Nucleocytoplasmic transfer of Cdk5 and its binding to puromycin-sensitive aminopeptidase in Dictyostelium discoideum

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ORIGINAL PAPER

Nucleocytoplasmic transfer of cyclin dependent kinase 5 and its binding to puromycin-sensitive aminopeptidase in *Dictyostelium discoideum*

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Abstract The *Dictyostelium discoideum* homolog of mammalian cyclin dependent kinase 5 (Cdk5) has previously been shown to be required for optimal growth and differentiation in this model organism, however, the subcellular localization of the protein has not previously been studied. In this study, immunolocalizations and a GFP fusion construct localized Cdk5 predominantly to the nucleus of vegetative cells. Western blots showed that Cdk5 was present in both nuclear and non-nuclear fractions, suggesting a functional role in both cellular locales. During the early stages of mitosis, Cdk5 gradually moved from a punctate nucleoplasmic distribution to localize adjacent to the inner nuclear envelope. During anaphase and telophase, Cdk5 localized to the cytoplasm and was not detected in the nucleoplasm. Cdk5 returned to the nucleus during cytokinesis. Proteolytic activity has been shown to be a critical regulator of the cell cycle. Immunoprecipitations coupled with immunolocalizations identified puromycin-sensitive aminopeptidase A (PsaA) as a potential Cdk5 binding partner in *Dictyostelium*. Immunoprecipitations also identified two phosphotyrosine proteins (35 and 18 kDa) that may interact with Cdk5 in vivo. Together, this work provides new insight into the localization of Cdk5, its function during cell division, and its binding to a proteolytic enzyme in *Dictyostelium*.

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Keywords Cyclin dependent kinase 5 · Puromycin sensitive aminopeptidase A · Nucleocytoplasmic localization · Mitosis · *Dictyostelium discoiduem* · Proteolysis

Abbreviations

Cdk	Cyclin dependent kinase
Crp	Cdc2-related PCTAIRE
Psa	Puromycin sensitive aminopeptidase
KLH	Keyhole limpet hemocyanin
IP	Immunoprecipitation
GFP	Green fluorescent protein
NumA	Nucleomorphin A
Cbp4a	Calcium-binding protein 4a
BSA	Bovine serum albumin
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
kDa	Kilodalton

Introduction

Dictyostelium discoideum is a simple eukaryote that is used as a model system for studying a number of cell and developmental processes. A large number of proteins homologous to those found in higher organisms have been characterized in *Dictyostelium,* making it a valuable system for biomedical research (Williams et al. 2006; Escalante 2011). In *Dictyostelium*, the cyclin dependent kinase 5 (Cdk5) homolog Crp (Cdc2-Related PCTAIRE) regulates endocytosis and secretion and is required for optimal growth and differentiation (Sharma et al. 2002). Cdks are a family of serine/threonine protein kinases that are activated by cyclins and are involved in regulating eukaryotic cell

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cycle progression. Cdk5 functions as a regulatory kinase and has been implicated in a diverse number of cellular processes in many cell types (Dhavan and Tsai 2001; Rosales and Lee 2006; Giese 2007). Several studies have linked Cdk5 dysregulation to advanced melanoma and neurodegenerative diseases such as Alzheimer's disease and amyotrophic lateral sclerosis (Dhavan and Tsai 2001; Abdullah et al. 2011; Crews et al. 2011). While other members of the Cdk protein family have cell cycle-promoting functions, Cdk5 has been shown to hold the cell cycle in check in neocortical neurons (Cicero and Herrup 2005). Until only recently, Cdk5 was thought to function primarily in the cytoplasm; however, an increasing number of studies have begun to detail the nuclear functions of Cdk5 (Yang and Mao 2008). Cdk5 has been reported to have a death-promoting activity within the nucleus of neurons and a pro-survival activity when localized to the cytoplasm (O'Hare et al. 2005). In cycling NIH 3T3 cells, the localization of Cdk5 changes from predominantly nuclear to cytoplasmic suggesting that Cdk5 acts as a cell cycle suppressor when it is localized in the nucleus (Zhang et al. 2008).

Puromycin-sensitive aminopeptidases (Psa) are highly conserved metalloproteases that hydrolyze N-terminal amino acids from oligopeptides and are linked to a number of cellular processes (Taylor 1993). In COS and 3T3 fibroblasts, Psa has been shown to localize to both the nucleus and cytoplasm and to associate with the microtubules of the spindle apparatus during mitosis (Constam et al. 1995). Ubiquitin-mediated proteolytic activity has been shown to regulate the cell cycle by controlling the levels of cyclins as well as a number of other key cell cycle regulators, suggesting that proteolysis is a fundamental process involved in the regulation of the cell cycle (Koepp et al. 1999). Inhibitors of aminopeptidase activity have been reported to suppress cell proliferation (Takahashi et al. 1985, 1989). Puromycin and bestatin, two Psa inhibitors, have previously been shown to arrest the cell cycle resulting in an accumulation of cells in G2/M phase, suggesting that Psa activities are required to complete mitosis and are essential for cell division and viability (Hersh 1981; Constam et al. 1995). Bestatin, which inhibits the final step of intracellular protein degradation (i.e., degradation of small peptides into free amino acids), has also been shown to repress epidermal growth factor-induced DNA synthesis and cell division in rat hepatocytes (Takahashi et al. 1987, 1988, 1989). In addition, Psa activity has been shown to be required for the meiosis in *Arabidopsis thaliana* and *Caenorhabditis elegans* (Sánchez-Morán et al. 2004; Lyczak et al. 2006). *Dictyostelium* PsaA localizes to the nucleoplasm and bestatin binds to PsaA and inhibits cell division supporting a cell cycle role for this enzyme in this model system (Catalano et al. 2011).

Sequence analysis shows that *Dictyostelium* Cdk5 possesses characteristics of both cdc2 kinases and PCTAIRE proteins. The kinase activity of the protein has been verified with a Histone H1 kinase assay (Michaelis and Weeks 1993; Sharma et al. 1999); however, neither the subcellular localization nor the in vivo binding partners of the protein have previously been studied. To rectify this situation, we produced and verified the specificity of a polyclonal antibody that detects endogenous Cdk5 and Cdk5-GFP and observed the localization of Cdk5-GFP in live and fixed cells. We also investigated the binding of Cdk5 to phosphoproteins and established nuclear proteins, including PsaA. The results from this work fit with those observed in mammalian cells suggesting that *Dictyostelium* can serve as a useful model for the further study of Cdk5 function and localization.

Materials and methods

Cells, lysates, and chemicals

Dictyostelium discoideum strains AX3 and AX3/[act15]:cdk5:GFP were grown either in the presence of *Escherichia coli* on SM agar pH 6.5 at 22°C in the dark for 24–30 h or axenically in HL-5 medium at 22°C and 150 rpm. The ethical use of mice was approved by the University of Toronto Animal Care Committee. Mice were asphyxiated with carbon dioxide, dissected, and the following tissues harvested: brain, heart, kidney, liver, and lung. Samples were prepared as previously described (Poloz and O'Day 2009).

Polyclonal antibody production, peptide competition assay, and affinity purification

An antibody was generated in New Zealand white rabbits against a synthetic peptide (CKISSKLGEGTFGVVYKGL) equivalent to an amino acid sequence (663–680) from a putative tyrosine kinase-like protein from *Dictyostelium discoideum* (DDB0229955; http://www.dictybase.org). The peptide was conjugated to keyhole limpet hemocyanin (KLH; Sigma-Aldrich Canada Ltd., Oakville, ON, Canada) via the N-terminal cysteine residue that was added specifically to facilitate this linkage. The unconjugated and conjugated peptides were a gift from Dr. Yali Wang (Advanced Syntech Corporation, Mississauga, ON, Canada). The University of Toronto Animal Care Committee approved the use of rabbits for antibody production according to the Production of Polyclonal Antibodies in Rabbits Standard Operating Procedures of the University of Toronto. A peptide competition assay verified the specificity of the antibody. Crude serum (1:40 dilution) was

incubated with ±6.5 μg/μl peptide immunogen and spun at 37°C for 1.5 h. The solutions were then incubated overnight at 4°C with gently rotation. Immune complexes were pelleted by centrifugation at 12000×*g* for 15 min at 4°C. Supernatants were retained and used for western blotting. Crude serum was IgG purified with the Affi-Gel® Protein A MAPS® II Kit (Bio-Rad Laboratories Canada Ltd., Mississauga, ON, Canada) and then affinity purified against the peptide immunogen using a SulfoLink Coupling Resin according to the manufacturer's instructions (Fisher Scientific Company, Toronto, ON, Canada).

Immunoprecipitation

Cells grown axenically in HL-5 medium were harvested in the mid-log phase of growth (1–4 × 10^6 cells/ml) and lysed with NP-40 lysis buffer containing 50 mM Tris pH 8.0, 150 mM NaCl, 0.5% NP-40, 5 mM EDTA, 1 mM EGTA, 1 mM Na_3VO_4, 10 mM NaF, 10 μg/ml leupeptin, 1 μg/ml pepstatin A, and a protease inhibitor cocktail (Hoffmann-La Roche Limited, Mississauga, ON, Canada). Lysates were sonicated 3 times for 5 s each. Rabbit polyclonal anti-Cdk (15–25 μl) or mouse monoclonal anti-GFP (20 μl; Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA) was added to whole cell lysates (1 mg) and spun overnight at 4°C. Protein G PLUS agarose or Immuno-Cruz™ IP resins (B/C/E/F; Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA) were added to lysates which were then spun for 4 h at 4°C. Resins were spun down and supernatants removed and retained as protein-depleted fractions (PD). Resins were washed with NP-40 lysis buffer to eliminate non-specific interactions, re-suspended in 2× sample loading buffer, and boiled for 5 min. Supernatants were removed and retained as immunoprecipitate fractions (IP). Aliquots of IP fractions (15–35 μl) were loaded into the appropriate wells of polyacrylamide gels and separated by SDS-PAGE. Samples were stored at −80°C for future use.

Immunolocalization

Cells in the mid-log phase of growth (1–4 × 10^6 cells/ml) were placed on 13 × 0.1 mm glass coverslips (McCrone Microscopes & Accessories, Westmont, IL, USA) and allowed to adhere for 15–30 min. Cells were fixed in ultracold methanol (−80°C) as previously described (Catalano and O'Day 2011). Fixed cells were incubated with primary antibody for 1 h followed by incubation with the appropriate secondary antibody for 1 h. The following primary and secondary antibodies were used: rabbit polyclonal anti-Cdk (1:50), rabbit polyclonal anti-PsaA (1:20; Catalano et al. 2011), mouse monoclonal anti-tubulin (1:100; 12G10, Developmental Studies Hybridoma Bank, The University of Iowa, Iowa, USA), goat anti-rabbit Alexa-488 (1:100), goat anti-mouse Alexa 555 (1:100), goat anti-rabbit Alexa 555 (1:40). Secondary antibodies were purchased from Invitrogen Canada Incorporated (Burlington, ON, Canada). Coverslips were mounted on slides with Prolong™ Gold antifade reagent with DAPI (Invitrogen Canada Inc., Burlington, ON, Canada) and nail polish. Cells were viewed with epifluorescence microscopy using a Nikon Eclipse 50i microscope equipped with a Nikon DS-Ri1 12 megapixel color cooled digital camera (Nikon Canada, Mississauga, ON, Canada). Images were captured and viewed with NIS Elements BR 3.0 (Nikon Canada, Mississauga, ON, Canada). Images were merged with ImageJ (National Institutes of Health, Bethesda, MD, USA; http://rsb.info.nih.gov/ij).

Isolation of nuclei

Nuclei were isolated as described elsewhere with a few minor changes (Kaller et al. 2006; Pilcher et al. 2007). Cells (2–3 × 10^7 cells) in the mid-log phase of growth (1–4 × 10^6 cells/ml) were harvested from HL-5 medium and washed with 1× cold KK2 buffer. Cells were resuspended in nuclei buffer containing 20 mM Tris–HCl pH 7.4, 5 mM MgOAc, 5% (w/v) sucrose, 0.5 mM EDTA pH 8.0, 1 mM EGTA, 1 mM Na_3VO_4, 10 mM NaF, 1 μg/ml pepstatin A, 10 μg/ml leupeptin, and a protease inhibitor cocktail (Hoffmann-La Roche Limited, Mississauga, ON, Canada). Cells were lysed by adding Triton-X to a final concentration of 1% and incubating on ice for 5 min. Lysates were spun at 12,000×*g* for 5 min at 4°C. Supernatants were removed and retained as non-nuclear (cytoplasmic) fractions. Pelleted nuclei were resuspended in RIPA lysis buffer containing 50 mM Tris–HCl pH 7.4, 150 mM NaCl, 1% NP-40, 0.25% sodium deoxycholate, 0.5 mM EDTA pH 8.0, 1 mM EGTA, 1 mM Na_3VO_4, 10 mM NaF, 1 μg/ml pepstatin A, 10 μg/ml leupeptin, and a protease inhibitor cocktail (Hoffmann-La Roche Limited, Mississauga, ON, Canada). Samples were sonicated three times for 5 s each to completely lyse the nuclei. Samples were then spun at 12,000×*g* for 10 min at 4°C. Supernatants were removed and retained as nuclei fractions. All samples were stored at −80°C for future use.

SDS-PAGE and western blotting

SDS-PAGE and western blotting were carried out as previously described (Huber and O'Day 2009, 2011a). The following antibodies and dilutions were used: rabbit polyclonal anti-Cdk (1:400), rabbit polyclonal anti-BSA (1:1,000; Millipore, Billerica, MA, USA), mouse monoclonal anti-GAPDH (1:100; Millipore, Billerica, MA, USA), mouse monoclonal anti-actin (1:1,000; Santa Cruz

Biotechnology Inc., Santa Cruz, CA, USA), mouse monoclonal anti-tubulin (1:1,000; 12G10, Developmental Studies Hybridoma Bank, The University of Iowa, Iowa, USA), rabbit polyclonal anti-NumA (1:100; Myre and O'Day 2002), rabbit polyclonal anti-PsaA (1:400; Catalano et al. 2011), rabbit polyclonal anti-Cbp4a (1:100; O'Day et al. 2009), mouse monoclonal anti-GFP (1:800; Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA), mouse monoclonal anti-phosphotyrosine (1:1,000; New England Biolabs Canada, Pickering, ON, Canada). Membranes were developed with the Amersham^{TM} ECL Plus Western Blotting Detection System (GE Healthcare, Buckinghamshire, UK) and scanned using a Storm 860 Phosphorimager/Fluorimager (Molecular Dynamics Inc., Sunnyvale, CA, USA).

Construction of a cdk5-GFP expression vector

A *cdk5*-GFP expression vector was generated according to previously described procedures (Suarez et al. 2011). cDNA was amplified by PCR using the PfuUltra II Fusion HS DNA Polymerase master mix (Agilent Technologies Canada Inc., Mississauga, ON, Canada) and the following forward and reverse primers, respectively; 5′-CAAGATCTAAAATGGAGAAATATTCAAAAATTG-3′ and 5′-CAACTAGTATTAATAGGTTCTAAACCATC-3′. PCR primers were designed to amplify the entire *cdk5* open reading frame (879 bp) encoding amino acids 1–292. BglII and SpeI recognition sites were added onto the forward and reverse primer, respectively, to facilitate insertion of the amplified fragment into the pDM323 vector, which when expressed, would generate a Cdk5 recombinant protein with a C-terminal GFP tag (http://dictybase.org/StockCenter/StockCenter.html; Veltman et al. 2009). Restriction enzymes were purchased from New England Biolabs Canada (Pickering, ON, Canada). Mini-prepped plasmids were digested and separated by agarose gel electrophoresis to confirm the presence of the *cdk5* insert. Plasmids were further verified by sequencing at the Analytical Genetics Technology Centre (Princess Margaret Hospital, Toronto, ON, Canada). The plasmid carrying the correct in-frame insertion was named pDM323-cdk5GFP. AX3 cells were transformed by electroporation with pDM323-cdk5GFP according to previously established procedures (Gaudet et al. 2007; Suarez et al. 2011). The resulting strain was named AX3/[act15]:cdk5:GFP.

Results

Antibody production and specificity

A polyclonal antibody was generated in rabbits against a synthetic 18 amino acid peptide (KISSKLGEGTFGVVYKGL) that was equivalent to an amino acid sequence (663–680) from a putative 103-kDa tyrosine kinase-like protein in *Dictyostelium discoideum* (DDB0229955; http://www.dictybase.org). The final bleed serum did not detect a 103-kDa protein as expected. Instead, the serum strongly detected a 33-kDa protein that was not detected by pre-immunization serum or by serum affinity purified against the peptide immunogen (Fig. 1a). In addition, the 33-kDa protein was not detected by sera from rabbits that had been injected with PBS or with the carrier protein KLH (data not shown). Both crude and affinity purified serums were able to detect different amounts of protein indicating that they could be used to detect changes in the expression of the unknown 33-kDa protein. A peptide competition assay verified the specificity of the crude serum toward the peptide immunogen (Fig. 1b). The 33-kDa protein and BSA-conjugated peptide immunogen were detected more strongly by unblocked antibody than by antibody incubated with the peptide. The successful conjugation of BSA to the peptide was verified by probing blots containing various concentrations of BSA-conjugated peptide with anti-BSA (Fig. 1c).

To determine the identity of the unknown 33-kDa protein, the peptide immunogen sequence was inputted into the BLAST server of the online *Dictyostelium* resource dictyBase (http://dictybase.org/tools/blast). The *E* value was set to the maximum (i.e., 1,000) to analyze all possible matches. All the proteins were either characterized or putative kinases (data not shown). The only exact match was the intended 103 kDa target protein (DDB0229955; http://www.dictybase.org). The only matches possessing a molecular weight close to 33 kDa were the *Dictyostelium discoideum* homologs of mammalian Cdk5 (33.2 kDa) and Cdk1 (33.8 kDa). The peptide sequence matched at the N-terminus of both proteins. The peptide also matched other *Dictyostelium* Cdks, however, they could be ruled out based on their significantly higher molecular weights (Cdk8, 42.8 kDa; Cdk9, 78.2 kDa; Cdk10, 42.0 kDa). The other two members of the Cdk protein family in *Dictyostelium*, Cdk7 (40.6 kDa) and Cdk11 (41.4 kDa), were not identified as matches in the BLAST analysis. Within a 12 amino acid region of similarity, there were 10 exact matches (83%) and 12 positive matches (100%) between the peptide immunogen and Cdk5 (Fig. 1d). There were 10 exact matches (83%) and 11 positive matches (92%) within a 12 amino acid region of similarity between the peptide immunogen and Cdk1 (Fig. 1d). Experiments were undertaken to determine which Cdk was likely detected by the antibody. The results of these experiments are described in the sections to follow.

Detection of a 33-kDa protein in mouse tissues

The sequence of the peptide immunogen is highly conserved in mouse Cdk5 and Cdk1 suggesting that the

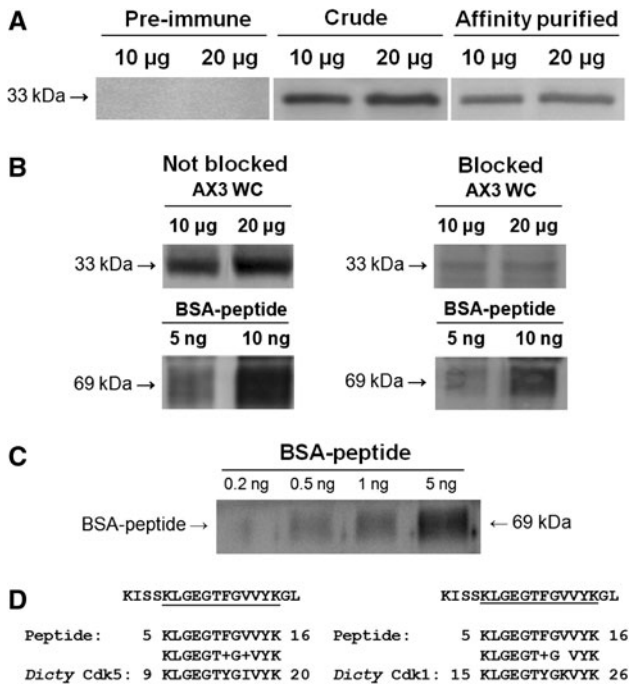

Fig. 1 Antibody production and specificity. **a** AX3 whole cell lysates (10 and 20 µg) were loaded into the appropriate lanes. Western blots probed with pre-immune, crude, and affinity purified serums. **b** Peptide competition assay. AX3 whole cell lysates (10 and 20 µg) and BSA-conjugated peptide immunogen (5 and 10 ng) were loaded into the appropriate lanes. Western blots probed with a 1:500 dilution of crude serum (Not blocked) or a 1:500 dilution of crude serum that was incubated with the peptide immunogen (Blocked). **c** Verification that the peptide immunogen was conjugated to BSA. BSA-conjugated peptide immunogen was loaded into the appropriate lanes at the indicated concentrations. Western blot probed with anti-BSA. **d** Alignment of the peptide immunogen with *Dictyostelium* Cdk5 and Cdk1

antibody could detect Cdks of mouse origin (Fig. 2a). Mouse tissue samples were prepared and protein extracts separated by SDS-PAGE and analyzed by western blotting. Mouse Cdk5 and Cdk1 possess molecular weights of 33.3 and 34.1 kDa, respectively (UniProtKB; Leinonen et al. 2006). The antibody detected a 33-kDa protein (Fig. 2b) that was not detected by pre-immunization serum (data not shown). The 33-kDa protein was detected in all tissue samples; however, its highest expression was observed in the brain. Previous studies have shown that mouse Cdk5 is expressed in all tissues; however, its highest expression and activity are observed in the nervous system, specifically the brain (Dhavan and Tsai 2001; Hellmich et al. 1992; Tsai et al. 1993; Ino et al. 1994). In addition, several studies have linked Cdk5 dysregulation to neurodegenerative diseases such as Alzheimer's disease (Dhavan and Tsai 2001; Abdullah et al. 2011). Online expression datasets for various mouse tissues also show a relative abundance of *cdk5* mRNA in the mouse brain compared to other tissues (BioGPS online database; http://biogps.gnf.org; Lattin et al. 2008; Wu et al. 2009). In contrast, the expression profiles for mouse Cdk1 do not show a relative abundance in brain or neuronal tissues suggesting that the 33-kDa protein was mouse Cdk5.

Immunoprecipitation of the 33-kDa protein

Anti-Cdk immunoprecipitated the 33-kDa protein from AX3 whole cell lysates (Fig. 3a). A 50- and 25-kDa band were also strongly detected on western blots and corresponded to the heavy and light chains, respectively, of the denatured antibody present in the IP fraction. The 33-kDa protein was only weakly detected in the protein-depleted sample providing further evidence that the protein was pulled down with the antibody. Due to the nature of the IP protocol (i.e., potential for non-specific interactions) and the artifacts that are possible during western blotting, it was necessary to determine whether the 33-kDa band that was observed in the IP fraction was in fact an immunoprecipitated protein. Anti-Cdk was immunoprecipitated in the absence of lysate using a commercially available resin designed to eliminate or reduce the detection of non-specific bands by the primary or secondary antibody during western blotting (ImmunoCruz^{TM} F; Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA). The IP fractions were separated by SDS-PAGE and analyzed by western blotting. Anti-Cdk detected the 33-kDa protein in the whole cell lysate, but not in the IP fraction (Fig. 3b), showing that the 33-kDa protein was an immunoprecipitated protein and not a fragment of the IP antibody that was detected by either the primary or secondary antibody during western blotting. Since the mouse tissue analysis suggested that the antibody primarily detected Cdk5, a *cdk5*-GFP fusion construct was generated to analyze the ability of anti-Cdk to detect and pull-down Cdk5-GFP. Anti-Cdk detected the 33-kDa protein in both AX3 and AX3/[act15]:cdk5:GFP lysates (Fig. 3c). The antibody also detected a 60-kDa protein in AX3/[act15]:cdk5:GFP lysates, which closely matched the expected molecular weight of the Cdk5-GFP fusion protein (Cdk5, 33 kDa; GFP, 27 kDa). A 42-kDa band was also detected by anti-Cdk in AX3/[act15]:cdk5:GFP lysates. This band was not detected by anti-GFP or by anti-Cdk in AX3 lysates (Fig. 5c) and, therefore, most likely corresponded to a Cdk5-GFP degradation product. Anti-Cdk pulled down the 33-kDa protein and Cdk5-GFP providing further support for the specificity of the antibody toward the peptide immunogen sequence (Fig. 3c). Although anti-Cdk pulled down the 33-kDa protein and Cdk5-GFP, the antibody did not completely immunodeplete either protein (Fig. 3c).

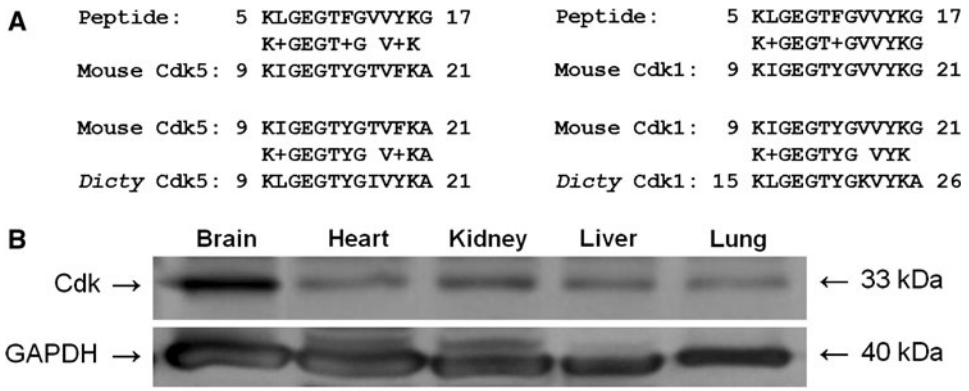

Fig. 2 Detection of a 33-kDa protein in mouse tissues. **a** Evolutionary conservation of the peptide immunogen sequence. Exact identities are indicated by the *corresponding letter*. Positive (i.e., similar) identities are indicated by *plus symbol*. **b** Protein lysates (25 µg) from the following mouse tissues were loaded into the appropriate lanes; brain, heart, kidney, liver, and lung. Western blots probed with anti-Cdk and anti-GAPDH (loading control)

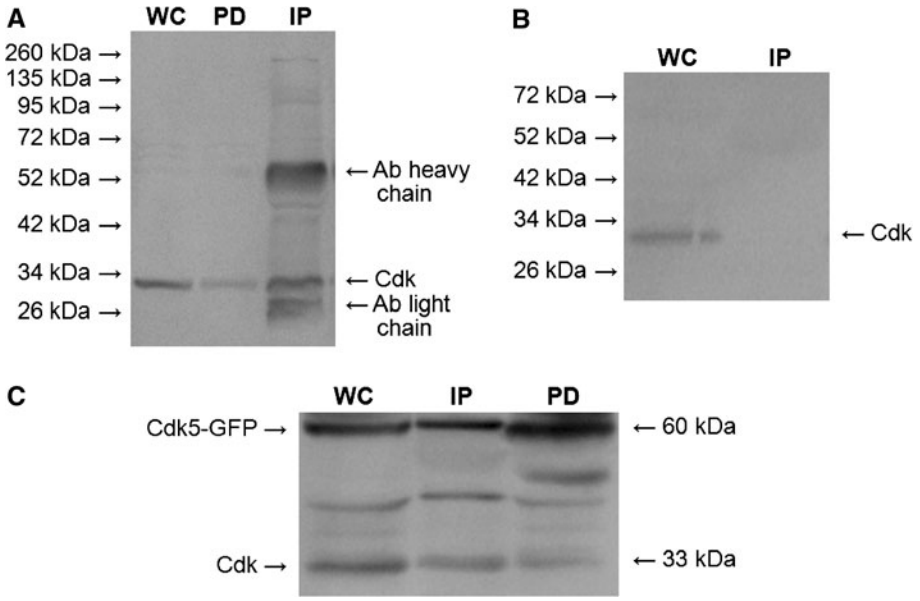

Fig. 3 Immunoprecipitation of the 33-kDa protein. **a** The 33-kDa protein was immunoprecipitated from AX3 whole cell lysates with anti-Cdk. Immunoprecipitates were pulled down with Protein G PLUS agarose beads. Western blot probed with anti-Cdk. **b** Immunoprecipitation of anti-Cdk in the absence of lysate. Anti-Cdk was pulled down with ImmunoCruz™ F IP resin. Western blot probed with anti-Cdk. **c** Immunoprecipitation of Cdk5 and Cdk5-GFP. Cdk5 and Cdk5-GFP were immunoprecipitated from AX3/[act15]:cdk5:GFP whole cell lysates with anti-Cdk. Immunoprecipitates were pulled down with ImmunoCruz™ B IP resin. Western blot probed with anti-Cdk. Whole cell lysate (WC, 25 µg), immunoprecipitate (IP, 15–25 µl), protein-depleted fraction (PD, 25 µg). Molecular weight markers (in kDa) are shown to the *left* of each blot in **a** and **b** and to the *right* of the blot in **c**

Subcellular localization

Anti-Cdk localized the protein primarily in the nuclei of fixed amoeba (Fig. 4a). Cells were co-stained with DAPI to reveal the nuclei and verify the nuclear localization. The entire cytoplasm was stained green; however, the nucleoplasm was particularly bright as compared to the rest of the cell. Nuclei were not observed in cells fixed and probed with pre-immunization serum or the Alexa 488 secondary antibody (data not shown). A western blot analysis of nuclear and non-nuclear (cytoplasmic) fractions obtained from a subcellular fractionation of *Dictyostelium* amoebae was performed to further analyze the subcellular localization of the protein. The 33-kDa protein was detected in both fractions in roughly equal amounts (Fig. 4b). To verify the efficiency of the fractionation, blots were probed with anti-tubulin, which has previously been used as a control for subcellular fractionations (Katsuno et al. 2003; Misawa et al. 2006). Tubulin was detected in non-nuclear (cytoplasmic) fractions, but not in nuclear fractions (Fig. 4b), adhering to previous studies (Katsuno et al. 2003). The localization of Nucleomorphin A (NumA),

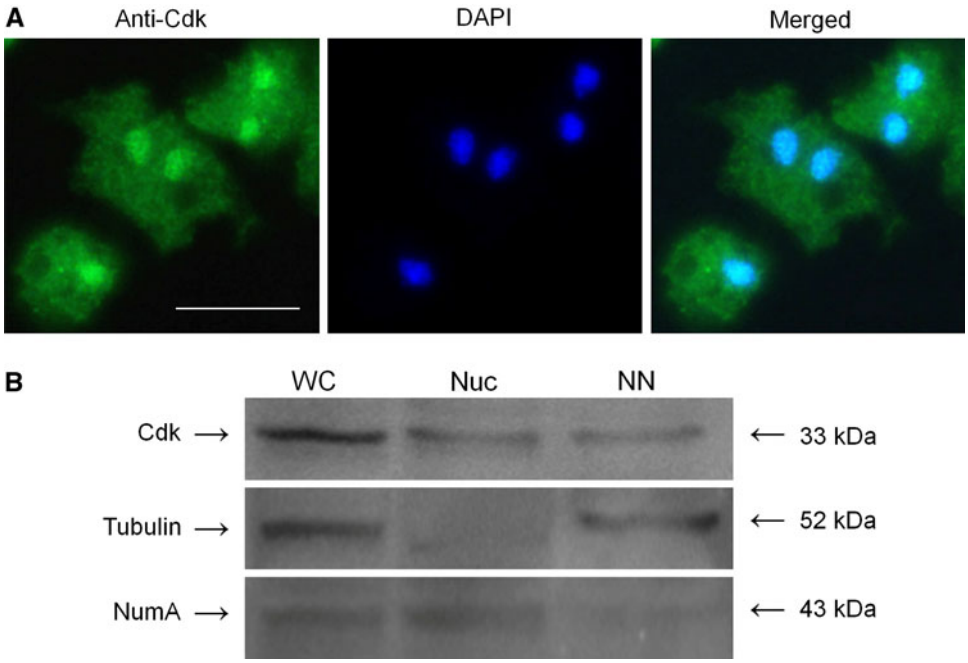

Fig. 4 Subcellular localization. **a** Axenically growing AX3 cells (1–4 × 10^6 cells/ml) were fixed and incubated with anti-Cdk followed by anti-rabbit Alexa-488 secondary antibody that fluoresced *green*. Cells were stained with DAPI to reveal nuclei (*blue*). Images were merged with ImageJ. **b** Nuclei from AX3 cells were isolated, lysed, and proteins (20 µg) separated by SDS-PAGE. Western blots probed with anti-Cdk, anti-tubulin (fractionation control), and anti-NumA (fractionation control). *WC* whole cell lysate, *Nuc* nuclei fraction, *NN* non-nuclei fraction. Molecular weight markers (in kDa) are shown to the *right* of each blot. *Scale bar* 10 µm

a characterized nuclear protein in *Dictyostelium*, was also analyzed (Myre and O'Day 2002). The detection of NumA in the nuclear fraction, but not in the non-nuclear fraction further verified the fractionation (Fig. 4b).

Due to the potential cross-reactivity of anti-Cdk with Cdk1, the localization of Cdk5-GFP was assessed and compared to the localization identified by the antibody. Cdk5-GFP localized predominantly in the nucleus of both live and fixed cells, however, the cytoplasm also fluoresced green (Fig. 5a, b). GFP alone did not predominantly localize to the nuclei of cells over-expressing GFP under the control of the same promoter (Fig. 5a). Whole cell lysates of AX3 and AX3/[act15]:cdk5:GFP were separated by SDS-PAGE and analyzed by western blotting (Fig. 5c). Anti-GFP detected Cdk5-GFP in AX3/[act15]:cdk5:GFP lysates, but not in AX3 lysates verifying the presence of the fusion protein in the transformed cells. Nuclei were isolated from AX3/[act15]:cdk5:GFP cells. Protein fractions were separated by SDS-PAGE and analyzed by western blotting with anti-GFP and anti-Cdk. Anti-GFP detected Cdk5-GFP in all fractions (Fig. 5d). Anti-Cdk detected Cdk5-GFP and the 33-kDa protein in all fractions as well (Fig. 5e). Blots probed with anti-tubulin verified the efficiency of the fractionations (Fig. 5d, e). Since the localization of Cdk5-GFP closely matched the localization identified by the antibody, this provided further evidence that the antibody primarily detected Cdk5.

Localization during mitosis

The immunolocalized protein was co-localized with tubulin to analyze its localization during mitosis (Roos et al. 1984; Kitanishi-Yumura and Fukui 1987). As shown in previous figures, the protein localized primarily in the nuclei of non-dividing cells (interphase; Fig. 6). The localization was punctate with several distinct spots visible within the nucleoplasm. During the early stages of mitosis (i.e., prophase, pro-metaphase, metaphase; Fig. 6), the punctate nucleoplasmic localization began to shift to the inner edges of the nucleus, eventually localizing to the spindle apparatus and as a ring at the inner nuclear periphery. During metaphase, there was also a depletion of staining in the nucleoplasm. The ring localization disappeared as cells entered anaphase, although the depletion of staining in the nucleoplasm was still evident (Fig. 6). During telophase, the protein localized to the cytoplasm and was absent from the nucleoplasm (Fig. 6). The protein returned to the nucleus during cytokinesis and formed larger granule-like accumulations in the nucleoplasm (Fig. 6). The immediate return of the protein to the nucleus during cytokinesis contrasted the gradual depletion of the initially punctate nucleoplasmic localization observed during the early stages of mitosis.

Co-immunoprecipitation of Cdk5 with characterized nuclear proteins

Although the functions of cytoplasmic Cdk5 have been extensively studied in mammalian systems, the nuclear functions of Cdk5 remain largely unknown. In addition, no previous study has investigated the nuclear functions of Cdk5 in *Dictyostelium*. To gain further insight into the functions of *Dictyostelium* Cdk5 in the nucleus, the protein was immunoprecipitated and analyzed for the binding to

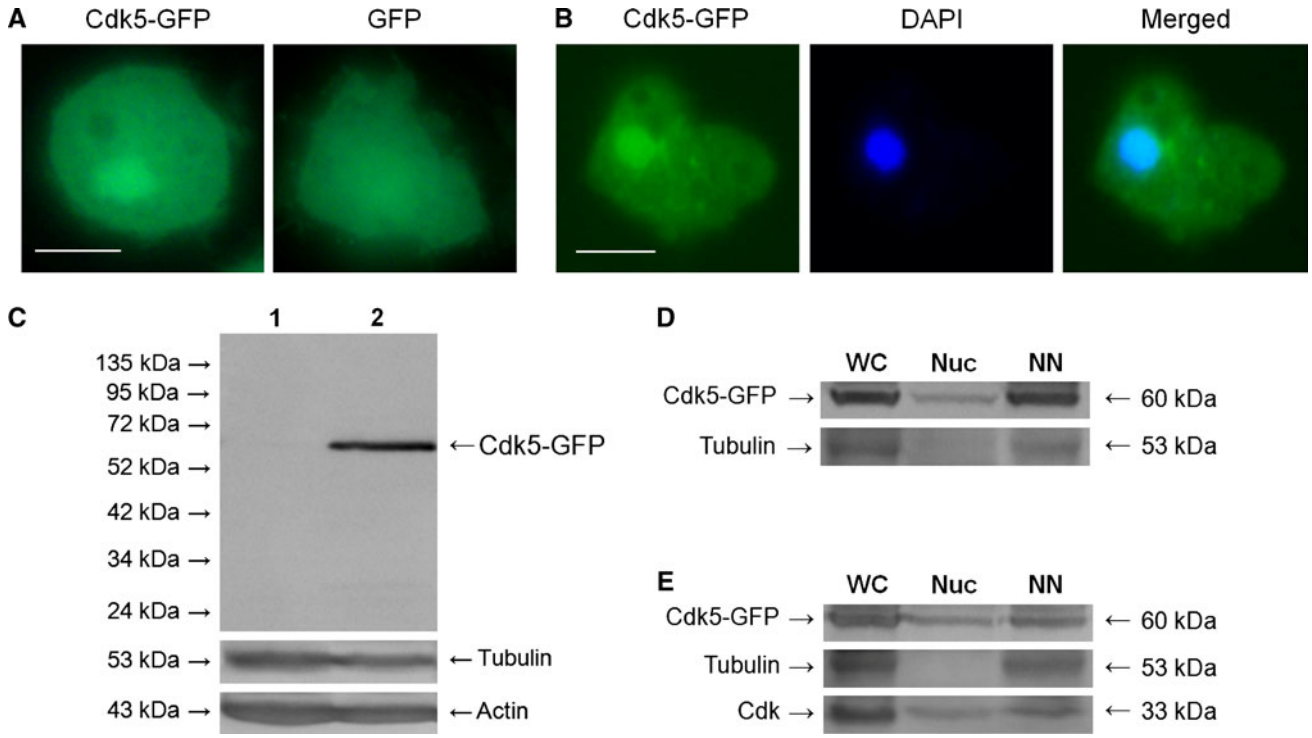

Fig. 5 Localization of Cdk5-GFP. **a** Live cell imaging of AX3/[act15]:cdk5:GFP and AX3/[act15]:GFP cells. *Scale bar* 5 µM. **b** AX3/[act15]:cdk5:GFP cells were fixed and stained with DAPI to reveal nuclei (*blue*). Images were merged with ImageJ. *Scale bar* 5 µm. **c** Whole cell lysates (20 µg) of AX3 (*1*) and AX3/[act15]:cdk5:GFP (*2*) were separated by SDS-PAGE and analyzed by western blotting. Western blot probed with anti-GFP, anti-tubulin, and anti-actin. **d**, **e** Nuclei from AX3/[act15]:cdk5:GFP cells were isolated, lysed, and proteins (20 µg) separated by SDS-PAGE. Western blots probed with anti-GFP (**d**), anti-Cdk (**e**), and anti-tubulin (fractionation control; **d**, **e**). *WC* whole cell lysate, *Nuc* nuclei fraction, *NN* non-nuclei fraction. Molecular weight markers (in kDa) are shown to the *left* of the blot in **c** and to the *right* of the blots in **d** and **e**

well-characterized nuclear proteins in *Dictyostelium*. NumA is a nuclear calmodulin-binding protein in *Dictyostelium* that regulates nuclear number (Myre and O'Day 2002). Yeast two-hybrid studies in combination with pull-down assays and western blotting, have identified calcium-binding protein 4a (Cbp4a) as a NumA binding partner (Myre and O'Day 2004). PsaA has recently been shown to localize to the nucleus and has been suggested to function during cell proliferation in *Dictyostelium* (Catalano et al. 2011). Yeast two-hybrid studies have also identified PsaA as a potential NumA binding partner (Myre 2005; Catalano et al. 2011). To investigate the binding of Cdk5 to these proteins, the 33-kDa protein was immunoprecipitated from AX3 lysates with anti-Cdk. NumA and Cbp4a were not detected in IP fractions; however, anti-PsaA detected a 98-kDa protein in immunoprecipitates (Fig. 7a). The detection of a 98-kDa protein adhered to the previously established molecular weight of PsaA (Catalano et al. 2011). To verify the co-immunoprecipitation of the 33-kDa protein and PsaA and to control for the potential cross-reactivity of anti-Cdk with Cdk1, Cdk5-GFP was immunoprecipitated from AX3/[act15]:cdk5:GFP whole cell lysates with anti-GFP. As expected, anti-GFP immunoprecipitated Cdk5-GFP, but not the 33-kDa protein (Fig. 7b). NumA and Cbp4a were not detected in Cdk5-GFP immunoprecipitates supporting their absence in anti-Cdk immunoprecipitates from AX3 whole cell lysates. PsaA was pulled down providing further evidence for the in vivo interaction of Cdk5 and PsaA and for the preferential detection of Cdk5 by anti-Cdk. The amount of PsaA that was pulled down from AX3 and AX3/[act15]:cdk5:GFP lysates was less than the amount that remained in the protein-depleted sample. As a final verification, the 33-kDa protein was pulled down from AX3/[act15]:cdk5:GFP lysates using anti-Cdk. Both Cdk5-GFP and the 33-kDa protein were pulled down (Fig. 7c). PsaA was also detected in IP fraction. To control for the detection of non-specific bands in the IP lane, anti-Cdk and anti-GFP were immunoprecipitated in separate reactions in the absence of lysate. The immunoprecipitates were separated by SDS-PAGE and analyzed by western blotting with anti-PsaA. Anti-PsaA detected PsaA in AX3 and AX3/[act15]:cdk5:GFP whole cell lysates, but not in the IP fractions providing further evidence that the 98-kDa band

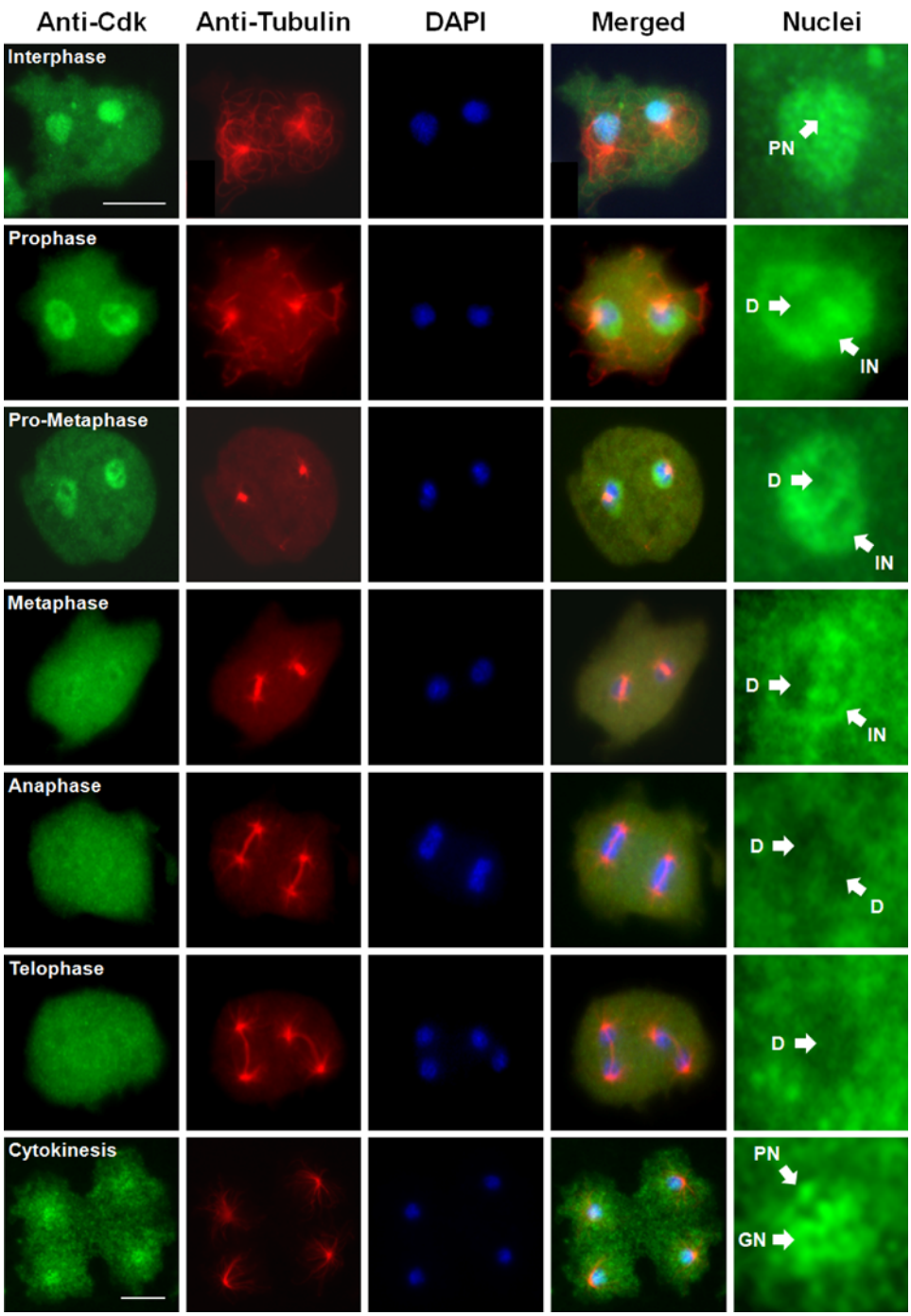

Fig. 6 Localization during mitosis. Axenically growing AX3 cells (1–4 × 10^6 cells/ml) were fixed and incubated with anti-Cdk followed by anti-rabbit Alexa-488 secondary antibody that fluoresced *green*. Cells were also incubated with anti-tubulin followed by anti-mouse Alexa-555 secondary antibody that fluoresced *red* to reveal cells undergoing mitosis. Cells were stained with DAPI to reveal nuclei (*blue*). Images were merged with ImageJ. *Scale bar* 5 µm. *Arrows* (*with letters*) in magnified nuclei images indicate localizations described in the "Results" and "Discussion" sections. *PN* punctate nuclear localization, *D* depletion of staining, *IN* localization toward inner edges of nucleus, *GN* granule-like nuclear localization

that was detected in the IP lane was indeed PsaA and not a non-specific band detected by either the primary or secondary antibody during western blotting (Fig. 7d).

Immunolocalization of PsaA in AX3/[act15]:cdk5:GFP cells

The co-localization of Cdk5 and PsaA was assessed by immunolocalizing PsaA in AX3/[act15]:cdk5:GFP cells (Fig. 8). Both proteins were found to localize predominantly in the nucleoplasm of *Dictyostelium* amoebae supporting data presented here and elsewhere (Catalano et al. 2011).

Co-immunoprecipitation of Cdk5 with phosphoproteins

Since Cdk5 has been shown to possess kinase activity, it was necessary to investigate whether Cdk5 bound any phosphoproteins. The 33-kDa protein was immunoprecipitated from AX3 cells. Anti-phosphothreonine or anti-phosphoserine did not detect any significant proteins in IP

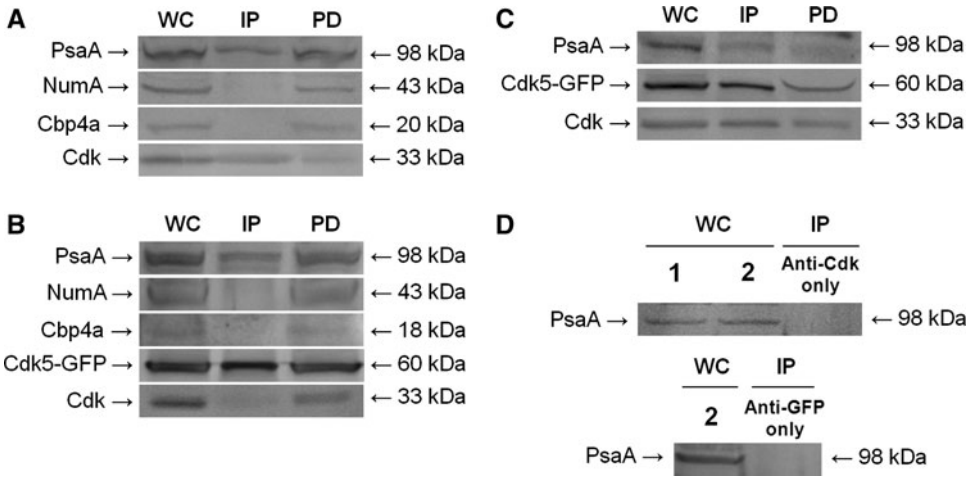

Fig. 7 Co-immunoprecipitation of Cdk5 with known nuclear proteins. **a** Cdk5 was immunoprecipitated from AX3 whole cell lysates with anti-Cdk. Immunoprecipitates were pulled down with ImmunoCruz™ F IP resin. Western blots probed with anti-PsaA, anti-NumA, anti-Cbp4a, and anti-Cdk. **b** Cdk5-GFP was immunoprecipitated from AX3/[act15]:cdk5:GFP whole cell lysates with anti-GFP. Immunoprecipitates were pulled down with ImmunoCruz™ C IP resin. Western blots probed with anti-PsaA, anti-NumA, anti-Cbp4a, anti-GFP, and anti-Cdk. **c** Cdk5 and Cdk5-GFP were immunoprecipitated from AX3/[act15]:cdk5:GFP whole cell lysates with anti-Cdk. Immunoprecipitates were pulled down with ImmunoCruz™ F IP resin. Western blots probed with anti-PsaA and anti-Cdk. Whole cell lysate (WC, 20–30 μg); Immunoprecipitate (IP, 25–35 μl), protein-depleted fraction (PD, 20–30 μg). **d** Verification of PsaA pull down. Anti-Cdk and anti-GFP were immunoprecipitated in the absence of lysate with ImmunoCruz™ F and ImmunoCruz™ C IP resin, respectively. AX3 (*1*) and AX3/[act15]:cdk5:GFP (*2*) whole cell lysates (WC; 25 μg) and anti-Cdk and anti-GFP immunoprecipitates (20 μl) were loaded into the appropriate lanes. Western blot probed with anti-PsaA. Molecular weight markers (in kDa) are shown on the *right* of each blot

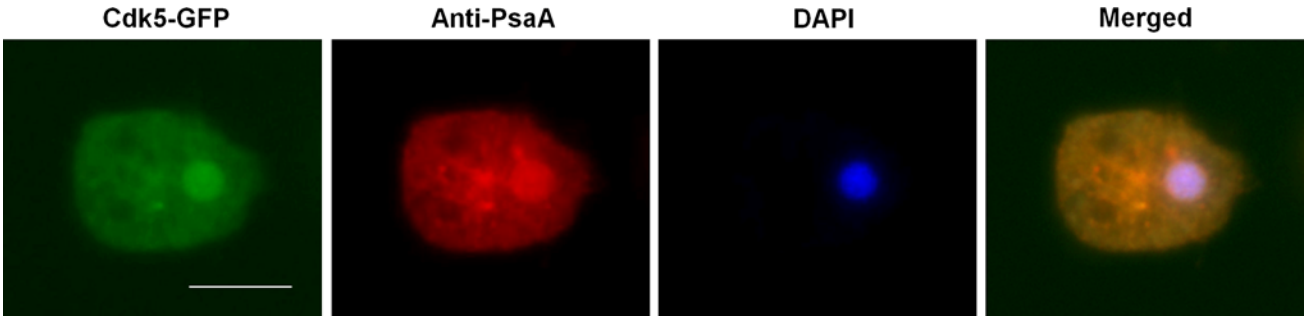

Fig. 8 Co-localization of Cdk5 and PsaA. Axenically growing AX3/[act15]:cdk5:GFP cells (1–4 × 10^6 cells/ml) were fixed and incubated with anti-PsaA followed by anti-rabbit Alexa-555 secondary antibody that fluoresced *red*. Cells were stained with DAPI to reveal nuclei (*blue*). Images were overlaid with ImageJ. *Scale bar* 5 μm

fractions (data not shown). Anti-phosphotyrosine strongly detected four proteins with molecular weights of 35, 28, 22, and 18 kDa in AX3 whole cell lysates (Fig. 9a). The 35-, 28-, and 18-kDa proteins were also detected in the IP fraction. The 18-kDa protein was not detected in the protein-depleted sample. To verify the pull-down of these proteins, Cdk5-GFP was immunoprecipitated from AX3/[act15]:cdk5:GFP lysates with anti-GFP. Anti-phosphotyrosine strongly detected six proteins with molecular weights of 35, 32, 28, 26, 22, and 18 kDa in whole cell lysates (Fig. 9b). The 35- and 18-kDa proteins were also detected in IP fractions, but only weakly detected in protein-depleted samples.

Discussion

This study reports the verification and use of an antibody that strongly detects a 33-kDa protein on western blots of *Dictyostelium* and mouse tissue samples. A peptide competition assay, an affinity purification against the peptide immunogen, and a BLAST analysis showed that the antibody was specific to the peptide and detected a conserved region in a large number of characterized or putative tyrosine kinase-like or serine/threonine kinases in *Dictyostelium*, most notably Cdk5, Cdk1, and the tyrosine kinase-like protein that was the intended target. This suggests that the sequence may be part of a conserved domain

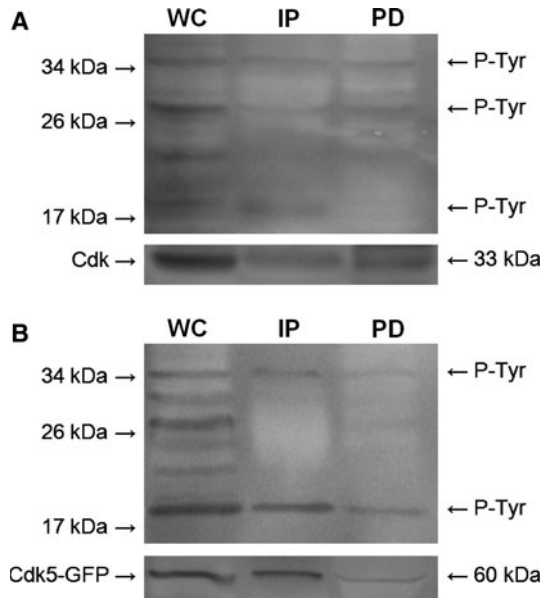

Fig. 9 Co-immunoprecipitation of Cdk5 with phosphotyrosine proteins. **a** Cdk5 was immunoprecipitated from AX3 whole cell lysates with anti-Cdk. Immunoprecipitates were pulled down with ImmunoCruz™ B IP resin. Western blots probed with anti-phosphotyrosine and anti-Cdk. **b** Cdk5-GFP was immunoprecipitated from AX3/[act15]:cdk5:GFP whole cell lysates with anti-GFP. Immunoprecipitates were pulled down with ImmunoCruz™ B IP resin. Western blots probed with anti-phosphotyrosine and anti-GFP. Whole cell lysate (WC, 25 μg), immunoprecipitate (IP, 35 μl), protein-depleted fraction (PD, 25 μg)

required for kinase activity in this model organism. The other Cdks of *Dictyostelium* that aligned with the peptide immunogen could be eliminated as targets based on significantly higher molecular weights. The results presented in this study suggest that the antibody primarily detects *Dictyostelium* Cdk5; however, the potential for cross-reactivity with *Dictyostelium* Cdk1 was high given the sequence similarity between the two proteins. This issue was partially rectified by showing that Cdk5-GFP, like the 33-kDa protein, localized to both the nucleus and cytoplasm of *Dictyostelium* amoebae. The nucleocytoplasmic localization was supported by live and fixed cell imaging and subcellular fractionations of AX3 and AX3/[act15]:cdk5:GFP cells. These results, together with the reported nucleocytoplasmic localization of mammalian Cdk5 (O'Hare et al. 2005; Yang and Mao 2008), suggest that the antibody primarily detects Cdk5. In addition, anti-Cdk was able to pull-down Cdk5-GFP and could detect the Cdk5-GFP recombinant protein. The conclusions drawn from experiments utilizing anti-Cdk were all verified with equivalent experiments performed with anti-GFP and the AX3/[act15]:cdk5:GFP strain.

The nuclear localization of mammalian Cdk5 has been known for some time now, however, the functions of nuclear Cdk5 are still being elucidated (Ino and Chiba 1996). A recent study has shown that in cycling NIH 3T3 cells, the localization of Cdk5 changes from predominantly nuclear to cytoplasmic suggesting that when localized to the nucleus, Cdk5 acts as a cell cycle suppressor (Zhang et al. 2008). Zhang et al. (2008) reported that the change in localization occurred before or shortly after the initiation of the cell cycle and blocking the migration of Cdk5 out of the nucleus retained cell cycle suppression. In this study, nuclear localization was also lost as cells proceeded through mitosis providing further evidence that Cdk5 was primarily being detected. The loss of nuclear localization was gradual fitting with previous work and indicating that *Dictyostelium* Cdk5 may act as a cell cycle suppressor when it is localized in the nucleus and that nuclear Cdk5 may function to maintain the cell in a non-dividing state. The data presented in this study suggests there are two pools of Cdk5 within the cell, one in the nucleus and one in the cytoplasm. A previous study has shown that the number of cells that undergo mitosis at any one time during the exponential phase of growth is extremely limited ($\sim$1.5%; Zada-Hames and Ashworth 1978). Therefore, the significant amount of cytoplasmic Cdk5 that was observed likely cannot be attributed to the relatively small number of cells undergoing mitosis, suggesting that cytoplasmic Cdk5 possesses functions outside of the nucleus in *Dictyostelium*. This hypothesis seems plausible since Cdk5 has previously been implicated in fluid-phase endocytosis, phagocytosis, and cytoskeletal function in *Dictyostelium* (Sharma et al. 2002). Cdk5 activity has previously been shown to be required for optimal cell proliferation in *Dictyostelium* (Sharma et al. 2002). The expression of Cdk5 remained relatively constant during axenic growth (data not shown) suggesting that the protein is constitutively expressed during vegetative conditions and may function during all stages of axenic growth (i.e., early-log, mid-log, late-log). Since previous work was unable to generate *cdk5* knock-outs, other approaches are required to define the functions of this kinase (Sharma et al. 2002). In this regard, a recent pharmacological approach using the Cdk inhibitor roscovitine further supports a role for Cdk5 during axenic growth (Huber and O'Day 2011b).

Co-immunoprecipitations showed that Cdk5 did not interact with NumA or Cbp4a, two established nuclear proteins. PsaA was detected in anti-Cdk immunoprecipitates from AX3 and AX3/[act15]:cdk5:GFP lysates and in anti-GFP immunoprecipitates from AX3/[act15]:cdk5:GFP lysates. Both proteins also localized primarily to the nucleoplasm. These findings, therefore, suggest an in vivo interaction between the two proteins. Furthermore, although *psa* mRNA is detected in a number of mouse tissues, its highest expression is observed in the brain, which is consistent with the hypothesis that Psa regulates neuropeptide activity (Constam et al. 1995). This finding was interesting

since larger amounts of Cdk5 were also detected in the mouse brain compared to other tissues, therefore, providing further support for the interaction of Cdk5 and PsaA. This finding also suggests an evolutionary conserved interaction between the two proteins. The amount of PsaA that was pulled down from AX3 and AX3/[act15]:cdk5:GFP lysates was lower than the amount that remained in the protein-depleted sample, suggesting that only a portion of the total cellular pool of PsaA associates with Cdk5.

Like Cdk5, a recent characterization of PsaA in this model organism suggests this protein also possesses a functional role in regulating cell proliferation and mitosis in *Dictyostelium* (Catalano et al. 2011). The association of Cdk5 with PsaA suggests that PsaA proteolytic activity may regulate the function and/or localization of Cdk5 during mitosis thereby allowing for progression through the cell cycle. Since both proteins were detected in the cytoplasm and nucleus, it is equally possible that interactions between these two proteins occur outside of the nucleus. This possibility and whether the association between Cdk5 and PsaA is an indirect or a direct interaction remain to be investigated.

Three phosphotyrosine proteins (35, 28, and 18 kDa) were pulled down from AX3 lysates with anti-Cdk, however, only the 35- and 18-kDa proteins were detected in Cdk5-GFP immunoprecipitates, suggesting that the 28-kDa protein was a non-specific pull-down. While this work has identified a 35- and an 18-kDa phosphotyrosine protein that may interact with Cdk5 in *Dictyostelium* in vivo, the identity of the proteins and the kinases mediating this phosphorylation remain to be elucidated. Several potential phosphorylation sites have been identified in mammalian Psa (Constam et al. 1995). Although the possible phosphorylation of Psa has been suggested previously, a 98-kDa phosphoprotein was not detected in immunoprecipitates from AX3 or AX3/[act15]:cdk5:GFP lysates, suggesting that *Dictyostelium* PsaA is not phosphorylated in vivo.

Together, this study provides new insight into the localization and function of Cdk5 in *Dictyostelium*. The nucleocytoplasmic localization of the protein provides further evidence that it functions as a true homolog of mammalian Cdk5. This work has also identified PsaA as a Cdk5-binding partner. Since Cdk5 and PsaA have previously been shown to be involved in mediating cell division and proliferation in *Dictyostelium* and since Cdk5 and PsaA exhibit similar localizations during *Dictyostelium* mitosis, this data suggests that these proteins may function as part of a protein complex or signaling pathway to regulate cell proliferation in *Dictyostelium*.

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