Molecular characterization and immunolocalization of *Dictyostelium discoideum* protein phosphatase 2A

M.B. Murphy, S.K. Levi, T.T. Egelhoff*

Physiology and Biophysics, School of Medicine, Case Western Reserve University, Cleveland, OH 44106-4970, USA

Received 6 May 1999; received in revised form 11 June 1999

Abstract Protein phosphatase 2A (PP2A) was previously purified from *Dictyostelium* and biochemically characterized. The purified PP2A holoenzyme was composed of a 37 kDa catalytic 'C-subunit', a 65 kDa 'A-subunit' and a 55 kDa 'B-subunit'. We report here the characterization of the genes encoding the *Dictyostelium* PP2A subunits as well as the immunolocalization of the PP2A subunits in *Dictyostelium*. The cDNAs encoding the B- and C-subunits were isolated from a *Dictyostelium* library and the deduced amino acid sequences reveal strong conservation with the mammalian PP2A homologues. Southern blot analysis suggests that each of the PP2A subunit genes is present in a single copy. The PP2A subunits were localized mainly to the cytosol in *Dictyostelium* cells. However, immunofluorescence confocal microscopy demonstrates that the B-subunit of PP2A is highly enriched in centrosomes, suggesting a potential role for this PP2A regulatory subunit in the centrosomal function.

Key words: Protein phosphatase 2A; Centrosome; Confocal immunofluorescence microscopy; *Dictyostelium*

1. Introduction

Many of the signal transduction cascades essential for mammalian cell proliferation, differentiation and motility, such as G-protein-coupled signaling and the mitogen-activated protein kinase cascade, have been found to play important roles in the simple eukaryotic amoeba *Dictyostelium discoideum* [1,2]. While there is an abundance of biochemical and molecular information about the numerous roles of kinases in *Dictyostelium*, little is known about the roles of serine/threonine phosphatases in these cells. In general, serine/threonine phosphatases can be classified into four pharmacologically distinct types: protein phosphatase (PP) 1, PP2A, PP2B and PP2C. In *Dictyostelium*, gene fragments have been identified by PCR which encode eight amino acid stretches that exhibit similarity to known PP1 and PP2A phosphatases [3]. More recently, partial cDNAs of the *Dictyostelium* PP2A C-subunit, PP1c, PPV and PP5 have been identified as expressed sequence tags. Although a gene encoding a PP1 catalytic subunit has been reported, it has also been reported that PP1 activity could not be detected in *Dictyostelium* lysates using okadaic acid and inhibitor 1 to characterize activities toward phosphorylase [4]. The Ca^{2+}/calmodulin dependent PP2B, also known as calcineurin, has been cloned and overexpressed in *Dictyostelium*. However, the cellular function of this phosphatase is not known [5].

We have biochemically characterized a myosin heavy chain phosphatase from *Dictyostelium* that is a heterotrimeric PP2A holoenzyme composed of a 37 kDa catalytic subunit (C-subunit) and associated regulatory subunits of 65 kDa (A-subunit) and 55 kDa (B-subunit) [6]. This phosphatase was identified as an activity that dephosphorylated threonine residues of the myosin II heavy chain that regulate filament assembly in *Dictyostelium*. Dephosphorylation of the myosin heavy chain by the purified PP2A holoenzyme promotes myosin II bipolar filament assembly in vitro. In order to improve the understanding of the structural and functional properties of the PP2A holoenzyme in *Dictyostelium* cells, we have pursued the molecular characterization as well as the immunolocalization of the phosphatase. We describe here the characterization of the genes encoding the 37 kDa catalytic C-subunit and the 55 kDa regulatory B-subunit of PP2A in *Dictyostelium*, as well as further characterization of a recently reported PP2A A-subunit gene. Additionally, we describe the localization of the PP2A subunits by immunofluorescence confocal microscopy.

2. Materials and methods

2.1. Identification of the PP2A catalytic C-subunit and B-subunit genes

Similar strategies were employed to identify the cDNAs encoding the 37 kDa catalytic C-subunit and the 55 kDa B-subunit of PP2A. The cDNA encoding the 65 kDa A-subunit was kindly provided by Bill Loomis. Initially, the genomic clones of the C- and B-subunits from *Dictyostelium* were generated by PCR. For this purpose, an alignment was generated of the amino acid sequences from 55 kDa B-subunits and 37 kDa catalytic subunits of PP2A from several organisms and PCR primers were synthesized matching highly conserved sequences near the amino-terminus and near the carboxyl-terminus of the sequences in the alignment. When PCR amplifications were performed with *Dictyostelium* genomic DNA as the template, these primers generated products of the appropriate size. A subsequent sequence analysis confirmed strong similarity to known 55 kDa B-subunits and 37 kDa catalytic subunits of PP2A. These genomic DNA PCR products were used to generate random primed probes (Boehringer Mannheim Biochemical) to screen a *Dictyostelium* lambda GT11 cDNA library. The DNA from two positive lambda plaques was isolated for each subunit, subcloned into pGEM7 as a *Bam*HI fragment and sequenced. The introns were identified by comparison of the cDNA sequence to the genomic sequence of initial PCR products. The cDNA sequences of the *Dictyostelium* PP2A catalytic subunit and the 55 kDa B-subunit have been deposited in GenBank, accession numbers AF138278 (catalytic subunit) and AF138279 (B-subunit).

2.2. Southern blot analysis

12 μg genomic DNA from the axenic *Dictyostelium* cell line NC4 was either single-digested with the restriction enzymes *Eco*RI and *Hin*dIII or double-digested with *Eco*RI and *Hin*dIII. The products

*Corresponding author. Fax: (1) (216) 368-1693.
E-mail: ette@po.cwru.edu

PII: S0014-5793(99)00835-2

of these restriction digests were separated on a 0.8% agarose gel. The DNA was transferred to GeneScreen Plus membranes (Dupont NEN) using the alkaline transfer protocol described by the manufacturer. The probe DNA specific for each of the three subunits of PP2A was generated as follows. A plasmid containing the PP2A A-subunit cDNA (pDd-PR65) was digested with the restriction enzymes *Pst*I and *Nhe*I to generate a 1.7 kb fragment. A plasmid containing the PP2A B-subunit cDNA (pGEM-B55) was digested with the restriction enzyme *Bam*HI to generate a 1.5 kb fragment. A plasmid containing the PP2A C-subunit was digested with the restriction enzyme *Bam*HI to generate a 1 kb fragment. Random-primed DNA probes were prepared from the above fragments using a labelling kit from Boehringer Mannheim Biochemical. After pre-hybridization for 1 h at 65°C, the probes were hybridized to the membranes for 16 h at 65°C. The membranes were then washed at 60°C with 2×SSC (or 5×SSC for a lower stringency) and then at room temperature with 0.1×SSC (or 5×SSC for the lower stringency). The bands were identified by autoradiography.

2.3. Expression of PP2A during Dictyostelium development

The multicellular development of NC4A2 cells [7] was achieved by harvesting vegetative amoeba in starvation buffer (20 mM MES pH 6.8, 0.2 mM $CaCl_2$, 2 mM $MgSO_4$). The cells were washed in starvation buffer, counted with a hemocytometer and then centrifuged at 2000×*g*. The cell pellet was gently resuspended to a density of 4×10^7 cells/ml. An aliquot of 10^7 cells was spotted onto a pre-sterilized HA-type filter pad (Millipore) which was placed on starvation buffer-soaked Whatman paper in a petri dish. The cells were lysed at time points by submerging the filter pad in a tube containing SDS-PAGE sample buffer. An aliquot from each time point was subjected to 10% SDS-PAGE and the gel was stained with Coomassie. Densitometry of the Coomassie-stained gel allowed for normalization of the amounts of sample from each time point. Identical amounts of sample from each time point were then used for Western blot analysis.

2.4. Production of antibodies and Western blot analysis

Antibodies were generated as follows. The *Dictyostelium* cDNAs encoding the 65 kDa A-subunit and the 37 kDa catalytic subunit of PP2A were expressed in bacteria and purified via 6×His tags. The recombinant proteins were used to immunize New Zealand white rabbits. IgG specific for the A-subunit was purified from the highest titer bleed over a protein A agarose column. IgG specific for the catalytic subunit was purified over an affinity matrix containing the recombinant catalytic subunit cross-linked to Bio-gel. For the B-subunit, a peptide corresponding to a highly conserved epitope, residues 83–96 (LKSLEIEEKINKIK), was synthesized with an amino-terminal cysteine for coupling to a column. Peptide synthesis and subsequent antibody production were performed by Quality Controlled Biochemicals. IgG from the serum containing the highest titer against the peptide, as determined by an enzyme-linked immunosorbent assay, was affinity-purified over a Sulfo-link column (Pierce) containing the coupled peptide antigen. A commercial monoclonal antibody that recognizes the carboxyl-terminal of the PP2A C-subunit was used for Western blot analysis.

Following SDS-PAGE, the gels were transferred to a PVDF membrane and probed with each primary antibody diluted 1:2000 in TBS containing 2.5% milk, 0.15% Tween 20, overnight at 4°C. After incubation with the primary antibodies, the blots were washed in TBS containing 0.3% Tween and then probed with an alkaline phosphatase-conjugated anti-rabbit secondary antibody diluted 1:4000 in TBS, 0.3% Tween. The blots were developed with Western Star chemiluminescence (Tropix) and exposed to film.

A.

```
M G E F Q D V D K Y I S I L K E C K P L S E S E V R D L C E   30
K A R E I L S K E S N V Q P V R C P V T V C G D I H G Q F H   60
D L M E L F K I G G N C P D T N Y L F M G D Y V D R G F Y S   90
V E T V T L L V A L K V R Y K D R V T I L R G N H E S R Q I  120
T Q V Y G F Y D E C L R K Y G N P N V W K L F T D L F D Y L  150
P L T A L I E N Q V F C L H G G L S P S I D T L D H I E N L  180
D R V Q E V P H E G A M C D L L W S D P D D R L G F G Y S P  210
R G A G Y T F G K D I S E Q F N H N N G L T L V A R A H Q L  240
V M E G Y N W C H D Q N V V T I F S A P N Y C Y R C G N L A  270
A I M E I D E K M K H T F L Q F D P A P R R G E P H V T R R  300
T P D Y F L * 306
```

B.

```
S E P L D W R F S Q T F G G K G N E D A S D A D V V S A I E   30
F D Q T G D F I A V G D R G G R V L L L E R T H D K Q S S K   60
K S K L P E Y R F Y S E F Q S H E P E F D Y L K S L E I E E   90
K I N K I K W C P K Q N D A Q F L L T T N D K T I K L W K V  120
Y E K K I K Q V S T S A T T T G P S Y N G S L A S N N T R S  150
P S H T T Y I Y N S S G A H N M N N N M N N S N N N N N L N  180
N F K I P K L T T R E T V V T A T P R K I F Q N A H A Y H I  210
N S I S L N S D G E T Y I S S D D L R I H L W N L N I N T E  240
C F N V V D I K P T N M E D L T E V I T S A E F H P T S C N  270
I F M Y S S S K G T I K L G D L R S S A L C D N H A K V F E  300
E Y E D P S N K S F F S E I I S S I S D I K F S R D G R Y I  330
L S R D F L T L K L W D I N M E N K P V K T I Q I H D Y L K  360
P K L C D L Y E N D C I F D K F E C T L N H D G T Q M L T G  390
S Y H N Y L H I Y D R N S K Q D V C L E A S K Q A T K S K T  420
K T L T T K M K L R S S K K E P K K P E D I H P D A I E Y T  450
K K T L H C A W H P K D N L I A V G A A N T V Y L Y A A T E  480
N K * 482
```

Fig. 1. Deduced amino acid sequences of the catalytic C-subunit (A) and the regulatory 55 kDa B-subunit (B). Identical residues to the human gene products are highlighted. The six WD-repeat motifs of the B-subunit are indicated with underlines.

2.5. Subcellular fractionation

Vegetative cells were harvested from petri dishes and washed with starvation buffer. The cells were lysed in 10 mM TES, pH 7, 1 mM EDTA and protease inhibitors, with 10 mM KCl or 100 mM KCl by gentle sonication on ice at a density of 5×10^7 cells/ml. Lysates were then centrifuged at $40\,000\times g$ or $400\,000\times g$ for 10 min at 4°C. The resulting supernatants and pellets were separated and the pellets were resuspended in an equal volume of lysis buffer. Western blot analysis was performed as described above.

2.6. Localization by Immunofluorescence microscopy

Vegetative NC4A2 cells were harvested in starvation buffer (20 mM MES, pH 6.8, 0.2 mM $CaCl_2$, 2 mM $MgSO_4$) at a density of 10^6 cells/ml and incubated at room temperature for 20 min prior to plating on a cover slip. Cells were allowed to settle and adhere onto the cover slip for approximately 20 min and were then fixed with a 5 min incubation in −10°C methanol or they were fixed in 2% formaldehyde, 15 mM phosphate buffer, pH 7, for 5 min, followed by a 5 min incubation in −10°C methanol. After washing the coverslips in TBS, samples were incubated with the affinity-purified primary polyclonal antibodies (0.02 mg/ml in 3% BSA, TBS) for 45 min at room temperature. The monoclonal antibody, YL1/2, was used for the tubulin staining. Secondary antibodies conjugated to FITC, TRITC or Alexa red were incubated for 45 min at room temperature. The samples were washed in several changes of TBS and, lastly, incubated in water containing DAPI to stain the nuclei. Images were captured and processed using a Zeiss LSM410 Confocal Laser Scanning microscope (Zeiss). All microscopy was performed using a 100× Plan-Neofluor objective (numerical aperature 1.3, oil objective) and an argon/krypton laser (excitation lines 488 nm and 568 nm, respectively). Images shown represent collapsed projections reconstructed from Z-sections of an optical thickness of 0.65 μm.

3. Results and discussion

3.1. Analysis of the Dictyostelium PP2A C-subunit and B-subunit genes

We have identified genomic sequences and cloned corresponding cDNAs encoding a 37 kDa C-subunit and a 55 kDa B-subunit of the serine/threonine phosphatase PP2A from *Dictyostelium*. A *Dictyostelium* cDNA library was screened with a random-primed genomic DNA PCR product specific to the C-subunit and B-subunit. Two positive clones were isolated for each subunit and subcloned into pGEM7. Database searches and Clustal alignments of the deduced amino acid residues revealed that the clones did indeed encode proteins very similar to PP2A genes from other species. Both the C-subunit and the B-subunit contain two introns that were identified by comparison of the genomic DNA sequence with each of the cDNAs (not shown). The *Dictyostelium* C-subunit shares 85% identity with the human C-subunit gene product, the B-subunit shares 55% identity with the human B-subunit gene product (Fig. 1, shaded residues).

The 55 kDa B-subunit of PP2A is a WD-repeat motif containing protein. WD-repeat motifs are tandem repeats of about 40 amino acids which are thought to generate a tertiary structure known as a β-propeller, similar to the β-subunit of heterotrimeric G-proteins [8]. The *Dictyostelium* B-subunit contains six recognizable WD-repeat motifs that are indicated with underlines and were identified by multiple sequence alignments with known WD-repeat containing proteins (Fig. 1B). Interestingly, the B-subunit gene contains an A,T-rich stretch of sequence just 5′ of intron 2 which gives rise to an asparagine-rich insert not found in other organisms (residues 165–178). Two independent cDNAs encoding the B-subunits were sequenced. Although neither cDNA contained an ATG start codon, Clustal alignments indicated that all the conserved portions of the B-subunit were present. Furthermore, the mass of the deduced amino acid sequence is predicted to be 55 kDa.

3.2. Southern blot analysis of the PP2A genes

Genomic DNA isolated from *Dictyostelium* cells was single- or double-digested with *Bam*HI and *Eco*RI. Southern blot analysis of the restriction-digested *Dictyostelium* genomic DNA suggests that each of the three subunits of PP2A is present in a single copy based on the restriction maps predicted from the cDNA sequences (Fig. 2). We have made several attempts to produce knock-out cells lines for the PP2A genes in *Dictyostelium*, however, targeted gene disruption has not been successful. This suggests that PP2A may have an essential role is these cells. PP2A has been shown to have many important cellular functions in higher eukaryotes, functions that are likely to be conserved and essential in *Dictyostelium*, including roles in cell cycle control [9]. The strong conservation of the *Dictyostelium* gene products with the mammalian homologues further supports this conclusion.

3.3. Expression of the three PP2A subunits during multicellular development

The specificity of the antibodies used in all subsequent studies is shown by Western blot analysis of total *Dictyostelium* cell lysates (Fig. 3A). Each of these antibodies also recognizes each of the subunits of the biochemically purified PP2A holoenzyme and the purified PP2A bands co-migrate with the bands shown in Fig. 3A. [6]. A commercial anti-PP2A C-sub-

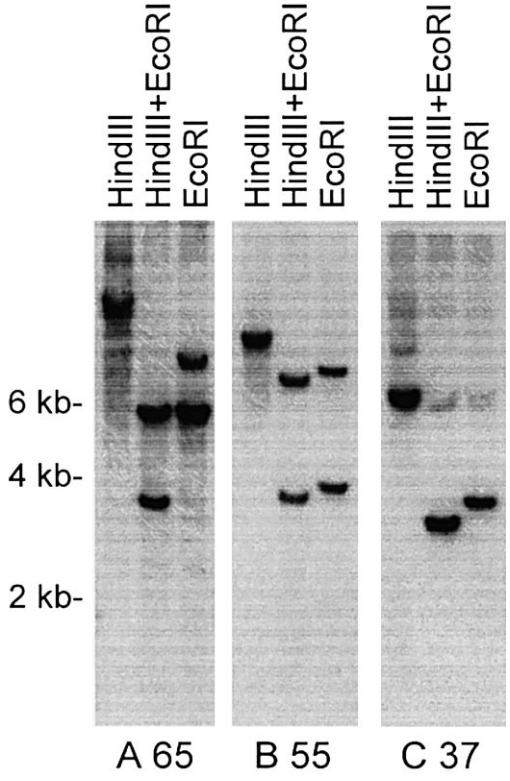

Fig. 2. Southern analysis of genomic *Dictyostelium* DNA digested with the indicated restriction enzymes. The panels from the left show blots incubated with probes specific to the A-subunit, B-subunit and C-subunit as described in Section 2.

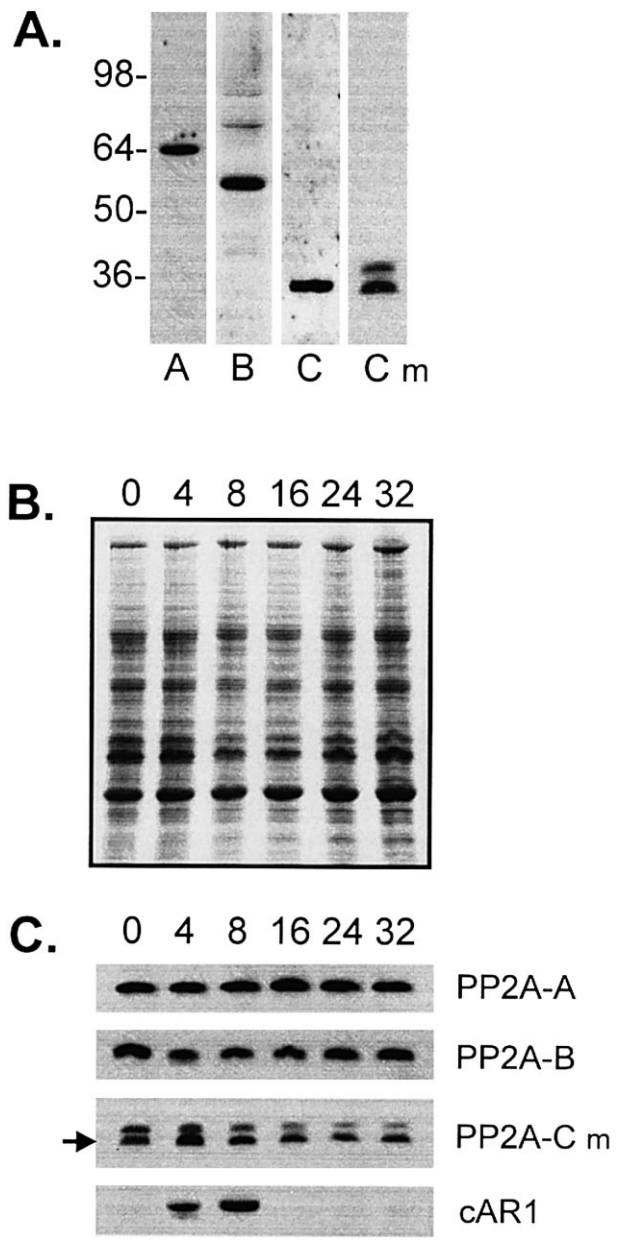

Fig. 3. Western blot analysis showing the expression levels of each of the three PP2A subunits during the multicellular development of NC4A2 *Dictyostelium* cells. The specificity of the antibodies used is demonstrated by Western blot analysis of total *Dictyostelium* cell lysates. Lanes A, B and C were probed with affinity-purified polyclonal antibodies for each of the PP2A subunits and lane 'Cm' was probed with a commercial monoclonal antibody for the PP2A catalytic subunit (A). Coomassie-stained gel (B) and corresponding Western blot showing expression of each of the PP2A subunits in total lysates of developed cells (C). Time points during multicellular development are indicated above each lane and proceed as follows: streaming by 4 h, mounds by 8 h, crawling slugs by 16 h and fruiting bodies by 24–32 h. The expression of the cAMP receptor (cAR1) is shown as a control. The amount of material loaded in each lane was normalized by densitometry of the Coomassie-stained gel (B).

unit monoclonal antibody recognizes two bands in *Dictyostelium* cell lysates. Only the lower band co-purifies with the PP2A holoenzyme [6]. The upper band may be a related phosphatase such as PPV or PPX that shares the carboxyl-terminal epitope recognized by the monoclonal anti-C-subunit antibody.

Dictyostelium is an organism that undergoes multicellular development when confronted with conditions of starvation. Some genes are differentially expressed during development such as the cAMP receptor 1 (cAR1) [10], as well as a calcineurin phosphatase [5]. It was of interest to determine if the PP2A genes have altered expression levels during development using Western blot analysis. Development was initiated by the removal of cells from nutrient media and subsequent incubation in starvation buffer on a filter pad. The expression levels of each of the PP2A genes do not change during development as shown by a Western blot (Fig. 3B and C). This result is consistent with a report on the expression levels of PP2A in resting and growing mammalian cells throughout the cell cycle [11].

3.4. Subcellular localization of the PP2A subunits

Dictyostelium cell lysates were subjected to high speed centrifugation in the presence of 10 or 100 mM KCl. The resulting pellet and supernatant materials were examined for the PP2A subunits by a Western blot (Fig. 4). Although some material was observed in the particulate fractions in the $400\,000 \times g$ samples, PP2A was mainly found in the cytosolic fraction in these subcellular fractionation studies. It is not known if the small amount of particulate material is associated with a cytoskeletal component or plasma membrane-associated protein. MHCK A, a protein that associates with the cytoskeletal fraction in low ionic strength but not at higher ionic strength, is shown as a control [12].

3.5. Immunofluorescence microscopy of the PP2A subunits

PP2A activity has been implicated in many aspects of the cellular function including signal transduction, cell cycle control, metabolism and regulation of transcription factors [13]. A regulatory mechanism common to the PP1 and PP2A serine/threonine phosphatases that may provide specificity in these diverse cellular functions involves the formation of multimeric complexes composed of different regulatory protein subunits. Several of these regulatory subunits have been shown to target the phosphatase activity to a subcellular location or to a specific substrate [14].

In order to identify potential regulatory roles for the 55 kDa B-subunit in *Dictyostelium*, we investigated the subcellular localization of the PP2A subunits by immunofluorescence microscopy using affinity-purified polyclonal antibodies spe-

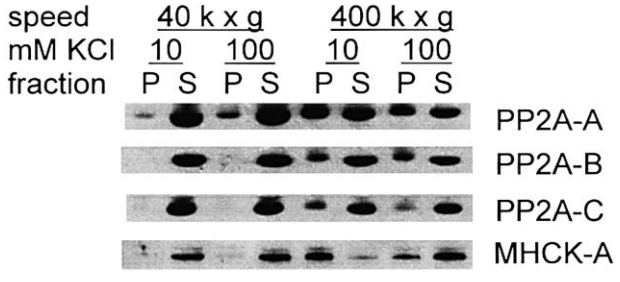

Fig. 4. Subcellular localization of the PP2A subunits in lysates fractionated by centrifugation. The centrifugation speed, ionic strength and fraction are indicated at the top. Each of the subunits labelled on the right were identified by Western blot analysis using the antibodies described above. MHCK A is shown as a control.

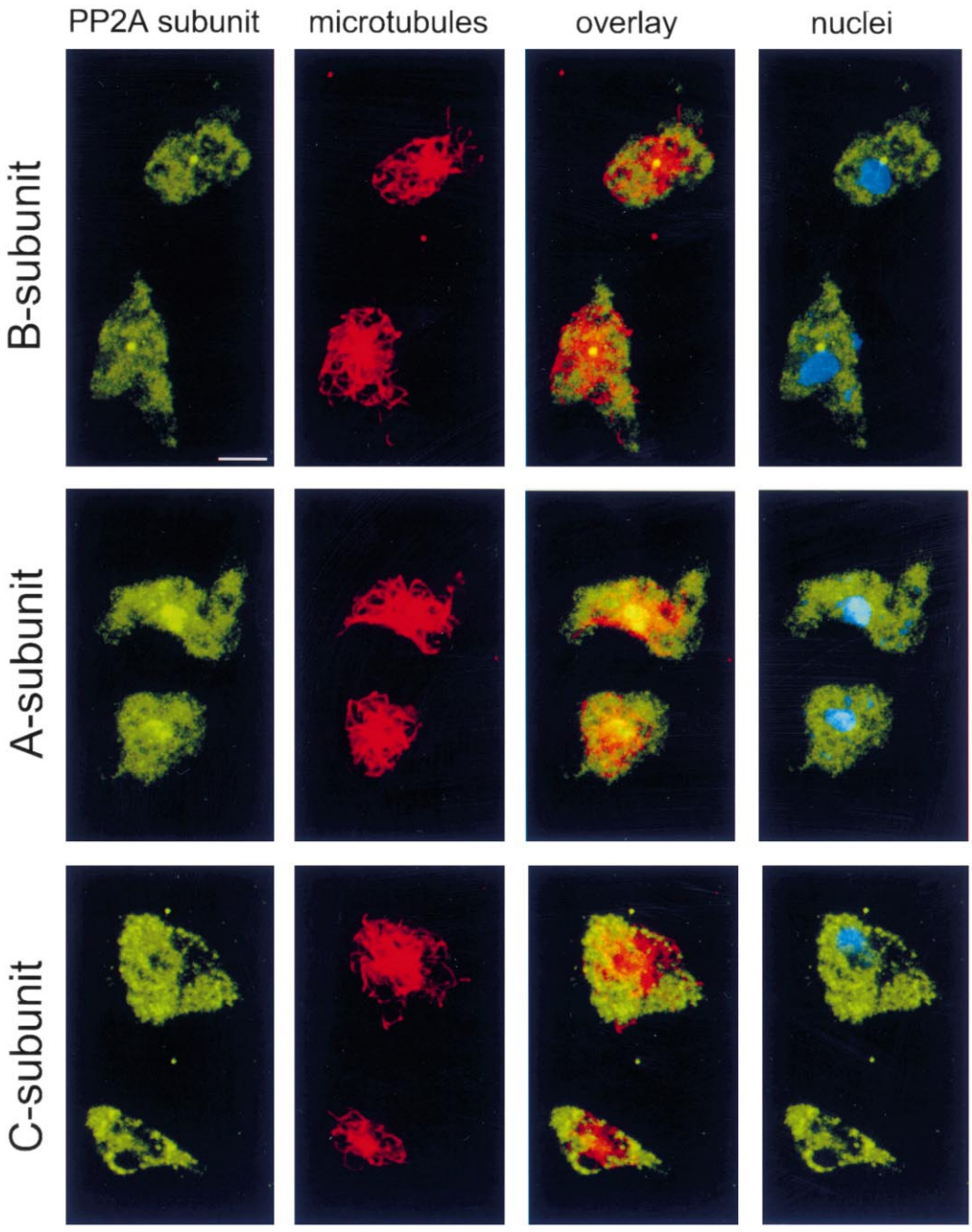

Fig. 5. Confocal images showing the immunolocalization of the PP2A subunits in *Dictyostelium* cells fixed in methanol. The PP2A subunit column shows the immunolocalization patterns of the B-subunit, A-subunit and C-subunit, respectively, using affinity-purified polyclonal primary antibodies and FITC-labelled secondary antibody. Note the bright green spot of the B-subunit. The microtubules column shows immunofluorescent staining of microtubules using monoclonal anti-tubulin primary and Alexa red-labelled secondary. An overlay image of these two fluorescence channels invariably revealed the bright spot of the B-subunit staining to coincide with the microtubule organizing center (yellow spot in overlay). The last column shows overlay images of DAPI-stained nuclei (blue) and each of the PP2A subunits. Note the peri-nuclear, centrosomal localization of the B-subunit, as well as the concentration of the A-subunit in the nucleus. Note the diffuse and speckled localization of the C-subunit.

cific to each subunit. Diffuse cytosolic localization is evident for each of the PP2A subunits. However, confocal images show the B-subunit highly enriched in centrosomes (Fig. 5, B-subunit). Also, the confocal images clearly show the A-subunit concentrated in the nucleus (Fig. 5, A-subunit). The C-subunit localization was diffuse and speckled but not concentrated in either the nucleus or the centrosome (Fig. 5, C-subunit). Identical results were obtained when cells were fixed with either formaldehyde or methanol. Additionally, immunofluorescence was not detected when cells were probed under identical conditions with only the secondary antibody. Interestingly, the three PP2A subunits do not share identical localizations in *Dictyostelium* cells, despite the purification of the three subunits as a multimeric holoenzyme complex from the soluble phase of cell lysates.

Recently, cyclin E has been demonstrated by immunofluorescence microscopy to be localized to centrosomes where it is thought to be involved in centrosomal reproduction [15,16].

We speculate that substrates of the cyclin E dependent kinase localized at the centrosomes may be dephosphorylated by PP2A to control centrosomal reproduction during the cell cycle. It is also possible that the B-subunit recruits PP2A to the centrosome to function in microtubule nucleation. In *Drosophila*, multiple centrosomes and disorganized microtubules are observed in mutants lacking PP2A activity [17]. Additionally, a role for the 55 kDa B-subunit in cell cycle control has been identified in *Drosophila* based upon the mitotic defects observed in B-subunit mutants [18]. The association of the three PP2A subunits with microtubules has been demonstrated in monkey kidney cells using immunofluorescence microscopy. However, these results depended upon a selective cell extraction with detergent [19]. This fixation method did not preserve the microtubule morphology in *Dictyostelium* cells (not shown). PPX, a divergent serine/threonine phosphatase that is 65% identical to PP2A, has shown to be localized to peri-centriolar material in mammalian cells [20]. Perhaps, PPX and PP2A serve overlapping functions. Lastly, studies have been initiated in our lab to observe the dynamic properties of PP2A in *Dictyostelium* cells expressing GFP fusion proteins of these PP2A subunits.

Acknowledgements: This work was supported by a Grant (RPG-97-015-01-CB) from the American Cancer Society. The authors would like to thank Bill Loomis for kindly providing the cDNA of the PP2A A-subunit, Rex Chisholm for providing helpful immunomicroscopy suggestions and a polyclonal anti-tubulin antibody, Marianne Pendergast for confocal microscopy expertise and Paul Steimle for critical review of the manuscript.

References

[1] Van Haastert, P.J.M. (1995) Experientia 51, 1144–1154.
[2] Segall, J.E., Kuspa, A., Shaulsky, G., Ecke, M., Maeda, M., Gaskins, C., Firtel, R.A. and Loomis, W.F. (1995) J. Cell Biol. 128, 405–413.
[3] Haribabu, B. and Dottin, R.P. (1991) Dev. Genet. 12, 45–49.
[4] Simon, M.N., Winckler, T., Mutzel, R., Veron, M. and da Costa Maia, J.C. (1992) Biochem. Biophys. Res. Commun. 184, 1142–1151.
[5] Dammann, H., Hellstern, S., Husain, Q.Y. and Mutzel, R. (1996) Eur. J. Biochem. 238, 391–399.
[6] Murphy, M.B., Egelhoff, T.T. (1999) Eur. J. Biochem. (in press).
[7] Shelden, E. and Knecht, D.A. (1995) J. Cell Sci. 108, 1105–1115.
[8] Neer, E.J., Schmidt, C.J., Nambudripad, R. and Smith, T.F. (1994) Nature 371, 297–300.
[9] Wera, S. and Hemmings, B.A. (1995) Biochem. J. 311, 17–29.
[10] Saxe, C.L., Johnson, R.L., Devreotes, P.N. and Kimmel, A.R. (1991) Genes Dev. 5, 1–8.
[11] Ruediger, R., Van Wart Hood, J.E., Mumby, M. and Walter, G. (1991) Mol. Cell Biol. 11, 4282–4285.
[12] Medley, Q.G., Lee, S.F., Cates, G.A. and Côté, G.P. (1991) J. Cell Biol. 115, 29a.
[13] Mumby, M.C. and Walter, G. (1993) Physiol. Rev. 73, 673–699.
[14] Hubbard, M.J. and Cohen, P. (1993) Trends Biochem. Sci. 18, 172–177.
[15] Lacey, K.R., Jackson, P.K. and Stearns, T. (1999) Proc. Natl. Acad. Sci. USA 96, 2817–2822.
[16] Hinchcliffe, E.H., Li, C., Thompson, E.A., Maller, J.L. and Sluder, G. (1999) Science 283, 851–854.
[17] Snaith, H.A., Armstrong, C.G., Guo, Y., Kaiser, K. and Cohen, P.T. (1996) J. Cell Sci. 109, 3001–3012.
[18] Mayer Jaekel, R.E., Ohkura, H., Ferrigno, P., Andjelkovic, N., Shiomi, K., Uemura, T., Glover, D.M. and Hemmings, B.A. (1994) J. Cell Sci. 107, 2609–2616.
[19] Sontag, E., Nunbhakdi Craig, V., Bloom, G.S. and Mumby, M.C. (1995) J. Cell Biol. 128, 1131–1144.
[20] Brewis, N.D., Street, A.J., Prescott, A.R. and Cohen, P.T. (1993) EMBO J. 12, 987–996.