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Protein Kinase A Phosphorylation Modulates Transport of the Polypyrimidine Tract-Binding Protein

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Abstract

The hnRNP proteins play important roles in mRNA processing in eukaryotes, but little is known about how they are regulated by cellular signalling 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'-cyclic AMP-dependent protein kinase (PKA). PKA directly phosphorylates PTB on conserved Serine 16, and PKA activation in PC12 cells induces Ser16 phosphorylation. PTB carrying a Serine 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 co-expression 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-Ser16-PTB is restricted to the cytoplasm, whereas the non-Ser16-phosphorylated PTB is nuclear. Thus, direct PKA phosphorylation of PTB at Ser16 modulates the nucleo-cytoplasmic distribution of PTB. This phosphorylation likely plays a role in the cytoplasmic function of PTB.

Introduction

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 signalling 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 signalling 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 (IRES) (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 amino-terminal domain. The N-terminal 55 amino acid 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 since the protein also has cytoplasmic functions. However, little is known about this regulation and what cellular signalling pathways impact it.

The 3', 5'-cyclic AMP dependent protein kinase (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 the supplemental data and in references (25) and (26).

Phospho-peptide analysis. Phospho-amino acid and phospho-peptide analyses were performed according to (27). In vitro- or vivo-labelled PTB protein was blotted to PVDF membranes. For phospho-amino acid analysis, the protein was hydrolysed with 6N HCl at 110^0C for 1 hour. For phospho-peptide analysis, the protein was digested overnight with trypsin and chymotrypsin (50 μg/ml each) in 50 mM $NaHCO_3$ (pH 8.0). The samples were vacuum-dried, washed three times

with 500 µl water, resuspended in 10 µl water, and dotted onto cellulose TLC plates (10 X 10 cm, EM Science). The plates were wetted with pH 1.9 running buffer (formic acid, 25ml; glacial acetic acid, 78 ml; in 1000 ml) and run at 1,000 Volts for 20 minutes. For phospho-amino acid analysis, the plates were then turned 90^0 clockwise and run in pH 3.5 buffer (glacial acetic acid, 50 ml; pyridine, 5 ml; in 1000 ml) at 1,500 Volts for 37 minutes. For phospho-peptide analysis, the plates were air-dried and subjected to chromatography in separation buffer (n-Butanol 37.5 ml, Pyridine, 25 ml, Glacial acetic acid, 7.5 ml, in 100 ml). Radioactive spots were visualized by phosphorimager. The standard phospho-amino acids was visualized with ninhydrin.

Heterokaryon assay. Heterokaryon assays were performed according to (17) with slight modifications. HeLa or HEK 293 human cells were fused with mouse NIH/3T3 cells 2 hours after initiating cycloheximide (100 µg/ml) treatment. Fusion was induced by addition of 50% polyethylene glycol (PEG) 3350 and incubation for 2 to 5 minutes. The cells were washed with DMEM, incubated for 5 hours 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 homogenized in 1x Laemmli sample buffer, incubated at 95°C for 3 minutes, 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 1:1000). Blots were washed and incubated with secondary antibody at 1:160,000 for 1 hour at room temperature. All incubations and washes were in TBS (20mM Tris (pH7.5), 0.5M NaCl, 1mM Na_3VO_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 using chemiluminescence.

Results

Serine 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 human embryonic kidney (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. 1A). A similar result was obtained from WERI-1

retinoblastoma cells (data not shown). This 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 Ser16 in the motif RGS near the N-terminus (Fig. 1B), and Ser525 in the motif RVS at the COOH-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. 2A). 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. 2B). Mutation of Serine 16 to Alanine (S16A) nearly abolished the PKA phosphorylation, but a control mutation, S21A, did not (Fig. 2A). The neuronal protein, nPTB, is also phosphorylated by PKA (Fig. 1B & 2A). These data indicate that Ser16 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 thin-layer chromatography showed that wild type PTB had three phospho-peptides after PKA phosphorylation (Fig. 2C). 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 to 20 (GTKRGSDELF) with Ser16 in either the phosphorylated or unphosphorylated state (Fig. 2D). The anti-phospho-Ser16 antibody (anti-pS16) specifically immunoprecipitated phospho-peptide 1 (Fig. 2C, far right panel). Peptide 2 was not immunoprecipitated by the anti-pS16 (Fig. 2C). 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 Ser16 (peptide 1).

Significantly, the anti-phospho-PTB antibodies also allowed us to confirm that Ser16 phosphorylation occurs in vivo upon 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. 2E). 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 Ser16.

Ser16 is critical for nuclear export of PTB. We next investigated the effect of Ser16 phosphorylation on PTB function. The N-terminal 55 amino acids 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. 3A & 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 indicates that Ser16 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 hours, 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. 3C). 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. 3C & D). In contrast, for the S16A mutant, mouse nuclear staining

was only about 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 one hour after heterokaryon formation rather than at 5 hours as done here. It takes at least 4 hours after cell fusion for PTB shuttling to reach equilibrium (17). Our own measurements of this time course indicate that one hour after fusion, the PTB concentration in the mouse nucleus has reached about 17% that of the equilibrium state (± 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 due to the difference in the post-fusion time points examined in the two studies. At 5 hours, the S16A mutant was dramatically reduced in the mouse nuclei relative to wild type PTB (Fig. 3C & D).

Since the S16A mutant can be imported into the HEK 293 nucleus (Fig. 3B), 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 about 57% that of human nuclei. The close proximity of Ser21 to the critical residues (aa 11-15) (16) of the NES may cause its mutation to also affect this process. Taken together, these data suggest that phosphorylation of Ser16 is important for the export of PTB from the nucleus to the cytoplasm, although some shuttling can occur without it.

PKA co-expression leads to increased phosphorylation at Ser16 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 co-expressed with the Myc-PTB in HEK 293 cells and the resulting subcellular distribution and phosphorylation of Myc-PTB was monitored.

In contrast to the predominantly nuclear localization observed when PTB was expressed alone (Fig. 3B), co-expression with PKA led to a large amount of wild type PTB localized in the cytoplasm (Fig. 4B). Unlike wild type PTB, the S16A mutant remains largely nuclear when co-expressed with PKA (Fig. 4B & 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. 4D). Thus, PKA specifically causes a shift in PTB localization to the cytoplasm, and the Ser16 in PTB is required for this shift.

To further confirm that phosphorylation of Ser16 was affecting the nucleo-cytoplasmic distribution of the protein, the N-terminal 55 amino acids (aa 1-55) of PTB were fused to 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 (WT) and its

mutants (S16A, S21A) were all localized to the nucleus when expressed alone (Fig. 5). As with full length PTB (Fig. 4B), this localization changed when co-expressed with PKA (Fig. 5B & C). The cytoplasmic accumulation of the wild type fusion protein PTB-(aa1-55)-EGFP (WT) was increased about 2 fold by PKA co-expression. This increase in cytoplasmic protein is less than that seen with the full length PTB (Fig. 4B), possibly due to 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 amino acids of PTB, including Ser16, 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 Ser16 by PKA co-expression leads to greater cytoplasmic accumulation of PTB. Conversely, blocking this phosphorylation by mutation of Ser16 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 can not 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 co-expression experiments that Ser16 was the major site of

phosphorylation in PTB, we carried out phospho-peptide mapping. Western blots of immunoprecipitated PTB showed that phosphorylation of Ser16 in Myc-PTB was indeed stimulated by PKA co-expression (Fig. 6A). To confirm that Ser16 is the predominant site of phosphorylation, we isolated Myc-PTB from transfected cells labeled with ^{32}P-orthophosphoric acid. PKA co-expression strongly increased the amount of ^{32}P-labelled PTB present in anti-Myc immunoprecipitates (Fig. 6B). 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. 6B). Phospho-peptide analysis showed that the wild type PTB had 3 primary phospho-peptides, a, b, and c (Fig. 6C), 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 Ser16 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 Ser16. We next wanted to know whether the phosphorylated protein was cytoplasmic. In cells where Myc-PTB and PKA were co-expressed, the majority of the phosphorylated PTB was cytoplasmic (data not shown). However, this could be because the overexpressed PKA was predominantly cytoplasmic (Fig. 4B). It is difficult to examine

endogeneous phosphorylated PTB in HEK 293 cells due to 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 antibodies (Fig. 7). PTB phosphorylated at Ser16 is detectable only in the cytoplasmic fraction, whereas non-Ser16-phosphorylated PTB is seen only in the nuclear fraction (Fig. 7). Thus, in *Xenopus* oocytes, only cytoplasmically localized PTB is phosphorylated at Ser16. 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 Ser16.

Discussion

We have shown that Ser16 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 overexpressed 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 Lys13, Arg14 and the downstream amino acids KKFK (aa 45-48). These two sets of basic groups flank the PKA phosphorylation site Ser16 and both are required for efficient nuclear localization (14, 15). This NLS has been shown to bind to the import receptor importin alpha (15). Importin alpha probably binds to unphosphorylated PTB, as the S16A mutant is imported normally. It is possible that phosphorylation of Ser16 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). Since 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 Ser312 increases its binding affinity to importin and is accompanied by increased nuclear import (22). In the opposite direction, PKA signalling has been shown to relocalize the yeast transcription factor Msn2p to cytoplasm (23). The shuttling signals of several other RNA binding proteins contain 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 signalling 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 snRNA 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 upon NGF treatment of PC12 cells (46). Whether Ser16 phosphorylation is involved in this process has not been examined.

In tissue culture cells, the minority of the endogeneous PTB is cytoplasmic (~14% ± 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% ± 2%) and this fraction is virtually all phosphorylated at Ser16. In the oocyte cytoplasm, much of the PTB is at the vegetal cortex where it is co-

localized 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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Figure legends:

Figure. 1. Endogeneous PTB is a phosphoprotein. **A**. Immunoprecipitation using anti-PTB (BB7) or anti-Flag control antibody from HEK 293 cells labelled in ^{32}P-orthophosphoric acid. An SDS-PAGE gel of the immunoprecipitated proteins is shown. The molecular size markers are to the left (M). **B**. PTB domains and the position of the PKA target Ser16 (left). S16A and control S21A mutants are shown below the sequence. The alignment of the Ser16 region from several species is shown to the right. The peptide sequence used for making antibodies is boxed.

Figure. 2. **PKA phosphorylates PTB at Serine 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. Bands for phosphorylated PTB and nPTB, autophosphorylated PKA are indicated in the upper panel. Coomassie blue stained PTB is shown in the lower panel. **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**. Phospho-peptide mapping of the PKA-phosphorylated PTB and its mutants. The far right panel shows a TLC of the phospho-peptides immunoprecipitated with the pS16-specific antibody. The wild type (WT) 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 Ser16 in PC 12 cells. Total protein of PC12 cells treated with or without forskolin (10 μM) for 6 hours, 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.

Figure. 3. **Ser16 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), with wild type Myc-PTB, or with the S16A or S21A mutants. This was probed with anti-Myc antibodies to control for equal expression of the transfected clones as compared to the endogeneous 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 position of the mouse nuclei in heterokaryons. **D**. Bar graph of the average (± SD) intensity of the Myc-PTB staining in the mouse nuclei relative to the human nuclei of the same heterokaryons (n = 4, 10, 9 heterokaryons, respectively).

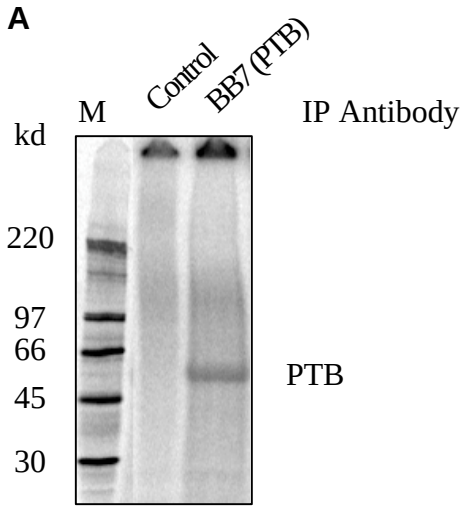
Figure. 4. **PKA co-expression relocalizes PTB to the cytoplasm and the S16A mutation blocks this effect. A**. A Western blot of protein from HEK 293 cells transfected with Flag-tagged PKA. **B**. Confocal microscopy of anti-Myc-stained PTB or anti-Flag-stained PKA in HEK 293 cells. **C**. To distinguish 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. This data is presented in a bar graph at the right as the percentage of cells with predominantly nuclear staining of Myc-PTB with PKA co-expression. This data is from the co-transfection 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 co-transfected 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.

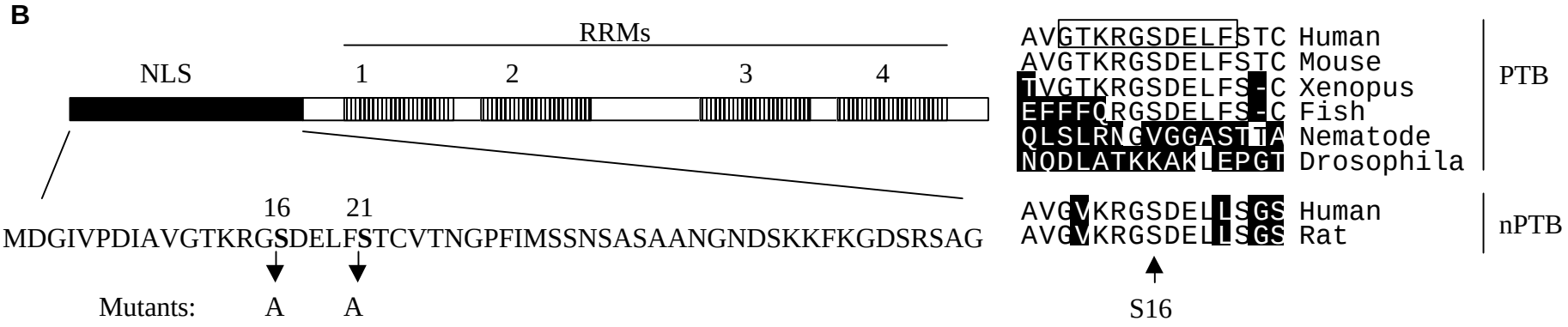
Figure. 5. **The N-terminus of PTB is sufficient to mediate PKA-dependent relocalization**. **A**. Diagrams of the PTB (aa 1-55)-EGFP fusion proteins. **B**. The EGFP fluorescence intensity in transfected cells was measured with the program NIH Image 1.62b7 for the cytoplasm and nucleus of each cell and the ratio

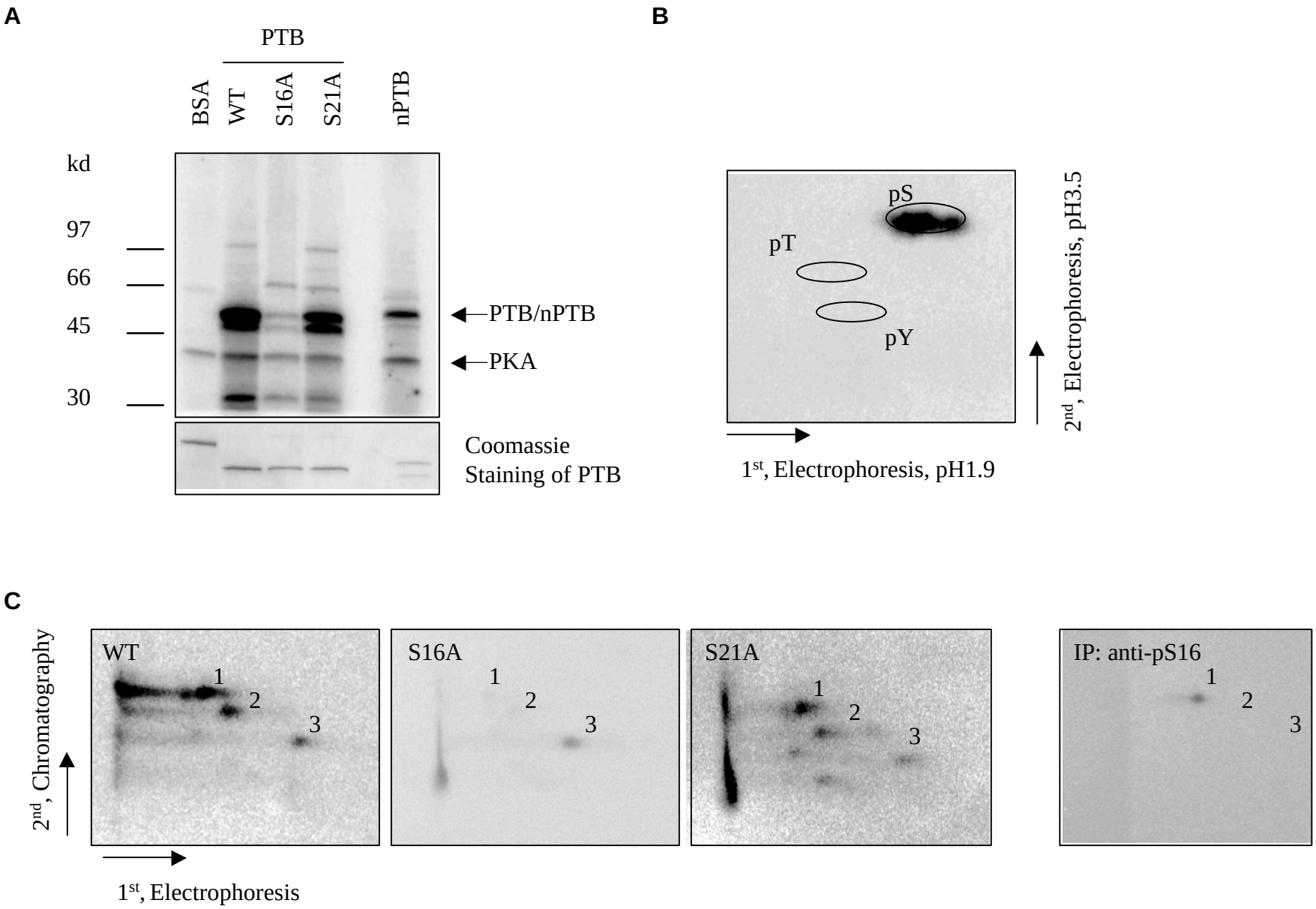
determined (C/N). The average increase in the C/N ratio upon PKA expression is plotted (n = 6, 5, and 5 cells respectively). The C/N ratio approximately doubles upon PKA expression for the wild type and S21A PTBs, but is unchanged for the S16A mutant. **C**. Confocal images of HEK 293 cells transfected with the constructs in **A.** These are co-expressed either with (Bottom Row) or without (Top Row) PKA. Note that the localization of EGFP itself was not affected by PKA co-expression.

Figure. 6. Ser16 is phosphorylated by PKA in vivo. A. Western blot of immunoprecipitated Myc-PTB from transfected HEK 293 cells. Note that the phospho-Ser16 band is only detectable in the PKA co-transfected sample. **B & C:** Ser16 is the major site of PKA phosphorylation. **B**. Immunoprecipitation of radio-labeled PTB from transfected HEK 293 cells grown in ^{32}P-orthophosphoric acid (left panel). 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 (far right panel). The wild type (WT) 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 1st dimension.

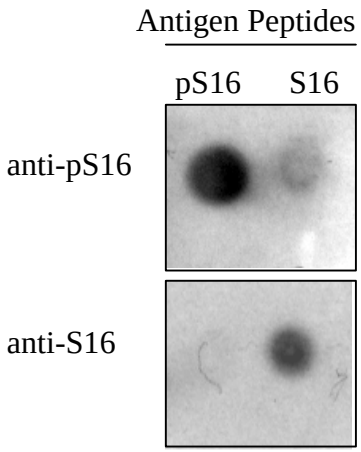
Fig. 7. Phospho-Ser16-PTB is restricted to the cytoplasm in *Xenopus laevis* oocytes. Nuclear or cytoplasmic fractions of stage III/IV oocytes were analyzed by Western blot using either anti-phospho-Ser16 (pS16), anti-non-phosphorylated PTB (S16), or anti-*Xenopus* PTB (R3B3) antibodies. Oocytes were 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.


A
M
Control
BB7 (PTB)
IP Antibody
kd
220
97
66
45
30
PTB

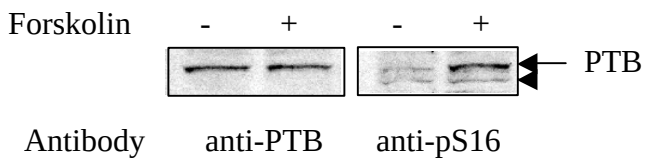

B
RRMs
NLS
1
2
3
4
MDGIVPDIAVGTKRGSDELFSTCVTNGPFIMSSNSASAANGNDSKKFKGDSRSAG
16
21
Mutants:
A
A
AVGTKRGSDELFSTC Human
AVGTKRGSDELFSTC Mouse
TVGTKRGSDELFS-C Xenopus
EFFFQRGSDELFS-C Fish
QLSLRNGVGGASTTA Nematode
NQDLATKKAKLEPGT Drosophila
PTB
AVGVKRGSDELLSGS Human
AVGVKRGSDELLSGS Rat
nPTB
S16


A
PTB
BSA
WT
S16A
S21A
nPTB
kd
97
66
45
30
PTB/nPTB
PKA
Coomassie
Staining of PTB
B
pS
pT
pY
2nd, Electrophoresis, pH3.5
1st, Electrophoresis, pH1.9
C
2nd, Chromatography
WT
S16A
S21A
IP: anti-pS16
1
2
3
1st, Electrophoresis

D


Antigen Peptides
pS16
S16
anti-pS16
anti-S16

E


Forskolin
-
+
-
+
PTB
Antibody
anti-PTB
anti-pS16

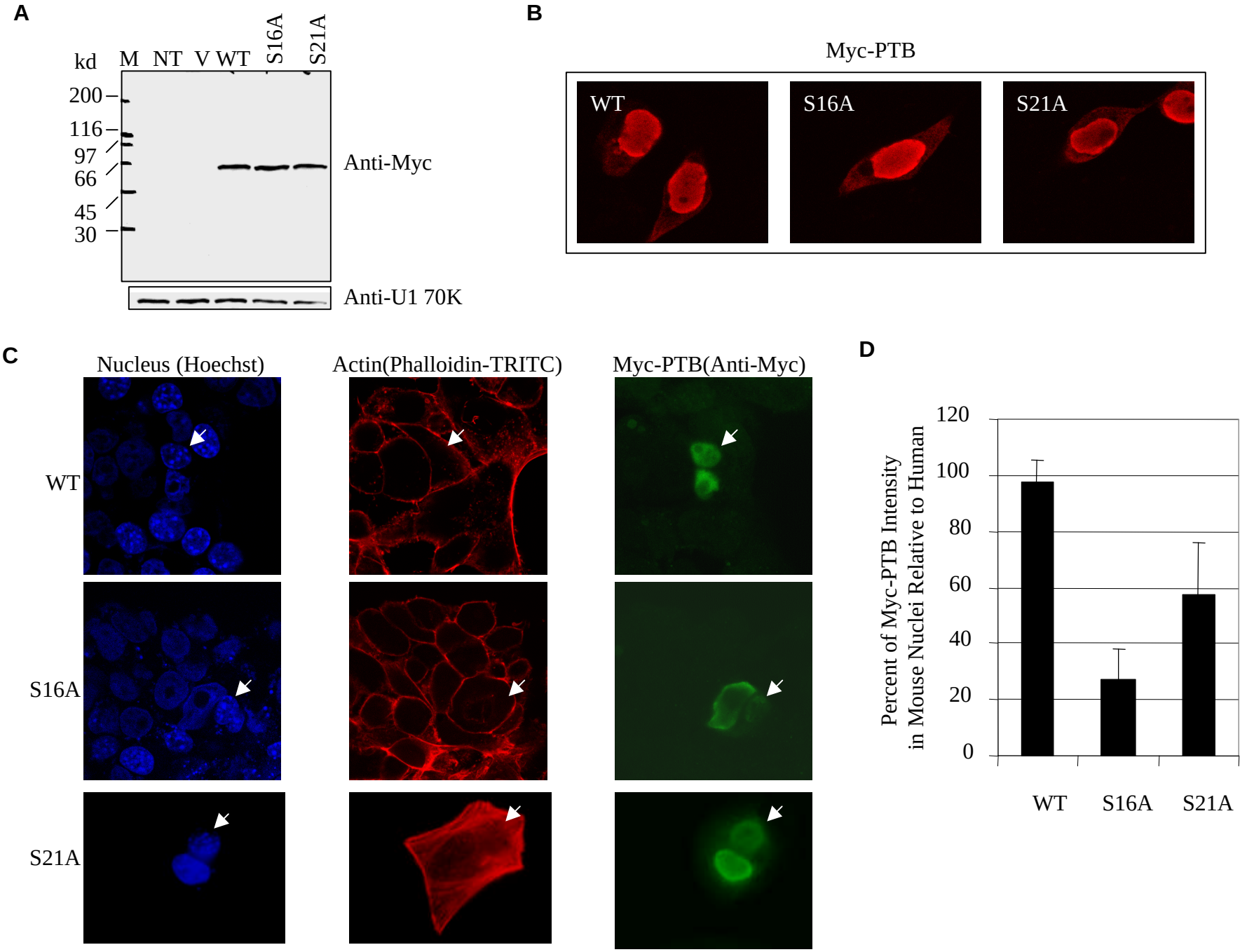

A
kd
M
NT
V
WT
S16A
S21A
200
116
97
66
45
30
Anti-Myc
Anti-U1 70K
B
Myc-PTB
WT
S16A
S21A
C
Nucleus (Hoechst)
Actin(Phalloidin-TRITC)
Myc-PTB(Anti-Myc)
WT
S16A
S21A
D
Percent of Myc-PTB Intensity in Mouse Nuclei Relative to Human
120
100
80
60
40
20
0
WT
S16A
S21A

Fig. 4

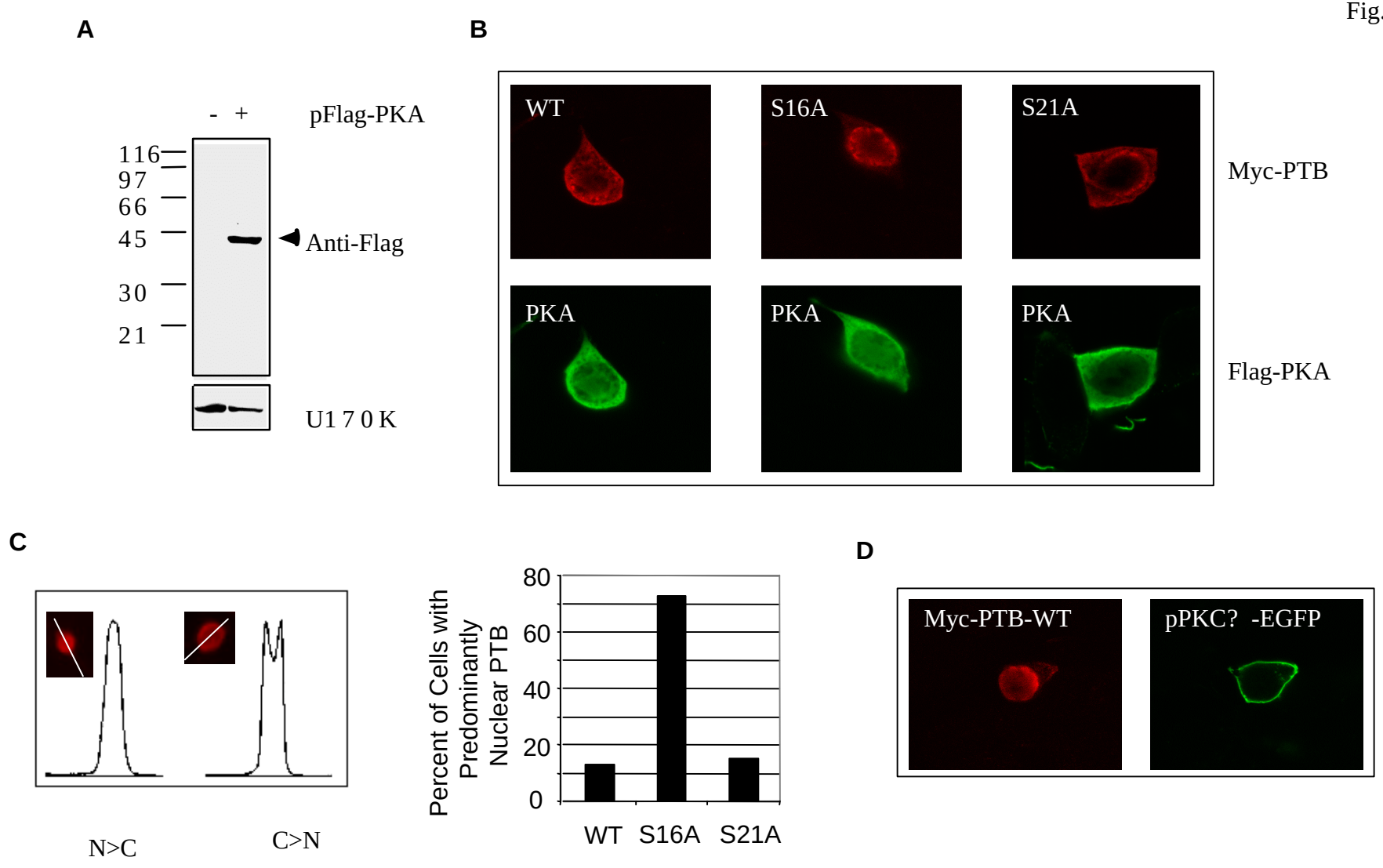
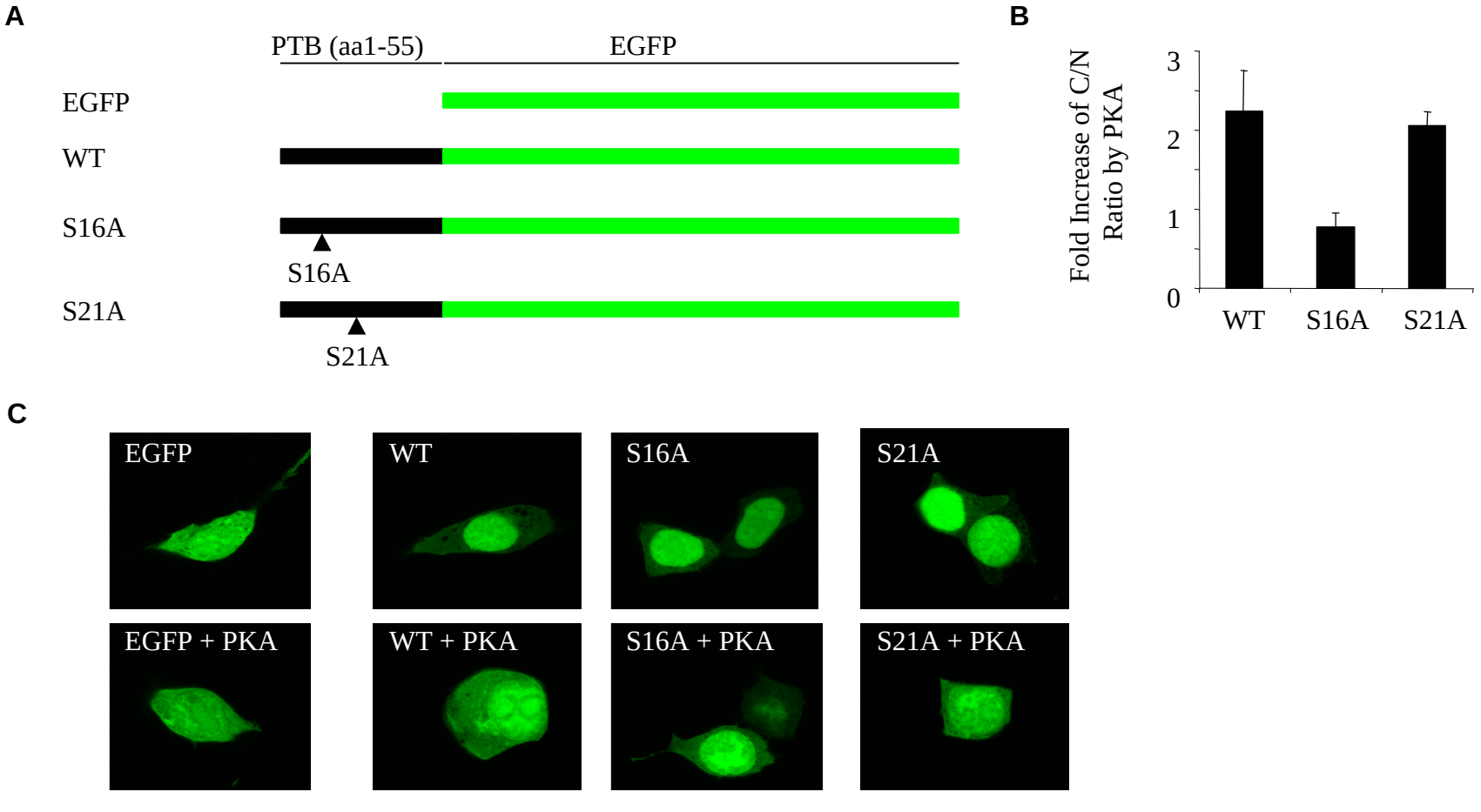

A
- +
pFlag-PKA
116
97
66
45
30
21
Anti-Flag
U1 7 0 K
B
WT
S16A
S21A
PKA
PKA
PKA
Myc-PTB
Flag-PKA
C
N>C
C>N
Percent of Cells with Predominantly Nuclear PTB
80
60
40
20
0
WT
S16A
S21A
D
Myc-PTB-WT
pPKC? -EGFP

Fig.5


A
PTB (aa1-55)
EGFP
EGFP
WT
S16A
S16A
S21A
S21A
B
Fold Increase of C/N Ratio by PKA
3
2
1
0
WT
S16A
S21A
C
EGFP
WT
S16A
S21A
EGFP + PKA
WT + PKA
S16A + PKA
S21A + PKA

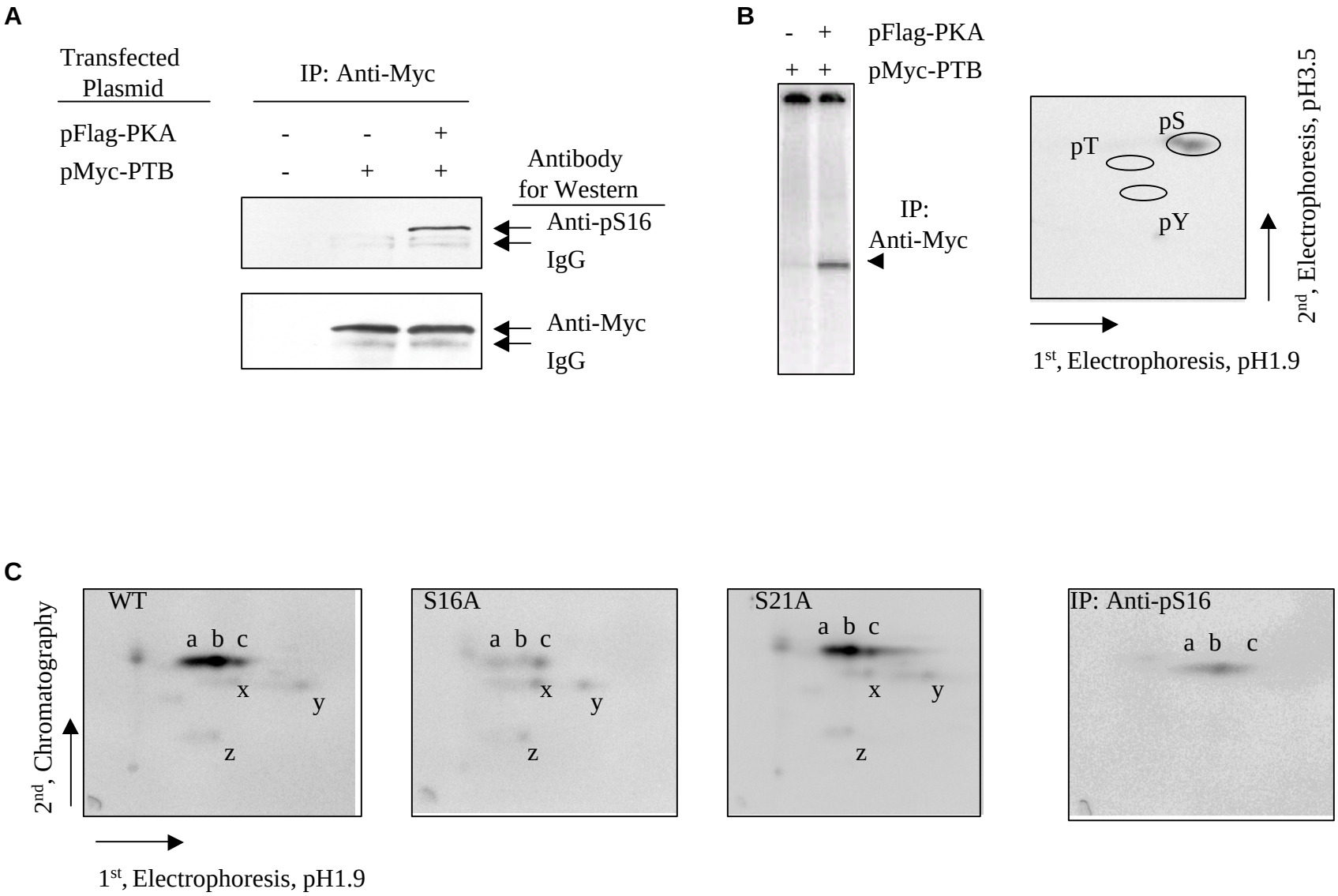
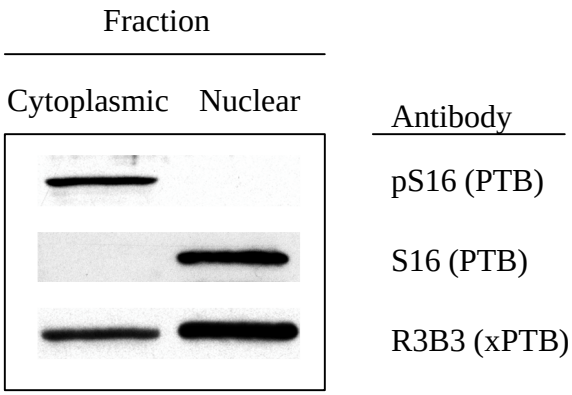

A
Transfected Plasmid
IP: Anti-Myc
pFlag-PKA
pMyc-PTB
- - +
- + +
Antibody for Western
Anti-pS16
IgG
Anti-Myc
IgG
B
- + pFlag-PKA
+ + pMyc-PTB
IP: Anti-Myc
pS
pT
pY
2nd, Electrophoresis, pH3.5
1st, Electrophoresis, pH1.9
C
WT
S16A
S21A
IP: Anti-pS16
a b c
x
y
z
2nd, Chromatography
1st, Electrophoresis, pH1.9

Fig. 7


Fraction
Cytoplasmic
Nuclear
Antibody
pS16 (PTB)
S16 (PTB)
R3B3 (xPTB)

Supplementary data:

Materials and Methods:

Cloning and expression of PTB and PKA. Clones for His-tagged PTB and mutants S16A and S21A were modified from the human PTB1 clone (a gift from Dr. James Patton) to remove a spacer between the His-tag and PTB sequence that contains two potential PKA target serines (RXS). Proteins were expressed in *E.Coli* strain BL21, purified using Ni-columns and stored in DG bufferFor mammalian expression, PTB sequences encoding amino acids 2 to 531 were inserted between the EcoR I and Kpn I sites of the pCMV-Myc vector (Clontech.). Flag-tagged PKA was made by amplifying codons 2 to 351 of the PKA catalytic alpha subunit (a gift from Dr. Harvey Herschman), with a 5'-Flag-tag (MDYKDDDDKAAA) primer and inserting this fragment into pcDNA3.1(+). Fusions of the human PTB N-terminus with EGFP were made by inserting the first 55 codons into the pEGFP-N1 vector. All PCR amplifications were done with Pfu DNA polymerase and resulting clones confirmed by sequencing the inserts.

In vitro phosphorylation. For PKA phosphorylation in vitro, 100 to 300 ng of bacterially expressed PTB were incubated with 10-20 ng of the alpha catalytic subunit of protein kinase A (CalBiochem), and 4 μCi [γ-^{32}P]ATP, in the kinase buffer (25 mM HEPES pH7.5, 10 mM $MgCl_2$), at 30^0C for 20 to 30 minutes.

Phospho-Ser16-specific antibody. A synthetic peptide GTKRGSDELF with Ser16 phosphorylated (PTB amino acids 11—20) was haptenized and used as the antigen by Alpha Diagnostics Intl. Inc., San Antonio, Texas. Non-phospho-Ser16 peptide antibodies were prepared similarly. Their specificity was confirmed on dot blots of the two peptides (Fig. 2E).

In vivo labelling and immunoprecipitation. HEK 293 cell cultures in 6 well plates were transfected with 2 µg plasmid. After 16-20 hours, cells were fasted in KRG buffer (128 mM NaCl, 5.2 mM KCl, 2.1 mM $CaCl_2$, 29 mM $MgSO_4$, 5 mM Glucose, and 10 mM Hepes, pH 7.5) for 1 hour, and then incubated with 250 µCi of ^{32}P-orthophosphoric acid in KRG buffer for 4 hours. The cells were lysed in 400 µl RIPA buffer (50 mM Tris-HCl, pH7.6; 150 mM NaCl; 1% Triton X-100; 0.5% deoxycholic acid; 0.1% SDS; 1 mM PMSF; 1 mM Na_3VO_4). For immunoprecipitation, 20 µl lysate was mixed with PBS-washed 100 µl protein A-sepharose 4B beads (3% slurry) and anti-Myc monoclonal antibody. The mixtures were incubated at room temperature with slight shaking for one hour and then washed three times in RIPA buffer. The immunocomplexes were recovered with SDS loading buffer, denatured at 100^0C for 5 minutes and subjected to SDS-PAGE. After exposure to phosphorimager plate, gel bands were excised, rehydrated in SDS buffer, blotted to PVDF membranes, and subjected to two-dimensional thin layer chromatography.

Cell culture, treatments. PC12 cells were grown in DMEM with 10% horse serum and 5% fetal bovine serum. HEK 293 cells were cultured in 15% bovine calf serum. The 8-(4-chlorophenylthio) adenosine-3', 5'-cyclic monophosphorothioate (cpt-cAMP) was prepared in DMEM and applied at 100 μM. Forskolin stock (10 mM) in ethanol was used at the final concentration of 10 μM.

Immunostaining. Cells were grown on coverslips, washed twice with 1% BSA in PBS, fixed in 4% paraformaldehyde (PFA) for 45 minutes, and permeabilized with 0.1% Triton X-100 for 10 minutes. Primary antibodies were added with 1% BSA in PBS and incubated at 4^0C for 16 to 20 hrs. Cells were then washed with PBS and incubated with the secondary antibody (Vector) at 1:1000 dilution for 1 hour at room temperature. The coverslips were washed three times with PBS before being mounted onto slides with VectorShield.

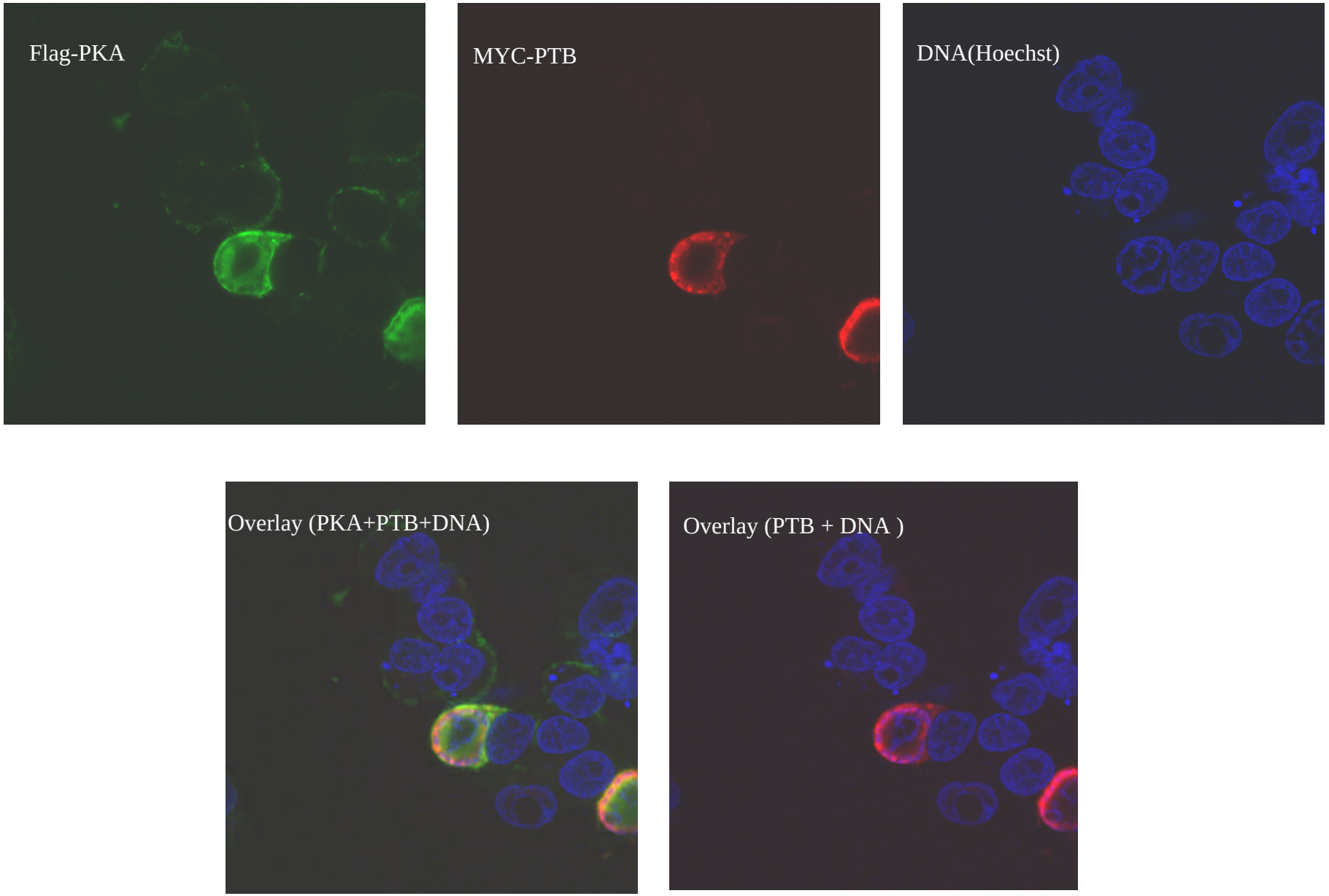
Confocal microscopy. Confocal microscopy was carried out using the Leica confocal microscope at the imaging facility of the Brain Research Institute at UCLA. The sections are at 0.5 μM in thickness, with a filter wavelength of 488 nm for flourescein or EGFP and 568 nm for Texas-Red.

Figure legend:

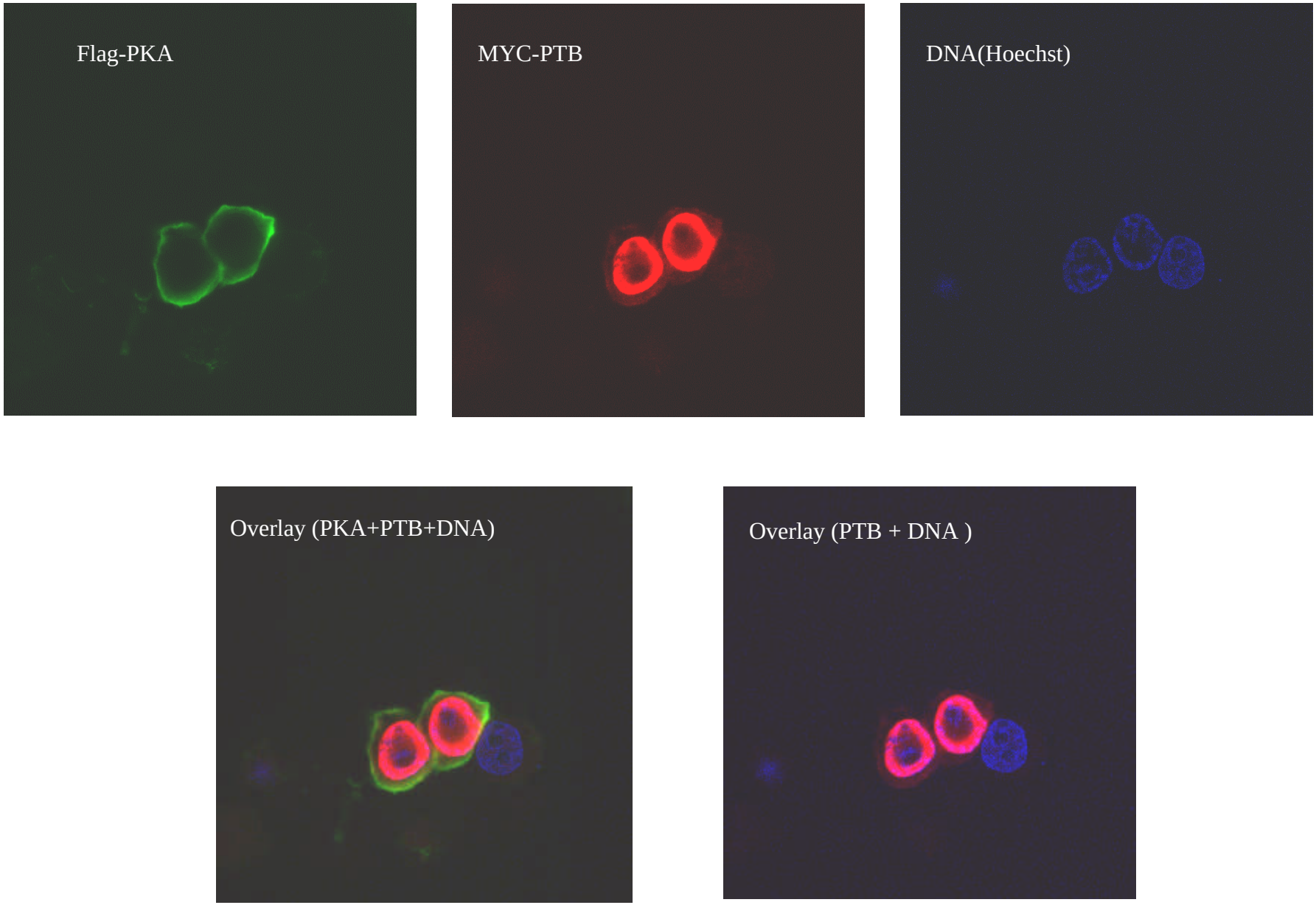
Fig. 1. Confocal images of Myc-PTB (**A**) and mutants (**B** & **C**) co-transfected with Flag-PKA. The nucleus is stained with Hoechst and the overlayed images are shown below individual ones in each panel. The nuclear rim staining of the Myc-PTB in S16A overlaps with that of the Hoechst staining, suggesting that the myc-PTB proteins are retained inside the nucleus.

Suppl_Fig. 1A

WT



S16A


Flag-PKA
MYC-PTB
DNA(Hoechst)
Overlay (PKA+PTB+DNA)
Overlay (PTB + DNA)

Suppl_Fig. 1C

S21A