Nuclear Localization of Protein Kinase U-α Is Regulated by 14-3-3*

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14-3-3 proteins are intracellular, dimeric molecules that bind to and modify the activity of several signaling proteins. We used human 14-3-3z as a bait in the yeast two-hybrid system to screen a murine embryonic cDNA library. One interacting clone was found to encode the carboxyl terminus of a putative protein kinase. The coding sequence of the human form (protein kinase Uα, PKUα) of this protein kinase was found in GenBankTM on the basis of sequence homology. The two-hybrid clone was also highly homologous to TOUSLED, an Arabidopsis thaliana protein kinase that is required for normal flower and leaf development. PKUα has been found by coimmunoprecipitation to bind to 14-3-3z in vivo. Our confocal laser immunofluorescence microscopic experiments revealed that PKUα colocalizes with the cytoplasmic intermediate filament system of cultured fibroblasts in the G1 phase of the cell cycle. PKUα is found in the perinuclear area of S phase cells and in the nucleus of late G2 cells. Transfection of cells with a dominant negative form of 14-3-3α promotes the nuclear localization of PKUα. These results suggest that the subcellular localization of PKUα is regulated, at least in part, by its association with 14-3-3.

14-3-3 proteins are intracellular, acidic dimeric molecules that play a role in signal transduction pathways (1, 2). They have been identified in many eukaryotic organisms, including plants and fungi, and are primarily found in the cytoplasmic compartment of eukaryotic cells. The biological function of 14-3-3 proteins is best modeled in the budding yeast Saccharomyces cerevisiae. Certain yeast strains that lack both 14-3-3 homologues, BMH1 and BMH2, are inviable (3). Furthermore, strains that lack BMH1 and BMH2 can be partially “rescued” by overexpression of the Ras-stimulated kinase TPK1 or by overexpression of clathrin heavy chain. These results suggest that BMH proteins play a role in both the Ras pathway and the cell cycle.

In an attempt to identify additional 14-3-3-binding partners, we performed a yeast two-hybrid screen with human 14-3-3z as a bait. One interacting clone was found to encode a serine/threonine kinase, named protein kinase U-α (PKUα). This protein kinase is homologous to a plant protein, TOUSLED, that is required for normal flower and leaf development (25). TOUSLED is constitutively localized in the nucleus of plant cells and is thought to play a role in cell cycle regulation (26).

EXPERIMENTAL PROCEDURES

Yeast Two-hybrid Screen—Full-length human 14-3-3z was inserted into the vector pAS1 (gift of Stephen Elledge, Baylor University) as an in-frame fusion with the transactivation domain of GAL4, as described previously (7). A mouse embryonic c12.5 DNA yeast two-hybrid library was screened (gift of Stan Hollenberg, Oregon Health Sciences University), and pAS1/14-3-3z was used as the bait. Yeast strain Y180 was cotransfected with pAS1/14-3-3z, and the mouse embryonic cDNA library and yeast were plated on media lacking histidine, tryptophan, and leucine. Colonies that grew in the absence of histidine were assayed for β-galactosidase activity by use of 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-gal) as a substrate. Positive colonies were plated onto media containing cycloheximide and cryptophan to expel the bait plasmid. Yeast that contained only the cDNA library plasmid were mated with yeast strain Y189 containing either pAS1/14-3-3z or pAS1/14-3-3z.

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† The abbreviations used are: PKUα, protein kinase U-α; PKUβ, protein kinase U-β; PAGE, polyacrylamide gel electrophoresis; GST, glutathione S-transferase; FITC, fluorescein isothiocyanate; kb, kilo-base pairs; DN, dominant negative; NES, nuclear export signal.
lamin that was grown on plates lacking leucine and tryptophan and reassembled for β-galactosidase activity.

Clones that specifically interacted with 14-3-3ζ were rescued and sequenced. DNA sequencing was performed with a Amersham Pharmacia Biotech 377 automated sequencer. BLAST searches (National Center for Biotechnology Information) were performed by use of the DNA and putative amino acid sequences of the two-hybrid clones.

14-3-3ζ Deletion Analysis—The amino-terminal portion of 14-3-3ζ (residues 1–78), the middle portion (residues 78–121), and the carboxy-terminal portion (residues 121–245) were inserted into pAS1-CYH as in-frame fusions with the DNA binding domain of GAL4 (residues 1–147) to make pAS1/14-3-3ζ, pAS1/C (residues 1–78), and pAS1/R (residues 121–245) (7). Clone 52b was used as an in-frame fusion with the transcription activator domain of GAL4 (residues 768–881) in the vector pGAD (7). Yeast strain Y190 was cotransformed with clone 52b and the 14-3-3ζ mutants or lamin. The efficiency of the interaction was assessed by β-galactosidase activity as assessed by the quantitative chlorophenyl-β-D-galactopyranoside assay (7).

Monoclonal Antibody Generation—A peptide corresponding to a region near the amino terminus of murine PKUζ (amino acids 670–684, sequence AYRKEDRIVQQLAC) was synthesized by standard Fmoc (N-9-fluorenylmethoxycarbonyl) chemistry. The peptide was purified by high pressure liquid chromatography, coupled to keyhole limpet hemocyanin, and injected into mice. After multiple injections, spleens were harvested, and splenic lymphocytes were isolated and fused to myeloma cells. Cell populations of fusion cells were tested for antibody production with the antigenic peptide in enzyme-linked immunosorbent assay reactions. Highly concentrated antibody was obtained from murine ascites after the intraperitoneal injection of hybridoma cells.

Northern Blot Analysis—Murine premade multiple tissue and embryonic poly(A) Northern blots were obtained from CLONTECH. The murine two-hybrid clone 52b, which corresponds to the carboxyl terminus of PKUζ and a human skeletal α-actin coding region cDNA (amino acids 202–374) were used to generate probes for Northern blot analysis. These probes were labeled with [α-32P]dCTP by use of random hexamers and the Klenow fragment of DNA polymerase I. Blots were prehybridized for 1 h at 42 °C in 50% formamide, 5× Denhardt's solution, 4× SSPE (0.6 M sodium chloride, 46 mM sodium phosphate, 5 mM EDTA), and 1% sodium dodecyl sulfate (SDS). Blots were washed under stringent conditions and were then visualized by autoradiography with Kodak XAR5 film. Equal loading of RNA was confirmed by ethidium bromide staining of the blots.

Transfection of Cultured Cells—NIH/3T3 fibroblasts were maintained in modified Eagle’s medium supplemented with 10% fetal calf serum. The KIAA0137 cDNA with a Myc epitope tag was inserted into pTARGET (Promega), a mammalian expression plasmid containing a cytomegalovirus promoter. The cDNA encoding a dominant-negative form of 14-3-3ζ (R56A and R60A) with an amino-terminal portion (residues 121–245) (7). Clone 52b was used as an in-frame fusion with the antigenic peptide in enzyme-linked immunosorbent assay reactions. Highly concentrated antibody was obtained from murine ascites after the intraperitoneal injection of hybridoma cells.

For in vitro binding assays, cell lysates were added to immobilized recombinant glutathione S-transferase (GST) fusion proteins. The entire coding regions of the human 14-3-3ζ and 14-3-3ζ cDNAs were subcloned into the bacterial expression vector pGEX-4T-3 (Amersham Pharmacia Biotech) and purified GST/14-3-3ζ and GST/14-3-3ζ fusion proteins were obtained by use of glutathione-agarose, as described (18). NIH/3T3 cell lysates obtained from clone 52b were incubated with 0.5 μg of immobilized GST/14-3-3ζ, GST/14-3-3ζ, or GST protein at 4 °C for 1 h. Immobilized proteins were washed extensively with lysis buffer with added NaCl (final concentration 1 M). Gel sample buffer was added, and boiled samples were separated by SDS-PAGE, transferred to nitrocellulose membranes, and analyzed by immunoblotting.

Immunoprecipitation—Immunoprecipitation assays, protein A/G-Plus agarose (Santa Cruz Biotechnology) was used to immobilize antibody-bound proteins. Immunoprecipitates were washed with lysis buffer with added NaCl (final concentration 1 M) and analyzed by SDS-PAGE as above.

Immunofluorescence Microscopy—NIH/3T3 fibroblasts were plated on chamber slides (Nunc, Inc.). After 2 days in culture, cells were fixed with 4% formaldehyde in phosphate-buffered saline (PBS), followed by permeabilization with 1% Triton X-100. Primary antibody was applied in phosphate-buffered saline with 10 mM glycine, was incubated with fixed, permeabilized cells for 1 h at room temperature. Murine monoclonal anti-FLAG epitope antibody (Santa Cruz Biotechnology) was used at a dilution of 1:300. Murine monoclonal anti-PKUζ antibody, rabbit polyclonal anti-14-3-3ζ (Santa Cruz Biotechnology), and murine IgG (Promega) were used at dilutions of 1:200. Murine monoclonal anti-Myc epitope antibody (Santa Cruz Biotechnology) and rabbit polyclonal anti-cyclin B1 antibody (Santa Cruz Biotechnology) were used at dilutions of 1:100. Goat polyclonal antivimentin antibody (Chemicon International Inc.) was used at a dilution of 1:40. After incubation with primary antibody, slides were washed three times with PBS. Fluorescein isothiocyanate (FITC)-conjugated anti-goat IgG secondary antibody (used at 1:200 dilution) (Santa Cruz Biotechnology), cyanine Cy3-conjugated anti-mouse IgG secondary antibody (used at 1:200 dilution) (Jackson Immunoresearch Laboratories), or FITC-conjugated anti-rabbit IgG secondary antibody (used at 1:200 dilution) (Jackson Immunoresearch Laboratories) was incubated for 1 h with cells at room temperature. Slides were washed three times with PBS. Coverslips were mounted with Vectashield mounting medium (Vector Laboratories Inc.), and cells were viewed in a confocal laser microscope (MRC 1024, Bio-Rad). To check for specificity of antibody binding, cells were treated with monoclonal or secondary antibody only, or with both an antigenic peptide (100 μM) was added to the anti-PKUζ antibody (0.1 mg/ml). Control slides did not display significant fluorescence in any case.

Cell Synchronization—Cells were treated with 5 μM aphidicolin, a DNA polymerase-a inhibitor, for 24 h to synchronize cells at G1/S (27, 28). Aphidicolin was washed off and replaced with fresh media for 0 h (G2/S-enriched cells), 6 h (G1/S-enriched cells), 12 h (G2/M-phases-enriched cells), and 18 h (G1/M-phases-enriched cells). synchronized cells were processed for immunofluorescence microscopy, as described above. The cell cycle distribution of parallel NIH/3T3 cells at these time points was confirmed by propidium iodine staining and fluorescent cell sorting.

Nuclear Extract Preparation—Nuclear extracts were prepared by an adaptation of the method of Heberlein and Tjian (29). In brief, cultured cells were lifted with a cell scraper in ice-cold phosphate-buffered saline, pelleted, and lysed in ice-cold detergent-free hypotonic lysis buffer I (15 mM Hepes, pH 7.6, 10 mM KCl, 5 mM MgCl₂, 0.1 mM EDTA, 1 mM dithiothreitol, 10 mM Na₃S₂O₃) by passing through a 22-gauge needle five times. Nuclei were collected by low speed centrifugation (2000 rpm for 10 min). Nuclei were resuspended in ice-cold buffer A (15 mM Hepes, pH 7.6, 115 mM KCl, 5 mM MgCl₂, 0.1 mM EDTA, 1 mM dithiothreitol, 10 mM Na₃S₂O₃) by passing through a 22-gauge needle five times. Nuclei were collected by low speed centrifugation (2000 rpm for 10 min). Nuclei were resuspended in ice-cold buffer A (15 mM Hepes, pH 7.6, 115 mM KCl, 5 mM MgCl₂, 0.1 mM EDTA, 1 mM dithiothreitol, 10 mM Na₃S₂O₃) by passing through a 22-gauge needle five times. Nuclei were collected by low speed centrifugation (2000 rpm for 10 min). Nuclei were resuspended in ice-cold buffer A (15 mM Hepes, pH 7.6, 115 mM KCl, 5 mM MgCl₂, 0.1 mM EDTA, 1 mM dithiothreitol, 10 mM Na₃S₂O₃) by passing through a 22-gauge needle five times. Nuclei were collected by low speed centrifugation (2000 rpm for 10 min).
RESULTS

Interactive Cloning of PKUα—To identify additional binding partners of 14-3-3 protein, the yeast two-hybrid system was used with 14-3-3z as bait to screen a mouse embryonic library. Approximately 2 million colonies were screened, and nine clones were found to specifically interact with 14-3-3z, with lamin as a negative control. One of the interacting clones, clone 52b, encoded the carboxyl-terminal portion of a putative serine/threonine kinase. Additional two-hybrid analysis demonstrated that clone 52b did not interact with the protein kinase Raf-1.

Clone 52b encoded a putative protein product of 94 amino acids. This clone was identical at the amino acid level to a portion of a human cDNA named protein kinase Uα (PKUα) (GenBankTM accession AB004884) (Fig. 1). Clone 52b was also identical at the amino acid level to a portion of a murine cDNA named multiple testes transcript 1 (mtt1) (GenBankTM accession AB004885) (30). In addition, it was found to share a high degree of sequence similarity with a portion of a human cDNA named protein kinase U-κ (PKUκ), and also with an open reading frame in the Caenorhabditis elegans genome (C07A9.3 in chromosome III, GenBankTM accession P34314).

PKUα and PKUβ each contain two potential coiled-coil domains. The kinase domains of these proteins share significant sequence similarity with protein kinase A and phosphorylase kinase. There is one potential 14-3-3-binding site in the carboxyl-terminal half of PKUα (amino acids 121–245) than to the amino-terminal region (amino acids 1–78) (Fig. 2). These results are consistent with previous observations of the interaction of 14-3-3z with phosphorylated troponin hydroxylase (31) but do not exclude the possibility that additional contact points exist between 14-3-3z and 52b. Indeed, mutational analysis of 14-3-3z has revealed that multiple residues in both the carboxyl- and amino-terminal portions of the protein are important for phosphoserine-mediated binding (15).

PKUα Expression in Embryonic and Adult Tissues—Northern blot analysis was performed to determine the gene expression pattern of PKUα. The clone 52b oligonucleotide probe was found to cross-react with an mRNA species of 4.3 kb in murine, rat, and human tissues (data not shown). PKUα was found to be highly expressed in whole murine embryos throughout development (Fig. 3A). In adult murine tissues, PKUα was widely expressed, with the highest level of expression found in testes (Fig. 3B). Although the clone 52b probe cross-reacted with a single mRNA species of 4.3 kb in most tissues, additional bands were detected in testes, including a major band of 3.7 kb.

Analysis of PKUα Protein—A monoclonal antibody was generated by use of a keyhole limpet hemocyanin-coupled peptide corresponding to the carboxyl terminus of murine PKUα (Fig. 1). When tested by enzyme-linked immunosorbent assay with the immunogenic peptide, the antibody was found to be efficient for both Western blotting and immunoprecipitation. PKUα protein levels were examined with protein lysates generated from cultured NIH/3T3 fibroblasts and 293 cells. The anti-PKUα monoclonal antibody specifically recognized a single species with a relative molecular mass of approximately 88 kDa that was detected in NIH/3T3 (Fig. 4) cells. This size corresponds to the molecular mass of TOUSLED (26). Anti-serum binding to the 88-kDa species was blocked by addition of the antigenic peptide (Fig. 4).

A fusion protein of GST and 14-3-3z was produced in bacteria and used to determine whether PKUα interacts with 14-3-3 in vitro. PKUα protein derived from NIH/3T3 cells bound to immobilized GST/14-3-3z fusion protein but not to GST protein alone (Fig. 5A). PKUα protein derived from NIH/3T3 cells also bound to immobilized GST/14-3-3z fusion protein (data not shown).

The ability of PKUα to interact with 14-3-3 in vivo was tested in immunoprecipitation experiments; protein lysates derived from subconfluent unsynchronized NIH/3T3 cells grown in the presence of 10% fetal calf serum were immunoprecipitated with anti-14-3-3z. A Western blot revealed that the immunoprecipitate contained PKUα, suggesting that PKUα and 14-3-3z form a complex in vivo (Fig. 5B).
Subcellular Localization of PKUα—To characterize the subcellular localization of PKUα, confocal laser immunofluorescence microscopy was performed with the monoclonal anti-PKUα antibody. Previous work has established that TOUSLED kinase is constitutively localized in the nucleus of plant cells (26). In addition, other investigators have shown that PKUβ is localized in the nucleus and, to a lesser extent, in the cytoplasm when overexpressed in COS1 cells (30). Analysis of nontransfected subconfluent NIH/3T3 cells grown in 10% fetal calf serum revealed that PKUα, in contrast to TOUSLED, was found in the cytoplasm in a wavy network pattern, characteristic of intermediate filaments, that extended throughout the cell (Fig. 6) (32, 33). Dual fluorescence experiments revealed that vimentin, an intermediate filament protein, and PKUα colocalized in NIH/3T3 fibroblasts (Fig. 6).

To determine whether the localization of PKUα is dependent on the cell cycle, transfected subconfluent NIH/3T3 fibroblasts were synchronized by exposure to aphidicolin, which causes cells to accumulate at the G1/S border by inhibiting DNA polymerase-α activity (27, 28). Subcellular localization of PKUα was examined by confocal laser immunofluorescence microscopy with the monoclonal anti-PKUα antibody at several time points after aphidicolin exposure (0, 6, 12, 18 h). These experiments demonstrated that PKUα was primarily localized in the cytoplasm at the G1/S border (0 and 18 h after release), but during S phase (6 h after release) it became perinuclear, and during late G2 (12 h after release) it became nuclear in distribution (Fig. 7).

The subcellular localization of PKUα in NIH/3T3 cells that were synchronized by aphidicolin exposure was next examined in parallel with cyclin B1. Previous work has demonstrated that cyclin B1 is primarily localized in the cytoplasm of cells in the G1, S, and early G2 phases of the cell cycle but that during late G2 cyclin B1 rapidly translocates into the nucleus (34). PKUα and cyclinB1 were localized in the cytoplasm of cells at the G1/S border (0 hours after aphidicolin release) (Fig. 8). Both PKUα and cyclin B1 became nuclear in distribution during late G2 (12 h after aphidicolin release) (Fig. 8).

In addition, NIH/3T3 cells were transfected with a mammalian expression plasmid encoding Myc epitope-tagged KIAA0137 that comprises amino acids 239–787 of PKUα and that includes the entire kinase domain. Confocal laser immunofluorescence microscopy with a monoclonal anti-Myc epitope antibody demonstrated the KIAA0137 also translocated into the nucleus of G2 phase cells after aphidicolin exposure (data not shown).

14-3-3 Inhibits the Nuclear Translocation of PKUα—14-3-3 binding is thought to promote the cytoplasmic localization of several proteins, including BAD, Raf-1, and Cdc25c (17, 19, 24). To examine whether 14-3-3 binding inhibits the nuclear local-
zephes were analyzed by immunoblotting with a monoclonal anti-PKUα antibody. This dominant negative form has mutations at two of the amphipathic groove that binds to phosphoserine (15).

3rd lane

GST

1st lane

GST alone

3rd lane, NIH/3T3 cell lysates were added to immobilized, recombinant GST/GST/3T3 lys), and after extensive washing, bound proteins were analyzed by immunoblotting. 4th lane, NIH/3T3 cell lysates were added to immobilized, recombinant GST/14-3-38 fusion protein (1433-GST/3T3 lys), and after extensive washing, bound proteins were analyzed by immunoblotting. 5th lane, an extract of NIH/3T3 cells (3T3 lys). This immunoblot is representative of results of four independent experiments. B, PKUα associates with 14-3-3ζ protein in vitro. Protein samples were analyzed by immunoblotting with a monoclonal anti-PKUα antibody and by a polyclonal anti-14-3-3ζ antibody that specifically recognizes the ζ isoform. 1st lane, a pooled extract of NIH/3T3 cells (3T3 lys) (10 μl). 2nd lane, an immunoprecipitate obtained from 500 μl of pooled 3T3 lysate with a monoclonal anti-14-3-3ζ antibody (IP 1433ζ). 3rd lane, an immunoprecipitate obtained from 500 μl of pooled 3T3 lysate with monoclonal anti-mitogen-activated protein kinase (IP MAPK). 4th lane, an immunoprecipitate obtained from 500 μl of pooled 3T3 lysate with murine IgG (IP IgG). This immunoblot is representative of results of three separate experiments.

Fig. 6. Subcellular localization of PKUα in unsynchronized cells. Confocal laser immunofluorescence microscopy was used to evaluate the intracellular localization of PKUα and vimentin. A, laser confocal immunofluorescence image of a NIH/3T3 cell by use of a murine monoclonal anti-PKUα primary antibody and a Cy3-conjugated anti-murine IgG secondary antibody. This appearance is consistent with the localization of PKUα to the intermediate filament system. No cellular staining was observed when mouse IgG was used as a primary antibody or when antigenic peptide (100 μM) was added to the anti-PKUα monoclonal antibody (data not shown). B, laser confocal immunofluorescence image of an NIH/3T3 cell by use of a goat polyclonal antivimentin primary antibody and a FITC-conjugated anti-goat IgG secondary antibody. C, dual fluorescence laser confocal image by use of murine monoclonal anti-PKUα and goat polyclonal antivimentin primary antibodies. Dual fluorescence is signified by a yellow color.

Fig. 7. Cell cycle-dependent subcellular localization of PKUα. Cultured NIH/3T3 cells were synchronized at G1/S by use of aphidicolin treatment. Cells were examined at various time points after release from aphidicolin exposure, fixed, and analyzed by confocal laser immunofluorescence microscopy to determine the intracellular localization of PKUα. A, 0 h after release from aphidicolin (G1/S border). B, 6 h after release from aphidicolin (S phase). C, 12 h after release from aphidicolin (G2/M border). D, 18 h after release from aphidicolin (G0 phase). The cell cycle distribution of parallel NIH/3T3 cells at these time points after release from aphidicolin was confirmed by propidium iodine staining and fluorescent cell sorting.

blotting with an anti-14-3-3β polyclonal antibody that recognizes most 14-3-3 isoforms (Fig. 9A). The ability of DN-14-3-3 to inhibit the association of PKUα with native 14-3-3 was tested in communoprecipitation experiments; protein lysates derived from subconfluent transfected NIH/3T3 cells grown in the presence of 10% fetal calf serum were immunoprecipitated with anti-PKUα. A Western blot revealed that the anti-PKUα immunoprecipitate derived from DN-14-3-3-transfected cells did not contain 14-3-3, in contrast to an immunoprecipitate derived from untransfected cells (Fig. 9B). Propidium iodine staining and fluorescent cell sorting was performed, and this showed that transfection of NIH/3T3 cells with DN-14-3-3 did not result in a significant enrichment of cells arrested at G2.

Nuclear extracts were obtained from DN-14-3-3-transfected NIH/3T3 cells and were analyzed by immunoblotting with the monoclonal anti-PKUα antibody. Transfected cells exhibited a marked increase in nuclear PKUα protein compared with untransfected controls (Fig. 9, C and D). Confocal laser immunofluorescence microscopy was also performed on DN-14-3-3-transfected cells with the monoclonal anti-PKUα antibody, and this revealed a significant increase in nuclear PKUα protein compared with untransfected controls (Fig. 10). DN-14-3-3 localization was also examined by confocal laser immunofluorescence microscopy experiments with a monoclonal anti-Myc epitope antibody, and these revealed that DN-14-3-3 was located primarily in the cytoplasm of transfected cells (Fig. 10E).

DISCUSSION

14-3-3 proteins are ubiquitously expressed intracellular dimeric proteins that regulate several aspects of cellular phys-
ology and bind to signaling, cell cycle, cytoskeletal, and apoptotic proteins (1, 2). The varied biochemical functions of 14-3-3 are dependent on binding to a partner protein; this binding may alter the enzymatic activity of the partner (e.g. tyrosine hydroxylase, protein kinase C, and Raf-1) (1, 2, 7–10), seques-

ter it (e.g. BAD) (19), enhance its solubility (e.g. keratin K8) (35), link it to other signaling proteins (e.g. BCR and Raf-1) (23), or promote its nuclear export (e.g. Cdc25) (24). 14-3-3 preferentially binds to proteins that contain serine-phosphorylated residues (14, 15, 20–22, 36, 37), a requirement that suggests that serine kinases play a critical role in the regulation of 14-3-3 binding. Indeed, in the case of the apoptosis-promoting protein BAD, the serine kinase Akt or other serine kinases may be required for BAD phosphorylation that leads to 14-3-3 binding (38). Not only do serine kinases regulate 14-3-3 binding, but it also appears that 14-3-3 regulates the activity of a variety of serine kinases, such as Raf-1 and protein kinase C.

In this work, we sought new binding partners of 14-3-3 by performing a yeast two-hybrid screen. The carboxyl-terminal portion of a murine serine/threonine kinase, named PKUα, was found to interact with 14-3-3γ. PKUα is homologous to an A. thaliana protein, TOUSLED, that is required for normal flower and leaf development (25). The identity of the signal transduction cascade in which TOUSLED participates is unclear; there is a homologue of TOUSLED in C. elegans, but the function of the worm protein is unknown.

In this study, we documented in Northern blot experiments that the PKUα gene is highly expressed throughout murine embryonic development and is widely expressed in adult murine tissues. GST/14-3-3β and GST/14-3-3γ fusion proteins were used to determine that PKUα binds to 14-3-3 in vitro. Coimmunoprecipitation experiments demonstrated that PKUα and 14-3-3 form a complex in vivo.

PKUα is found in the cytoplasmic intermediate filament system of cells at the G1/S border, in the perinuclear area of S

![Fig. 8. Similar subcellular localization of PKUα and cyclin B1. Cultured NIH/3T3 cells were synchronized at G1/S by use of aphidicolin treatment. Cells were examined at various time points after release from aphidicolin exposure, fixed, and analyzed by confocal laser immunofluorescence microscopy with a murine monoclonal anti-PKUα primary antibody or a rabbit polyclonal anti-cyclin B1 primary antibody. A, PKUα localization 0 h after release from aphidicolin (G1/S border). C, cyclin B1 localization 0 h after release from aphidicolin. B, PKUα localization 12 h after release from aphidicolin (G2/M border) in the same cells depicted in A. D, cyclin B1 localization 12 h after release from aphidicolin in the same cells depicted in B. The cell cycle distribution of parallel NIH/3T3 cells at these time points after release from aphidicolin was confirmed by propidium iodine staining and fluorescent cell sorting.](image)

![Fig. 9. Dominant negative 14-3-3γ promotes the nuclear localization of PKUα. A, expression of dominant negative 14-3-3γ in transfected cells. NIH/3T3 fibroblasts were transfected with a mammalian expression vector encoding a Myc epitope-tagged version of dominant negative 14-3-3γ (R56A and R60A). Cell lysates were analyzed by anti-pan-14-3-3 antibody immunoblotting. Equal amounts of total protein were loaded in each lane. 1st lane, an extract of untransfected NIH/3T3 cells (Untx. 3T3 lys.). 2nd lane, an extract of cells that was transfected with dominant negative 14-3-3γ (DN-1433). B, dominant negative 14-3-3γ inhibits the association of PKUα with 14-3-3. Protein samples were analyzed by immunoblotting with a rabbit polyclonal anti-pan-14-3-3 antibody or a mouse monoclonal anti-PKUα antibody. 1st lane, an extract of NIH/3T3 cells transfected with DN-14-3-3γ. 2nd lane, an extract of untransfected NIH/3T3 cells. 3rd lane, a control immunoprecipitate (IP) obtained with murine IgG from DN-14-3-3γ-transfected cell lysate. 4th lane, a control immunoprecipitate obtained with murine IgG from untransfected cell lysate. 5th lane, an immunoprecipitate obtained with monoclonal anti-PKUα antibody from DN-14-3-3γ-transfected cell lysate. 6th lane, an immunoprecipitate obtained with monoclonal anti-PKUα antibody from untransfected cell lysate. Equal amounts of total protein were used to generate each immunoprecipitate. Faint anti-PKUα immunoreactive bands were observed in 1st and 2nd lanes after prolonged chemiluminescent exposure of the blot. This immunoblot is representative of results of two separate experiments. C, anti-PKUα epitope immunoblot analysis of nuclear and cytoplasmic fractions of cell lysates. Nuclear (Nuc) and cytoplasmic (Cyto) extracts were obtained from untransfected (Untx.) NIH/3T3 cells or from cells that were transfected with Myc epitope-tagged dominant negative 14-3-3γ (DN-1433). Extracts were analyzed by immunoblotting with a monoclonal anti-Myc epitope antibody. Equal amounts of total protein were loaded in each lane. Equal loading of nuclear extracts was confirmed by immunoblotting with a monoclonal anti-polymerase cell nuclear antigen antibody. D, densitometric analysis of anti-PKUα immunoblots described in B by use of NIH Image software. Each column represents the average ± S.E. of three determinations.](image)
phase cells, and in the nucleus of late G2 cells. This localization differs from that of TOUSLED protein, which is found entirely in the nuclei of plant cells at all phases of the cell cycle (26). TOUSLED lacks a putative 14-3-3-binding site and this may explain the difference in subcellular localization. In transfected COS1 cells, previous work has demonstrated that overexpressed PKUα is found in the nucleus with some cytoplasmic localization (30), but the subcellular localization of native PKUβ and PKUα has not been previously determined.

In order to test the ability of 14-3-3 to regulate the subcellular localization of PKUα, NIH/3T3 cells were transfected with a dominant negative form of 14-3-3η that is mutated at two arginine residues (R56A and R60A). Dominant negative forms of 14-3-3 are located on one side of the amphipathic groove that is mutated at two arginine residues (R56A and R60A). Dominant negative forms of 14-3-3, and crystallographic analysis suggests that several key residues, including leucine-216 and leucine-220 of 14-3-3ζ, are located on one side of the amphipathic groove that binds to phosphoserine-containing peptide motifs (39). Mutation of leucine 220 of 14-3-3ζ to aspartic acid abrogates binding to Raf-1 kinase, and this demonstrates that residues in the NES-like sequence are important for phosphoserine motif binding (39). One hypothetical model that explains our results is that wild type 14-3-3 forms a oligomeric complex with PKUα and Crm1 in the nucleus of cultured cells that mediates PKUα export into the cytoplasm and that DN-14-3-3 forms inactive heterodimers that are unable to bind simultaneously to both PKUα and Crm1. Experiments are ongoing to test this model of 14-3-3-mediated nuclear export.

The intranuclear substrates of PKUα and TOUSLED, if any, have not been identified. The intranuclear biochemical function of TOUSLED is obscure, although its role in proliferative events in plant development suggests that it may have a cell cycle-related activity (25, 26). Further studies are needed to investigate this possibility.

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