

ERK is a novel regulatory kinase for poly(A) polymerase

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ABSTRACT

Poly(A) polymerase (PAP), which adds poly(A) tails to the 3' end of mRNA, can be phosphorylated at several sites in the C-terminal domain. Phosphorylation often mediates regulation by extracellular stimuli, suggesting PAP may be regulated by such stimuli. In this study, we found that phosphorylation of PAP was increased upon growth stimulation and that the mitogen-activated protein kinase ERK was responsible for the increase in phosphorylation. We identified serine 537 of PAP as a unique phosphorylation site by ERK. PAP phosphorylation of serine 537 by ERK increased its nonspecific polyadenylation activity *in vitro*. This PAP activity was also activated by stimulation of ERK with phorbol-12-myristate-13-acetate *in vivo*. These data suggest that ERK is a novel regulatory kinase for PAP and further, that PAP activity could be regulated by extracellular stimuli through an ERK-dependent signaling pathway(s).

INTRODUCTION

Primary mRNA transcripts are processed to their mature forms by a series of coordinated events in the nucleus including capping, splicing, cleavage and poly(A) addition. Cleavage and poly(A) addition at the 3' end of the mRNA marks the end of mRNA synthesis. Cleavage takes place between the upstream hexamer (AAUAAA) (1,2) and the downstream GU rich sequences (3), and a poly(A) tail of 200–250 residues in length is then synthesized at the cleavage site by the 3' end formation machinery. The 3' end formation machinery is composed of multiple transacting factors, including cleavage- and polyadenylation-specificity factor (CPSF), cleavage-stimulation factor (CstF), two cleavage factors (CFI and CFII), poly(A) polymerase (PAP) and poly(A)-binding protein II (4–8). PAP is the enzyme responsible for the synthesis of the poly(A) tails. In addition to nuclear polyadenylation, polyadenylation of mRNA can also occur in the cytoplasm. Additional

cis-elements such as cytoplasmic polyadenylation element (CPE) and adenylation control element (ACE) (9–11), and other transacting factors such as CPSF and cytoplasmic polyadenylation element binding protein (CPEB) (12) are involved in cytoplasmic polyadenylation.

Because the poly(A) tail plays essential roles in mRNA stability, transport and translation (13,14), polyadenylation may be an important regulatory step in diverse cellular processes (15–17) and PAP is a likely target for regulation. The catalytic domain of PAP is in the N-terminus (18,19). The C-terminal region carries an RNA-binding domain, two nuclear localization signals and a serine/threonine-rich regulatory domain (20), and also serves as a platform for regulatory proteins whose binding modulates the activity or cellular localization of PAP (21–23).

Activity of PAP could also be controlled through phosphorylation of the C-terminal domain (CTD). The serine/threonine-rich region in CTD contains multiple cyclin-dependent kinase (cdk) sites, which are phosphorylated *in vitro* and *in vivo* by p34cdc2/cyclin B (24). Growing evidence suggests that PAP can be regulated during several phases of the cell cycle. In M-phase, PAP is hyperphosphorylated by p34cdc2/cyclin B and the hyperphosphorylated PAP shows reduced activity *in vitro*. Increased polyadenylation activity has been reported during the G₀ to S phase transition (25,26). Furthermore, PAP can be phosphorylated by G₁ and G₂ type cyclin-dependent kinases and interacts with both G₁ and G₂ type cyclins (27). However, the biological relevance of phosphorylation of PAP and the specificity of the phosphorylation sites during the cell cycles remain unclear. Importantly, despite extracellular stimuli usually trigger protein phosphorylation in the cell, regulation of PAP by extracellular stimuli has not been examined.

The present study was designed to determine, first, how phosphorylation of PAP changes upon growth stimulation and, second, which kinase is responsible for phosphorylation of PAP and, finally, whether this phosphorylation regulates PAP activity. We found that serum stimulation of G₀ quiescent cells increases phosphorylation of PAP and that extracellular signal-regulated kinase (ERK) is

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responsible for the phosphorylation. Quadrupole time of flight (Q-TOF) mass spectrometry and the site-directed mutagenesis studies indicated that serine 537 of PAP is the major target site of ERK. PAP phosphorylation at serine 537 led to the increase of the nonspecific polyadenylation activity *in vitro*. Therefore, our results suggest that ERK functions as a novel regulatory kinase for PAP and that PAP could be regulated by extracellular stimuli through an ERK signaling pathway(s).

MATERIALS AND METHODS

DNA construction

Full length or defined regions of mouse PAP were cloned into pGEX4T-1 (Amersham) for expression as GST fusions in *Escherichia coli*, and into pEBG (22) and p3Flag (Invitrogen) for expression as GST fusions and Flag fusions, respectively, in mammalian cells. Mutations (S537A and S534A) were introduced into vectors by the QuickChange site-directed mutagenesis (Stratagene) as per the manufacturer's instructions.

Cell culture and transfection

HeLa cells were cultured in DMEM (Dulbecco's modified Eagle's medium, Invitrogen) supplemented with 10% fetal bovine serum (FBS) and antibiotics (penicillin 100 U/ml, streptomycin 100 µg/ml and amphotericin B 0.25 µg/ml). Mammalian expression vectors (1 µg for 35 mm plates and 5 µg for 100 mm plates) were transfected by Lipofectamine (Invitrogen) as per the manufacturer's instructions.

Preparation of recombinant PAP derivatives

GST-PAP or Flag-PAP constructs or their truncation derivatives were expressed in HeLa cells by transfection of mammalian expression vectors. Cell lysates were harvested from cells after wash with cold phosphate-buffered saline (PBS, pH 7.2) by scrapping with 300 µl of EBC solution containing 50 mM Tris-HCl, pH 7.5, 120 mM NaCl, 0.5% Nonidet P-40, 50 mM NaF, 200 µM sodium orthovanadate, 1 mM phenylmethylsulfonyl fluoride and protease inhibitor (CompleteTM; Roche). Cell lysates were incubated at 4°C for 1 h with 20 µl of glutathione affinity Sepharose (50% slurry, Amersham Pharmacia Biotech) for GST-PAP. Recombinant protein G agarose (Invitrogen) and 500 ng of anti-Flag antibody were incubated with lysates for Flag-PAP, respectively and PAP-bound beads were extensively washed with EBC solution.

Recombinant GST-PAP truncation constructs were expressed in *E. coli* and purified using glutathione-Sepharose. Protein synthesis in *E. coli* cells containing the GST-PAP constructs was induced by adding isopropyl β-D-thiogalactoside (IPTG) at 0.1 mM and the cells were further grown for 5 h at 23°C. After harvesting the cells, the cell pellet was suspended in PBS buffer and sonicated. Soluble fraction was incubated with glutathione affinity Sepharose. After repeated washes, proteins were eluted from beads with elution buffer (10 mM reduced glutathione in 50 mM Tris-HCl, pH 8.0).

Antibodies and chemicals

Antiserum to phosphoserine 537 of PAP was generated by Peptron. Antibodies specific for PAP (Santa Cruz), GST (Santa Cruz), ERK (Santa Cruz), phospho-ERK (Santa Cruz), phospho-serine (Chemicon) and Flag (Sigma) were purchased. Phorbol-12-myristate-13-acetate (PMA) and PD98059 were purchased from Calbiochem. The recombinant protein kinases (Raf, MEK and ERK) were purchased from Upstate Biotechnology.

Analysis of PAP phosphorylation

For *in vitro* analysis, PAP-bound beads or 200 ng of purified proteins were phosphorylated using 2 ng/µl of ERK, 0.1 unit of Raf or 0.1 unit of mitogen-activated protein kinase kinase (MEK) with 10 mM ATP or 0.5 µl of [γ -³²P]ATP (3000 µCi/mmol) at 37°C for 1 h in Assay Dilution Buffer (Upstate biotechnology) containing 20 mM 3-(N-morpholino) propanesulfonic acid (MOPS), pH 7.2, 25 mM β-glycerophosphate, 5 mM ethylene glycol tetraacetic acid (EGTA), 1 mM sodium orthovanadate and 1 mM dithiothreitol. The phosphorylated products were separated by SDS-PAGE for analysis of phosphorylation or washed by EBC for further use.

For analysis of the phosphorylation status of PAP *in vivo*, HeLa cells transfected with PAP constructs were synchronized at G₀, by incubating them in serum-free medium for 12 h. Quiescent cells were serum-stimulated by changing the media to DMEM containing 20% FBS. Alternatively, unsynchronized cells, cells were harvested 12 h after transfection and PMA was added to media at 100 ng/ml for activating ERK. In both cases, the cell lysates were resolved on a 10% SDS-polyacrylamide gel and subject to immunoblot analysis. For the quantitation of the amounts of GST-PAP or Flag-PAP proteins, the cell lysates were treated with λ-protein phosphatase (NEB, 1000 unit/ml) before gel electrophoresis.

Q-TOF analysis

For Q-TOF analysis, the GST-PAP truncation derivatives purified from *E. coli* were phosphorylated in 10 mM ATP with ERK (2 ng/µl) and separated by SDS-PAGE. The band corresponding to the phosphorylated form was excised from the gel and analyzed by nano-ESI on a Q-TOF2 mass spectrometer (Micromass).

In vitro polyadenylation assay

Flag-PAP-bound beads were phosphorylated by ERK, as described earlier. Briefly, the lysates were treated with λ-protein phosphatase (1000 U/ml) in the presence of 2 mM MnCl₂ (final concentration) at 30°C for 1 h before binding to protein G agarose beads to create phosphate-free protein. When necessary, PAP was phosphorylated by ERK, as described earlier. We determined the activity of Flag-PAP by measuring incorporation of AMP from [α -³²P] ATP into 120 nt-length RNA in the presence of MnCl₂, as described previously (28). The DNA template for the 120 nt RNA was obtained by PCR from cDNA for prothrombin 3' untranslated region with a pair of primers 5'PT and 3'PT: 5'PT, 5'-CGA ATT CGG GCC ACT CAT

ATT CTG; 3'PT, 5'-GGG TAC CGC TGA GAG TCA CTT TTA TTG G. The PCR product was cloned to the BamHI/SmaI site of pGEM3zf (Promega). The resulting recombinant DNA was linearized with FokI and used for *in vitro* transcription by T7 RNA polymerase. Polyadenylation reaction products were extracted with phenol extraction and precipitated by ethanol. The precipitates were dissolved in water and spotted in a PEI thin layer chromatography (TLC) plate. The residual free [α - 32 P]ATP was separated from RNA by TLC with 1.5 M KH_2PO_4 buffer and the [α - 32 P]AMP-incorporated RNA were analyzed by scintillation. Alternatively, the products were analyzed on a 5% polyacrylamide gel containing 8 M urea. We also assayed the activity of Flag-PAP in the presence of MgCl_2 instead of MnCl_2 with the RNA substrate containing a SV40 late mRNA poly(A) signal, as described previously (29)

RESULTS

Phosphorylation of CTD of PAP was increased by serum induction

We examined the phosphorylation status of PAP in response to growth stimuli using HeLa cells expressing the CTD of PAP fused to a GST tag (GST-CTD). Addition of serum to the quiescent HeLa cells, which were prepared by serum starvation for 12 h, increased phosphorylation of GST-CTD as indicated by western blotting with phosphoserine-specific antibody (Figure 1). Phosphorylation of CTD reached a maximum 10 min after the stimulation. We thought it unlikely that Cdks participated in this phosphorylation because they are known to be activated several hours after serum stimulation; Cdk4 and Cdk6 are activated in mid- G_1 and Cdk2

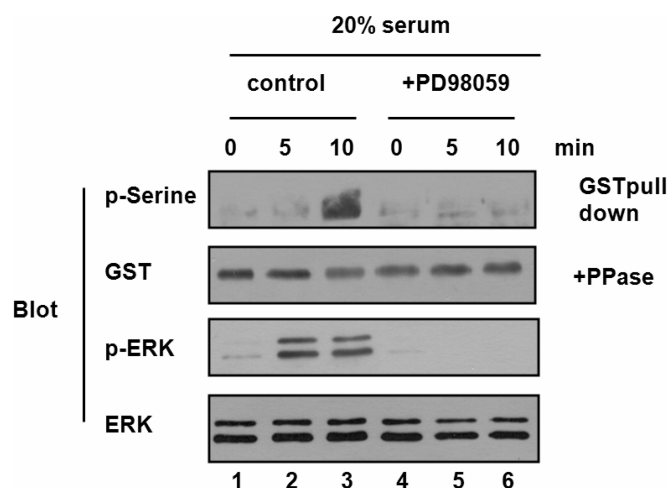


Figure 1. Phosphorylation of after serum stimulation. Phosphorylation of the CTD of PAP fused to GST (GST-CTD) in transiently transfected HeLa cells was analyzed by immunoblot using anti-phosphoserine antibody after the cell lysates were GST pull-downed. Cells transfected with GST-CTD were cultured in serum free media for 12 h and then stimulated with 20% serum (lanes 1–3) or 20% serum containing 50 μM PD98059 (lanes 4–6) for the indicated time (lanes 1 and 4, 0 min; lanes 2 and 5, 5 min; lanes 3 and 6, 10 min). The amounts of GST-CTD present in the lysates were evaluated by immunoblot using anti-GST antibody after the treatment with λ protein phosphatase to convert multiple bands of GST-CTD to a single band.

is activated in late- G_1 (30). Therefore, we tested whether a mitogen-activated protein kinase (MAPK) pathway is involved in PAP phosphorylation. The phosphorylation induced by serum stimulation was blocked by PD98059, a MAPK pathway inhibitor (Figure 1), suggesting that MAPKs participate in PAP phosphorylation.

ERK phosphorylated serine 537 of PAP *in vitro*

To identify the MAPK responsible for phosphorylation of PAP, we attempted to phosphorylate PAP using purified MAPKs Raf, MEK and ERK *in vitro*. Of these three, only ERK, phosphorylated PAP (Figure 2A), defining PAP as a substrate for ERK kinase. To locate the ERK phosphorylation site in PAP, we expressed truncated derivatives of PAP as GST-fusions in HeLa cells and purified them with glutathione resin. The resin-bound GST fusions were phosphorylated by ERK *in vitro* using [γ - 32 P]ATP. Phosphorylated signals were observed with all PAP fragments containing residues 488–542, but not with the 488–520 fragments (Figure 2B). An additional phosphorylated band running at a similar size to CTD corresponded to auto-phosphorylated ERK, whose gel mobility relative to that of CTD was observed to be slightly changed by minor perturbations of the electrophoresis condition, such as gel percentage and temperatures. We also purified the 488–520, 488–543 and 543–595 fragments from *E. coli* as GST fusion forms and used them for ERK phosphorylation (Figure 2C). The 488–542 fragment was phosphorylated, but the 488–520 and 543–595 were not. All these data allowed us to infer that the phosphorylation site is located in residues 521–542 (Figure 2D). The 521–542 region contains eight serines and one threonine. However, the well-known ERK substrate recognition site of PXS/P (X; a neutral or basic amino acid) is not present (31).

To identify the ERK phosphorylation site, we designed an experiment using Q-TOF mass spectrometry. We expressed and purified the CTD of PAP, which consists of residues 488–739, from *E. coli*, and phosphorylated it *in vitro* with ERK. Phosphorylated CTD was analyzed by Q-TOF ESI-MS analysis. Mascot analysis (32) of the Q-TOF ESI-MS data (Table 1) showed that the phosphorylation increased the molecular weight of the 515–543 peptides by 80 Da, which corresponds to a single phosphate group. Except that the 515–543 peptide, no additional phosphorylated peptides were deduced from Mascot analysis. These data indicate that ERK phosphorylates a single site in the 515–543 region. Furthermore, phosphorylated CTD and unphosphorylated control CTD were analyzed by Q-TOF MS/MS analysis and the peaks were compared. We observed peaks corresponding to fragments containing phosphorylated serine 537 in the phosphorylated sample (Figure 3A). Serine 537 was previously reported as a potential nonconsensus cdk site (24). To confirm ERK phosphorylation of serine 537, we mutated it to alanine and examined phosphorylation of the mutant CTD fragment. The S537A mutant CTD was not phosphorylated by ERK, while control S534A CTD in which serine 534 had been converted to alanine was phosphorylated (Figure 3B). We conclude that serine 537 of PAP is a unique phosphorylation site for ERK.

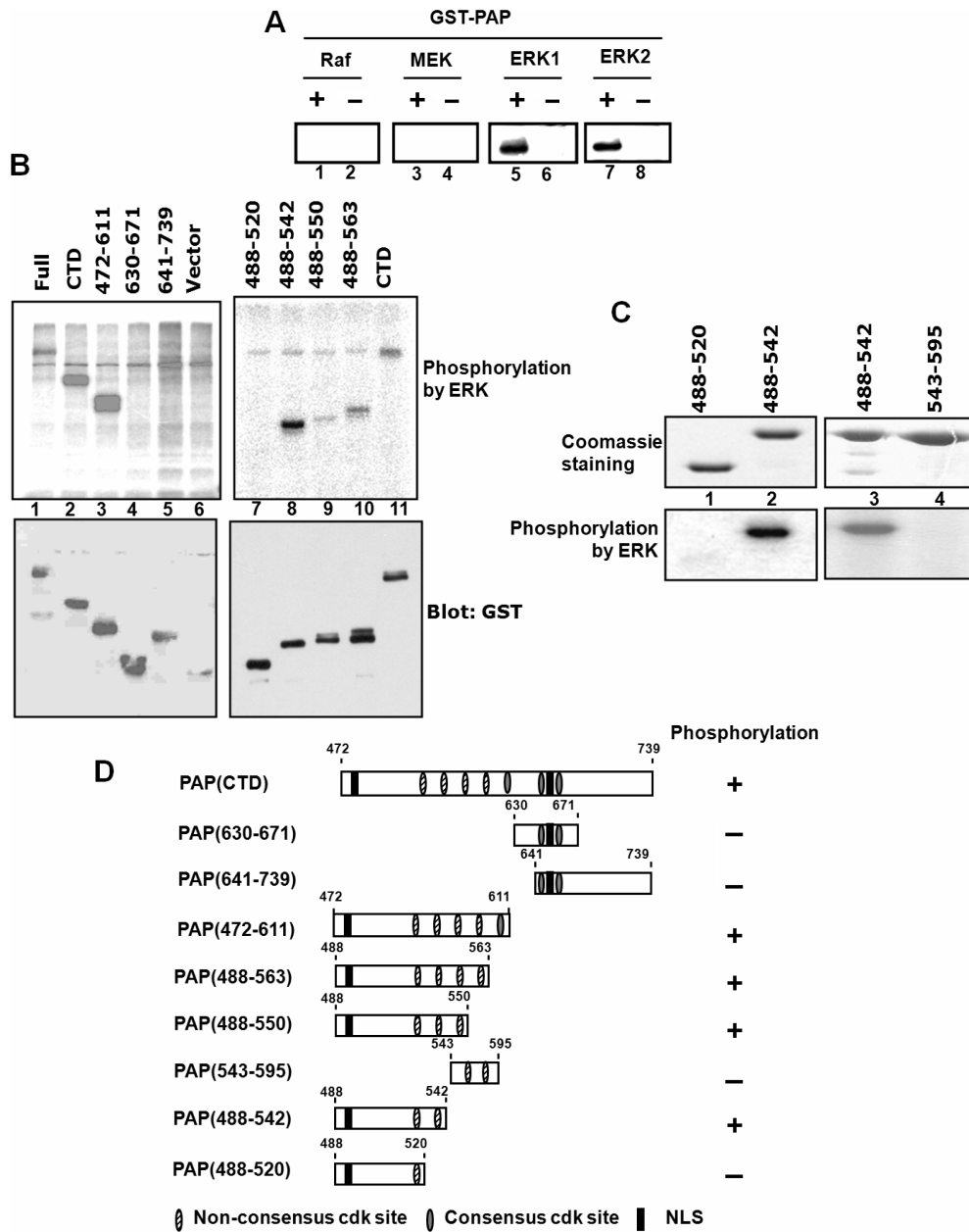


Figure 2. Phosphorylation of PAP by ERK *in vitro*. Lysates of HeLa cells transfected with (A) full-length GST-PAP or (B) GST-PAP truncation derivatives were incubated with glutathione-Sepharose beads. Glutathione beads were phosphorylated by constitutively active kinases in the presence of [γ - 32 P]ATP for 1 h at 37°C. Sample buffer was added to stop the reaction and proteins were separated by SDS-PAGE. Phosphorylated bands were visualized by autoradiography. Expression of PAP derivatives were confirmed by immunoblot on the lysates with GST antibody. (C) GST fusion derivatives were expressed in *E. coli*. Fusion proteins were eluted from glutathione-Sepharose beads and each proteins were phosphorylated by constitutively active ERK in the presence of [γ - 32 P]ATP for 1 h at 37°C. The products were analyzed as in (B) and input proteins were also visualized by Coomassie staining. (D) Data of (B) and (C) are schematically presented to show the region of PAP required for the phosphorylation by ERK.

Anti-phosphoserine 537 serum recognizes phosphoserine 537 of PAP

To examine the phosphorylation status of serine 537 of PAP *in vivo*, we used a phosphorylated peptide corresponding to the region of PAP around serine 537 (SMSVPpSPTSAT) to generate anti-phosphoserine 537 specific antibody. The peptide, encoded in exon 17, is shared by PAP II, PAP I and other isoforms (Figure 4A), and is well conserved in PAP from various vertebrates

(Figure 4B). To confirm the specificity of the antibody, we expressed and purified the 488–543 region of PAP fused to GST and a S537A mutant derivative from *E. coli* and treated them with ERK *in vitro*. Then they were analyzed by western blot using the phospho-specific antibody. The ERK-treated 488–542 substrate was recognized by the phosphoserine antibody, but the untreated 488–542 substrate and the ERK-treated S537A mutant were not (Figure 4C). These results demonstrate the specificity of

Table 1. Mascot search result of phosphorylated PAP-CTD^a

| Query | Observed ^b | Mr(expt) ^c | Mr(calc) ^d | Delta ^e | Miss ^f | Score ^g | Expect ^h | Rank ⁱ |
|-------|-----------------------|-----------------------|-----------------------|--------------------|-------------------|--------------------|---------------------|-------------------|
| 1 | 1005.52 | 3013.52 | 3013.36 | 0.17 | 0 | 44 | 0.58 | 1 |
| 2 | 1010.85 | 3029.51 | 3029.35 | 0.16 | 0 | 66 | 0.0034 | 1 |
| 3 | 1032.21 | 3093.59 | 3093.32 | 0.27 | 0 | 17 | 2.8e + 02 | 1 |
| 4 | 1032.51 | 3094.49 | 3093.32 | 1.17 | 0 | 26 | 36 | 1 |
| 5 | 1037.51 | 3109.49 | 3109.32 | 0.18 | 0 | 39 | 2.1 | 1 |

| Query | Peptide ^j |
|-------|---|
| 1 | LTALNDSSLDLSDNSMSPVPSPTSAMK |
| 2 | LTALNDSSLDLSDNSMSPVPSPTSAMK + Oxidation (M) |
| 3 | LTALNDSSLDLSDNSMSPVPSPTSAMK + Phospho (ST) |
| 4 | LTALNDSSLDLSDNSMSPVPSPTSAMK + Phospho (ST) |
| 5 | LTALNDSSLDLSDNSMSPVPSPTSAMK + Oxidation (M); Phospho (ST) |

^aThe C-terminal domain from 488 to 739 of PAP was overexpressed in *E. coli* and purified. The purified CTD was phosphorylated by ERK *in vitro*. Untreated and phosphorylated CTD were compared by Q-TOF Electrospray Ionization Mass Spectrometry (ESI/MS). Fragment ion spectra generated from ESI/MS were analyzed using MASCOT tools.

^bExperimental *m/z* value.

^cTransformed relative molecular mass from experimental *m/z*.

^dCalculated relative molecular mass of the matched peptide.

^eDifference between the experimental and calculated masses.

^fNumber of missed trypsin cleavage sites.

^gIons score.

^hExpectation value for the peptide match.

ⁱRank of the ions match (1–10, where 1 is the best match).

^jMatched peptide.

the antibody and provide another line of evidence that serine 537 of PAP is phosphorylated by ERK.

ERK phosphorylates serine 537 of PAP *in vivo*

To determine whether ERK phosphorylates PAP *in vivo* at serine 537, we stimulated ERK activity in HeLa cells and assessed PAP phosphorylation. We tested whether PAP was phosphorylated at serine 537 when the growth of the quiescent HeLa cells was stimulated by the addition of serum. GST-CTD was first examined for phosphorylation using the anti-phosphoserine 537 specific antibody (Figure 5A). The phosphorylation signals were quantified against the amount of GST-CTD. Phosphorylation at serine 537 peaked at 10 min with the concomitant increase of the phosphorylated forms of ERK after the serum addition, but the co-treatment with PD98059 inhibited the phosphorylation. Next, we further tested whether that full-length PAP is phosphorylated by ERK using GST-PAP (Figure 5B). HeLa cells transfected with the GST-PAP construct were treated with phorbol-12-myristate-13-acetate (PMA), a stimulatory agonist, which activates ERK. Phosphorylation of GST-PAP was examined with anti-phosphoserine 537 specific antibody. We observed that the PMA treatment increased the phosphorylation and that the increase was correlated with the activation of ERK. In addition, the co-treatment with PD98059 reduced the PMA effect on phosphorylation. However, PD98059 did not entirely inhibit phosphorylation at serine 537. It remains to be demonstrated whether this incomplete inhibition was due to the residual activity of ERK even in the presence of PD98059 or it reflects the presence of another kinase that can phosphorylate serine 537 of PAP.

We also investigated whether endogenous PAP is phosphorylated at serine 537 by ERK. HeLa cell lysates

were prepared after the PMA stimulation, immunoprecipitated with anti-phosphoserine 537 antibody and the immunoprecipitates were immunoblotted with anti-PAP antibody. When this co-immunoprecipitation experiment was employed with cells treated with PMA for 10 min, a 106 kD protein band (33) appeared in the immunoprecipitates by anti-phosphoserine 537 antibody, but not by preimmune antiserum (Figure 6A). Therefore, we conclude that this band is the phosphorylated PAP at serine 537. The 106 kD band signal increased and reached to the maximum in 10 min after the PMA addition, but its signal was not increased by the co-treatment with PD98059 (Figure 6B). Taken together, our data suggest that ERK phosphorylates serine 537 of PAP *in vivo*. A 100 kD band was detected with pre-immune serum in lane 3 of Figure 6A. Although we did not further examine its identity, we conclude that this band is not related to phosphorylation of serine 537 because it was not observed with anti-phosphoserine 537 antibody.

Effect of phosphorylation at serine 537 on PAP activity

To examine effects of phosphorylation of PAP at serine 537, we measured the nonspecific polyadenylation activity of PAP in a Mn²⁺-containing reaction buffer (28). Flag-PAP was prepared from HeLa cell lysates by immobilization using anti-Flag antibody and protein G beads. It is likely that Flag-PAP purified from unsynchronized cells is partially phosphorylated. This partial phosphorylation might make it difficult to evaluate the effect of ERK on *in vitro* PAP activity. We treated the HeLa cell lysates with protein phosphatase prior to the purification of Flag-PAP and then treating with ERK. The polyadenylation activity of the purified Flag-PAP was assayed before and after ERK treatment and reaction products were analyzed by gel electrophoresis

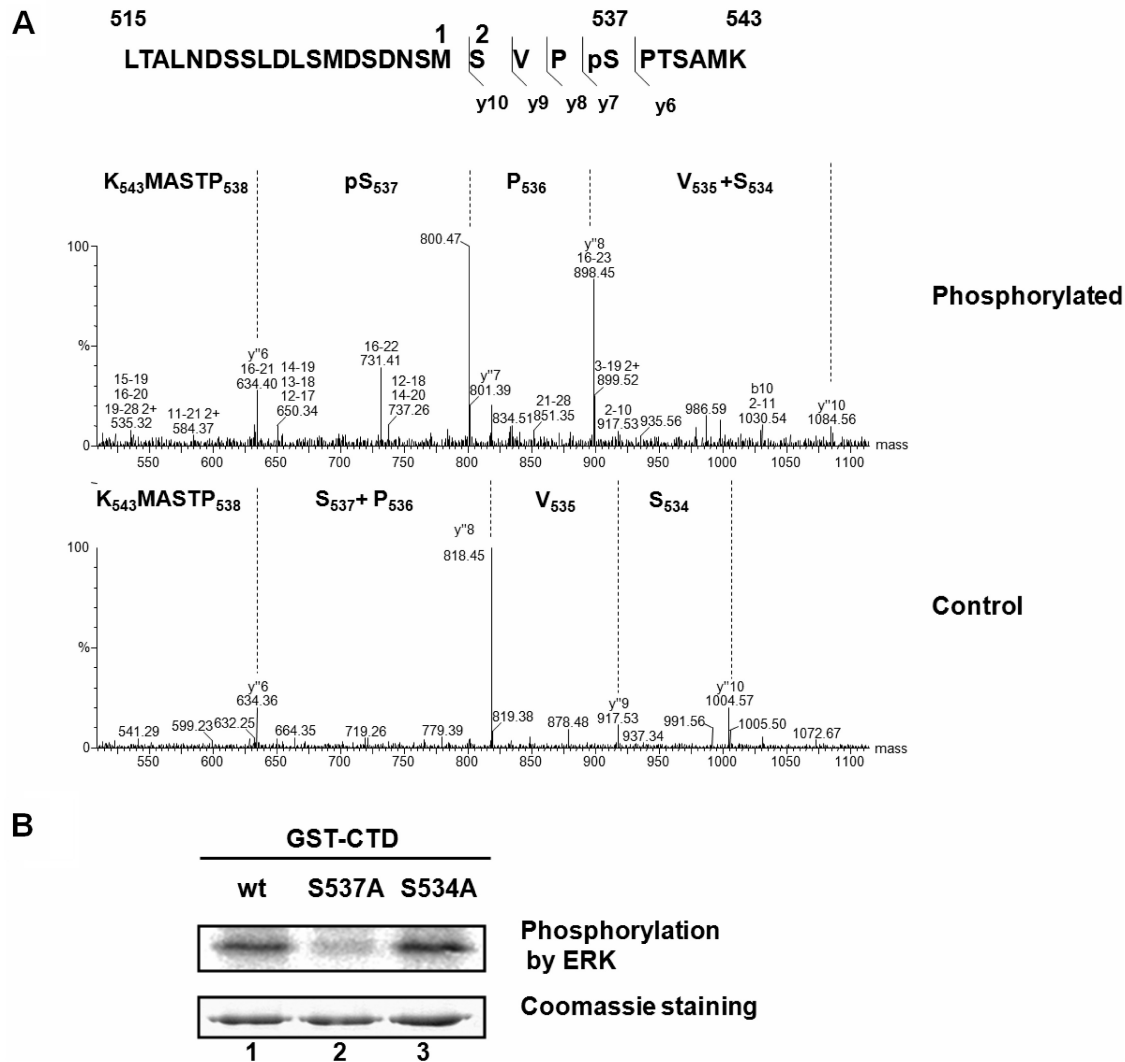


Figure 3. Analysis of phosphorylation site. (A) MS/MS spectrum of the doubly charged ion of the peptide including S537. CTD of PAP (residues 488–739) was overexpressed in *E. coli* and purified. The purified CTD was phosphorylated by ERK using 10 mM ATP *in vitro*. The untreated (control) and phosphorylated CTD forms were excised from gel and compared by Q-TOF MS/MS analysis. The spectra correspond to the y-ion fragment series. The sequences (C- to N-terminus) within the spectrum correspond to the signals indicated by the vertical lines. (B) The wild-type GST-CTD (lane 1) and mutants S537A (lane 2) and S534A (lane 3) were expressed in *E. coli* and purified. The purified GST-CTD proteins were phosphorylated by ERK *in vitro* using [γ - 32 P]ATP. Phosphorylated band were visualized by autoradiography after SDS-PAGE. Input proteins (200 ng) were also visualized by Coomassie staining.

and thin layer chromatography (Figure 7). The activity of Flag-PAP increased by about 40% with the ERK treatment, while that of the S537A mutant was unaffected, suggesting that phosphorylation of PAP at serine 537A by ERK increased its poly(A) addition activity. These results indicate that PAP activity is increased by single-site phosphorylation at serine 537 by ERK.

Since the PMA treatment causes phosphorylation of PAP at serine 537 in the cell, we also used GST-PAP purified from the PMA-treated cells for the *in vitro* phosphorylation assay. We found that the PAP activity increased with the PMA induction, but that of the S537A mutant did not (Figure 8A). Furthermore, the increase of the PAP activity by the PMA induction disappeared when treated concomitantly with an ERK inhibitor, PD98059

(Figure 8B). These results further support that phosphorylation of PAP at serine 537 increases its activity.

We also carried out the polyadenylation assays in the presence of MgCl₂ using a substrate carrying the polyadenylation signals. However, we did not observe the specific polyadenylation products (data not shown), possible due to lack of other 3' processing factors in the purified Flag-PAP or GST-PAP.

DISCUSSION

In this study, we show that PAP is phosphorylated at a serine 537 in response to extracellular growth stimuli. Since the PAP phosphorylation increases its poly(A) addition activity *in vitro*, we conclude that PAP is

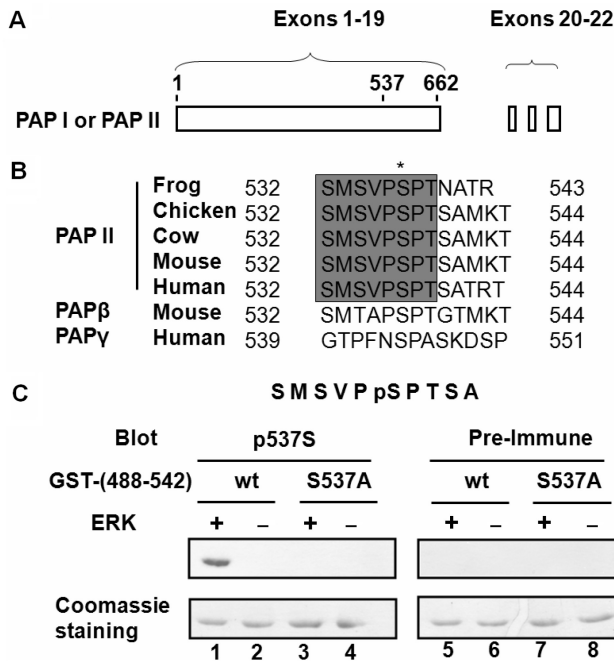


Figure 4. Generation of anti-phosphoserine 537 antibody. (A) The position of serine 537 is shown in exons of PAP. PAP I and PAP II share the region exons 1–19. (B) Amino acid sequences near 537 serine are conserved in vertebrate PAPs. The sequences of frog, cow, human, chicken and mouse PAP were shown aligned. The best alignments for PAP paralogs, PAPβ and PAPγ were also shown. (C) Antisera against the phosphospecific-peptide corresponding to 10 amino acids around serine 537 was generated from rat. The GST-488-542 PAP derivative (lanes 1, 2, 5 and 6) and mutant S537A (lanes 3, 4, 7 and 8) were purified from *E. coli* and phosphorylated by ERK *in vitro* using 10 mM ATP. Odd-numbered lanes, ERK-treated; even-numbered lanes, no ERK-treated. The products were analyzed by immunoblot with anti-phosphoserine 537 (lanes 1–4) or pre-immune serum for control (lanes 5–8).

regulated through an ERK-dependent signaling pathway. However, we do not exclude the possibility that PAP phosphorylation at serine 537 differently affects specific polyadenylation *in vivo* because phosphorylation may participate in modulation of interaction between PAP and other factors involved in 3' processing of mRNA. Since poly(A) tails are implicated in maintaining mRNA stability, exporting mRNA from nucleus to cytoplasm, and enhancing translation efficiency (13,14), regulation of PAP could be an important mechanism by which cells can alter gene expression.

Our finding that PAP activity can be regulated by phosphorylation through an ERK-dependent pathway supports the hypothesis that PAP activity responds to extracellular growth stimuli because ERK is a member of the MAPK family of kinases (34). Upon mitogenic growth factor activation, ERK promotes both the proliferation of quiescent cells in G0 and the progression of cell cycling through the restriction point in G1 phase (35). Our results show that the increase of phosphorylation at serine 537 occurs in about 10 min after serum induction, which coincides with the timing of ERK activation, implying that PAP activity is activated during cell proliferation

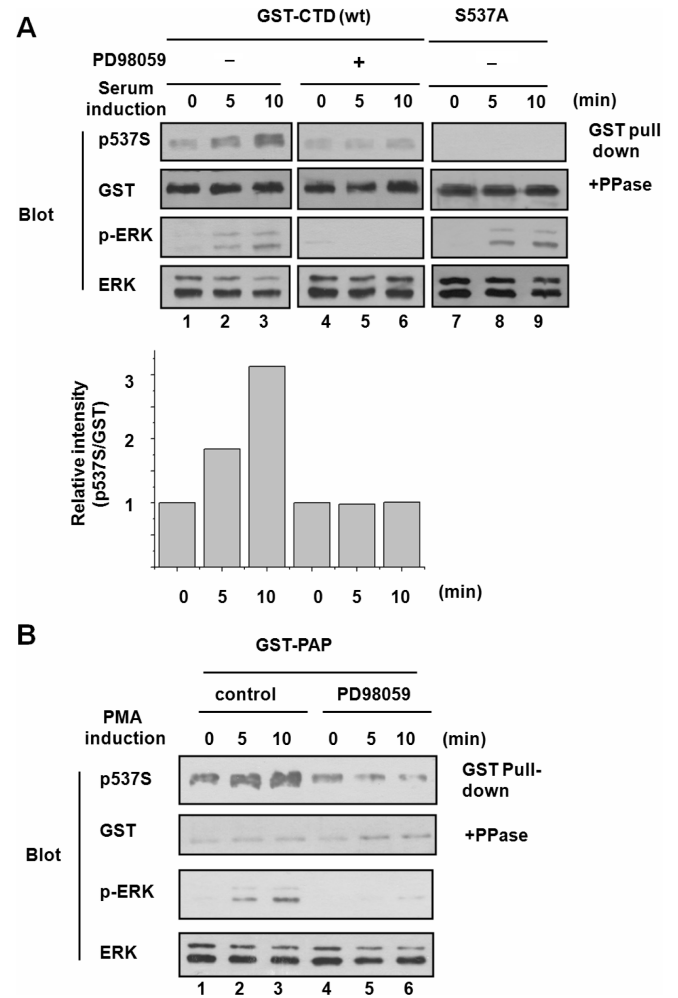


Figure 5. Phosphorylation of serine 537 by stimulation of ERK *in vivo*. (A) HeLa cells were transfected with GST-CTD (residues 472–739) (lanes 1–6) and its S537 mutant (lanes 7–9). Cells were cultured in serum free media for 12 h and then stimulated with 20% serum (lanes 1–3 and 7–9) or 20% serum containing PD98059 (50 μM) (lanes 4–6) for the indicated time (lanes 1, 4 and 7, 0 min; lanes 2, 5 and 8, 5 min; lanes 3, 6 and 9, 10 min). To examine the phosphorylation status of serine 537, GST pull-downed complexes were visualized by immunoblot with anti-phosphoserine 537 antiserum. Activation of ERK in total lysates was confirmed by immunoblot with anti-p-ERK antibody. The phosphorylation signals were quantified against the amount of GST-CTD treated with λ protein phosphatase, and shown below the immunoblots. (B) HeLa cells were transfected with full-length GST-PAP. PMA alone (lanes 1–3) or with PD98059 (50 μM) (lanes 4–6) was added to 100 ng/ml at 12 h after transfection and the cells were further incubated (lanes 1 and 4, 0 min; lanes 2 and 5, 5 min; lanes 3 and 6, 10 min). GST pull-downed complexes from the cell lysates were visualized by immunoblot with anti-phosphoserine 537 antiserum. The amounts of GST-CTD present in the lysates were evaluated with anti-GST antibody after the treatment with λ protein phosphatase.

upon serum induction, which coincides with the timing of ERK activation, implying that PAP activity is activated during cell proliferation upon serum induction. In addition to its important role in the proliferation of quiescent cells, ERK is involved in a various cellular functions including apoptosis, proliferation and differentiation (36,37). Furthermore, ERK participates in regulating the somatic cell cycle in G2 and M phase (38) and

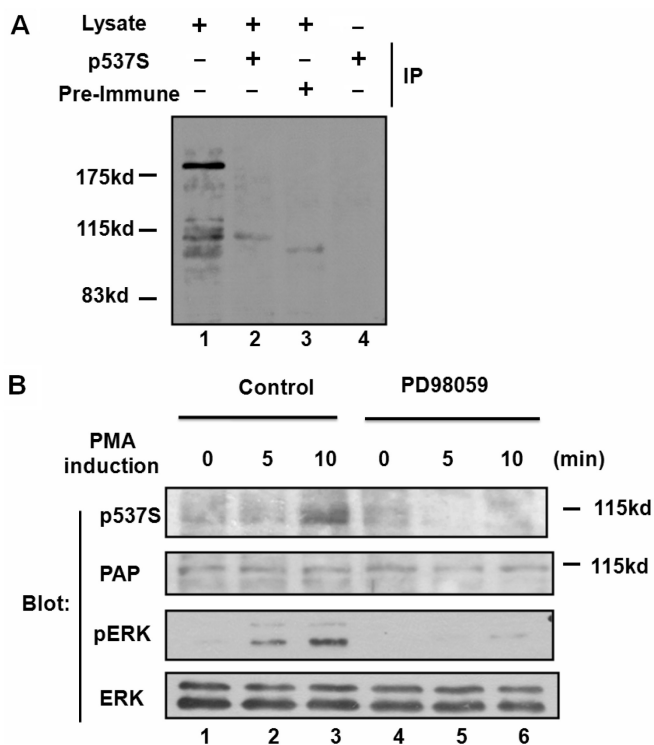


Figure 6. Phosphorylation of serine 537 of endogenous PAP. HeLa cells were cultured in media with 10% serum. When cell confluence reaches 70–80%, PMA was added to 100 ng/ml. At 10 min after the PMA treatment, cell extracts were prepared. They were immunoprecipitated with anti-phosphoserine 537 antibody (lane 2) or pre-immune serum (lane 3). The immunoprecipitates were blotted with anti-PAP antibody. Lysates (5% input) alone (lane 1) or anti-phosphoserine 537 antibody alone (lane 4) before the immunoprecipitation was electrophoresed and blotted with anti-PAP antibody for controls. **(B)** Alternatively, cell extracts were prepared at the indicated time (lanes 1 and 4, 0 min; lanes 2 and 5, 5 min; lanes 3 and 6, 10 min) after PMA alone (lanes 1–3) or with PD98059 (50 μ M) (lanes 4–6) was added to 100 ng/ml. The lysates were blotted with anti-phosphoserine 537, anti-PAP, anti-pERK or anti-ERK antibody.

is also activated in *Xenopus* oocyte maturation (39). Therefore, PAP regulation by ERK could be used as a general means to modulate gene expression by varying poly(A) mRNA tail length during those varied and critical cellular events.

Phosphorylation of PAP at serine 537 by ERK had not been predicted previously, because serine 537 is one of several PAP nonconsensus cdk sites, which can be phosphorylated p34cdc/cyclin B. Furthermore, PAP has no apparent ERK recognition site (PXS/P, where X is a neutral or basic amino acid) (31) or ERK docking site (DEF: docking site for ERK, FxFP) (40). Serine or threonine followed by a proline (S/TP) was suggested as the minimal consensus sequence for ERK (41,42). In the case of PAP, in fact, serine 537 is followed by proline, but it still remains obscure how ERK recognizes PAP. The kinase interaction motif (KIM) of ERK serves as a common docking domain for interactions with other proteins such as activators, regulators and substrates (43). Interaction between positively charged amino acid clusters of substrates and aspartic acid in the KIM site of ERK is

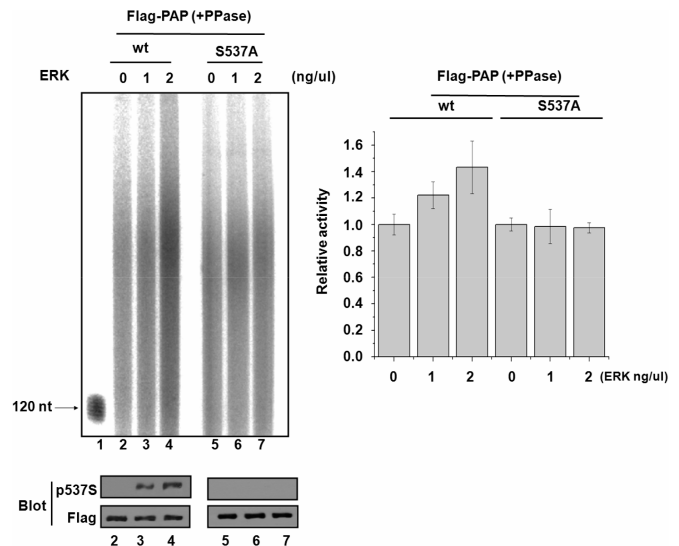


Figure 7. Effect of phosphorylation of serine 537 on nonspecific activity of PAP. **(A)** HeLa cells were transfected with fusions of Flag-PAP wt (lanes 2–4) or S537A (lanes 5–7) and cell lysates were treated with λ -protein phosphatase. Flag-PAPs were precipitated by incubating cell lysates with protein G agarose beads and anti-Flag antibody. Flag-PAP-bound beads were phosphorylated with the indicated amounts of ERK (lanes 2 and 5, 0 ng/ μ l; lanes 3 and 6, 1 ng/ μ l; lanes 4 and 7, 2 ng/ μ l) *in vitro* and used for the *in vitro* polyadenylation assay. We determined the activity of Flag-PAP by measuring incorporation of AMP from [α - 32 P] ATP into 120 nt-length RNA. Polyadenylated RNAs were analyzed on a 5% polyacrylamide gel containing 8 M urea (left). The 120 nt-length RNA labeled at the 3' end with [32 P]pCp was electrophoresed as a control (lane 1). The Flag-PAP present in the purified fraction was semiquantitatively determined by immunoblotting with anti-Flag. Phosphorylation of PAP at serine 537 was visualized by immunoblot with anti-phospho-537 serine. Alternatively, polyadenylated RNAs were separated from free [α - 32 P] ATP in PEI membrane by TLC and analyzed by scintillation (right). Relative PAP activities are expressed as the quantities of polyadenylated RNAs relative to that of RNA polyadenylated by Flag-PAP(wt) alone after normalization to immunoblot signals of PAP. The values are calculated from three independent experiments.

known to be required for recognition (44). There is a cluster of positively charged residues (bold and underlined) near the serine 537 residue, 489 **KR KQLHQ LLPSHV LQKRK KKHST** 500. It is possible that ERK recognizes PAP through an interaction between KIM and positively charged amino acids near the serine 537 residue. Interestingly, PAP β , which is identified as a testis-specific PAP (45,46), has the conserved sequences near serine 537 and the KIM site, suggesting that PAP β can be phosphorylated by ERK at serine 537 (Figure 4). On the other hand, PAP γ , which is another PAP paralog (47), does not show any similarity with the sequences near serine 537 of PAP (Figure 4) or the KIM site.

It is well known that phosphorylation of a protein can lead to a conformational change of the protein that results in an increase or decrease in activity. Protein phosphorylation is also able to change the affinity of binding proteins. There are several lines of evidence that PAP activity is modulated by phosphorylation. In the M-phase of the cell cycle, hyperphosphorylation of PAP by p34cdc/cyclin B on cdk sites represses PAP activity (24), as

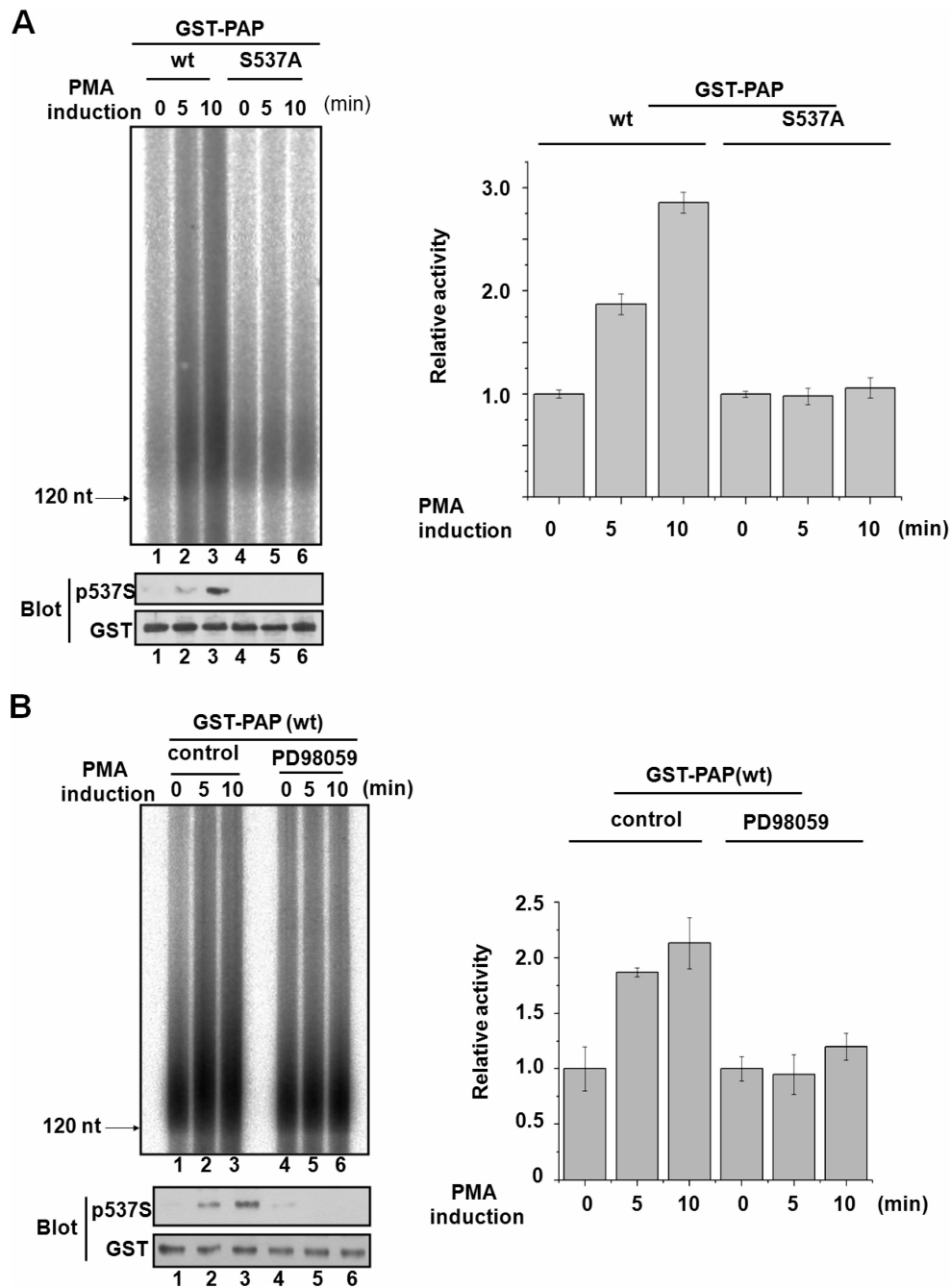


Figure 8. Nonspecific activity of PAP isolated from HeLa cells treated with PMA. **(A)** Non-specific activities of PAP from HeLa cells transfected with GST-PAP wt [lanes 1–3] or S537A [lanes 4–6] were measured prior to and following the treatment with PMA (100 ng/ml). The cells were harvested at the indicated time (lanes 1 and 4, 0 min; lanes 2 and 5, 5 min; lanes 3 and 6, 10 min) after the treatment with PMA (100 ng/ml). GST-PAPs were precipitated by incubating cell lysates with glutathione beads. GST-PAP-bound beads were used for the *in vitro* polyadenylation assay. We determined the activity of Flag-PAP by measuring incorporation of AMP from [α - 32 P] ATP into 120 nt-length RNA. Polyadenylated RNAs were analyzed on a 5% polyacrylamide gel containing 8 M urea (left). The position of the 120 nt-length substrate RNA is indicated by an arrow. The GST-PAP present in the purified fraction was semiquantitatively determined by immunoblotting with anti-GST. Phosphorylation of PAP at serine 537 was visualized by immunoblot with anti-phospho-537 serine. Alternatively, polyadenylated RNAs were separated from free [α - 32 P] ATP in PEI membrane by TLC and analyzed by scintillation (right). Relative PAP activities are calculated as in Figure 7. **(B)** HeLa cells were transfected with GST-PAP. The cells were harvested at the indicated time (lanes 1 and 4, 0 min; lanes 2 and 5, 5 min; lanes 3 and 6, 10 min) after the treatment with PMA (100 ng/ml) alone (lanes 1–3) or with PD98059 (50 μ M) (lanes 4–6). GST-PAPs were precipitated and used for the *in vitro* polyadenylation assay, as in (A).

discussed in the Introduction. Recombinant PAP purified from insect cells shows lower activity than the recombinant enzyme purified from *E. coli*, as a feature attributed to phosphorylation: insect recombinant enzymes have

about 5-fold higher K_m -values for the substrate (48). When molt-3 cells are infected with human immunodeficiency virus-1 (HIV-1), phosphorylation of PAP increases PAP activity although the kinase responsible for the

phosphorylation is unclear (49,50). Therefore, a picture had emerged that phosphorylation of different sites resulted in different effects on the activity of PAP, but the effect of phosphorylation at any specific site was not defined until this work. Here, we show that single site-phosphorylation at serine 537 is responsible for the increase of PAP activity. Colgan *et al.* (24) previously suggested that that full phosphorylation at both consensus and nonconsensus cdk sites represses PAP activity. Since serine 537 was previously considered a nonconsensus cdk site, it is likely that the effect of single-site phosphorylation on PAP activity differs from that of hyperphosphorylation at the multiple sites. Phosphorylation at other nonconsensus or consensus cdk sites may interfere with that at serine 537 in modulation of PAP activity.

In summary, we have shown that PAP is phosphorylated in response to extracellular stimuli. This phosphorylation occurs by action of ERK. ERK phosphorylates the serine 537 residue of PAP specifically and the phosphorylation stimulates PAP activity. These data suggest that ERK is a regulatory kinase for PAP. Given that ERK is an important downstream component in the MAPK cascade that serves to integrate a variety of extracellular signals, our findings indicate that PAP activity can be modulated through ERK signaling pathways by extracellular signals.

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REFERENCES

1. Sheets, M.D., Ogg, S.C. and Wickens, M.P. (1990) Point mutations in AAUAAA and the poly (A) addition site: effects on the accuracy and efficiency of cleavage and polyadenylation *in vitro*. *Nucleic Acids Res.*, **18**, 5799–5805.
2. Zarkower, D., Stephenson, P., Sheets, M. and Wickens, M. (1986) The AAUAAA sequence is required both for cleavage and for polyadenylation of simian virus 40 pre-mRNA *in vitro*. *Mol. Cell Biol.*, **6**, 2317–2323.
3. Gil, A. and Proudfoot, N.J. (1987) Position-dependent sequence elements downstream of AAUAAA are required for efficient rabbit beta-globin mRNA 3' end formation. *Cell*, **49**, 399–406.
4. Keller, W., Bienroth, S., Lang, K.M. and Christofori, G. (1991) Cleavage and polyadenylation factor CPF specifically interacts with the pre-mRNA 3' processing signal AAUAAA. *EMBO J.*, **10**, 4241–4249.
5. Barabino, S.M. and Keller, W. (1999) Last but not least: regulated poly(A) tail formation. *Cell*, **99**, 9–11.
6. Zhao, J., Hyman, L. and Moore, C. (1999) Formation of mRNA 3' ends in eukaryotes: mechanism, regulation, and interrelationships with other steps in mRNA synthesis. *Microbiol. Mol. Biol. Rev.*, **63**, 405–445.
7. Shatkin, A.J. and Manley, J.L. (2000) The ends of the affair: capping and polyadenylation. *Nat. Struct. Biol.*, **7**, 838–842.
8. Takagaki, Y. and Manley, J.L. (2000) Complex protein interactions within the human polyadenylation machinery identify a novel component. *Mol. Cell Biol.*, **20**, 1515–1525.
9. McGrew, L.L., Dworkin-Rastl, E., Dworkin, M.B. and Richter, J.D. (1989) Poly(A) elongation during *Xenopus* oocyte maturation is required for translational recruitment and is mediated by a short sequence element. *Genes Dev.*, **3**, 803–815.
10. Salles, F.J., Darrow, A.L., O'Connell, M.L. and Strickland, S. (1992) Isolation of novel murine maternal mRNAs regulated by cytoplasmic polyadenylation. *Genes Dev.*, **6**, 1202–1212.
11. West, M.F., Verrotti, A.C., Salles, F.J., Tsirka, S.E. and Strickland, S. (1996) Isolation and characterization of two novel, cytoplasmically polyadenylated, oocyte-specific, mouse maternal RNAs. *Dev. Biol.*, **175**, 132–141.
12. McGrew, L.L. and Richter, J.D. (1990) Translational control by cytoplasmic polyadenylation during *Xenopus* oocyte maturation: characterization of cis and trans elements and regulation by cyclin/MPF. *EMBO J.*, **9**, 3743–3751.
13. Beelman, C.A. and Parker, R. (1995) Degradation of mRNA in eukaryotes. *Cell*, **81**, 179–183.
14. Tarun, S.Z. Jr and Sachs, A.B. (1996) Association of the yeast poly(A) tail binding protein with translation initiation factor eIF-4G. *EMBO J.*, **15**, 7168–7177.
15. Gunderson, S.I., Vagner, S., Polycarpou-Schwarz, M. and Mattaj, I.W. (1997) Involvement of the carboxyl terminus of vertebrate poly(A) polymerase in U1A autoregulation and in the coupling of splicing and polyadenylation. *Genes Dev.*, **11**, 761–773.
16. Wells, D.G., Dong, X., Quinlan, E.M., Huang, Y.S., Bear, M.F., Richter, J.D. and Fallon, J.R. (2001) A role for the cytoplasmic polyadenylation element in NMDA receptor-regulated mRNA translation in neurons. *J. Neurosci.*, **21**, 9541–9548.
17. Richter, J.D. (1999) Cytoplasmic polyadenylation in development and beyond. *Microbiol. Mol. Biol. Rev.*, **63**, 446–456.
18. Martin, G. and Keller, W. (1996) Mutational analysis of mammalian poly(A) polymerase identifies a region for primer binding and catalytic domain, homologous to the family X polymerases, and to other nucleotidyltransferases. *EMBO J.*, **15**, 2593–2603.
19. Martin, G., Keller, W. and Doublet, S. (2000) Crystal structure of mammalian poly(A) polymerase in complex with an analog of ATP. *EMBO J.*, **19**, 4193–4203.
20. Raabe, T., Murthy, K.G. and Manley, J.L. (1994) Poly(A) polymerase contains multiple functional domains. *Mol. Cell Biol.*, **14**, 2946–2957.
21. Phillips, C., Jung, S. and Gunderson, S.I. (2001) Regulation of nuclear poly(A) addition controls the expression of immunoglobulin M secretory mRNA. *EMBO J.*, **20**, 6443–6452.
22. Kim, H., Lee, J.H. and Lee, Y. (2003) Regulation of poly(A) polymerase by 14-3-3epsilon. *EMBO J.*, **22**, 5208–5219.
23. Vagner, S., Vagner, C. and Mattaj, I.W. (2000) The carboxyl terminus of vertebrate poly(A) polymerase interacts with U2AF 65 to couple 3'-end processing and splicing. *Genes Dev.*, **14**, 403–413.
24. Colgan, D.F., Murthy, K.G., Zhao, W., Prives, C. and Manley, J.L. (1998) Inhibition of poly(A) polymerase requires p34cdc2/cyclin B phosphorylation of multiple consensus and non-consensus sites. *EMBO J.*, **17**, 1053–1062.
25. Hauser, H., Knippers, R. and Schafer, K.P. (1978) Increased rate of RNA-polyadenylation. An early response in concanavalin A activated lymphocytes. *Exp. Cell Res.*, **111**, 175–184.
26. Martincic, K., Campbell, R., Edwards-Gilbert, G., Souan, L., Lotze, M.T. and Milcarek, C. (1998) Increase in the 64-kDa subunit of the polyadenylation/cleavage stimulatory factor during the G0 to S phase transition. *Proc. Natl Acad. Sci. USA*, **95**, 11095–11100.
27. Bond, G.L., Prives, C. and Manley, J.L. (2000) Poly(A) polymerase phosphorylation is dependent on novel interactions with cyclins. *Mol. Cell Biol.*, **20**, 5310–5320.
28. Zhao, W. and Manley, J.L. (1996) Complex alternative RNA processing generates an unexpected diversity of poly(A) polymerase isoforms. *Mol. Cell Biol.*, **16**, 2378–2386.
29. Lutz, C.S., Murthy, K.G., Schek, N., O'Connor, J.P., Manley, J.L. and Alwine, J.C. (1996) Interaction between the U1 snRNP-A protein and the 160-kD subunit of cleavage-polyadenylation specificity factor increases polyadenylation efficiency *in vitro*. *Genes Dev.*, **10**, 325–337.

30. Aktas,H., Cai,H. and Cooper,G.M. (1997) Ras links growth factor signaling to the cell cycle machinery via regulation of cyclin D1 and the Cdk inhibitor p27KIP1. *Mol. Cell Biol.*, **17**, 3850–3857.
31. Clark-Lewis,I., Sanghera,J.S. and Pelech,S.L. (1991) Definition of a consensus sequence for peptide substrate recognition by p44mpk, the meiosis-activated myelin basic protein kinase. *J. Biol. Chem.*, **266**, 15180–15184.
32. Lubec,G., Afjehi-Sadat,L., Yang,J.W. and John,J.P. (2005) Searching for hypothetical proteins: theory and practice based upon original data and literature. *Prog. Neurobiol.*, **77**, 90–127.
33. Thuresson,A.C., Astrom,J., Astrom,A., Gronvik,K.O. and Virtanen,A. (1994) Multiple forms of poly(A) polymerases in human cells. *Proc. Natl Acad. Sci. USA*, **91**, 979–983.
34. Blenis,J. (1993) Signal transduction via the MAP kinases: proceed at your own RSK. *Proc. Natl Acad. Sci. USA*, **90**, 5889–5892.
35. Pardee,A.B. (1989) G1 events and regulation of cell proliferation. *Science*, **246**, 603–608.
36. Pearson,G., Robinson,F., Beers Gibson,T., Xu,B.E., Karandikar,M., Berman,K. and Cobb,M.H. (2001) Mitogen-activated protein (MAP) kinase pathways: regulation and physiological functions. *Endocr. Rev.*, **22**, 153–183.
37. Wang,X., Martindale,J.L. and Holbrook,N.J. (2000) Requirement for ERK activation in cisplatin-induced apoptosis. *J. Biol. Chem.*, **275**, 39435–39443.
38. Dangi,S., Chen,F.M. and Shapiro,P. (2006) Activation of extracellular signal-regulated kinase (ERK) in G2 phase delays mitotic entry through p21CIP1. *Cell Prolif.*, **39**, 261–279.
39. Carnero,A., Jimenez,B. and Lacal,J.C. (1994) Progesterone but not ras requires MPF for in vivo activation of MAPK and S6 K11: MAPK is an essential connexion point of both signaling pathways. *J. Cell Biochem.*, **55**, 465–476.
40. Jacobs,D., Glossip,D., Xing,H., Muslin,A.J. and Kornfeld,K. (1999) Multiple docking sites on substrate proteins form a modular system that mediates recognition by ERK MAP kinase. *Genes Dev.*, **13**, 163–175.
41. Davis,R.J. (1993) The mitogen-activated protein kinase signal transduction pathway. *J. Biol. Chem.*, **268**, 14553–14556.
42. Songyang,Z., Lu,K.P., Kwon,Y.T., Tsai,L.H., Filhol,O., Cochet,C., Brickey,D.A., Soderling,T.R., Bartleson,C. *et al.* (1996) A structural basis for substrate specificities of protein Ser/Thr kinases: primary sequence preference of casein kinases I and II, NIMA, phosphorylase kinase, calmodulin-dependent kinase II, CDK5, and Erk1. *Mol. Cell Biol.*, **16**, 6486–6493.
43. Kolch,W. (2000) Meaningful relationships: the regulation of the Ras/Raf/MEK/ERK pathway by protein interactions. *Biochem. J.*, **351**(Pt 2), 289–305.
44. Tanoue,T., Adachi,M., Moriguchi,T. and Nishida,E. (2000) A conserved docking motif in MAP kinases common to substrates, activators and regulators. *Nat. Cell Biol.*, **2**, 110–116.
45. Lee,Y.J., Lee,Y. and Chung,J.H. (2000) An intronless gene encoding a poly(A) polymerase is specifically expressed in testis. *FEBS Lett.*, **487**, 287–292.
46. Kashiwabara,S., Zhuang,T., Yamagata,K., Noguchi,J., Fukamizu,A. and Baba,T. (2000) Identification of a novel isoform of poly(A) polymerase, TPAP, specifically present in the cytoplasm of spermatogenic cells. *Dev. Biol.*, **228**, 106–115.
47. Kyriakopoulou,C.B., Nordvarg,H. and Virtanen,A. (2001) A novel nuclear poly(A) polymerase (PAP), PAP gamma. *J. Biol. Chem.*, **276**, 33504–33511.
48. Wittmann,T. and Wahle,E. (1997) Purification and characterization of full-length mammalian poly(A) polymerase. *Biochim. Biophys. Acta*, **1350**, 293–305.
49. Schroder,H.C., Rottmann,M., Wenger,R. and Muller,W.E. (1990) Dramatic increase in poly(A) synthesis after infection of Molt-3 cells with HIV. *Virus Res.*, **15**, 251–266.
50. Stetler,D.A., Seidel,B.L. and Jacob,S.T. (1984) Purification and characterization of a nuclear protein kinase from rat liver and a hepatoma that is capable of activating poly(A) polymerase. *J. Biol. Chem.*, **259**, 14481–14485.