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Cyclin-dependent kinase 5 is a calmodulin-binding protein that associates with puromycin-sensitive aminopeptidase in the nucleus of *Dictyostelium*

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ABSTRACT

Cyclin-dependent kinase 5 (Cdk5) is a serine/threonine kinase that has been implicated in a number of cellular processes. In *Dictyostelium*, Cdk5 localizes to the nucleus and cytoplasm, interacts with puromycin-sensitive aminopeptidase A (PsaA), and regulates endocytosis, secretion, growth, and multicellular development. Here we show that Cdk5 is a calmodulin (CaM)-binding protein (CaMBP) in *Dictyostelium*. Cdk5, PsaA, and CaM were all present in isolated nuclei and Cdk5 and PsaA co-immunoprecipitated with nuclear CaM. Although nuclear CaMBPs have previously been identified in *Dictyostelium*, the detection of CaM in purified nuclear fractions had not previously been shown. Putative CaM-binding domains (CaMBDs) were identified in Cdk5 and PsaA. Deletion of one of the two putative CaMBDs in Cdk5 (^{132}LLINRKGELKLADFGLARAFGIP^{154}) prevented CaM-binding indicating that this region encompasses a functional CaMBD. This deletion also increased the nuclear distribution of Cdk5 suggesting that CaM regulates the nucleocytoplasmic transport of Cdk5. A direct binding between CaM and PsaA could not be determined since deletion of the one putative CaMBD in PsaA prevented the nuclear localization of the deletion protein. Together, this study provides the first direct evidence for nuclear CaM in *Dictyostelium* and the first evidence in any system for Cdk5 being a CaMBP.

1. Introduction

Cyclin-dependent kinase 5 (Cdk5) is a member of a family of serine/threonine protein kinases and has been shown to function in a diversity of cellular processes such as axon guidance, membrane transport, myogenesis, insulin secretion, and lens differentiation among others [1,2]. Cdk5 dysregulation has been linked to advanced melanoma and neurodegenerative diseases such as amyotrophic lateral sclerosis and Alzheimer's disease [1,3,4]. Initial characterizations of Cdk5 focused its cytoplasmic functions however recent studies have begun to reveal its nuclear functions [5,6]. When NIH3T3 cells enter the cell cycle, the localization of Cdk5 changes from predominantly nuclear to cytoplasmic, suggesting that nuclear Cdk5 acts as a cell cycle suppressor [7]. Cdk5 then returns to the nucleus at the end of the cell cycle. Cdk5 has also been shown to suppress the cell cycle in neocortical neurons and to possess a death-promoting function within the nucleus of neurons and a pro-survival function when localized to the cytoplasm [8,9].

The *Dictyostelium discoideum* homologue of mammalian Cdk5 regulates endocytosis, phagocytosis, and secretion and is required for the optimal growth, differentiation, and multicellular development of this model organism [10–12]. Fitting with observations in mammals, the protein localizes to both the nucleus and cytoplasm of growing cells and translocates from the nucleus to the cytoplasm during mitosis [12,13]. Cdk5 interacts with puromycin-sensitive aminopeptidase (PsaA) whose function has also been linked to cell proliferation and mitosis in *Dictyostelium* [13,14]. PSAs are highly conserved metalloproteases that hydrolyze N-terminal amino acids from oligopeptides [15]. In mammals, PSA localizes to both the nucleus and cytoplasm and associates with the microtubules of the spindle apparatus during mitosis [16]. Inhibitors of aminopeptidase activity arrest the cell cycle and suppress cell proliferation [16–19]. PSA activity has also shown to be required for meiosis in *Arabidopsis thaliana* and *Caenorhabditis elegans* [20,21].

Calmodulin (CaM) is the primary sensor of calcium (Ca^{2+}) within the cell and binds to a diversity of CaM-binding proteins (CaMBPs) that localize throughout the cell in various subcellular compartments [22–24]. Several CaM-binding motifs that contain either a specific positioning of hydrophobic amino acids, a clustering of basic amino acids, or an IQ or IQ-like motif, have been reported, however not all CaM-binding motifs have been resolved [25–28]. In addition, some

Abbreviations: CaM, calmodulin; Ca^{2+}, calcium; CaMBP, CaM-binding protein; CaMBD, CaM-binding domain; Cdk5, cyclin-dependent kinase 5; PsaA, puromycin-sensitive aminopeptidase A; GFP, green fluorescent protein; NLS, nuclear localization sequence

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proteins have been shown to contain more than one type of motif [29]. A well-established and accepted method for identifying CaMBPs and their CaM-binding domains (CaMBDs) involves the deletion of the putative domain(s), followed by an analysis of whether the deletion protein can bind CaM [30,31]. In *Dictyostelium*, CaM localizes predominantly to membranes of the osmoregulatory system (i.e., contractile vacuole) and has been shown to interact with a number of CaMBPs [28,32–35]. Using immunofluorescence, a previous study suggested that CaM also localizes to both the nucleus and cytoplasm but issues of antibody specificity and the absence of appropriate controls common in today's research left these results in question [36]. Like Cdk5 and PsaA, CaM function in *Dictyostelium* has also been linked to mitosis and cell proliferation [33,37]. Nuclear CaMBPs have been identified in both mammalian and *Dictyostelium* cells [28,29,38–40]. In *Dictyostelium*, the most well studied nuclear CaMBP is NumA, a nucleoplasmic/nucleolar protein that regulates nuclear number and binds to PsaA [29,41]. Although nuclear CaM has been studied in mammals, the presence of CaM in *Dictyostelium* nuclei has not previously been shown [28,42,43].

A number of CaM-dependent kinases have been characterized in mammals and some of these kinases have been shown to localize and function within the nucleus [44,45]. In HEK293T and PC12 cells, p35 activity has been shown to be regulated by both CaM-binding and Cdk5 phosphorylation [46]. Studies in *Dictyostelium* have reported the CaM-dependent phosphorylation and dephosphorylation of proteins involved in fertilization, germination, and chemotaxis [47–49]. However the identity of the proteins and of the CaM-dependent kinases and phosphatases has yet to be reported. In this study, co-immunoprecipitations showed that Cdk5 is a CaMBP in *Dictyostelium* and that Cdk5, PsaA, and CaM interact with each other in *Dictyostelium* nuclei, thus providing the first direct evidence for nuclear CaM in this model organism. Putative CaMBDs were identified in Cdk5 and PsaA. The ability of these domains to mediate CaM-binding was assessed using deletion constructs and co-immunoprecipitations, which ultimately identified a region in Cdk5 than encompasses a functional CaMBD.

2. Materials and methods

2.1. Cells and chemicals

All strains were grown either in the presence of *Escherichia coli* on SM agar pH 6.5 ± G418 (100 μg/ml) at 22 °C in the dark for 24–30 h or axenically in HL-5 medium ± G418 (10 μg/ml) at 22 °C and 150 rpm [13]. The following strains were used: AX3 (parental), AX3/[act15]:cdk5:GFP, AX3/[act15]:cdk5ΔCaMBD1:GFP, AX3/[act15]:cdk5ΔCaMBD2:GFP, AX3/[act15]:cdk5ΔCaMBD1 + 2:GFP, AX3/[act15]:psaA:GFP, AX3/[act15]:psaAΔCaMBD:GFP. W7 was purchased from Sigma-Aldrich Canada Limited (Oakville, ON, Canada). The QIAquick™ PCR Purification Kit, QIAquick™ Gel Extraction Kit, and QIAprep™ Spin Miniprep Kit were used for all PCR purifications, gel extractions, and plasmid isolations respectively and were all purchased from Qiagen Incorporated (Toronto, ON, Canada). Restriction enzymes were purchased from New England BioLabs Limited (Pickering, ON, Canada). Sequence alignments were performed using the dictyBase BLAST server (http://www.dictybase.org/tools/blast).

2.2. Axenic growth assay

Vegetative cells ($1–4\times10^6$ cells/ml) grown in HL-5 medium + G418 (10 μg/ml) were diluted to 2×10^5 cells/ml, transferred to 6-well plates, and then incubated at 22 °C and 150 rpm. Cell concentrations were measured every 24 h over a 96 h period using a hemocytometer. In separate experiments, AX3/[act15]:cdk5:GFP cells ($1–2\times10^6$ cells/ml) were grown in HL-5 + G418 (10 μg/ml) ± the CaM antagonist W7 (50 μM) for 24 h.

2.3. Isolation of nuclei

Nuclei were isolated as previously described with minor modifications [12,13,50–52]. Cells (2×10^8) in the mid-log phase of growth ($1–5\times10^6$ cells/ml) were harvested from HL-5 medium and washed one time with ice-cold KK2 buffer (2.3 g/l KH_2PO_4, 1.3 g/l K_2HPO_4, pH 6.5). Cells were resuspended in 10 ml of 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 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 2 ml of 20% Triton-X and incubating on ice for 10 min. Lysates were spun at 2000 rpm for 15 min at 4 °C. Supernatants were removed and retained as non-nuclear (i.e., cytoplasmic) fractions. Pelleted nuclei were resuspended in 1.5 ml of NP-40 lysis buffer containing 50 mM Tris pH 8.0, 150 mM NaCl, 0.5% NP-40, 5 mM EDTA, 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). Samples were sonicated two times for 10 s each to completely lyse the nuclei and then spun at 12,000 rpm for 10 min at 4 °C. Supernatants were removed and retained as nuclei fractions. All samples were stored at −80 °C for future use.

2.4. Immunoprecipitation

Immunoprecipitations were performed as previously described [13]. Rabbit polyclonal anti-Cdk5 (15–25 μl) [13], mouse monoclonal anti-GFP (green fluorescent protein, 30 μl; Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA), or mouse monoclonal anti-CaM (5–10 μl; EMD Chemicals Inc., Gibbstown, NJ, USA) was added to whole cell lysates or nuclei fractions (1–2 mg). Immunoprecipitates were pulled-down with Immunocruz™ IP resins (B/C/E; 100 μl slurry; Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA). Supernatants were removed and retained as protein-depleted (PD) fractions. Resins were washed 2–3 times with NP-40 lysis buffer, resuspended in 2× sample loading buffer, and then boiled for 5 min. Supernatants were removed and retained as the immunoprecipitate (IP) fraction. Aliquots of IP fractions (e.g. 25 μl) were separated by SDS-PAGE and analyzed by Western blotting. Samples were stored at −80 °C for future use.

2.5. SDS-PAGE and Western blotting

SDS-PAGE and Western blotting were performed as previously described [13,53]. The following primary antibodies were used: rabbit polyclonal anti-Cdk5 (1:400) [13], mouse monoclonal anti-tubulin (1:1000; 12G10, Developmental Studies Hybridoma Bank, The University of Iowa, IA, USA), rabbit polyclonal anti-PsaA (1:400) [14], mouse monoclonal anti-GFP (1:1000; Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA), mouse monoclonal anti-CaM (1:1000; EMD Chemicals Inc., Gibbstown, NJ, USA), mouse monoclonal anti-phosphotyrosine and mouse monoclonal anti-phosphothreonine (1:1000; New England Biolabs Canada, Pickering, ON, Canada). Membranes were developed with the Amersham™ ECL Plus Western Blotting Detection System (GE Healthcare, Buckinghamshire, UK) and scanned using a Storm 860 Phosphorimager/Fluorimager (Molecular Dynamics Inc., Sunnyvale, CA, USA).

2.6. Live cell imaging, fixation, and immunolocalization

Cells in the mid-log phase of growth ($1–4\times10^6$ cells/ml) were viewed live after adhering to a coverslip for 30 min. Fixation in ultra-cold methanol and immunolocalization were performed as previously described [14]. The following primary and secondary antibodies were used: mouse monoclonal anti-GFP (1:5000; Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA), mouse monoclonal anti-tubulin (1:100; 12G10, Developmental Studies Hybridoma Bank, The

University of Iowa, IA, USA), goat anti-mouse Alexa Fluor™ 488 and goat anti-mouse Alexa Fluor™ 555 (1:100; Life Technologies Inc., Burlington, ON, Canada). The localization of tubulin was used to assess cellular integrity. Prolong™ Gold anti-fade reagent with DAPI (Life Technologies Inc., Burlington, ON, Canada) was placed on the slide prior to mounting and coverslips were then sealed using nail polish. Cells were viewed with a Nikon 50i epifluorescence microscope equipped with a Nikon Digital-Sight DS-Ri1 camera (Nikon Canada, Mississauga, ON, Canada) and images were analyzed (e.g., intensity measurements) using Nikon Imaging Software Elements Basic Research 3.0 (Nikon Canada, Mississauga, ON, Canada). For intensity measurements, at least one hundred cells were counted per replicate and at least 4 replicates were performed. The Student's *t*-test ($\alpha = 0.05$) was used to calculate significance for all graphs. Significant differences are indicated with an asterisk.

2.7. Generation of GFP expression vectors and cell lines

Vector pDM323, which incorporates C-terminal GFP, was used to generate all GFP-fusion protein constructs [54]. Full length Cdk5 and psaA were amplified from cDNA using the primers CAGAGCTCAAA ATGGAGAAATATTCAAAA (forward) and CAACTAGTATTAATAGGTTC TAAACCATCG (reverse) for cdk5 and CAGAGCTCAAAATGTGTAAATA TAATGATC (forward) and CAACTAGTTTTTTTAATCCAATTTAATAAATC (reverse) for psaA, which incorporated a Kozak site as well as the appropriate restriction sites (underlined) as previously described [14]. The Cdk5 fragment was incorporated into pDM323 with BglII and SpeI cut sites. For psaA, a SacI site was inserted into pDM323 between the BglII and SpeI sites prior to incorporation of the psaA fragment with SacI and SpeI cut sites. The deletion constructs cdk5ΔCaMBD1-GFP, cdk5ΔCaMBD2-GFP, cdk5ΔCaMBD1 + 2-GFP, and psaAΔCaMBD-GFP were generated in a similar manner, however only the regions before and after the deletion were amplified (Table 1). The amplification products were ligated together and then amplified by PCR using the same primers used to amplify full-length Cdk5 or psaA. cdk5ΔCaMBD1 + 2-GFP was generated by amplifying the region before and after CaMBD2 using cdk5ΔCaMBD1-GFP as a template. Purified fragments were then incorporated into pDM323 using BglII and SpeI cut sites for cdk5 constructs and SacI and SpeI cut sites for psaA constructs. cdk5ΔCaMBD1-GFP and cdk5ΔCaMBD2-GFP possessed a BamHI and HindIII restriction site, respectively, in place of the CaMBD, while cdk5ΔCaMBD1 + 2-GFP possessed a BamHI site in place of CaMBD1 and a HindIII site in place of CaMBD2. psaAΔCaMBD-GFP possessed a HindIII site in place of the CaMBD. The constructs were transformed into AX3 cells as previously described [14]. After four days of selection, colonies were transferred to HL-5 containing 10 μg/ml G418 within which they were maintained. In total six strains were generated; AX3/[act15]:cdk5:GFP (encoding Cdk5-GFP), AX3/[act15]:cdk5ΔCaMBD1:GFP (encoding Cdk5ΔCaMBD1-GFP), AX3/[act15]:cdk5ΔCaMBD2:GFP (encoding Cdk5ΔCaMBD2-GFP), AX3/[act15]:cdk5ΔCaMBD1 + 2:GFP (encoding Cdk5ΔCaMBD1 + 2-GFP), AX3/[act15]:psaA:GFP (encoding PsaA-GFP), and AX3/[act15]:psaAΔCaMBD:GFP (encoding PsaAΔCaMBD-GFP).

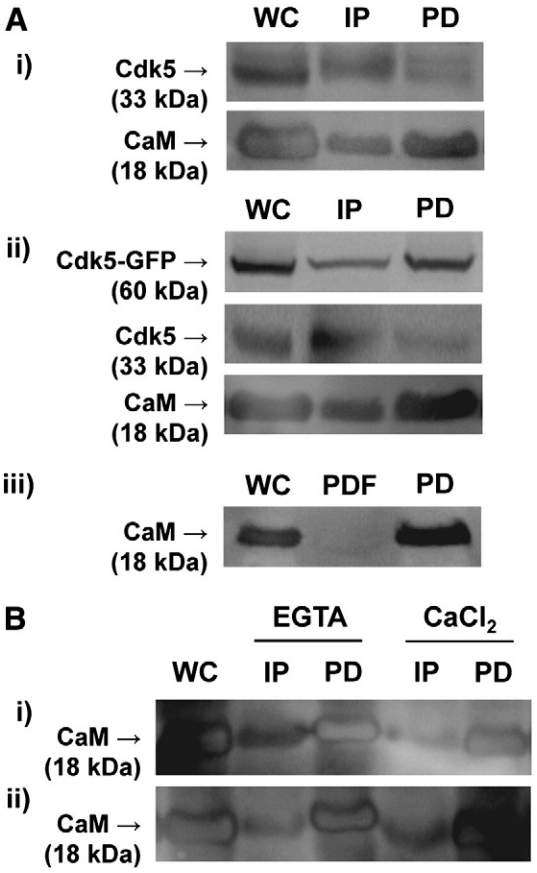

Fig. 1. Co-immunoprecipitation of Cdk5 and CaM. (A) Cdk5 was immunoprecipitated from AX3 (i) or AX3/[act15]:cdk5:GFP (ii) whole cell lysates with anti-Cdk5. Immunoprecipitates were pulled-down with Immunocruz B™ IP resin. Western blots probed with anti-CaM or anti-Cdk5. (iii) Incubation of the pull-down resin with a whole cell lysate in the absence of antibody. Western blot probed with anti-CaM. (B) Effect of Ca^{2+} and EGTA on the co-immunoprecipitation of Cdk5 and CaM. Cdk5 was immunoprecipitated from AX3 (i) or AX3/[act15]:cdk5:GFP (ii) whole cell lysates with anti-Cdk5 ± EGTA (5 mM) or $CaCl_2$ (2 mM). Immunoprecipitates were pulled-down with Immunocruz™ B IP resin. Western blots probed with anti-CaM. WC, whole cell lysate (25 μg); IP, immunoprecipitate (25 μl); PD, protein-depleted fraction (25 μg); PDF, pull-down fraction (25 μl aliquot). Molecular weights (in kDa) are indicated in parentheses under each protein label.

Table 1
List of primers used to generate GFP-fusion CaMBD deletion constructs.

Construct	Region before/after deletion	For./Rev.	Primers	Restriction site
cdk5ΔCaMBDl-GFP	Before	For.	CAGAGCTCAAAATGGAGAAATATTCAAAA	BglII
		Rev.	CAGGATCCACCATATGTACCTTC	BamHI
	After	For.	CAGGATCCAAAAGAATAAGATTAGACTCTG	BamHI
		Rev.	CAACTAGTATTAATAGGTTCTAAACCATCG	SpeI
cdk5ΔCaMBD2-GFP	Before	For.	CAGAGCTCAAAATGGAGAAATATTCAAAA	BglII
		Rev.	CAAAGCTTATTTTGTGGTTTTAAATCTCTATG	HindIII
	After	For.	CAAAGCTTGTTAGAACATATTCACATGAAG	HindIII
		Rev.	CAACTAGTATTAATAGGTTCTAAACCATCG	SpeI
psaAΔCaMBD-GFP	Before	For.	CAGAGCTCAAAATGTGTAAATATAATGATC	SacI
		Rev.	CAAAGCTTACAATATTCACCAACTGATAATTC	HindIII
	After	For.	CAAAGCTTAAAGGTGAAGATCGTTATTTAG	HindIII
		Rev.	CAACTAGTTTTTTTAATCCAATTTAATAAATC	SpeI

Restriction sites are underlined. For a diagrammatic representation of each GFP-fusion protein see Fig. 5.

3. Results

3.1. Co-immunoprecipitation of CaM with Cdk5 and PsaA

Cdk5 was immunoprecipitated from AX3 and AX3/[act15]:cdk5:GFP whole cell lysates with anti-Cdk5 and the immunoprecipitates were analyzed by Western blotting with anti-CaM. The ability of anti-Cdk5 to immunoprecipitate both Cdk5 and Cdk5-GFP was detailed in a previous study and confirmed in this study (Fig. 1Ai,ii) [13]. CaM was strongly detected in Cdk5 immunoprecipitates from AX3 and AX3/[act15]:cdk5:GFP whole cell lysates (Fig. 1Ai,ii). To control for the non-specific binding of proteins to the pull-down resins, a whole cell lysate was incubated with the pull-down resin in the absence of antibody. The pull-down fraction was separated by SDS-PAGE and analyzed by Western blotting with anti-CaM. CaM was detected in the whole cell lysate and protein-depleted fraction, but not in the pull-down fraction (Fig. 1Aiii).

CaM is the primary sensor of Ca^{2+} within the cell; however binding of CaM to its CaMBPs can be either Ca^{2+}-dependent or -independent. To investigate the dependence of Ca^{2+} on the Cdk5/CaM interaction, Cdk5 was immunoprecipitated from AX3 whole cell lysates with anti-Cdk5 in the presence of $CaCl_2$ or the Ca^{2+} chelator EGTA. In addition, Cdk5 and Cdk5-GFP were immunoprecipitated from AX3/[act15]:cdk5:GFP whole cell lysates with anti-Cdk5 in the presence of $CaCl_2$ or EGTA. CaM was detected in Cdk5 immunoprecipitates from both treatments and both strains (Fig. 1B). To verify the interaction between Cdk5 and CaM, CaM was immunoprecipitated from AX3 and AX3/[act15]:cdk5:GFP whole cell lysates with anti-CaM. Immunoprecipitates were separated by SDS-PAGE and analyzed by Western blotting with anti-Cdk5. CaM was detected in CaM immunoprecipitates from AX3 and AX3/[act15]:cdk5:GFP whole cell lysates confirming the pull-down of

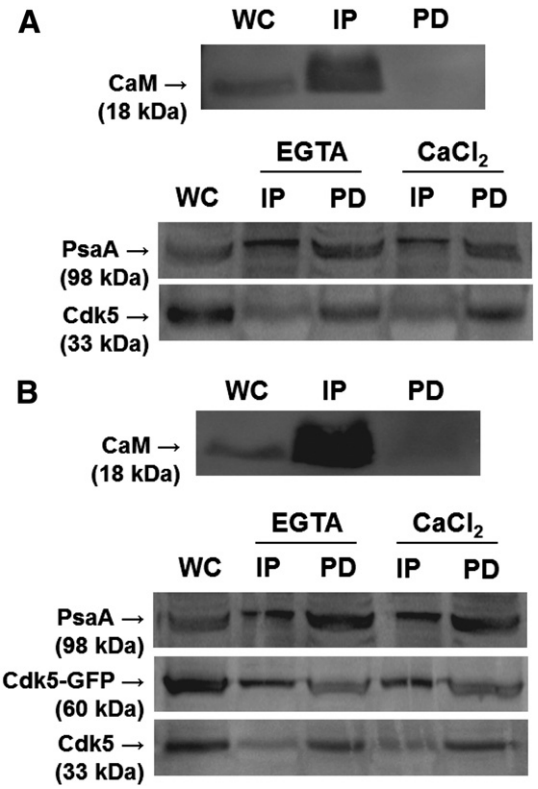

Fig. 2. Co-immunoprecipitation of CaM, Cdk5, and PsaA. (A) CaM was immunoprecipitated from AX3 whole cell lysates with anti-CaM ± EGTA (5 mM) or $CaCl_2$ (2 mM). Immunoprecipitates were pulled-down with Immunocruz™ C IP resin. Western blots probed with anti-CaM, anti-PsaA, or anti-Cdk5. (B) CaM was immunoprecipitated from AX3/[act15]:cdk5:GFP AX3 whole cell lysates with anti-CaM ± EGTA (5 mM) or $CaCl_2$ (2 mM). Immunoprecipitates were pulled-down with Immunocruz™ C IP resin. Western blots probed with anti-CaM, anti-PsaA, or anti-Cdk5. WC, whole cell lysate (25 μg); IP, immunoprecipitate (25 μl); PD, protein-depleted fraction (25 μg). Molecular weights (in kDa) are indicated in parentheses under each protein label.

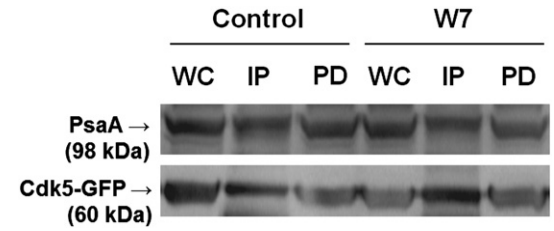

Fig. 3. Effect of CaM inhibition on the co-immunoprecipitation of Cdk5 and PsaA. AX3/[act15]:cdk5:GFP cells ($1–2\times10^6$ cells/ml) were grown in HL-5 medium ± W7 (50 μM) for 24 h. Cdk5-GFP was immunoprecipitated from whole cell lysates with anti-GFP. Immunoprecipitates were pulled-down with Immunocruz™ C IP resin. Western blots probed with anti-PsaA or anti-GFP. WC, whole cell lysate (25 μg); IP, immunoprecipitate (25 μl); PD, protein-depleted fraction (25 μg). Molecular weights (in kDa) are indicated in parentheses under each protein label.

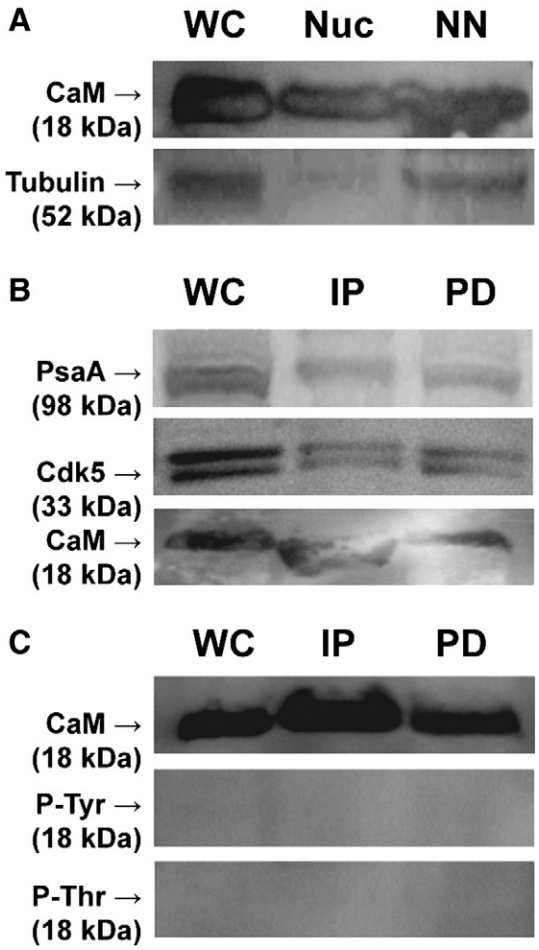

Fig. 4. Interactions between Cdk5, PsaA, and CaM in isolated nuclei. (A) Subcellular localization of CaM. Nuclei were isolated from AX3 cells as described in the Materials and methods section. Protein fractions (25 μg) were separated by SDS-PAGE and analyzed by Western blotting with anti-CaM and anti-tubulin (fractionation control). WC, whole cell lysate; Nuc, nuclei fraction; NN, non-nuclei fraction. (B) Immunoprecipitation of nuclear CaM. CaM was immunoprecipitated from nuclear fractions with anti-CaM and immunoprecipitates were pulled-down with Immunocruz™ E IP resin. Western blots probed with anti-PsaA, anti-Cdk5, or anti-CaM. WC, whole cell lysate (20 μg); IP, immunoprecipitate (25 μl); PD, protein-depleted fraction (20 μg). (C) CaM was immunoprecipitated from AX3 whole cell lysates with anti-CaM. Immunoprecipitates were pulled-down Immunocruz™ E IP resin. Western blots probed with anti-CaM, anti-phosphotyrosine, or anti-phosphothreonine. WC, whole cell lysate (25 μg); IP, immunoprecipitate (25 μl); PD, protein-depleted fraction (25 μg). Molecular weights (in kDa) are indicated in parentheses under each protein label.

the protein (Fig. 2A,B). Cdk5 was detected in CaM immunoprecipitates from AX3 whole cell lysates and there was no effect of $CaCl_2$ or EGTA on the pull-down (Fig. 2A). In addition, both Cdk5 and Cdk5-GFP were detected in CaM immunoprecipitates from AX3/[act15]:cdk5:GFP whole cell lysates with no apparent effect of $CaCl_2$ or EGTA treatment (Fig. 2B). Like Cdk5, PsaA was also detected in CaM immunoprecipitates from AX3 and AX3/[act15]:cdk5:GFP whole cell lysates, with no apparent effect of $CaCl_2$ or EGTA on the pull-down (Fig. 2A,B).

W7, a well-established antagonist of CaM, has previously been used to inhibit CaM activity in *Dictyostelium* [55]. Since Cdk5 and PsaA were shown to co-immunoprecipitate with CaM, the effect of CaM inhibition on the Cdk5/PsaA interaction was analyzed after a 24 hour axenic growth period. After 24 h, W7 had inhibited axenic growth by 40–50%, but did not affect the co-immunoprecipitation of Cdk5-GFP and PsaA (Fig. 3).

3.2. Interaction between Cdk5, PsaA, and CaM in isolated nuclei

To provide direct evidence for nuclear CaM in *Dictyostelium*, AX3 cells were fractionated into nuclear and non-nuclear (i.e., cytoplasmic) fractions, which were then separated by SDS-PAGE and analyzed by Western blotting with anti-CaM. CaM was detected in both nuclear and non-nuclear fractions, whereas tubulin, a previously reported control for nuclear/non-nuclear fractionations, was not (Fig. 4A) [13,56,57].

Since Cdk5, PsaA, CaM have all been shown to localize to the nucleus and since the three proteins co-immunoprecipitate with each other in whole cell lysates [12–14,58], the interactions between them in nuclear fractions was analyzed. CaM was immunoprecipitated from AX3 nuclear fractions with anti-CaM. The immunoprecipitates were separated by SDS-PAGE and analyzed by Western blotting with anti-Cdk5, anti-PsaA, and anti-CaM. CaM was detected in immunoprecipitates confirming the pull-down of the protein (Fig. 4B). Cdk5 and PsaA were detected in all fractions, including the immunoprecipitate (Fig. 4B). Interestingly, two protein bands of molecular weights 33 and 37 kDa were detected on Western blots probed with anti-Cdk5 (Fig. 4B).

A previous study reported the detection of an 18 kDa phosphotyrosine protein in Cdk5 and Cdk5-GFP immunoprecipitates [13]. Since CaM was also detected in Cdk5 immunoprecipitates and since the protein possesses a molecular weight of 18 kDa, the intracellular phosphorylation of CaM was analyzed. CaM was immunoprecipitated from AX3 whole cell lysates with anti-CaM and analyzed by Western blotting with anti-phosphotyrosine and anti-phosphothreonine. An 18 kDa protein was not detected in CaM immunoprecipitates by either antibody (Fig. 4C). Anti-phosphotyrosine and anti-phosphothreonine both detected phosphoproteins on Western blots of whole cell extracts (data not shown).

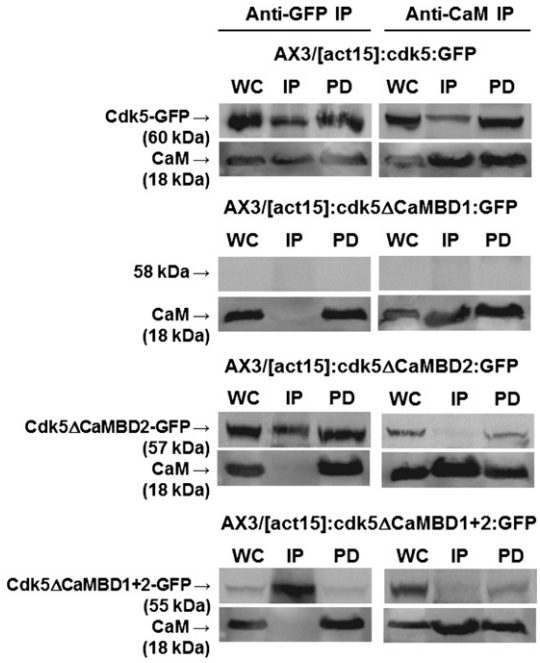

Fig. 6. Effect of putative CaMBD deletion on the binding of Cdk5-GFP to CaM. Whole cell lysates were incubated with anti-GFP or anti-CaM. Immunoprecipitates were pulled-down with Immunocruz™ B IP resin, separated by SDS-PAGE, and analyzed by Western blotting with anti-GFP and anti-CaM. WC, whole cell lysate (20 μg); IP, immunoprecipitate (25 μl); PD, protein-depleted fraction (20 μg). Molecular weights (in kDa) are indicated in parentheses under each protein label.

3.3. Identification of putative CaMBDs in Cdk5 and PsaA

Since both Cdk5 and PsaA were detected in CaM immunoprecipitates, it was necessary to investigate the ability of these proteins to bind directly to CaM. Putative CaMBDs were identified in both Cdk5 and PsaA using the online CaM Target Database (http://

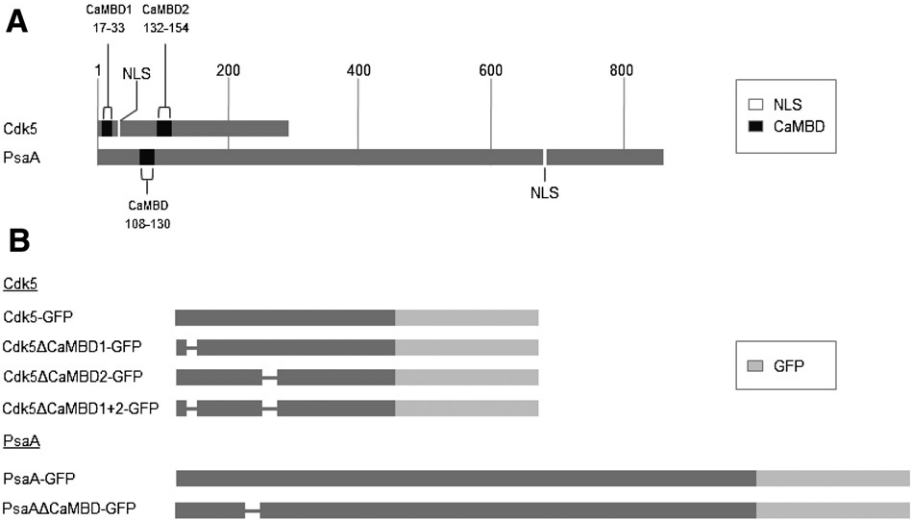

Fig. 5. Domain architecture of Cdk5, PsaA, and GFP-fusion proteins. (A) Location of putative CaMBDs and nuclear localization sequences (NLSs) in Cdk5 and PsaA. Amino acid positions of putative CaMBDs are indicated. (B) Domain architecture of the GFP-fusion proteins.

calcium.uhnres.utoronto.ca/ctdb/ctdb/home.html). Two putative domains were identified in Cdk5 and one in PsaA (Fig. 5A). The putative CaMBDs in Cdk5, ^{17}IVYKAKNRETGEIVALK^{33} (i.e., CaMBD1) and ^{132}LLINRKGELKLADFGLARAFGIP^{154} (i.e., CaMBD2), were identified in the N-terminal and middle regions, respectively, of the Cdk5 amino acid sequence (Fig. 5A). The putative CaMBD in PsaA, ^{108}LSLVFTGLLNDKLKGFYRSKYTV^{130} (i.e., CaMBD), was identified in the N-terminal region of the protein (Fig. 5A). Deletion vectors that encoded proteins lacking these domains were constructed to investigate the ability of these domains to mediate CaM-binding (Fig. 5B). The subcellular localization of Cdk5 and PsaA has been investigated in detail in previous studies [12–14,58]. Since we were also interested in investigating the effect of domain deletion on the subcellular localization of the proteins, they were tagged with GFP (Fig. 5B).

3.4. Effect of putative CaMBD deletion on the binding of Cdk5-GFP to CaM and its localization

Proteins were immunoprecipitated from whole cell lysates with anti-GFP or anti-CaM; however the antibodies did not completely immunodeplete any of the proteins (Fig. 6). All of the GFP-fusion proteins were detected in GFP immunoprecipitates indicating that they were all successfully pulled-down (Fig. 6; left panel). CaM was also successfully pulled-down since it was detected in CaM immunoprecipitates (Fig. 6; right panel). Immunoprecipitates were separated by SDS-PAGE and analyzed by Western blotting with anti-GFP and anti-CaM. Cdk5ΔCaMBD1-GFP was not detected in any of the fractions that were analyzed (e.g., whole cell, immunoprecipitate, and protein-depleted) indicating that the protein was either not expressed

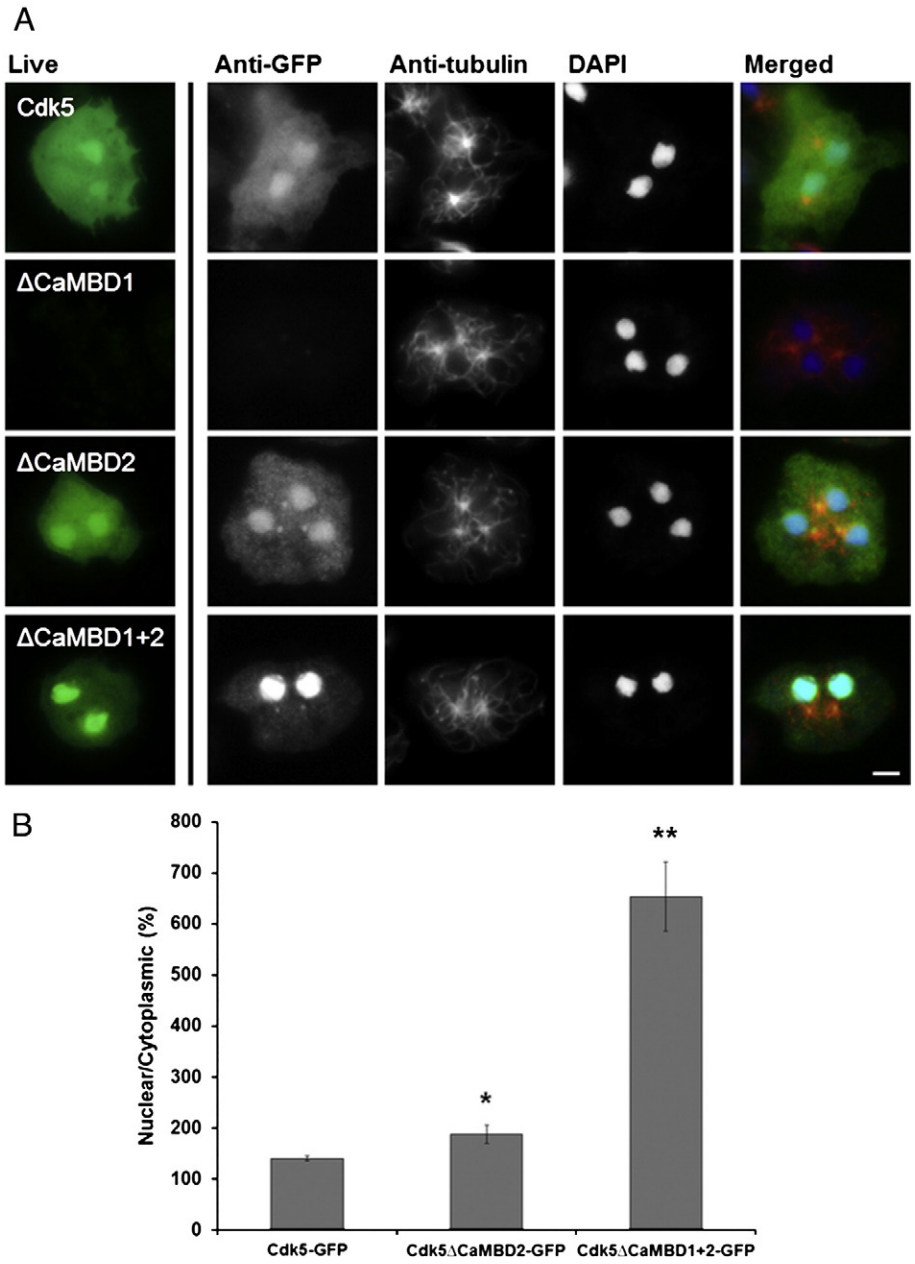

Fig. 7. Effect of putative CaMBD deletion on the localization of Cdk5-GFP. (A) Localization of Cdk5-GFP, Cdk5ΔCaMBD1-GFP, Cdk5ΔCaMBD2-GFP, and Cdk5ΔCaMBD1 + 2-GFP in live and fixed cells. Cells were stained with DAPI to reveal nuclei. All images shown are representative of a typical cell of that strain. Scale bar = 3 μm. (B) Nuclear/cytoplasmic distribution of Cdk5-GFP, Cdk5ΔCaMBD2-GFP, or Cdk5ΔCaMBD1 + 2-GFP in live cells. Data presented as mean nuclear/cytoplasmic distribution ± s.e.m. (n = 4). At least 100 cells were analyzed for each replicate. *p-value ≤ 0.05 vs. Cdk5-GFP; **p-value ≤ 0.05 vs. Cdk5-GFP and Cdk5ΔCaMBD2-GFP.

or degraded shortly after translation (Fig. 6). The other GFP-fusion proteins were all expressed and detected at the correct molecular weight (Fig. 6).

CaM was detected in Cdk5-GFP immunoprecipitates, but not in Cdk5ΔCaMBD2-GFP or Cdk5ΔCaMBD1 + 2-GFP immunoprecipitates (Fig. 6; left panel). To confirm these results, CaM was immunoprecipitated from AX3/[act15]:cdk5:GFP, AX3/[act15]:cdk5ΔCaMBD2:GFP, and AX3/[act15]:cdk5ΔCaMBD1 + 2:GFP whole cell lysates. Cdk5-GFP was detected in CaM immunoprecipitates, however Cdk5ΔCaMBD2-GFP and Cdk5ΔCaMBD1 + 2-GFP were not (Fig. 6; right panel).

Cdk5-GFP localized to both the nucleus and cytoplasm of live and fixed cells adhering to previous findings (Fig. 7A,B) [12,13]. The amount of Cdk5-GFP in the nucleus was 40 ± 5% higher than the amount in the cytoplasm (Fig. 7B). No intracellular fluorescence was observed in live or fixed AX3/[act15]:cdk5ΔCaMBD1:GFP cells, which was consistent with the inability to detect Cdk5ΔCaMBD1-GFP on Western blots (Figs. 6,7A). Like Cdk5-GFP, Cdk5ΔCaMBD2-GFP also localized to both the nucleus and cytoplasm of live and fixed cells (Fig. 7A,B). The amount of Cdk5ΔCaMBD2-GFP in the nucleus was 88 ± 18% higher than in the cytoplasm, which was significantly higher than the nuclear distribution of Cdk5-GFP (p-value ≤ 0.05; Fig. 7B). Cdk5ΔCaMBD1 + 2-GFP also localized to both the nucleus and cytoplasm of live and fixed cells, however the distribution of the protein in the nucleus relative to the cytoplasm was significantly higher than Cdk5-GFP and Cdk5ΔCaMBD2-GFP (553 ± 68%; p-value ≤ 0.05; Fig. 7A,B).

3.5. Effect of putative CaMBD deletion on the binding of PsaA-GFP to CaM and its localization

Proteins were immunoprecipitated from whole cell lysates with anti-GFP or anti-CaM, separated by SDS-PAGE, and analyzed by Western blotting with anti-GFP and anti-CaM. Anti-GFP and anti-CaM successfully pulled-down their target proteins (i.e., GFP-fusion proteins and CaM respectively) and the GFP-fusion proteins were detected at the correct molecular weight (Fig. 8A). However, the antibodies did not completely immunodeplete any of the proteins (Fig. 8A). CaM was detected in PsaA-GFP immunoprecipitates, but not in PsaA ΔCaMBD-GFP immunoprecipitates (Fig. 8A; left panel). These results were verified by showing that PsaA-GFP could be detected in CaM immunoprecipitates, while PsaAΔCaMBD-GFP could not (Fig. 8A; right panel).

PsaA-GFP localized primarily to the nucleus of live and fixed cells adhering to previous findings (Fig. 8B) [14,58]. Although PsaAΔCaMBD-GFP could be detected on Western blots, no intracellular fluorescence was observed in live or fixed AX3/[act15]:psaAΔCaMBD:GFP cells (Fig. 8B).

Previous studies have shown that the overexpression of either PsaA-GFP or Cdk5-GFP in AX3 cells does not affect the rate of cell proliferation [12,14]. In this study, overexpression of PsaAΔCaMBD-GFP, Cdk5ΔCaMBD1-GFP, Cdk5ΔCaMBD2-GFP, or Cdk5ΔCaMBD1 + 2-GFP also had no effect on axenic growth (Fig. 9).

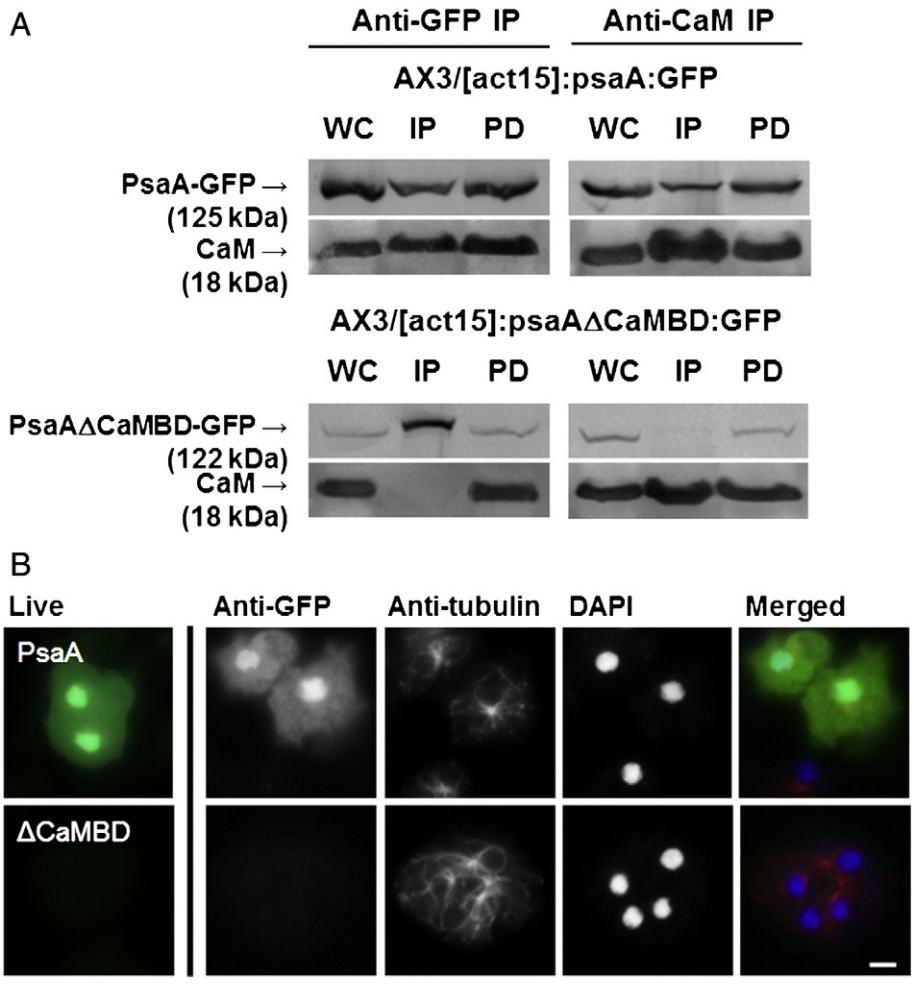

Fig. 8. Effect of putative CaMBD deletion on the binding of PsaA-GFP to CaM and its localization. (A) Whole cell lysates were incubated with anti-GFP or anti-CaM. Immunoprecipitates were pulled-down with Immunocruz™ B IP resin, separated by SDS-PAGE, and analyzed by Western blotting with anti-GFP and anti-CaM. WC, whole cell lysate (20 μg); IP, immunoprecipitate (25 μl); PD, protein-depleted fraction (20 μg). Molecular weights (in kDa) are indicated in parentheses under each protein label. (B) Localization of PsaA-GFP and PsaAΔCaMBD-GFP in live and fixed cells. Cells were stained with DAPI to reveal nuclei. All images shown are representative of a typical cell of that strain. Scale bar = 3 μm.

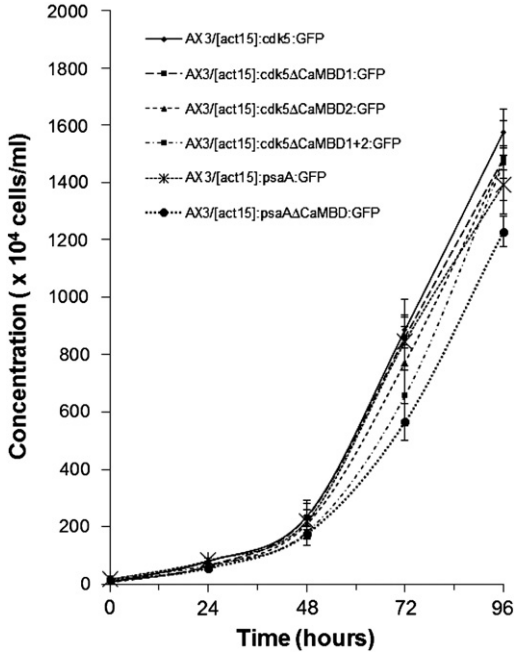

Fig. 9. Axenic growth of cells over-expressing Cdk5-GFP, PsaA-GFP, or GFP-fusion proteins lacking putative CaMBDs. Vegetative cells ($1–4\times10^6$ cells/ml) grown in HL-5 medium were diluted to 2×10^5 cells/ml and then transferred to 6-well plates. Cell concentrations were measured every 24 h over a 96 hour period. Data presented as mean concentration ± s.e.m. (n = 4).

4. Discussion

This study identified Cdk5 as a CaMBP in *Dictyostelium* and determined a region of the protein that is required for CaM-binding. It also provided the first direct evidence for nuclear CaM and revealed new insight into the nuclear relationship between Cdk5, PsaA and CaM in this model organism. The results of this study set the stage for further research into the function and interaction of nuclear CaM with its associated nuclear CaMBPs.

In mammals, p35 function has been shown to be regulated by CaM-binding and Cdk5 phosphorylation; however a direct binding between Cdk5 and CaM was not shown [46]. CaM-dependent kinases have been identified in mammals and some of these kinases have been shown to function in the nucleus [44,45]. Previous studies in *Dictyostelium* had implicated CaM-dependent phosphorylation and dephosphorylation in fertilization, germination, and chemotaxis, however the targeted proteins as well as the proteins and phosphatases involved were not identified [47–49]. In this study, Cdk5 was identified as a CaM-dependent kinase that interacts with CaM and PsaA in *Dictyostelium* nuclei (Fig. 10).

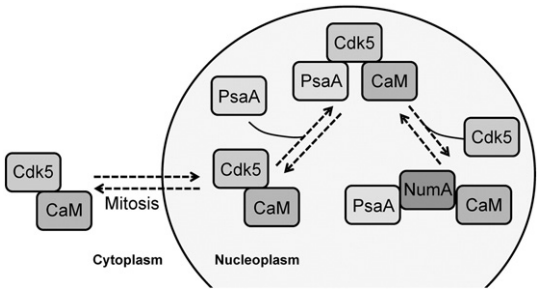

Fig. 10. Interaction of Cdk5 with CaM and PsaA in *Dictyostelium* nuclei. Cdk5 is a CaMBP that associates with CaM and PsaA in the nucleoplasm. CaM regulates the nucleocytoplasmic transport of Cdk5 during mitosis. PsaA also interacts with NumA, a previously identified CaMBP that regulates nuclear number.

Table 2
Sequence homology between the CaMBDs of *Dictyostelium* Cdk5 and human Cdk5.

CaMBD	Sequence homology	
CaMBDI	Dicty Cdk5	^{17}IVYKAKNRETGEIVALK^{33}
		V + KAKNRET EIVALK
	Human Cdk5	^{17}TVFKAKNRETHEIVALK^{33}
CaMBD2	Dicty Cdk5	^{132}LLINRKGELKLADFGLARAFGIP^{154}
		LLINR GELKLADFGLARAFGIP
	Human Cdk5	^{132}LLINRNGELKLADFGLARAFGIP^{154}

Binding of Cdk5 to CaM occurred both in the presence and absence of Ca^{2+} indicating that the binding was Ca^{2+}-independent. The region of amino acids that was deleted in Cdk5ΔCaMBD1-GFP contained 1-10, 1-12, 1-14, and 1-16 hydrophobic motifs. The region deleted in Cdk5ΔCaMBD2-GFP contained all these motifs as well as a 1-8-14 hydrophobic motif. Deletion of CaMBD2 in Cdk5 prevented CaM-binding indicating that this region encompasses a functional CaMBD (i.e., ^{132}LLINRKGELKLADFGLARAFGIP^{154}). Since Cdk5ΔCaMBD1-GFP could not be detected either in cells (i.e., via GFP) or on Western blots, we cannot rule out the possibility that the deleted region also contains a functional CaMBD, especially considering that it does possess 1-10, 1-12, 1-14, and 1-16 hydrophobic motifs. However, since Cdk5 ΔCaMBD2-GFP and Cdk5ΔCaMBD1 + 2-GFP were not able to bind to CaM, our data confirms that CaMBD2 contains a functional CaMBD. Interestingly, the two CaMBDs in *Dictyostelium* Cdk5 are strongly conserved in human Cdk5 indicating that the interaction between CaM and Cdk5 in human cells warrants investigation (Table 2).

In *Dictyostelium*, Cdk5 function has been linked to endocytosis, phagocytosis, secretion, cell proliferation, cell differentiation, and multicellular development [10–12]. The protein has also been characterized as a nucleocytoplasmic protein that exits the nucleus during mitosis, only to return to the nucleus during cytokinesis [13]. Interestingly, the nucleocytoplasmic translocation of Cdk5 has also been reported to occur in mammalian cells [7]. Deletion of Cdk5 CaMBD2 significantly increased the nuclear distribution of the GFP-fusion protein. This effect was dramatically enhanced by deletion of both CaMBD1 and CaMBD2. Since Cdk5ΔCaMBD2-GFP and Cdk5ΔCaMBD1 + 2-GFP could not bind CaM, and since CaM has been shown to function at multiple points in the cell cycle and to be required for cell proliferation in mammals [59,60], these results indicate that CaM may regulate the nucleocytoplasmic transport of Cdk5, which may ultimately regulate mitosis and cell proliferation (Fig. 10). Another interesting finding was the detection of two proteins by anti-Cdk5 in nuclear fractions (33 and 37 kDa) suggesting that Cdk5 may be post-translationally modified in the nucleus.

Although both CaM and Cdk5 function have been linked to mitosis and cell proliferation in *Dictyostelium* [11–13,33,37], axenic growth was unaffected by the over-expression of the Cdk5ΔCaMBD-GFP fusion proteins. Axenic growth has also been shown to be unaffected by the over-expression of Cdk5-GFP [12], indicating that in general, *Dictyostelium* may be able to tolerate the overexpression of GFP-fusion proteins. The non-effect on axenic growth could also be attributed to the ability of endogenous Cdk5 to still bind CaM. The inability to detect an 18 kDa phosphotyrosine or phosphothreonine protein in CaM immunoprecipitates indicates that the unknown phosphotyrosine protein that was previously identified in Cdk5 immunoprecipitates was not CaM [13], that intracellular CaM is not phosphorylated in vivo, and that CaM is likely not a direct target of Cdk5 kinase activity.

PsaA was detected in CaM immunoprecipitates however a direct binding of PsaA to CaM could not be confirmed (Fig. 10). PsaA-GFP localized primarily to the nucleus and was detected in CaM immunoprecipitates. In contrast, PsaAΔCaMBD-GFP was absent from CaM immunoprecipitates and could not be localized in either live or fixed cells. Despite the deleted region in PsaAΔCaMBD-GFP containing 1-10, 1-12, 1-14, 1-16, and 1-5-10 hydrophobic motifs, the absence of PsaA ΔCaMBD-GFP from CaM immunoprecipitates was likely due to the

mis-localization of the protein and its absence from *Dictyostelium* nuclei. Like Cdk5 and CaM, PsaA function has been linked to cell proliferation and mitosis in *Dictyostelium* [14]. Although PsaA has also been shown to interact with and co-localize with Cdk5, a previous study showed that PsaA was not phosphorylated in vivo indicating that the protein is likely not a target of Cdk5 kinase activity [13]. By treating axenically grown cultures with W7, this study showed that CaM activity is not required for the Cdk5/PsaA interaction, however it remains to be determined whether the association between Cdk5 and PsaA is an indirect or a direct interaction.

CaM localizes predominantly to the contractile vacuole in *Dictyostelium*, however nuclear and cytoplasmic CaM have also been reported [32,33,36]. Nuclear CaMBPs have been identified in both mammalian and *Dictyostelium* cells, and while nuclear CaM has been studied in mammals, nuclear localization of CaM in *Dictyostelium* had not previously been confirmed by subcellular fractionation and Western blotting [28,29,38–40,42,43]. The most well studied nuclear CaMBP in *Dictyostelium* NumA regulates nuclear number and binds PsaA (Fig. 10) [29,41]. By showing that CaM was present in nuclear fractions of *Dictyostelium* and that it co-immunoprecipitated with Cdk5 and PsaA, this study has provided the first direct evidence for nuclear CaM and has validated previous studies that have characterized nuclear CaMBPs [29].

5. Conclusions

This study is the first to identify Cdk5 as a CaMBP in any system. Since Cdk5, PsaA, and CaM have all been shown to be involved in mediating cell proliferation in *Dictyostelium* and since Cdk5 and PsaA exhibit similar localizations during *Dictyostelium* mitosis, the data suggest that these proteins may function as part of a signaling pathway to regulate cell proliferation in *Dictyostelium*. The effect of CaMBD deletion on the nucleocytoplasmic localization of Cdk5 indicates that the Cdk5/CaM interaction is necessary for the proper localization of Cdk5. Together these findings set the stage for further research into the function of nuclear CaM and its associated CaMBPs, the functional details of the Cdk5, PsaA, and CaM interaction, and the binding of Cdk5 to CaM in other systems.

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