Protein kinase A phosphorylation modulates transport of the polypyrimidine tract-binding protein

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The heterogeneous nuclear ribonucleoprotein particle (hnRNP) proteins play important roles in mRNA processing in eukaryotes, but little is known about how they are regulated by cellular signaling pathways. The polypyrimidine-tract binding protein (PTB, or hnRNP I) is an important regulator of alternative pre-mRNA splicing, of viral RNA translation, and of mRNA localization. Here we show that the nucleo-cytoplasmic transport of PTB is regulated by the 3′,5′-cAMP-dependent protein kinase (PKA). PKA directly phosphorylates PTB on conserved Ser-16, and PKA activation in PC12 cells induces Ser-16 phosphorylation. PTB carrying a Ser-16 to alanine mutation accumulates normally in the nucleus. However, export of this mutant protein from the nucleus is greatly reduced in heterokaryon shuttling assays. Conversely, hyperphosphorylation of PTB by coexpression with the catalytic subunit of PKA results in the accumulation of PTB in the cytoplasm. This accumulation is again specifically blocked by the S16A mutation. Similarly, in Xenopus oocytes, the phospho-Ser-16-PTB is restricted to the cytoplasm, whereas the non-Ser-16-phosphorylated PTB is nuclear. Thus, direct PKA phosphorylation of PTB at Ser-16 modulates the nucleo-cytoplasmic distribution of PTB. This phosphorylation likely plays a role in the cytoplasmic function of PTB.

The heterogeneous nuclear ribonucleoprotein particle (hnRNP) proteins are involved in a variety of processes in mRNA metabolism including pre-mRNA splicing, mRNA transport, and translation (1). These processes are often regulated by cellular signaling pathways, but how this dynamic control is achieved is mostly unknown. Some hnRNP proteins are known to be phosphorylated, but in most cases the particular kinase that modifies the protein is not known. Moreover, it is generally not clear how such modifications affect protein function. HnRNP proteins localize primarily in the nucleus at steady state but some of them engage in nucleo-cytoplasmic shuttling (2, 3). There are examples of hnRNP localization being altered by specific signaling pathways (4, 5).

The polypyrimidine tract-binding protein (PTB, or hnRNP I) has both nuclear and cytoplasmic functions. In the nucleus, it is a splicing repressor of a number of alternative exons (6, 7). In the cytoplasm, PTB plays a role in viral RNA translation through internal ribosome entry sites (8–11). Also in the cytoplasm, PTB is implicated in mRNA localization in Xenopus oocytes (12). The protein contains four RNA-recognition-motif type RNA binding domains (RRMs) and a conserved N-terminal domain. The N-terminal 55-aa segment of PTB contains both nuclear import and export signals and is sufficient to allow some nucleo-cytoplasmic shuttling in heterokaryon assays (13–17). There is an additional sequence within RRM2 that enhances nuclear export (17). At steady state, PTB is highly enriched in the nucleus, but its distribution must be regulated because the protein also has cytoplasmic functions. However, little is known about this regulation and what cellular signaling pathways impact it.

The 3′,5′-cAMP-dependent protein kinase A (PKA) transduces the signals of many extracellular factors including hormones and neurotransmitters (18). Activation of this kinase has a variety of downstream effects, including the phosphorylation of transcription factors in the nucleus (19), and of ion channels in the cytoplasm (20, 21). Activation of the PKA pathway can also regulate the subcellular localization of proteins, increasing their presence in either cytoplasm or nucleus (22–24).

In this report, we show that PKA phosphorylates PTB in vitro and in vivo and that this phosphorylation modulates the subcellular localization of the protein.

Materials and Methods

Cloning and Expression of PTB and PKA, Protein Phosphorylation Assay, Antibody Production, and Cell Culture. These methods are described in *Supporting Materials and Methods*, which is published as supporting information on the PNAS web site, www.pnas.org, and in refs 25 and 26.

Phospho-Peptide Analysis. Phospho-amino acid and phospho-peptide analyses were performed according to ref. 27. In vitro- or vivo-labeled PTB protein was blotted to poly(vinylidene difluoride) membranes. For phospho-amino acid analysis, the protein was hydrolysed with 6 M HCl at 110°C for 1 h. For phospho-peptide analysis, the protein was digested overnight with trypsin and chymotrypsin (50 μg/ml each) in 50 mM NaHCO₃ (pH 8.0). The samples were vacuum-dried, washed three times with 500 μl of water, resuspended in 10 μl of water, and dotted onto cellulose TLC plates (10 × 10 cm, EM Science). The plates were wetted with pH 1.9 running buffer (formic acid, 25 ml; glacial acetic acid, 78 ml; in 1,000 ml) and run at 1,000 Volts for 20 min. For phospho-amino acid analysis, the plates were then turned 90° clockwise and run in pH 3.5 buffer (50 ml glacial acetic acid/5 ml pyridine; in 1,000 ml) at 1,500 Volts for 37 min. For phospho-peptide analysis, the plates were air-dried and subjected to chromatography in separation buffer (37.5 ml 1-butanol/25 ml pyridine/7.5 ml glacial acetic acid; in 100 ml). Radioactive spots were visualized by phosphorimager. The standard phospho-amino acids were visualized with ninhydrin.

Heterokaryon Assay. Heterokaryon assays were performed according to (17) with slight modifications. HeLa or human embryonic kidney (HEK) 293 cells were fused with mouse NIH/3T3 cells 2 h after initiating cycloheximide (100 μg/ml) treatment. Fusion was induced by addition of 50% polyethylene glycol 3350 and incubation for 2–5 min. The cells were washed with DMEM, incubated for 5 h in culture media plus cycloheximide, and fixed for immunostaining. Human and mouse nuclei were distinguished by staining with Hoechst 33342 at ≈0.4 μg/ml in PBS.

Xenopus Oocyte Nuclear, Cytoplasmic Extracts, and Western Blots. Xenopus laevis stage III/IV oocytes (28) were defolliculated in 2 mg/ml type I collagenase (Sigma). Nuclei and cytoplasm were manually isolated at 4°C in nuclear separation buffer [50 mM Tris, pH 8.0/50 mM NaCl/0.05% IGEPAL CA-630 (Sigma)/100 units/ml RNasin ribonuclease inhibitor (Promega)/0.1 μg/ml leupeptin/0.1 μg/ml aprotonin/0.1 μg/ml trypsin inhibitor/0.4 mM Pefabloc/1.0 mM DTT]. Nuclear or cytoplasmic fractions were
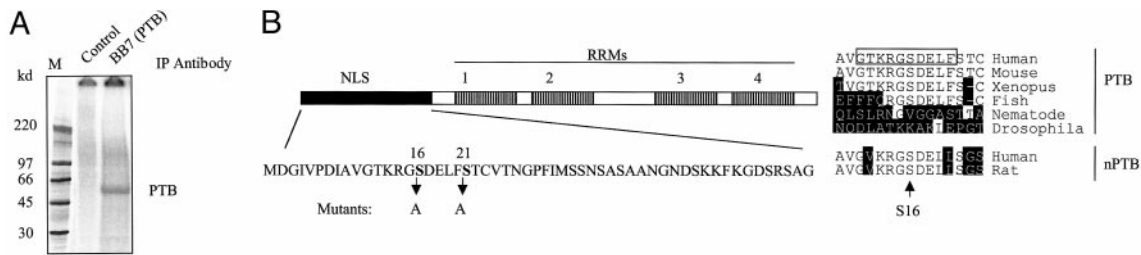
Fig. 1. Endogeneous PTB is a phospho-protein. (*A*) Immunoprecipitation using anti-PTB (BB7) or anti-Flag control antibody from HEK 293 cells labeled in ^{32}P-orthophosphoric acid. An SDS/PAGE gel of the immunoprecipitated proteins is shown. The molecular mass markers are to the left (M). (*B*) PTB domains and the position of the PKA target Ser-16 (*Left*). S16A and control S21A mutants are shown below the sequence. The alignment of the Ser-16 region from several species is shown (*Right*). The peptide sequence used for making antibodies is boxed.

homogenized in 1× Laemmli sample buffer, incubated at 95°C for 3 min, loaded directly onto a 10% SDS/polyacrylamide gel, and subsequently transferred to nitrocellulose membrane. The blots were incubated overnight at room temperature in primary antibody [either rabbit anti- pS16 at 1:250 dilution, anti-S16 at 1:500, or R3B3 (anti-*Xenopus* PTB) at 1:1,000]. Blots were washed and incubated with secondary antibody at 1:160,000 for 1 h at room temperature. All incubations and washes were in TBS (20 mM Tris, pH 7.5/0.5 M NaCl/1 mM Na$_3$VO$_4$) plus 5% powdered milk, except for the final wash (TBS without powdered milk) before detection. Peroxidase-conjugated goat anti-rabbit IgG (Sigma) was used as a secondary antibody, and was detected by chemiluminescence.

Results

Ser-16 of PTB Is Phosphorylated by PKA *in Vitro* and *in Vivo*. Phosphorylation is a common modulator of the activity of many proteins. To see whether PTB is a phospho-protein, we cultured HEK 293 cells in phosphate-free medium supplemented with ^{32}P-orthophosphoric acid to label cellular proteins. PTB was then immunoprecipitated with antibody BB7 (29). The PTB protein brought down by BB7 antibody was indeed labeled with orthophosphate (Fig. 1*A*). A similar result was obtained from WERI-1 retinoblastoma cells (data not shown). This finding indicates that a portion of the PTB in cells is phosphorylated.

Preliminary experiments indicated that the phosphorylation of PTB was on serine residues (data not shown and see below). In examining the PTB sequence, we found two potential PKA target serines that are conserved among vertebrate PTBs [(K/R)-X-S] (30, 31). These sites are also conserved in the neuronal PTB homologue, nPTB (32, 33). The two sites are Ser-16 in the motif RGS near the N terminus (Fig. 1*B*), and Ser-525 in the motif RVS at the C terminus.

To examine whether these sites could be directly phosphorylated by PKA, we incubated recombinant PTB with the purified catalytic subunit of PKA in the presence of [γ-^{32}P] ATP. We found that PKA phosphorylated PTB *in vitro* (Fig. 2*A*). Acid hydrolysis followed by separation of the phospho-amino acids by two-dimensional electrophoresis showed that PTB was phosphorylated only at serine residues, and not threonine or tyrosine residues (Fig. 2*B*). Mutation of Ser-16 to alanine (S16A) nearly abolished the PKA phosphorylation, but a control mutation, S21A, did not (Fig. 2*A*). The neuronal protein, nPTB, is also phosphorylated by PKA (Figs. 1*B* and 2*A*). These data indicate that Ser-16 is likely the major target site of PKA phosphorylation.

To confirm the site of PKA phosphorylation, we performed phospho-peptide analysis. Trypsin-chymotrypsin cleavage and phospho-peptide analysis by two-dimensional TLC showed that wild-type PTB had three phospho-peptides after PKA phosphorylation (Fig. 2*C*). Phospho-peptide 1 is by far the most prominent. The phosphorylation of both peptide 1 and peptide 2 was abolished by the S16A mutation and not by the S21A mutation, suggesting

that both peptides 1 and 2 contain the same PKA target serine residue.

To characterize these peptides further, we raised antibodies to a synthetic PTB peptide containing amino acids 11–20 (GTKRGS-DELF) with Ser-16 in either the phosphorylated or unphosphorylated state (Fig. 2*D*). The anti-phospho-Ser-16 antibody (anti-pS16) specifically immunoprecipitated phospho-peptide 1 (Fig. 2*C Right*). Peptide 2 was not immunoprecipitated by the anti-pS16 (Fig.
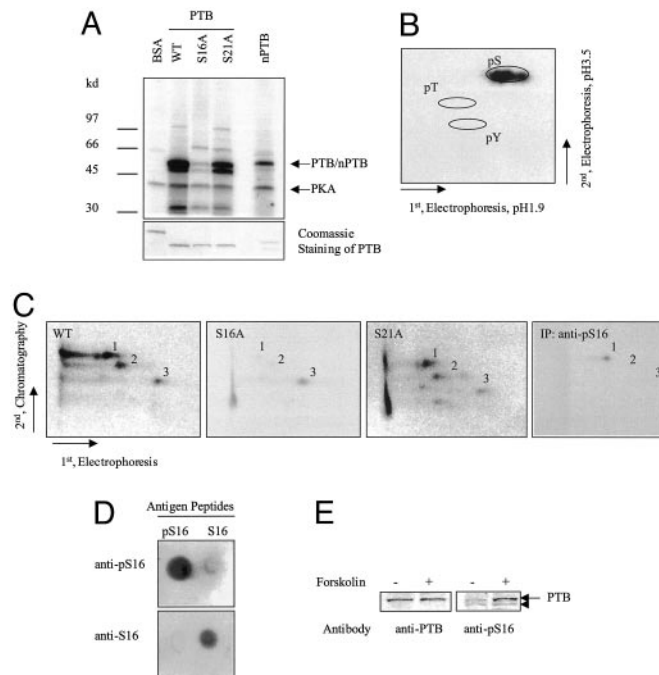

Fig. 2. PKA phosphorylates PTB at Ser-16 *in vitro* and *in vivo*. (*A*) Purified recombinant PTB, nPTB, and mutant proteins were incubated with purified PKA and [γ-^{32}P]ATP and separated on the gel shown. (*Upper*) Bands for phosphorylated PTB and nPTB, autophosphorylated PKA are indicated. (*Lower*) Coomassie blue-stained PTB is shown. (*B*) Phospho-amino acid analysis by two-dimensional electrophoresis. Positions of standard phospho-serine (pS), phospho-tyrosine (pY), or phospho-threonine (pT) are shown as ellipses. (*C*) (*Left*) Phospho-peptide mapping of the PKA-phosphorylated PTB and its mutants. (*Right*) TLC of the phospho-peptides immunoprecipitated with the pS16-specific antibody is shown. The wild-type and anti-pS16 IP samples were run simultaneously in the same tank to allow accurate alignment of the plates. (*D*) Dot blot showing the specificity of the pS16 and S16 antibodies for phosphorylated or unphosphorylated peptides. (*E*) Stimulation of the PKA pathway increases phosphorylation of PTB Ser-16 in PC12 cells. Total protein of PC12 cells treated with or without forskolin (10 μM) for 6 h, was probed with the antibodies as indicated. The arrow indicates the PTB band. The arrowhead indicates a band weakly recognized by the anti-pS16 antibody but not by the PTB antibody BB7. This band may be the neuronal PTB homologue nPTB.
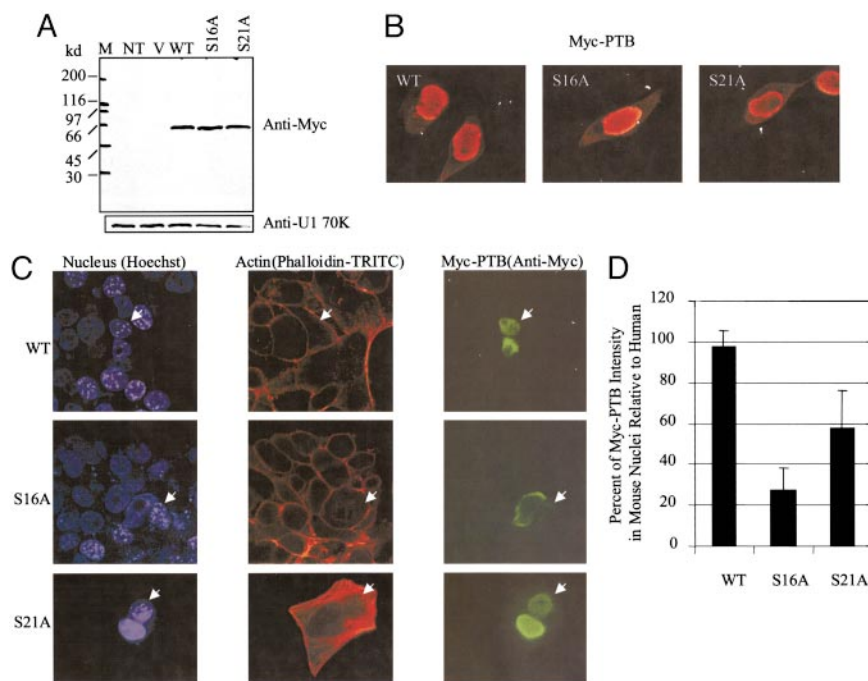
Fig. 3. Ser-16 is required for nuclear export of PTB. (*A*) A Western blot of the total protein from HEK 293 cells either not transfected (NT), or transfected with vector (V), wild-type Myc-PTB, or the S16A or S21A mutants. This was probed with anti-Myc antibodies to control for equal expression of the transfected clones as compared with the endogenous U1 70K protein. (*B*) Confocal microscopy of HEK 293 cells transfected with Myc-PTB plasmids and stained with anti-Myc. All of these proteins show strongly nuclear staining. (*C*) Heterokaryon assay of wild-type Myc-PTB or its mutants expressed in human HEK 293 cells that were fused with mouse NIH 3T3 cells. Mouse nuclei are distinguished from human nuclei by their punctate Hoechst staining. Actin staining of the fused cells with phalloidin-TRITC confirmed the cell fusion by determining that the actin cytoskeleton surrounded the multiple nuclei. Arrowheads indicate the positions of the mouse nuclei in heterokaryons. (*D*) Bar graph of the average ($\pm$SD) intensity of the Myc-PTB staining in the mouse nuclei relative to the human nuclei of the same heterokaryons ($n = 4$, 10, and 9 heterokaryons, respectively).

2*C*). This peptide could have a different serine residue whose phosphorylation is affected by the S16A mutation. Alternatively, it could be a peptide containing pS16 whose structure prevents immunoprecipitation. In summary, these experiments identify the primary site of PKA phosphorylation on PTB *in vitro* as residue Ser-16 (peptide 1).

Significantly, the anti-phospho-PTB antibodies also allowed us to confirm that Ser-16 phosphorylation occurs *in vivo* after PKA activation. We treated PC12 cells with the PKA inducer forskolin. We then probed the total cellular protein on immuno-blots for the presence of phospho-PTB using the anti-pS16 antibody (Fig. 2*E*). Although the total PTB in the cell is equivalent with or without forskolin, phospho-PTB was strongly stimulated by forskolin treatment. Similar results were obtained with another PKA stimulator, 8-(4-chlorophenylthio) adenosine-3′,5′-cyclic mono-phosphorothioate (cpt-cAMP) (data not shown). Thus, PKA activation *in vivo* leads to phosphorylation of PTB on Ser-16.

Ser-16 Is Critical for Nuclear Export of PTB. We next investigated the effect of Ser-16 phosphorylation on PTB function. The N-terminal 55 aa of PTB contain both nuclear localization signals (NLS) and nuclear export signals (NES) (13, 15–17). We thus tested the effect of the S16A mutation on the nuclear import or export of PTB.

Myc-tagged wild-type PTB or PTB mutants were expressed in HEK 293 cells and their subcellular localization was examined by immunostaining with anti-Myc antibody (Fig. 3 *A* and *B*). We found that wild-type PTB and its two mutants S16A and S21A were all predominantly nuclear, similar to endogeneous PTB. A small amount of cytoplasmic staining was seen with all three proteins, but no obvious differences were observed between them. This finding indicates that Ser-16 is not required for the nuclear import of PTB.

Next we examined the effect of the S16A mutation on PTB export in heterokaryon assays. HEK 293 cells were transfected with wild-type or mutant Myc-PTB. After 16–20 h, these cells were fused with mouse NIH/3T3 cells to form heterokaryons in the presence of the protein synthesis inhibitor cycloheximide. The human and mouse nuclei can be distinguished in a heterokaryon by the punctate pattern of staining seen in the mouse nuclei with Hoechst dye. The Myc-PTB transported to the mouse nucleus of a heterokaryon was detected by immunostaining with anti-Myc antibody

(Fig. 3*C*). Five hours after fusion, the intensity of wild-type Myc-PTB staining in mouse nuclei was nearly 100% that of the human nuclei of the same heterokaryons (4 heterokaryons measured, Fig. 3 *C* and *D*). In contrast, for the S16A mutant, mouse nuclear staining was only ≈25% that of the human nuclei (10 heterokaryons measured). Thus, the S16A mutation greatly decreases but does not eliminate PTB shuttling.

A previous report did not observe a difference in the shuttling of the wild-type PTB protein and the S16A mutant (16). However, these measurements were done 1 h after heterokaryon formation rather than at 5 h as done here. It takes at least 4 h after cell fusion for PTB shuttling to reach equilibrium (17). Our own measurements of this time course indicate that 1 h after fusion, the PTB concentration in the mouse nucleus has reached ≈17% that of the equilibrium state ($\pm$2.4%, $n = 10$ fusions; data not shown). At this earlier time point, the difference in shuttling between the two proteins is necessarily smaller. Thus the discrepancy between our results and the earlier report is likely caused by the difference in the postfusion time points examined in the two studies. At 5 h, the S16A mutant was dramatically reduced in the mouse nuclei relative to wild-type PTB (Fig. 3 *C* and *D*).

Because the S16A mutant can be imported into the HEK 293 nucleus (Fig. 3*B*), its failure to efficiently relocalize into the mouse nucleus in the heterokaryon assay is presumably due to a defect in export from the human nucleus. The other mutant, S21A, gave a partial effect, with mouse nuclear staining at ≈57% that of the human nuclei. The close proximity of Ser-21 to the critical residues (amino acid 11–15) (16) of the NES may cause its mutation to also affect this process. Taken together, these data suggest that phosphorylation of Ser-16 is important for the export of PTB from the nucleus to the cytoplasm, although some shuttling can occur without it.

PKA Coexpression Leads to Increased Phosphorylation at Ser-16 and Cytoplasmic Accumulation of PTB. To further examine the role of phosphorylation in PTB nuclear export, we tested the effect of PKA overexpression on PTB localization. In these experiments, a Flag-tagged, catalytic subunit of PKA was coexpressed with the Myc-PTB in HEK 293 cells and the resulting subcellular distribution and phosphorylation of Myc-PTB was monitored.
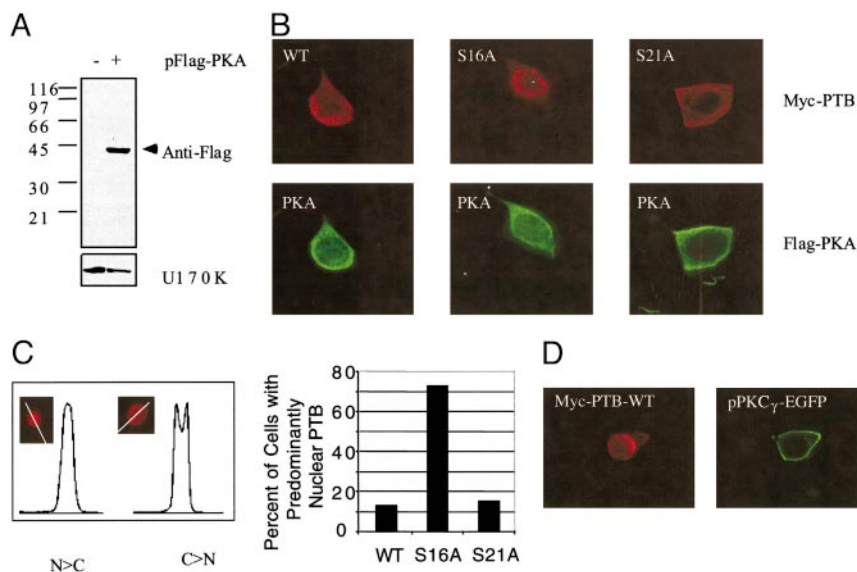
Fig. 4. PKA coexpression relocalizes PTB to the cytoplasm and the S16A mutation blocks this effect. (*A*) A Western blot of protein from HEK 293 cells transfected with Flag-tagged PKA. (*B*) Confocal microscopy of anti-Myc-stained PTB or anti-Flag-stained PKA in HEK 293 cells. (*C*) To distinguish cells with strong nuclear staining from those with stronger cytoplasmic Myc-PTB staining, anti-Myc immunoflourescence intensity was plotted along a line drawn across the cells. Cells showing a single peak of flourescence centered on the nucleus were counted as predominantly nuclear stainings. Cells showing a bimodal distribution with a dip over the nucleus were counted as predominantly cytoplasmic. These data are presented in a bar graph at the right as the percentage of cells with predominantly nuclear staining of Myc-PTB with PKA coexpression. These data are from the cotransfection of 2 μg of Myc-PTB with 6 μg of PKA plasmid, where *n* for each bar is 178, 107, and 78 cells, respectively. (*D*) Activating the PKC pathway does not relocalize Myc-PTB to cytoplasm. Shown are the confocal images of Myc-PTB cotransfected with pPKCγ-EGFP. The cells were stimulated with TPA (40 ng/ml) to activate PKCγ. Note that the activated PKCγ proteins are typically localized at the cell membrane.

In contrast to the predominantly nuclear localization observed when PTB was expressed alone (Fig. 3*B*), coexpression with PKA led to a large amount of wild-type PTB localized in the cytoplasm (Fig. 4*B* and Fig. 8, which is published as supporting information on the PNAS web site). Unlike wild-type PTB, the S16A mutant remains largely nuclear when coexpressed with PKA (Fig. 4 *B* and *C*). Note that the nuclear rim staining seen in this figure is frequently observed with this mutant. The control mutant S21A showed a cytoplasmic immunostaining pattern similar to wild-type PTB. Another activated kinase, protein kinase Cγ (PKCγ), did not have this effect on PTB localization (Fig. 4*D*). Thus, PKA specifically causes a shift in PTB localization to the cytoplasm, and the Ser-16 in PTB is required for this shift.

To further confirm that phosphorylation of Ser-16 was affecting the nucleo-cytoplasmic distribution of the protein, the N-terminal 55 aa (amino acids 1–55) of PTB were fused to enhanced GFP (EGFP) and tested for localization (Fig. 5). This portion of PTB was previously shown to be sufficient for nuclear localization and to be capable of shuttling (13, 14, 16, 17). The PTB-(aa1–55)-EGFP fusion protein (wild type) and its mutants (S16A, S21A) were all localized to the nucleus when expressed alone (Fig. 5). As with full length PTB (Fig. 4*B*), this localization changed when coexpressed with PKA (Fig. 5 *B* and *C*). The cytoplasmic accumulation of the wild-type fusion protein PTB-(aa1–55)-EGFP (wild type) was increased ≈2-fold by PKA coexpression. This increase in cytoplasmic protein is less than that seen with the full-length PTB (Fig. 4*B*), possibly because of the lack of an additional NES element present in RRM2 that increases the export of full-length PTB (17). Importantly, the nuclear localization of the S16A mutant EGFP fusion was not changed by PKA, whereas the response of the S21A mutant to PKA was similar to the wild type. Thus, the N-terminal 55 aa of PTB, including Ser-16, are sufficient to mediate PKA-dependent protein relocalization, albeit at a lower level than the full-length protein.

These experiments indicate that increasing phosphorylation of Ser-16 by PKA coexpression leads to greater cytoplasmic accumulation of PTB. Conversely, blocking this phosphorylation by mutation of Ser-16 prevents cytoplasmic accumulation. It is important to note that PKA is present in both the nucleus and cytoplasm of the transfected cells, with higher concentrations in the cytoplasm. This means that we cannot determine where the phosphorylation is taking place. The PKA could be phosphorylating PTB in the nucleus and increasing its export or it could phosphorylate PTB in the cytoplasm to block its import into the nucleus.

Overexpressed PKA may also activate other kinases in these cells. To confirm in these coexpression experiments that Ser-16 was the major site of phosphorylation in PTB, we carried out phospho-peptide mapping. Western blots of immunoprecipitated PTB
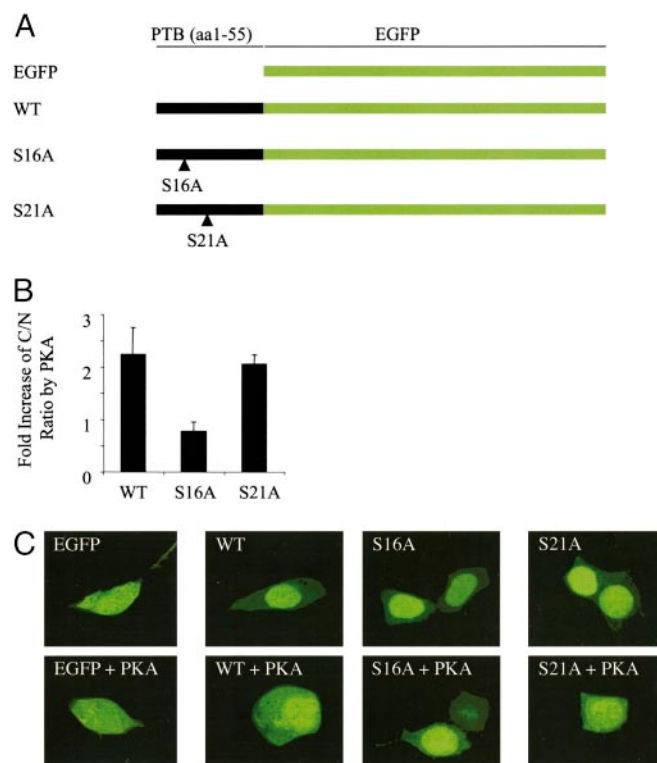

Fig. 5. The N terminus of PTB is sufficient to mediate PKA-dependent relocalization. (*A*) Diagrams of the PTB (amino acids 1–55)-EGFP fusion proteins. (*B*) The EGFP fluorescence intensity in transfected cells was measured with the program NIH IMAGE 1.62b7 for the cytoplasm and nucleus of each cell and the ratio determined (C/N). The average increase in the C/N ratio after PKA expression is plotted (*n* = 6, 5, and 5 cells, respectively). The C/N ratio approximately doubles after PKA expression for the wild-type and S21A PTBs, but is unchanged for the S16A mutant. (*C*) Confocal images of HEK 293 cells transfected with the constructs in *A*. These are coexpressed either with (*Lower*) or without (*Upper*) PKA. Note that the localization of EGFP itself was not affected by PKA coexpression.
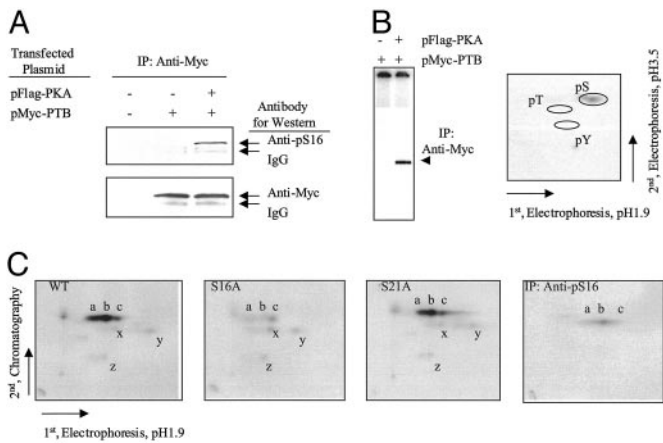
Fig. 6. Ser-16 is phosphorylated by PKA *in vivo*. (*A*) Western blot of immunoprecipitated Myc-PTB from transfected HEK 293 cells. Note that the phospho-Ser-16 band is only detectable in the PKA cotransfected sample. (*B* and *C*) Ser-16 is the major site of PKA phosphorylation. (*B*) Immunoprecipitation of radiolabeled PTB from transfected HEK 293 cells grown in ^{32}P-orthophosphoric acid (*Left*). This phosphorylated protein was subjected to phospho-amino acid analysis as in Fig. 2. (*C*) Phospho-peptide mapping of the immunoprecipitated Myc-PTB showed a major peptide (*b*) and two less intense spots (*a* and *c*). There were also three minor spots (*x*, *y*, and *z*) that were used to align the panels. The major spot *b* is eliminated by the S16A mutation. This spot is immunoprecipitated by the pS16 antibody (*Right*). The wild-type and anti-pS16 IP samples were run in the same tank, and the spot *b* in both samples is at the same distance from the starting point in the first dimension.

showed that phosphorylation of Ser-16 in Myc-PTB was indeed stimulated by PKA coexpression (Fig. 6*A*). To confirm that Ser-16 is the predominant site of phosphorylation, we isolated Myc-PTB from transfected cells labeled with ^{32}P-orthophosphoric acid. PKA coexpression strongly increased the amount of ^{32}P-labeled PTB present in anti-Myc immunoprecipitates (Fig. 6*B*). The majority of PTB phosphorylation in these cells is thus due to PKA. Phospho-amino acid analysis showed that the protein was phosphorylated at Ser residues only (Fig. 6*B*). Phospho-peptide analysis showed that the wild-type PTB had three primary phospho-peptides, *a*, *b*, and *c* (Fig. 6*C*), with three background spots *x*, *y*, and *z*. Of these, phospho-peptide *b* is by far the most prevalent. The S16A mutation specifically eliminated the major phospho-peptide *b*. Moreover, this phospho-peptide could be immunoprecipitated by the pS16 antibody. Thus Ser-16 is the major site of Myc-PTB phosphorylation by PKA in the transfected HEK 293 cells.

Phosphorylated PTB Is Highly Restricted to the Cytoplasm of Stage III/IV *Xenopus* Oocytes. The relocalization of PTB to the cytoplasm induced by PKA is accompanied by increased phosphorylation of Ser-16. We next wanted to know whether the phosphorylated protein was cytoplasmic. In cells where Myc-PTB and PKA were coexpressed, the majority of the phosphorylated PTB was cytoplasmic (data not shown). However, this could be because the overexpressed PKA was predominantly cytoplasmic (Fig. 4*B*). It is difficult to examine endogeneous phosphorylated PTB in HEK 293 cells because of the low levels of phosphorylated protein and the leakiness of the nuclei during nuclear-cytoplasmic separations. *Xenopus* oocytes contain significant amounts of cytoplasmic PTB that can be cleanly separated from the nuclear pool of protein. During *Xenopus* oogenesis, PTB binds to an RNA element that is required for the cytoplasmic localization of the Vg1 mRNA to the vegetal pole of the oocyte (12). PKA is thought to be active during these stages of oocyte development before maturation (34). To examine the PTB in the oocyte, we isolated cytoplasmic and nuclear fractions of stage III/IV *Xenopus* oocytes and probed these fractions on Western blots with the phosphorylation-sensitive antibod-

ies (Fig. 7). PTB phosphorylated at Ser-16 is detectable only in the cytoplasmic fraction, whereas non-Ser-16-phosphorylated PTB is seen only in the nuclear fraction (Fig. 7). Thus, in *Xenopus* oocytes, only cytoplasmically localized PTB is phosphorylated at Ser-16. These results support the data from cultured cells that phosphorylation controls the nucleo-cytoplasmic distribution of PTB. Moreover, these data suggest that the cytoplasmic accumulation of PTB seen in *Xenopus* oocytes is tied to the phosphorylation of Ser-16.

Discussion

We have shown that Ser-16 of PTB is a target of PKA phosphorylation *in vitro* and *in vivo*. A serine at this position is required for efficient nuclear export but not import, and over-expressed PKA increases cytoplasmic PTB. In *Xenopus* oocytes, phosphorylated PTB is exclusively cytoplasmic whereas non-phosphorylated protein is nuclear. Thus, PKA apparently controls the nucleo-cytoplasmic distribution of this important hnRNP protein through the phosphorylation of a specialized nuclear export signal.

The PTB NLS is of the bipartite type with two sets of basic residues including Lys-13, Arg-14 and the downstream amino acids KKFK (amino acids 45–48). These two sets of basic groups flank the PKA phosphorylation site Ser-16 and both are required for efficient nuclear localization (14, 15). This NLS has been shown to bind to the import receptor importin α (15). Importin α probably binds to unphosphorylated PTB, as the S16A mutant is imported normally. It is possible that phosphorylation of Ser-16 could inhibit this interaction to reduce import. In other studies, PTB residues 1–25 were shown to act as a nuclear export signal in constructs containing a separate NLS (16). There is also a sequence in RRM2 that increases PTB shuttling (17). Because the S16A mutant is nuclear but greatly reduced in shuttling, phosphorylation is presumably required for efficient export. Confirming this will await the identification of the PTB export receptor.

Some RNA-binding proteins require ongoing transcription to exhibit shuttling behavior. This is interpreted to mean that the protein is being exported as a component of an mRNP complex. For PTB, shuttling does not require transcription (17). This is in agreement with the result that the N-terminal domain of PTB lacking the RRMs is sufficient for PKA-dependent relocalization (Fig. 5).

Protein kinases, including PKA, are known to regulate the subcellular localization of a number of proteins (22, 24, 35–37). These phosphorylation events apparently modulate the interaction between a cargo protein and its transport factor (38). PKA phosphorylation of the Dorsal protein on Ser-312 increases its binding affinity to importin and is accompanied by increased nuclear import (22). In the opposite direction, PKA signaling has been shown to relocalize the yeast transcription factor Msn2p to cytoplasm (23). The shuttling signals of several other RNA binding proteins contain
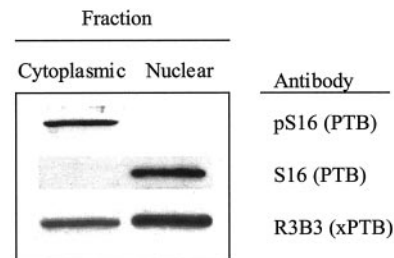

Fig. 7. Phospho-Ser-16-PTB is restricted to the cytoplasm in *X. laevis* oocytes. Nuclear or cytoplasmic fractions of stage III/IV oocytes were analyzed by Western blot using either anti-phospho-Ser-16 (pS16), anti-nonphosphorylated PTB (S16), or anti-*Xenopus* PTB (R3B3) antibodies. Oocytes were manually dissected into nuclear and cytoplasmic fractions. The pS16 and S16 lanes used 10 oocyte equivalents of proteins, and the R3B3 lanes used 5 oocyte equivalents.

PKA consensus sites (R-X-S), including the M9 sequence of hnRNP A1 and the HuR HNS (39, 40). Thus the relocalization of PTB by PKA may be part of a larger ensemble of RNA-binding proteins being affected by the PKA signaling.

Non-PKA signaling pathways are also known to affect the subcellular localization of RNA-binding proteins, including hnRNP A1, hnRNP K, and the arginine/serine-rich family of SR proteins (4, 5, 41–43). HnRNP A1 is relocalized to the cytoplasm in response to activation of the MKK3/6-p38 pathway by osmotic stress or UV irradiation (5). Under these conditions, inclusion of an adenovirus E1A exon is increased, consistent with the protein's effect as a splicing repressor of this exon. The kinase that directly phosphorylates the hnRNP A1 protein in this relocalization process and the site of modification remain unidentified. HnRNP K protein is directly phosphorylated by the mitogen-activated protein kinase/extracellular-signal-regulated kinase (MAPK/ERK). This leads to cytoplasmic accumulation of the protein and translational inhibition of target mRNAs (4). In this case, it is not clear whether the phosphorylation increases the cytoplasmic level of hnRNP K by blocking its nuclear import or enhancing its export. An example of phosphorylation being specifically required for nuclear export is the U small nuclear RNA system (44). The export of these RNAs requires the phosphorylated adaptor for RNA export (PHAX). PHAX is phosphorylated in the nucleus, where it mediates export, and then dephosphorylated in the cytoplasm. The kinase that modifies PHAX and its site of phosphorylation are currently unknown.

The effect of PKA stimulation on PTB function is not yet clear. We have shown that PTB phosphorylation is induced in PC12 cells after forskolin treatment. Under these conditions, we have not observed substantial changes in PTB localization or alternative splicing, presumably because only a fraction of the total PTB is being modified in response to the stimulus. Other groups have shown that PTB is relocalized to the cytoplasm after poliovirus infection (45). We have found no change in the phosphorylation of PTB over the course of poliovirus infection. Thus, the change in localization is likely due to other factors such as protein cleavage (45). The PTB homologue, nPTB, has been reported to relocalize to the cytoplasm on NGF treatment of PC12 cells (46). Whether Ser-16 phosphorylation is involved in this process has not been examined.

In tissue culture cells, the minority of the endogeneous PTB is cytoplasmic ($\approx 14 \pm 2\%$; data not shown). The phosphorylated protein is a very minor portion of the total PTB and it is not clear what fraction of the cytoplasmic protein is phosphorylated. In contrast, *Xenopus* oocytes have a larger fraction of PTB in the cytoplasm ($23 \pm 2\%$) and this fraction is virtually all phosphorylated at Ser-16. In the oocyte cytoplasm, much of the PTB is at the vegetal cortex where it is colocalized with Vg1 mRNA (12). The PTB bound to the localized Vg1 mRNA is thus likely the phosphorylated form of the protein. The phosphorylation or lack of dephosphorylation of PTB in oocytes may serve to increase the cytoplasmic pool of protein and allow its maintenance at the vegetal pole.

The results presented here couple the cAMP-dependent protein kinase pathway with PTB, a protein with important functions in alternative pre-mRNA splicing, viral RNA translation, and mRNA localization. It will be interesting to examine whether natural inducers of protein kinase A, such as the neurotransmitters dopamine and serotonin, can stimulate PTB phosphorylation and alter PTB transport. How this regulation of PTB localization affects the downstream cellular functions of the protein are important questions for the future.

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