. Ahn (University of Colorado, Boulder, CO). Expression plasmids for MAPK (wild type and kinase defective mutants) were obtained from Dr. M. Cobb (University of Texas, Dallas, TX).

Cell Culture and Transfection—COS-7 and NIH-3T3 cells were cultured in Dulbecco's modified Eagle's medium (Life Technologies, Inc.) supplemented with 10% fetal bovine serum, 100 units/ml penicillin, and 100 μg/ml streptomycin. EGF and PD098059 were purchased from Calbiochem. Transfections were performed using calcium phosphate precipitation as described previously (7). All transfections were performed in triplicate, and each DNA construct was tested in at least three independent experiments. Forty eight hours post-transfection cells were lysed in 0.25 M Tris, pH 7.8, with three freeze and thaw cycles. Cell lysates (50 μg/assay) were assayed for luciferase activity as described previously (7).

Immunoprecipitation and Western Blot Analysis—Transfected cells were lysed in the lysis buffer (20 mM Tris, pH 7.4, 140 mM NaCl, 1% Nonidet P-40, 10 mM NaF, 1 mM Na_3VaO_4, 1 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, and 5 μg/ml leupeptin). For immunoprecipitation, 0.5 mg of the cell lysate was incubated with 2 μg of anti-Express for 2 h at 4 °C. The protein A/G-agarose was then added and was incubated for 1 h at 4 °C. The immunocomplexes were washed 5 time with the lysis buffer, eluted in loading buffer, and fractionated on 10% SDS-PAGE. After electron transfer, the membrane was probed using anti-HA monoclonal antibody (Covance Research Product Inc.), diluted 1:2000. For Western blot analysis using anti-GAL4DBD antibody (Santa Cruz Biotechnology), 20 μg of cell lysate was fractionated on 10% SDS-PAGE, transferred to nylon membrane, and incubated with the antibody diluted 1:500. Detection was performed using ECL system.

In Vitro Kinase Assay—COS-7 cells were transfected with HA-MEK1 or HA-MPK expression vectors. MEK1 and MAPK were immunoprecipitated from 100 μg of lysate using 2 μg of anti-HA antibody as described above. The immunoprecipitates were washed 3 times with the lysis buffer and 3 times with PBS. They were then incubated in a 40-μl reaction mixture containing 25 mM HEPES, pH 7.2, 10 mM $MgCl_2$, 1 mM dithiothreitol, [γ-^{32}P]ATP (NEN Life Science Products, 10 μCi per assay), and GST-PTTG expressed in and purified from *Escherichia coli* (10 μg/reaction) as substrate at 30 °C for 30 min. Reactions were terminated by addition of sample buffer and analyzed by SDS-PAGE. The phosphorylated products were visualized by autoradiography.

GST-Pull-down Assay—Expression of GST fusion proteins was induced with 0.5 mM isopropyl-β-thiogalactopyranoside at 37 °C for 90 min. Cells were centrifuged, and the resulting pellet was resuspended in a lysis buffer containing 20 mM Tris, pH 7.4, 140 mM NaCl, 1% Nonidet P-40, 10 mM NaF, 1 mM Na_3VaO_4, 1 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, and 5 μg/ml leupeptin. Cells were lysed by three freeze-thaw cycles. Cell debris was removed by centrifugation, and the supernatant was added to Sepharose 4B beads (Amersham Pharmacia Biotech) and incubated at room temperature for 30 min. After three washes with the lysis buffer, the fusion proteins immobilized on the beads were incubated with 300 μg of cell lysate from cells transfected with HA-tagged MEK1 at 4 °C for 2 h. After 5 washes in the lysis buffer, the bound proteins were eluted in SDS sample buffer, separated on 12% SDS-PAGE, and blotted onto nylon membrane. The membrane was probed with anti-HA antibody (Covance Research Product Inc.), diluted 1:2000.

Fluorescence Microscopy—COS-7 cells were transfected with GFP-PTTG (wild type or mutants) and HA-MEK1, either alone or in combination. Cells were fixed 24 h post-transfection with 2% neutral buffered formaldehyde (2% formaldehyde, 20 mM $NaPO_4$, pH 7.4) for 15 min at 37 °C, washed 3 times with PBS, and blocked with 1% fetal calf serum in PBS. Cells were then incubated at 37 °C with 1:100 anti-HA antibody (Covance Research Product Inc.) for 1 h and with 1:50 anti-mouse Ig rhodamine (Chemicon) for 1 h at 37 °C, with 3 PBS washes after each incubation. Slides were examined with fluorescence microscope. The fluorescence data are based on multiple transfection experiments.

RESULTS

Characterization of PTTG Transcriptional Activation Domains—Previous studies showed that PTTG could function as a transcriptional activator (5) and activate transcription of bFGF gene (10). However, the amino acid sequences responsible for this function have yet to be defined. To localize the

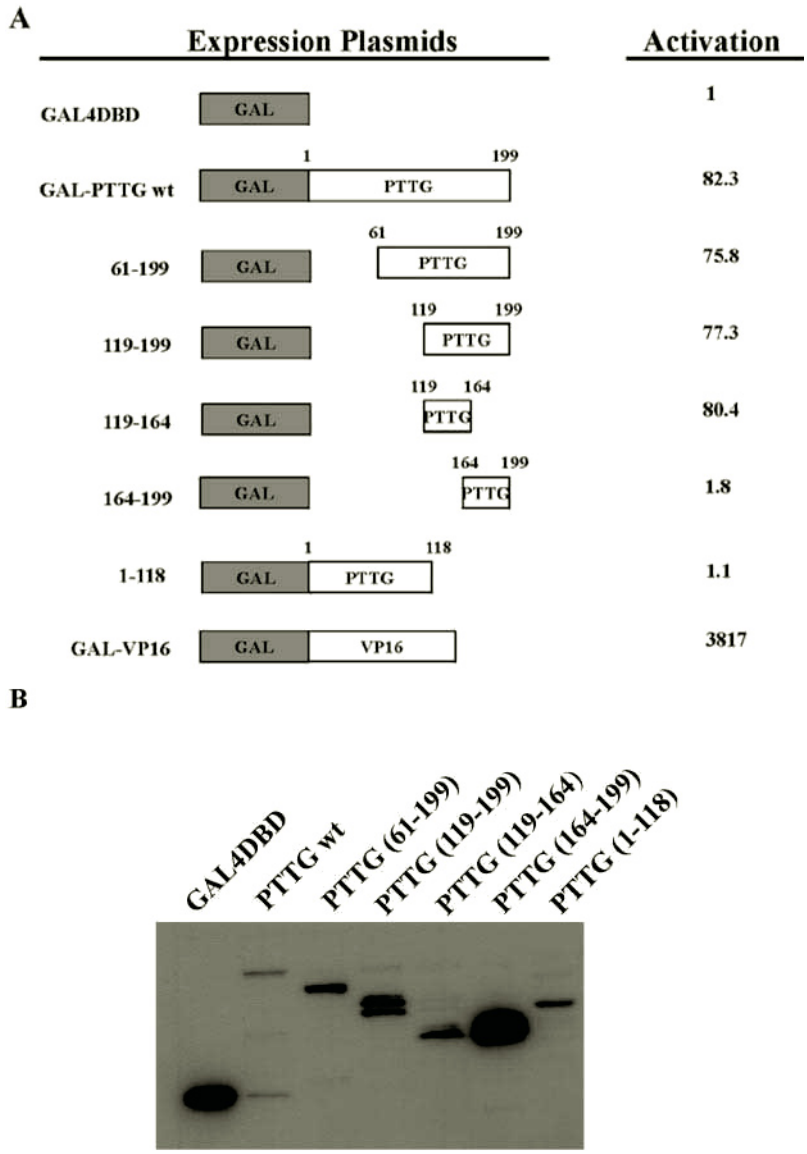

FIG. 1. **Mapping PTTG transactivation domain.** Various parts of PTTG were fused to the yeast GAL4 DBD-(GAL1–147). The fusion plasmids were transiently transfected into COS-7 cells and were assayed for the ability to activate a target gene. The reporter contains five GAL4-binding sites upstream of the E1B minimal promoter-luciferase fusion (5×GAL-TATA-LUC). *A,* activity of different GAL-PTTG fusion constructs. Values are represented as fold ctivation over GAL4BDB, which was set arbitrarily as 1.00. Values represent mean from three independent experiments. *B,* Western blot assay. Cell lysates from COS-7 cells transfected with various GAL-PTTG fusion constructs were fractionated on 10% SDS-PAGE and blotted onto nylon membrane. The membrane was probed with anti-GAL4DBD monoclonal antibody, and the expression of the fusion proteins was visualized by ECL. *wt,* wild type.

transcriptional activation domain of PTTG, fusion constructs were made between various regions of PTTG and the GAL4 DNA-binding domain (GAL4-DBD, amino acids 1–147). The GAL4-DBD contains signals for dimerization (26) and nuclear translocation (27) in addition to its specific DNA binding activity and shows no transactivation function (26, 27). The expression plasmids coding for GAL4-DBD fused to various parts of PTTG were transfected into COS-7 cells together with a reporter plasmid containing five GAL4-binding sites in front of E1B minimal promoter linked to luciferase (24). As shown in Fig. 1*A*, wild type PTTG activated reporter gene transcription about 82-fold (Fig. 1*A, construct wt*). Deletion of up to 118 amino acids from the N terminus does not affect PTTG transactivation activity (Fig. 1*A, constructs 61–199* and *119–199*). Additional deletion of the C-terminal 35 amino acids has little effect on PTTG transactivation function (Fig. 1*A, construct 119–164*). However, deletion of either 164 amino acids from the N terminus or 81 amino acids from the C terminus resulted in complete loss of transcriptional activation (Fig. 1*A, constructs 164–199* and *1–118*). The expression of all the GAL4-PTTG fusion constructs in transfected cells was verified by Western blot analysis (Fig. 1*B*). These results indicate that amino acids between positions 118 and 164 are responsible for PTTG transcriptional activation activity. Compared with the strong transcriptional activator VP16, PTTG is a relatively weak activator (Fig. 1*A*).

Activation of MAPK Cascade Potentiates PTTG Transactivation Function—The stimulation of cellular proliferation and differentiation involves the activation of signaling pathways that are initiated by specific receptors at the cell surface. Many of these signaling pathways converge on MAP kinase cascade and its downstream targets. MAP kinase was shown to phosphorylate and activate several nuclear transcription factors (18–21). The presence of a consensus phosphorylation site within the PTTG transactivation domain (amino acids 161–164 PPSP) (17) prompted us to investigate whether activation of the MAP kinase signal transduction pathway affects PTTG transcriptional activation function.

EGF is one of the growth factors that is known to activate MAPK signaling transduction pathway (28, 29). We therefore tested the effect of EGF on transcriptional activation function of PTTG. As shown in Fig. 2*A*, treatment of transfected cells with EGF resulted in 6-fold increase in GAL-PTTG transactivation activity. A primary pathway for MAP kinase activation by EGF consists of sequential activation of guanine exchange factor SOS, the guanosine triphosphate-binding protein Ras, and the protein kinases Raf-1, MEK1 or -2, and ERKs (30–33). To test whether EGF exerts its effect on PTTG transactivation

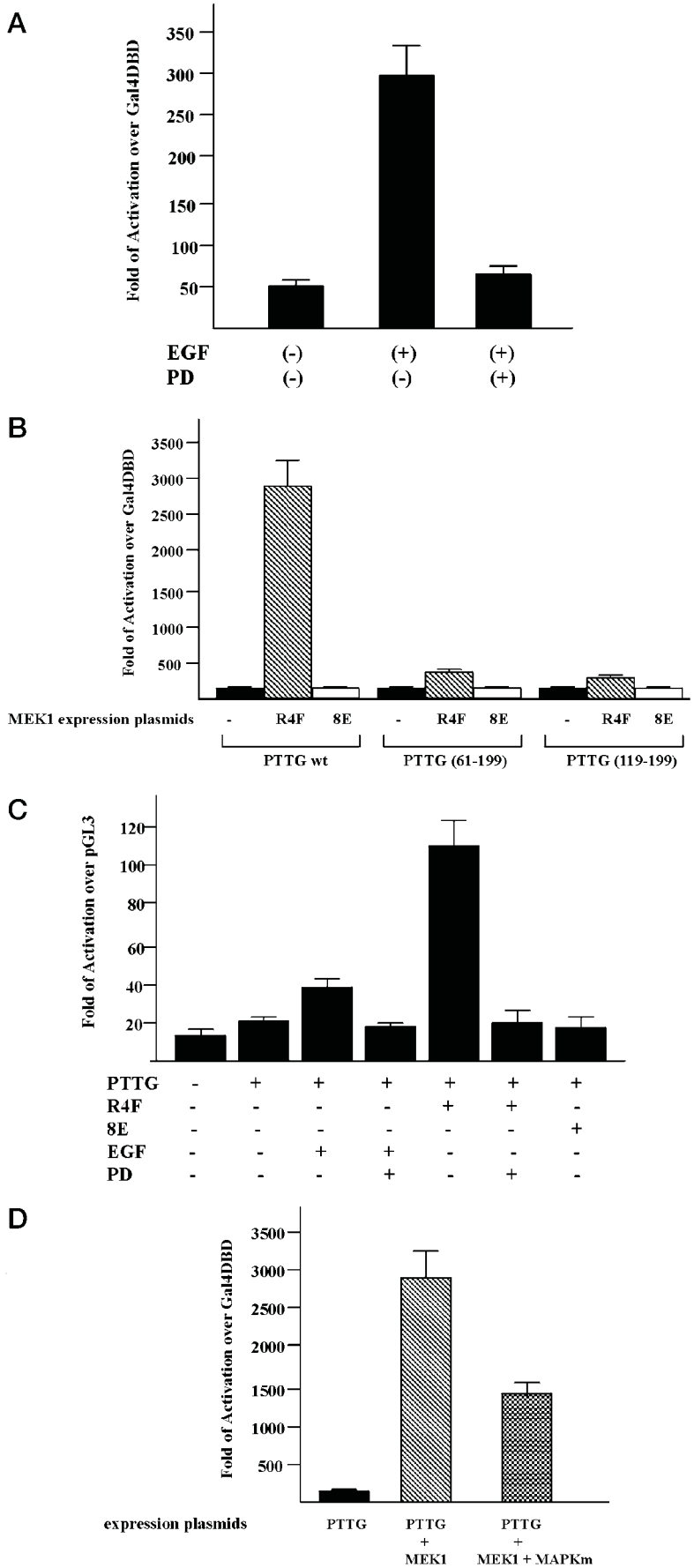

FIG. 2. **Activation of MAP kinase signal transduction pathway enhances PTTG transactivation function.** *A,* NIH-3T3 cells were transfected with GAL-PTTG fusion construct and reporter gene 5×GAL-TATALUC. Cells were starved in serum-free medium for 24 h and then treated with 50 ng/ml EGF with or without PD98059 for 16 h. *B,* cells were co-transfected with various GAL-PTTG fusion constructs together with either the constitutively activate MEK1 (*R4F*) or inactive MEK1 (*8E*). *C,* cells were co-transfected with bFGFLUC, pCMV-PTTG, and with either the constitutively activate MEK1 (*R4F*) or inactive MEK1 (*8E*). Treatments were the same as in *A*. *D,* cells were co-transfected with GAL-PTTG, GAL-PTTG + constitutively activate MEK1, or GAL-PTTG + constitutively activate MEK1 + kinase-defective MAPK (*MAPKm*). The transactivation activity of the PTTG expression constructs was assayed by measuring the luciferase activity of the reporter gene 5×GAL-TATA-LUC (*A, B,* and *D*), or bFGFLUC (*C*). Values are represented as fold activation over GAL4BDB (*A, B,* and *D*), or over pGL3 promoterless luciferase vector (*C*), which were set arbitrarily as 1.00. Values represent mean from three independent experiments.

function through this pathway, we initially asked whether PD098059, a specific inhibitor of MEK1, would affect EGF-stimulated transactivation function of PTTG. Fig. 2*A* shows that in the presence of 50 μM PD098059, EGF-stimulated transactivation activity of PTTG was inhibited. We then tested the ability of a constitutively active form of MEK1 to activate the MAP kinase phosphorylation cascade. This mutant form of MEK1 was shown to have basal activity several hundred times greater than that of the unphosphorylated wild type kinase and was able to stimulate MAP kinase activity (34). As shown in Fig. 2*B*, expression of constitutively active MEK1 (R4F) resulted in 40-fold increase in transactivation activity of the wild type PTTG, whereas expression of inactive MEK1 mutant (8E) had no effect on PTTG activity. Although expression of constitutively active MEK1 could also increase the transactivation function of the PTTG N-terminal deletion mutants, the effects were much less compared with wild type PTTG (3- *versus* 40-fold, Fig. 2*B*). These results indicate that activation of PTTG transactivation function by MAP kinase cascade requires not only PTTG transactivation domain but also residues within the N-terminal 60 amino acids.

We then tested whether activation of MAP kinase cascade also affects the ability of PTTG to transactivate bFGF transcription. Fig. 2*C* shows that either EGF treatment or co-transfection of the constitutively active form of MEK1 resulted in 2- or 5-fold increase in luciferase activity of bFGF-LUC, respectively, whereas the inactive MEK1 mutant had no effect. The stimulatory effect of EGF and MEK1 on PTTG transactivation of bFGF was also attenuated by treatment of cells with PD098059 (Fig. 2*C*).

To confirm that enhanced PTTG transactivation activity by expression of the constitutively active MEK1 is mediated by MAP kinase, we then tested the ability of expression vector for kinase-defective mutants of MAPK to function as inhibitors of MEK1-activated reporter gene activity. Although these mutants MAPK can be phosphorylated on activating site, they possess less than 5% of the wild type activity (35), and they appear to interfere with endogenous MAPK activity (36, 37). As shown in Fig. 2*D*, expression vector for kinase-defective MAPK was able to attenuate the ability of the constitutively active MEK1 to activate the GAL-PTTG fusion protein, consistent with role for MAPK in mediating activation signal from MEK1.

PTTG Is Phosphorylated by MAPK on Ser^{162} in Vitro—To determine whether modulation of PTTG transactivation function by activation of MAP kinase signal transduction pathway is a result of direct phosphorylation of PTTG, we tested the ability of MEK1 and MAPK to phosphorylate PTTG *in vitro*. COS-7 cells were transfected with either constitutively active MEK1 or MAPK expression plasmid. MEK1 and MAPK were immunoprecipitated, and the immunocomplexes were incubated with PTTG expressed in and purified from *E. coli* in the presence of [γ-^{32}P]ATP. As shown in Fig. 3, while MEK1 phosphorylated recombinant MAPK (Fig. 3, *lane 2*), it did not phosphorylate PTTG (Fig. 3, *lane 1*). MAPK, on the other hand, was able to phosphorylate PTTG (Fig. 3, *lane 3*). These results indicate that MAPK is kinase that phosphorylates PTTG.

To determine whether the consensus MAPK phosphorylation site located between amino acids 160 and 163 (PPSP) was responsible for PTTG phosphorylation by MAP kinase, site-directed mutagenesis was used to change this Ser^{162} to alanine. As shown in Fig. 4, wild type PTTG was phosphorylated by immunocomplexes from cells transfected with either MAPK alone (*lane 1*) or MAPK together with MEK1 (*lane 3*), whereas PTTG mutant containing Ser^{162} to Ala substitution was no longer phosphorylated by MAPK (*lanes 2* and *4*). We also tested

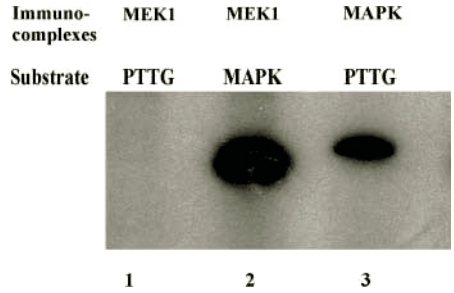

FIG. 3. **Phosphorylation of PTTG by MAP kinase *in vitro*.** COS-7 cells were transfected with either HA-tagged MEK1 or MAPK. Cells were lysed 48 h after transfection, and expressed MEK1 or MAPK was immunoprecipitated with monoclonal antibody 12CA5, which recognizes the HA tag. The immunocomplexes were used to phosphorylate PTTG expressed in and purified from *E. coli* or recombinant MAPK (Santa Cruz Biotechnology). *Lane 1,* MEK1 immunoprecipitate + PTTG; *lane 2,* MEK1 immunoprecipitate + MAPK; *lane 3,* MAPK immunoprecipitate + PTTG.

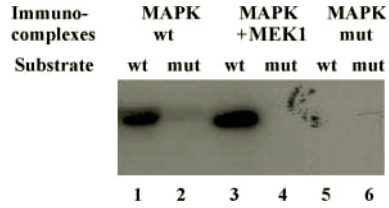

FIG. 4. **PTTG is phosphorylated by MAP kinase on Ser^{162}.** COS-7 cells were transfected with HA-tagged MAPK, MEK1 + MAPK, or kinase-defective MAPK. Cells were lysed 48 h after transfection, and expressed MEK1 or MAPK was immunoprecipitated with monoclonal antibody 12CA5, which recognizes the HA tag. The immunocomplexes were used to phosphorylate purified wild type PTTG or a mutant containing Ala substitutions of Ser^{162}. *In vitro* kinase assay was described under "Materials and Methods."

whether kinase-defective mutant of MAPK could phosphorylate PTTG. Fig. 4 shows that immunocomplexes from cells transfected with the kinase-defective MAPK could no longer phosphorylate either the wild type or mutant PTTG (*lanes 5* and *6*). These results indicate that Ser^{162} is phosphorylated by MAPK *in vitro*.

PTTG Interacts with MEK1 through an SH3 Domain Binding Site—Our transfection data indicated that activation of PTTG transactivation function by MAPK cascade requires the presence of the N-terminal 60 amino acids. We hypothesized that MEK1 may directly interact with PTTG and bring it to close contact with MAPK for phosphorylation. To test this hypothesis, we performed co-immunoprecipitation experiments. As shown in Fig. 5*A*, an immunocomplex was detected in COS-7 cells co-transfected with both PTTG and MEK1 expression plasmids (Fig. 5*A, lane 3*), whereas no immunocomplex was detected in cells transfected with either plasmid alone (Fig. 5*A, 1st* and *2nd lane*). Fig. 5*B* shows that MEK1 was expressed in COS-7 cells transfected with either MEK1 alone or co-transfected with both MEK1 and PTTG.

To determine which amino acids are involved in PTTG interaction with MEK1, we initially made a deletion mutant in which the N-terminal 60 amino acids of PTTG were deleted. Fig. 6 shows that this deletion mutant no longer interacts with MEK1 (Fig. 6, *upper panel*), suggesting that the interactive domain of PTTG with MEK1 resides within the N-terminal 60 amino acids. A motif search in this region revealed an SH3 domain interactive site between amino acid 51 and 54 (PGLP). To determine whether this motif is important in mediating interaction between PTTG and MEK1, site-directed mutagenesis was used to change Pro^{51} and Pro^{54} into alanine residues. As shown in Fig. 6, amino acid substitution of these proline residues resulted in loss of interaction between PTTG and

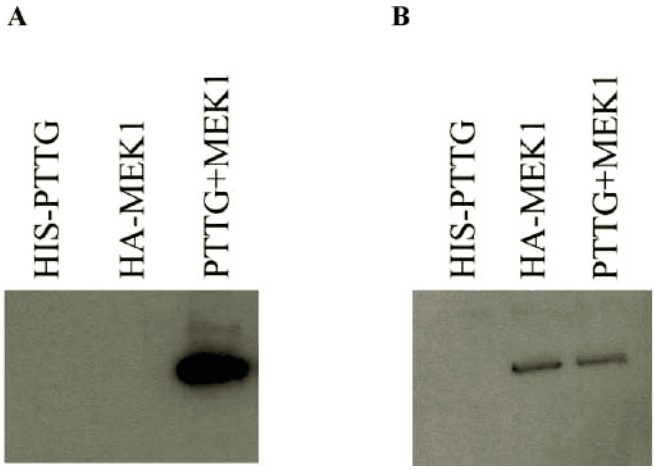

FIG. 5. **Interaction of PTTG with MEK1.** COS-7 cells were transfected with His-tagged PTTG and HA-tagged MEK1 expression vectors. His-PTTG immunoprecipitates were immunoblotted for MEK1 with antibody to HA (*A*). Identical amounts of cell lysate were blotted with anti-HA to verify expression of comparable amounts of MEK1 (*B*).

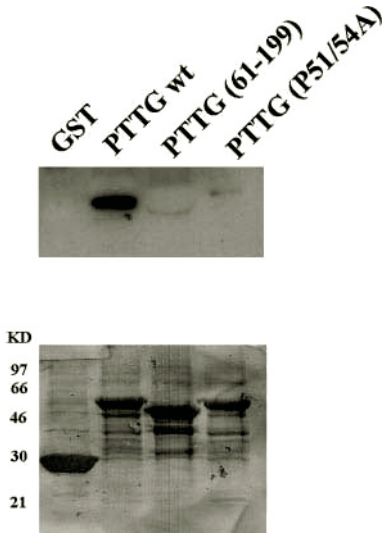

FIG. 6. **Interaction of PTTG with MEK1 through an SH3-binding site.** COS-7 cells were transfected with HA-tagged MEK1 expression vectors. The cell lysates were incubated with various GST-PTTG fusion proteins immobilized on Sepharose 4B. The bound proteins were analyzed on 10% SDS-PAGE, blotted, and probed with anti-HA antibody (*upper panel*). Ponceau S staining of the same membrane verified expression of comparable amounts of different GST-PTTG fusion protein (*lower panel*).

MEK1 (Fig. 6, *upper panel*). The expression of mutant PTTG fusion proteins was verified by staining the membrane for Western blot analysis with Ponceau S. As shown in Fig. 6, all fusion proteins were expressed to comparable levels (Fig. 6, *lower panel*). These results indicate that PTTG interacts with MEK1 via this SH3-binding site located between amino acids 51 and 54.

Point Mutations within the MEK1 Interaction and MAPK Phosphorylation Sites Abolished PTTG Transactivation Function—To test the effects of mutations in MEK1 interaction and MAPK phosphorylation sites on PTTG transcriptional activation function, point mutations were made in GAL-PTTG fusion plasmid. As shown in Fig. 7*A*, mutations within either the MEK1 interaction or the MAPK phosphorylation site resulted in almost complete loss of PTTG transactivation function. Co-transfection with constitutively active MEK1 could still induce a 3-fold increase in MEK1 interaction site-defective mutant (Fig. 7*A, P51/54A*), but it had no effect on MAPK phosphorylation site-defective mutant (Fig. 7*A, S162A*). Similarly, EGF treatment-stimulated PTTG transactivation function was also diminished in the mutants (Fig. 7*A*). Fig. 7*B* shows that both PTTG mutants were expressed in transfected cells. These results suggest that the MEK1 interaction and MAPK phosphorylation sites play essential roles in the PTTG transcriptional activation function.

A

Fold of Induction over GAL4DBD

	Control	MEK1 (+)	EGF (+)
PTTG wt	82.4	2987	445.1
PTTG (P51/54A)	4.8	14.2	6.4
PTTG (S162A)	3.7	4.3	3.5

B

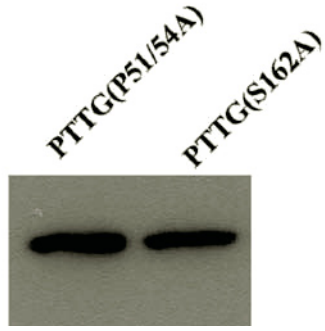

FIG. 7. **Effect of point mutations within MAPK phosphorylation and MEK1 interaction sites on PTTG transactivation function.** *A,* NIH-3T3 cells were co-transfected with wild type and mutants GAL-PTTG either alone (control) or with the constitutively activate MEK1 (*MEK1+*). For EGF treatment, cells were starved in serum-free medium for 24 h and then treated with 50 ng/ml EGF for 16 h. Transactivation activity of the fusion constructs was assayed by measuring the luciferase activity of the reporter gene 5×GAL-TATA-LUC. Values are represented as fold activation over GAL4BDB, which was set arbitrarily as 1.00. Values represent mean from three independent experiments. *B,* Western blot analysis to verify expression of GAL-PTTG mutants.

Activation of MAPK Cascade Enhances PTTG Nuclear Translocation—To determine the mechanism, by which MAPK cascade modulates PTTG transactivation function, we tested whether activation of this signal transduction pathway could affect PTTG intracellular localization. We transfected COS-7 cells with expression vector containing GFP fused to wild type PTTG or mutants containing defective sites for MAP kinase phosphorylation and MEK1 interaction. HA-tagged, constitutively active MEK1 was co-transfected in some cells to activate the MAPK cascade. As shown in Fig. 8, PTTG was localized mainly in the cytoplasm in cell transfected with PTTG alone (Fig. 8*A*). In cells co-transfected with MEK1, the wild type PTTG is expressed predominantly in the nucleus (Fig. 8*B*). However, in both MAPK phosphorylation and MEK1-binding sites defective mutants were mostly retained in the cytoplasm (Fig. 8, *C* and *D*). These results suggest that activation of MAP kinase cascade enhances PTTG nuclear translocation and that both the MAPK phosphorylation and the MEK1 interactive sites are necessary for this translocation.

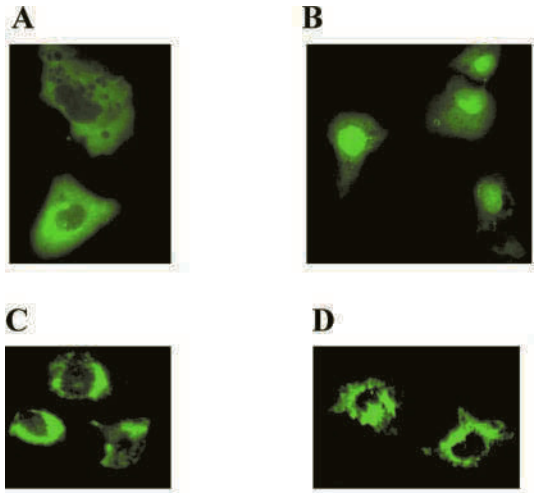

FIG. 8. **Activation of MAPK cascade enhances PTTG nuclear translocation.** COS-7 cells were transfected with GFP-PTTG wild type alone (*A*), GFP-PTTG wild type + MEK1 (*B*), GFP-PTTG (S162A/P163A) + MEK1 (*C*), and GFP-PTTG (P51/54A) + MEK1 (*D*). Twenty four hours after transfection, cells were fixed, and localization of wild type and mutant proteins were detected by the green fluorescence of GFP.

DISCUSSION

PTTG has been implicated in many cellular processes including cell proliferation, transformation, and tumorigenesis. In this study we demonstrated that the transcriptional activation function of PTTG is modulated by activation of MAPK cascade in response to mitogenic stimulus such as EGF.

We mapped the PTTG transactivation domain to a region between amino acids 119 and 164. The amino acid sequences within this region are acidic and proline-rich, characteristic of the transactivation domains of many transcriptional factors (38). Our results agree with a previous study that located the transactivation domain of human PTTG to amino acids 123–202 (5). This previous study showed that GAL4DBD-hPTTG and GAL4DBD-VP16 activated transcription of the reporter gene to the same level (about 12-fold over GAL4DBD) (5). In our study, however, we found that PTTG, under unstimulated conditions, is a much weaker transcriptional activator compared with VP16 (80- *versus* 3800-fold induction over control GAL4BDB). This discrepancy might be the result of using different reporter genes or a species difference between human and rat PTTG.

MAP kinase cascade is important in the regulation of cell growth, apoptosis, and differentiation, and an important target of MAP kinase cascade is the regulation of gene expression in the nucleus. We provide evidence that MAP kinases participate in regulating PTTG transactivation function. We demonstrated that EGF-stimulated PTTG transactivation function is mediated by activation of the MEK1-MAPK cascade, for blocking this signal transduction pathway with an MEK1-specific inhibitor abolished the stimulatory effect of EGF on PTTG. Another approach involved transfection of an expression vector for a constitutively active form of MEK1 which would be expected to activate MAP kinase. Our results show that the constitutive active form of MEK1 was able to enhance PTTG transactivation activity 40-fold, and this induced level of transactivation corresponds to almost the same level as that detected with the strong acidic activation domain of VP16 (39). In addition we found that expression vectors for kinase-defective MAPKs attenuated MEK1-induced transactivation activity of PTTG. The partial blocking effects of these vectors may result from insufficient disruption of activation of the endogenous MAPKs. These findings suggest that activation of MAP kinase cascade is an important modulator of PTTG transactivation function.

We have tested whether the effect of activation of MAP kinase signal transduction pathway on PTTG transactivation function is a result of PTTG phosphorylation by MAPK. The primary sequence of peptide substrates phosphorylated by MAP kinase has been examined, and a consensus sequence for phosphorylation has been defined as Pro-*X*-(Ser/Thr)-Pro (17, 40, 41). PTTG contains a consensus MAP kinase site (^{160}PPSP^{163}) within its activation domain. Our results show that PTTG is phosphorylated *in vitro* by MAPK at Ser^{162} and that mutant PTTG containing Ala substitution of Ser^{162} is no longer phosphorylated by MAPK and is unable to activate transcription. These results suggest that the MAPK phosphorylation site plays an essential role in PTTG transactivation function. Recently, Ramos-Morales *et al.* (9) showed that hPTTG was phosphorylated by cyclin-dependent kinase (Cdc2) on Ser^{165} which corresponds to Ser^{162} in rat PTTG. This is not surprising because the consensus phosphorylation site for Cdc2 (Ser-Pro-*X*-(Arg/Lys)) (42) in PTTG overlaps with the MAPK site. Activation of PTTG transactivation function is similar to that of c-Myc. c-Myc is phosphorylated by MAPK both *in vitro* and *in vivo* at Ser^{62} located within the N-terminal transactivation domain (17). Increased phosphorylation at this site is associated with enhanced transactivation of gene expression, and mutation of this site resulted in loss of transactivation function (18–20). Interestingly, like Ser^{165} in PTTG, Ser^{62} in c-Myc is also a substrate for Cdc-2 kinase (20). Although we have not tested whether PTTG is phosphorylated by MAPK *in vivo*, the functional data presented in this study indicate that PTTG is likely a physiological target for the MAP kinase cascade.

The transfection studies described here demonstrate that maximal induction of PTTG transactivation function following activation of MAPK cascade requires not only the transactivation domain but also amino acids within the N-terminal 60 amino acids. We showed that PTTG co-immunoprecipitates with MEK1 and that this interaction was mediated by an SH3-binding motif (Pro-*X*-*X*-Pro) located between amino acids 51 and 54.

Sequences in MEK1 between subdomains 9 and 10 of the conserved core are proline-rich, containing consensus sequences for potential SH3 domain interactions. This domain was shown to be important for interactions between MEK1 and Raf-1 (43, 44) as well as substrate recognition (45). A more recent study showed that although this proline-rich domain is required for efficient activation of MAPKs by MEK1, it is not the site of MEK1-Raf interaction (46). Recently, a MEK1-binding partner, MP1, was identified and shown to enhance enzymatic activation of MAP kinase cascade (47). PTTG represents another binding partner for MEK1. We propose that interaction between PTTG and MEK1 brings PTTG in close proximity with MAP kinase for efficient phosphorylation and activation.

Phosphorylation can induce nuclear translocation of transcription factors that are present in the latent state in the cytoplasm. A well known example is a family of proteins termed STATs (signal transducers and activators of transcription). STATs become activated by Janus kinases through tyrosine residues. The activated STAT proteins are translocated to the nucleus where they bind to specific DNA sequences and stimulate transcription (48–50). Our results showed that PTTG, a protein predominantly expressed in the cytoplasm, is translocated into the nucleus upon activation of the MAP kinase cascade. The nuclear translocation of PTTG requires the presence of the MAP kinase phosphorylation and MEK1 interactive sites. These sites are also important for PTTG transactivation function. The localization of MAPKs is predominantly cytoplas-

mic in quiescent cells (51, 52). However, upon mitogen stimulation, a fraction of MAPK translocates into nucleus (51, 52) where it phosphorylates and activates nuclear transcription factors such as Myc. PTTG is likely to be phosphorylated by cytoplasmic MAPK before its nuclear translocation, since mutation of the MAPK phosphorylation site resulted in cytoplasmic retention of PTTG. This evidence provided the second mechanism for PTTG nuclear translocation. In a previous study, we showed that PTTG translocates into the nucleus by interacting with PTTG-binding factor (10).

In summary, we have characterized the transactivation domain of PTTG. We have demonstrated that PTTG is linked to MAPK cascade through its interaction with MEK1 and that activation of this kinase cascade enhances PTTG nuclear translocation and transactivation function. This evidence indicates that MAPK cascade is one of the important mechanisms in regulation of PTTG biological functions.

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