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The Cyclin-Dependent Kinase Inhibitor Roscovitine Inhibits Kinase Activity, Cell Proliferation, Multicellular Development, and Cdk5 Nuclear Translocation in *Dictyostelium discoideum*

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ABSTRACT

Roscovitine, a cyclin-dependent kinase (Cdk) inhibitor, inhibited kinase activity and the axenic growth of *Dictyostelium discoideum* at micromolar concentrations. Growth was almost fully rescued in 50 μM and ~50% rescued in 100 μM roscovitine-treated cultures by the over-expression of Cdk5-GFP. This supports the importance of Cdk5 function during cell proliferation in *Dictyostelium* and indicates that Cdk5 is a primary target of the drug. Roscovitine did not affect the expression of Cdk5 protein during axenic growth but did inhibit its nuclear translocation. This novel result suggests that the effects of roscovitine could be due in part to altering Cdk5 translocation in other systems as well. Kinase activity was inhibited by roscovitine in assays using AX3 whole cell lysates, but not in assays using lysates from Cdk5-GFP over-expressing cells. At higher concentrations, roscovitine impaired slug and fruiting body formation. Fruiting bodies that did form were small and produced relatively fewer spores many of which were round. However, roscovitine did not affect stalk cell differentiation. Together with previous findings, these data reveal that roscovitine inhibits Cdk5 during growth and as yet undefined Cdks during mid-late development. J. Cell. Biochem. 113: 868–876, 2012.

KEY WORDS: Cdk5; ROSCOVITINE; *Dictyostelium discoideum*; CELL PROLIFERATION; DEVELOPMENT; NUCLEAR TRANSLOCATION

Cyclin-dependent kinases (Cdks) are a family of serine/threonine protein kinases that are activated by cyclins and are involved in regulating eukaryotic cell cycle progression. The Cdk protein family in *Dictyostelium* is comprised of homologues of mammalian Cdk1, Cdk5, and Cdk8. *Dictyostelium* also possesses genes that encode putative Cdks that have not yet been characterized (i.e., Cdk7, Cdk9, Cdk10, and Cdk11). Roscovitine, a potent cell-permeable Cdk inhibitor, inhibits kinase activity by binding to the ATP-binding pocket of Cdks [Meijer et al., 1997]. Roscovitine is currently in Phase 2 and Phase 2b clinical trials for the treatment of nasopharyngeal cancer and non-small cell lung cancer, respectively. Roscovitine is also being researched for its potential treatment of leukemia, herpes simplex infection, HIV infection, breast cancer, and chronic inflammation disorders such as cystic fibrosis and arthritis [Diwan et al., 2004; Agbottah et al., 2005; Goh et al., 2005; Pumfery et al., 2006; Rossi et al., 2006; Węsierska-Gądek et al., 2011ab]. Although roscovitine has been reported to inhibit the activity of a number of mammalian Cdks (e.g., Cdk1, Cdk2, Cdk5, Cdk7, and Cdk9), various studies have implicated it as a preferential Cdk5 inhibitor [Meijer et al., 1997; Goodyear and Sharma, 2007; Liebl et al., 2010; Jain et al., 2011]. In fact, it is the only established Cdk inhibitor shown to exhibit selectivity towards Cdk5 over other Cdks. In addition, roscovitine has been shown to be non-selective towards mammalian Cdk4, Cdk6, and Cdk8 [Pinhero et al., 2004;

Abbreviations used: Cdk, cyclin-dependent kinase; GFP, green fluorescent protein; ATP, adenosine triphosphate; DB, development buffer; MHC, myosin II heavy chain; PsaA, puromycin-sensitive aminopeptidase; NumA, nucleomorphin A; DcsA, cellulose synthase.

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Bach et al., 2005]. The ability of roscovitine to inhibit mammalian Cdk10 and Cdk11 has not been reported. Inhibition of Cdk5 with roscovitine has been shown to block both long-term potentiation induction and NMDA-induced currents in rat hippocampal neurons, attenuate morphine tolerance in rats, inhibit cell proliferation and induce apoptosis of MDA-MB-231 cells, and inhibit endothelial cell migration [Li et al., 2001; Wang et al., 2004; Goodyear and Sharma, 2007; Liebl et al., 2010]. Roscovitine has also been used to inhibit Cdk5 activity in mouse C2C12 myoblasts [Sahlgren et al., 2003]. Despite its use in mammalian systems, the effectiveness of this drug at inhibiting Cdk activity in *Dictyostelium* has not previously been analyzed.

Cdk5 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 amyotrophic lateral sclerosis and Alzheimer's disease [Dhavan and Tsai, 2001; Lee et al., 2010; Abdullah et al., 2011; Crews et al., 2011]. In *Dictyostelium*, Cdk5 has been shown to be required for optimal growth and differentiation, however, attempts to generate a knockout mutant have been unsuccessful [Sharma et al., 2002]. A recent study showed that Cdk5 localizes to both the nucleus and cytoplasm in *Dictyostelium* and binds to puromycin-sensitive aminopeptidase A [PsaA; Huber and O'Day, 2011a]. Here, cells were treated with roscovitine to assay the effectiveness of the drug at inhibiting Cdk5-dependent cellular processes and to gain further insight into the function of Cdks in *Dictyostelium*. The results from this work support previous research that implicates Cdk5 in *Dictyostelium* growth and development. They also show that roscovitine inhibits nuclear translocation of Cdk5, an event that has not been reported previously for any organism.

MATERIALS AND METHODS

CELLS AND STRAINS

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 [Huber and O'Day, 2011a].

AXENIC GROWTH ANALYSIS

AX3 and AX3/[act15]:cdk5:GFP cultures were grown to a concentration of 1-4 $\times 10^6$ cells/ml and then diluted to 1–2 $\times 10^5$ cells/ml ± roscovitine (Santa Cruz Biotechnology, Inc., Santa Cruz, CA) at the desired concentration. Cultures were shaken at 22°C and 150 rpm and their concentrations measured at 24-h time intervals. Cells were also harvested at 24-h intervals 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, Ontario, Canada). Samples were stored at −80°C for future use.

ASEXUAL DEVELOPMENT

Vegetative cells were harvested from SM plates and washed four times in development buffer (DB; 5 mM Na_2HPO_4, 5 mM KH_2PO_4, 1 mM $CaCl_2$, 2 mM $MgCl_2$). Washed cells (6×10^7 cells/ml) were evenly distributed on a Metricel® black membrane disc filter (0.45 μm pore size; Pall Canada Ltd, Mississauga, Ontario, Canada) overlaid on four Whatman #3 cellulose filters (Fisher Scientific Company, Toronto, Ontario, Canada) pre-soaked in DB ± roscovitine at the desired concentration. Cells were maintained in a humidity chamber at 22°C. Structures were viewed and photographed every 4 h with a Nikon Eclipse 50i microscope equipped with a Nikon DS-Ri1 12 mexapixel color cooled digital camera (Nikon Canada, Mississauga, Ontario, Canada). Images were captured and viewed with NIS Elements BR 3.0 (Nikon Canada). Fruiting bodies were stained with calcofluor (1:10,000; UVITEX, Ciba-Geigy Ltd, Baslestadt, Switzerland) and imaged as described above. Spore germination was determined by plating spores with *E. coli* on SM agar plates and assessing fruiting body formation after 5–6 days.

KINASE ASSAY

Cells (1×10^6 cells/ml) were grown in HL-5 medium ± roscovitine (200 μM) for 24 h after which time they were harvested and lysed with NP-40 lysis buffer containing 50 mM Tris, pH 8.0, 150 mM NaCl, 0.5% NP-40, 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). Lysates were sonciated three times for 5 s each. Whole cell lysates (10 μg) were incubated with 40 mM $MgCl_2$, 500 nM ATP (EMD Chemicals, Inc., Gibbstown, NJ), 10 μg histone H1 (Santa Cruz Biotechnology, Inc.), and 1 mM DTT for 20 min at room temperature. Kinase activity was assessed using the Kinase-Glo® Luminescent Kinase Assay Platform according to the manufacturer's instructions (Promega Corporation, Madison, WI). Luminescence was measured using a BioTek Synergy HT Multi-Mode Microplate Reader (BioTek Instruments, Inc., Winooski, VT). The sensitivity level and integration time per well of the microplate reader was set to 150 and 1 s, respectively.

ISOLATION OF NUCLEI

AX3 and AX3/[act15]:cdk5:GFP cultures (1×10^6 cells/ml) ± ros-roscovitine (200 μM) were shaken at 22°C and 150 rpm for 24 h. Nuclei were isolated after 24 h according to a protocol described elsewhere [Huber and O'Day, 2011a]. Additions to the protocol are described below. Pelleted nuclei were resuspended and sonicated in RIPA buffer. Samples were then spun at 12,000*g* for 10 min at 4°C. Supernatants were removed and retained as nuclei fractions. Insoluble pellets containing the actin/myosin cytoskeleton [Huber and O'Day, 2011b] were resuspended in RIPA buffer and sample loading buffer. All samples were stored at −80°C for future use. Aliquots (20–25 μl) of cytoskeletal fractions were separated by SDS–PAGE and analyzed by Western blotting. The enrichment of tubulin in non-nuclear samples and of nucleomorphin A (NumA) in nuclear samples served as a fractionation controls [Huber and O'Day, 2011a].

SDS–PAGE AND WESTERN BLOTTING

SDS–PAGE and Western blotting were carried out as previously described [Huber and O'Day, 2011a]. The following antibodies and dilutions were used: rabbit polyclonal anti-Cdk5 [1:400; Huber and O'Day, 2011a], mouse monoclonal anti-actin (1:1,000; Santa Cruz Biotechnology, Inc.), mouse monoclonal anti-tubulin (1:1,000; 12G10, Developmental Studies Hybridoma Bank, The University of Iowa, Iowa, USA), rabbit polyclonal anti-NumA [1:500; Myre and O'Day, 2002]. 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).

STATISTICAL ANALYSIS

Pairwise Wilcoxon rank sum tests were performed on the relevant data using R for Windows version 2.11.1 (http://www.R-project.org).

RESULTS

EFFECT OF ROSCOVITINE ON CELL PROLIFERATION, Cdk5 EXPRESSION, AND KINASE ACTIVITY

Roscovitine dose-dependently inhibited the axenic growth (i.e., cell proliferation) of AX3 cells (Fig. 1A). By 96 h, 50 μM roscovitine had inhibited cell numbers by over 50% while 100 μM roscovitine had inhibited growth by over 80% (P-value <0.006; Fig. 1C,D). Cdk5 activity had previously been shown to be required for optimal cell proliferation in *Dictyostelium* [Sharma et al., 2002]. Therefore, a strain over-expressing a Cdk5-GFP fusion protein [AX3/[act15]:cdk5:GFP; Huber and O'Day, 2011a] was used to analyze the effect of Cdk5-GFP over-expression on roscovitine-inhibited axenic growth. There was no significant difference between the growth rates of parental AX3 cells and AX3/[act15]:cdk5:GFP cells (Fig. 1A,B). However, Cdk5-GFP over-expression completely rescued the inhibitory effects of 50 μM roscovitine on growth after 24 and 96 h and almost completely rescued growth after 48 and 72 h (P-value <0.006; Fig. 1B,C). The growth of AX3/[act15]:cdk5:GFP cells treated with 100 μM roscovitine was also significantly increased at all time points compared to parental AX3 cells (P-value <0.002; Fig. 1B,D).

While these data strongly support a role for Cdk5 during growth and the specific effect of roscovitine on Cdk5, it was important to examine what effects the drug was having on the expression of Cdk5 and on other cellular components. For example, roscovitine has previously been shown to inhibit Cdk5 expression in MDA-MB-231 breast cancer cells [Goodyear and Sharma, 2007]. *Dictyostelium* Cdk5 was constitutively expressed during axenic growth and roscovitine (100 μM) did not affect this expression (Fig. 1E). Cdk5 expression was also unaffected by treatment with 50 μM roscovitine (data not shown). The expression of actin and tubulin protein as well their cellular organization and that of myosin II heavy chain (MHC) were also unaffected by roscovitine treatment (Fig. 1E and Fig. S1). Similarly, roscovitine treatment did not affect the number of nuclei per cell or the size or shape of cells (data not shown). An in vivo interaction between Cdk5 and PsaA was reported previously [Huber and O'Day, 2011a]; however, roscovitine did not have an effect on this interaction (Fig. S2).

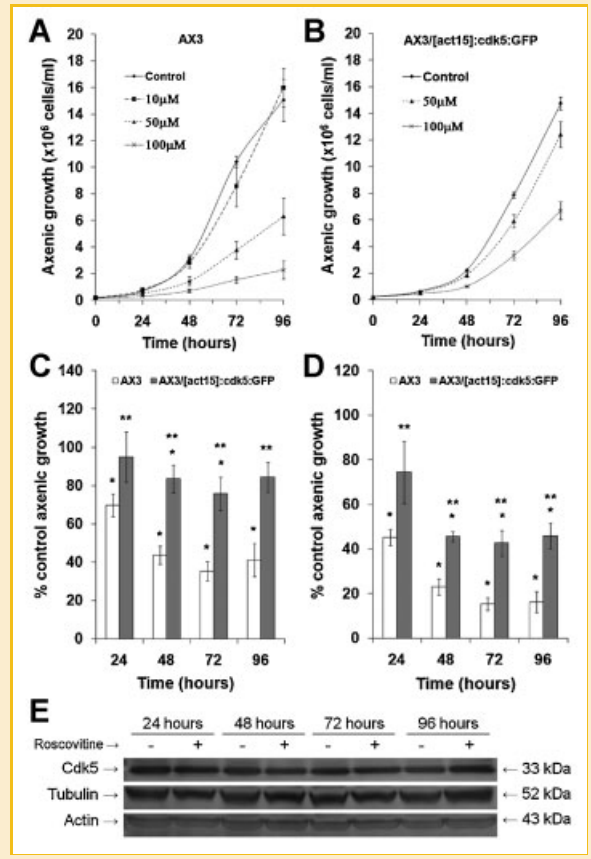

Fig. 1. Effect of roscovitine on the rate of cell proliferation of AX3 and AX3/[act15]:cdk5:GFP cells. A: AX3 cells treated with roscovitine. The concentration of both untreated and roscovitine-treated cultures was measured every 24 h over a 96-h period. Data presented as mean axenic growth ± SEM (n = 4–9). B: AX3/[act15]:cdk5:GFP cells treated with roscovitine. Data presented as mean axenic growth ± SEM (n = 4). C: Effect of roscovitine (50 μM) on the proliferation of AX3 and AX3/[act15]:cdk5:GFP cells. Data presented as mean % control axenic growth ± SEM (n = 4–7). D: Effect of roscovitine (100 μM) on the proliferation of AX3 and AX3/[act15]:cdk5:GFP cells. Data presented as mean % control axenic growth ± SEM (n = 4–9). *P-value <0.006 versus control. **P-value <0.02 versus AX3. E: Effect of roscovitine on the expression Cdk5. AX3 cells in the mid-log phase of growth (1–4 × 10^6 cells/ml) were diluted to 1–2 × 10^5 cells/ml and grown ± roscovitine (100 μM) for 96 h. Cells were harvested and lysed every 24 h. Western blots probed with anti-Cdk5, anti-tubulin, and anti-actin. Blots shown are representative of Western blots from two independent experiments. Molecular weight markers (in kDa) are shown to the right of each blot in (E).

The effect of roscovitine on kinase activity in *Dictyostelium* was assessed by measuring the amount of free ATP remaining in whole cell lysates after a kinase assay using the well-established Cdk substrate histone H1 [Swank et al., 1997]. The less ATP present in solution following an incubation the more kinase activity. Cells were pre-treated with 200 μM roscovitine for 24 h. A higher concentration of roscovitine (200 μM) was required since the starting concentration of the culture was higher than in the 96-h growth assay (Fig. 1). Roscovitine significantly inhibited the growth of AX3

cells by 65 ± 5% after 24 h (P-value <0.01; Fig. 2A). Growth was ~60% rescued in Cdk5-GFP over-expressing cells (P-value <0.05; Fig. 2A). Kinase assays using AX3/[act15]:cdk5:GFP lysates contained ~34% less ATP than assays using AX3 lysates suggesting that kinase activity was increased in Cdk5-GFP over-expressing cells (P-value <0.0002; Fig. 2B). Roscovitine significantly decreased kinase activity in AX3 lysates since assays using roscovitine-treated lysates contained ~32% more ATP than untreated lysates (P-value <0.005; Fig. 2B). There was no statistically significant effect of roscovitine on kinase activity in AX3/[act15]:cdk5:GFP lysates (Fig. 2B).

SUBCELLULAR LOCALIZATION OF Cdk5

Cdk5 has been shown to localize to both the nucleus and cytoplasm of *Dictyostelium* amoebae [Huber and O'Day, 2011a]. To further analyze the subcellular localization of Cdk5 and to quantify the amount of protein in each cellular locale, vegetative cells were fractionated to obtain nuclear, non-nuclear (i.e., cytoplasmic), and detergent-insoluble (i.e., actin/myosin cytoskeletons) fractions. The subcellular distribution of Cdk5 in AX3 cells was similar to the distribution in AX3/[act15]:cdk5:GFP cells (Fig. 3A). Relative to the amounts detected in whole cell lysates, there was an approximately equal amount of Cdk5 in the nuclear and non-nuclear fractions of both strains (Fig. 3A,B). Cdk5 was also detected in the cytoskeletal fractions of both strains; however, the amounts relative to whole cell lysates were much less than the amounts observed in nuclear and non-nuclear fractions (Fig. 3A,B).

EFFECT OF ROSCOVITINE ON THE SUBCELLULAR LOCALIZATION OF Cdk5

AX3 and AX3/[act15]:cdk5:GFP cells in the mid-log phase of growth (~1×10^6 cells/ml) were treated with 200 μM roscovitine for 24 h to analyze the effect of roscovitine on the nucleocytoplasmic localization of Cdk5 during periods of high cell division. Treatment with roscovitine decreased the nuclear/non-nuclear distribution ratio for Cdk5 by over 40% and 30% in AX3 and AX3/[act15]:cdk5:GFP cells, respectively (Fig. 4A,B). A lower distribution ratio indicated that there was less Cdk5 in the nucleus relative to the cytoplasm. Tubulin (53 kDa) was enriched in non-nuclear samples and NumA (38 kDa) was enriched in nuclear samples, confirming the efficiency of the fractionations [Fig. 4A; Huber and O'Day, 2011a]. The subcellular distribution of both proteins was unaffected by treatment with roscovitine (Fig. 4A,C). In addition, roscovitine increased the amount of Cdk5 detected in cytoskeletal fractions from AX3 and AX3/[act15]:cdk5:GFP cells by 104 ± 34% and 54 ± 13%, respectively (Fig. 4A,D). Roscovitine did not affect the whole cell expression levels of Cdk5 in either strain (Fig. 4A,D).

EFFECT OF ROSCOVITINE ON ASEXUAL DEVELOPMENT

Previous studies have reported a function for Cdks during *Dictyostelium* development [Sharma et al., 2002; Takeda et al., 2002; Lin et al., 2004; Greene et al., 2011]. Therefore, it was necessary to examine the effect of roscovitine on asexual development. At 50 and 100 μM roscovitine there were no visible effects on the timing of early or later developmental events (data not shown). Roscovitine (0.5 or 1 mM) also did not have any visible effects during the first 12 h of development but by 16 h, when

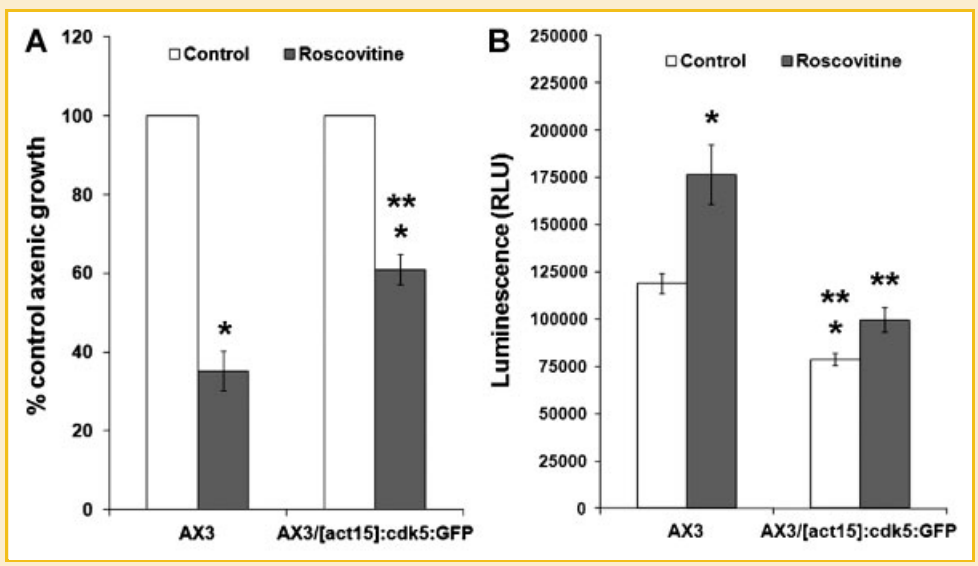

Fig. 2. Effect of roscovitine on kinase activity. A: Effect of roscovitine (200 μM) on the axenic growth of AX3 and AX3/[act15]:cdk5:GFP cells. Data presented as mean % control axenic growth ± SEM (n = 8–13). *P-value <0.01 versus control. **P-value <0.05 versus AX3. B: Cells (1×10^6 cells/ml) were treated with roscovitine (200 μM) for 24 h after which time they were lysed. Whole cells lysates (10 μg) of untreated (i.e., control) and roscovitine-treated cells were incubated with $MgCl_2$ (40 mM), ATP (500 nM), histone H1 (10 μg), and DTT (1 mM) as detailed in the Materials and Methods Section. Kinase activity was measured using the Kinase-Glo® Luminescence Kinase Assay Platform (Promega Corporation) which measured the amount of free ATP remaining in solution after a 20-min incubation at room temperature. Data presented as mean luminescence (RLU) ± SEM (n = 8). RLU = relative light units. *P-value <0.005 versus AX3 control. **P-value <0.0005 versus AX3 roscovitine.

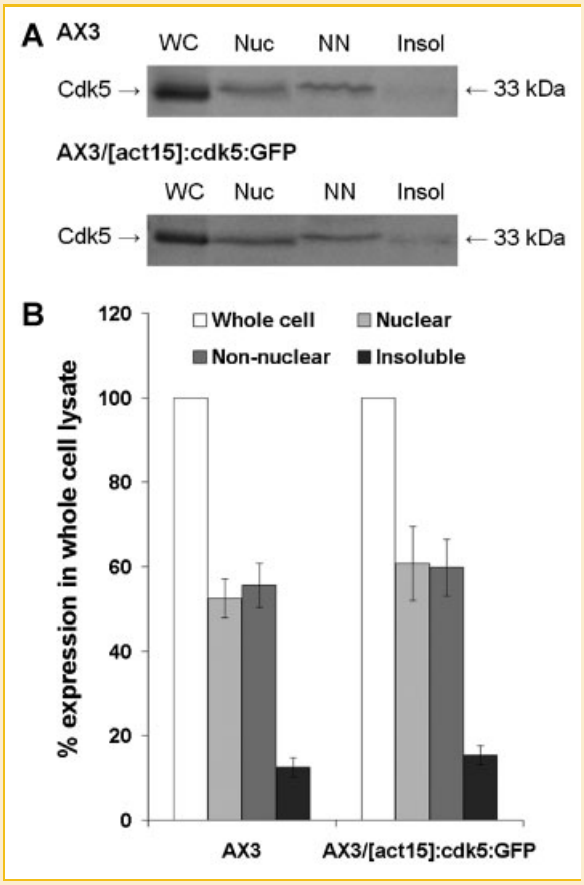

Fig. 3. Subcellular localization of Cdk5 in AX3 and AX3/[act15]:cdk5:GFP cells. Nuclei were isolated from AX3 and AX3/[act15]:cdk5:GFP cells. Proteins from whole cell lysates (WC, 15–20 μg) and nuclear (Nuc, 15–20 μg), non-nuclear (NN, 15–20 μg), and insoluble fractions (Insol; 20–25 μl) were separated by SDS–PAGE and analyzed by Western blotting. A: Subcellular distribution of Cdk5 in AX3 and AX3/[act15]:cdk5:GFP cells. Western blots probed with anti-Cdk5. B: Protein bands were quantified and plotted. Data presented as mean % expression in whole cell lysate ± SEM (n = 9–10). Molecular weight markers (in kDa) are shown to the right of each blot in (A).

untreated cells were at the slug stage, roscovitine-treated cells had not progressed past the mound stage (Fig. 5). By 20 h, untreated filters contained almost fully developed fruiting bodies while the roscovitine-treated cells were still mounds. By 24 h, when mature fruiting bodies were present on untreated filters, roscovitine-treated cells were still arrested at the mound/slug stage (Figs. 5 and 6A). There were no visible differences between the effects of 0.5 and 1 mM roscovitine. Fruiting bodies did eventually form after 30–40 h on roscovitine-treated filters, but the number and size of fruiting bodies was far less than controls (Fig. 5). After 48 h, roscovitine had significantly inhibited fruiting body formation by 67 ± 9% and 77 ± 7% at 0.5 and 1 mM, respectively (P-value <0.00003; Fig. 6A). In addition, there were a significant number of mounds, slugs, and abnormal developmental structures still present on roscovitine-treated filters after 48 h (Fig. 5).

Since development was effectively stopped at the mound stage, it appeared that roscovitine was acting at or shortly after mound formation. To test this, filters containing normally developing mounds at 12 h were transferred to fresh Whatman #3 cellulose filters pads containing the desired concentration of roscovitine (0.5 or 1 mM; Fig. S3). While roscovitine still inhibited development, the effect was reduced as some fruiting bodies were observed on roscovitine-treated filters after 24 h (Fig. S3). Roscovitine significantly inhibited fruiting body formation after 24 h by 72 ± 10% and 87 ± 5% at 0.5 and 1 mM concentrations, respectively (P-value <0.008; Fig. 6B) and by 46 ± 14% and 38 ± 16% after 48 h (P-value <0.03; Fig. 6B).

Untreated fruiting bodies possessed dense, opaque spore masses composed of a large number of spores (Fig. 7A). Spore masses from fruiting bodies that developed in the presence of roscovitine were translucent and contained relatively few spores. The spores that were present were located at the top of the spore mass. There were no apparent differences in stalk morphology (Fig. 7B); however, the majority of roscovitine-treated spores were round compared to the elliptical shape of spores from untreated fruiting bodies (Fig. 7C). This observation was statistically verified by measuring the length and width of spores from untreated and roscovitine-treated fruiting bodies where the closer the ratio is to 1.0 the more round the spore (Fig. 7D). Roscovitine-treated spores germinated and formed phenotypically normal fruiting bodies (data not shown).

DISCUSSION

Roscovitine is an effective inhibitor of kinase activity, cell proliferation, and multicellular development in *Dictyostelium*. The effective concentrations were higher than those used for mammalian cells; however, this is common for pharmacological studies in *Dictyostelium* using inhibitors designed to inhibit mammalian proteins. Over-expression of Cdk5-GFP dramatically rescued the inhibitory effect of roscovitine on growth and kinase activity revealing that Cdk5 is a primary target of roscovitine during cell proliferation. While roscovitine had no detectable effects on various parameters of normal cells at least part of the effect on cell proliferation could be due to the drug's effect on nuclear translocation. Roscovitine also inhibited the progress of multicellular development and affected the morphology of spore cells; however, the Cdks involved during development remain in question.

Current knowledge of Cdk5 function in *Dictyostelium* is based on a strain over-expressing a dominant negative form of the protein [Sharma et al., 2002]. That study reported the inability to generate a Cdk5 null mutant despite using a number of different knockout strategies, suggesting that a knockout mutation is lethal and that Cdk5 is a critical regulator of axenic growth in *Dictyostelium*. Here we attempted to advance our knowledge of Cdk5 using a pharmacological approach coupled with an expression vector for Cdk5-GFP. The expression of Cdk5 remained relatively constant during axenic growth showing that the protein is constitutively expressed during vegetative conditions and may function during all stages of axenic growth (e.g., early-log, mid-log, and late-log). While micromolar amounts of roscovitine significantly inhibited cell proliferation of *Dictyostelium*, this inhibitory effect could be partially (100 μM) or almost completely (50 μM) rescued by

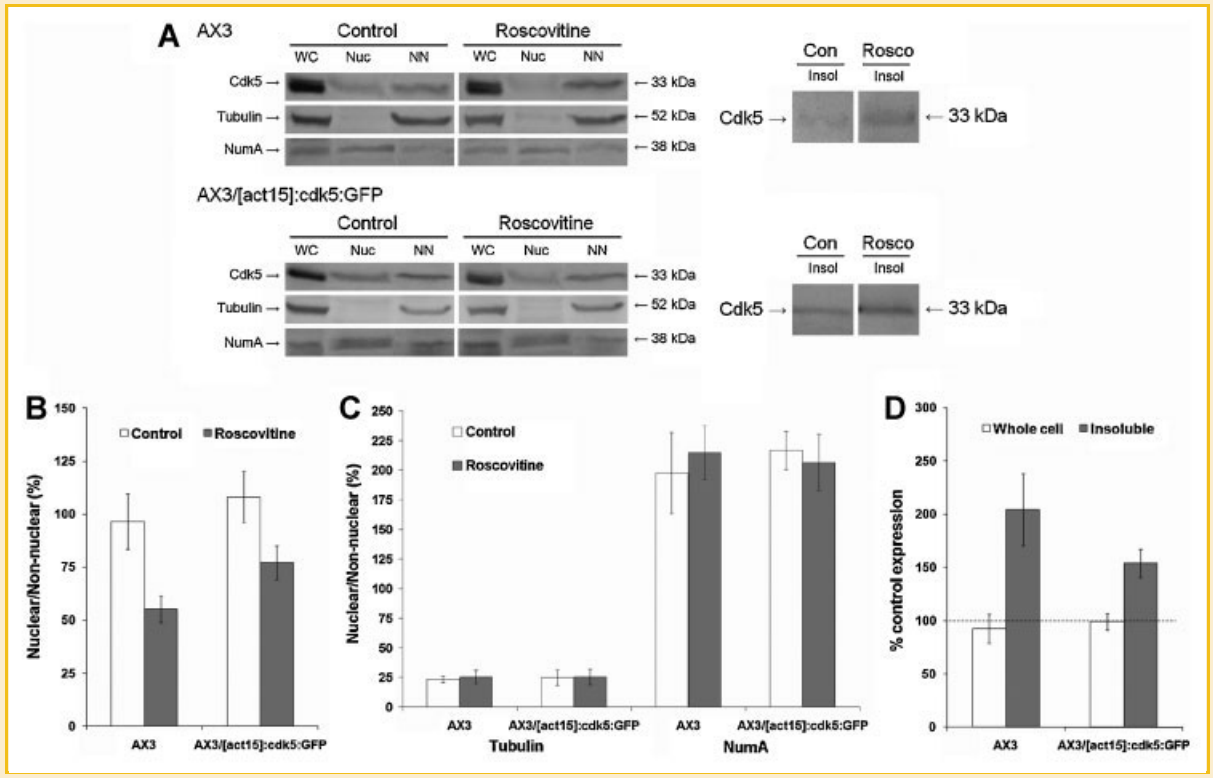

Fig. 4. Effect of roscovitine on the subcellular localization of Cdk5 in AX3 and AX3/[act15]:cdk5:GFP cells. A: Proteins from whole cell lysates (WC, 15–20 μg) and nuclear (Nuc, 15–20 μg), non-nuclear (NN, 15–20 μg), and insoluble fractions (Insol; 20–25 μl) were separated by SDS–PAGE and analyzed by Western blotting. Western blots probed with anti-Cdk5, anti-tubulin, and anti-NumA. Protein bands were quantified and plotted. B: Effect of roscovitine on the nuclear/non-nuclear distribution ratio of Cdk5 in AX3 and AX3/[act15]:cdk5:GFP cells. C: Effect of roscovitine on the nuclear/non-nuclear distribution ratio of tubulin and NumA in AX3 and AX3/[act15]:cdk5:GFP cells. Data in (B) and (C) presented as mean nuclear/non-nuclear (%) ± SEM (n = 4–8). D: Effect of roscovitine on the expression of Cdk5 in whole cell lysates and in insoluble fractions. Data presented as mean % control expression ± SEM (n = 5–8).

Cdk5-GFP over-expression. This data supports the previously reported function for Cdk5 during axenic growth and suggests that roscovitine predominantly if not specifically inhibits Cdk5 during cell proliferation [Sharma et al., 2002]. Cdk8 is likely not a target during growth since roscovitine does not inhibit Cdk8 activity in mammals and Cdk8 has been shown to be dispensable for *Dictyostelium* growth [Takeda et al., 2002; Pinhero et al., 2004; Bach et al., 2005]. Cdk1 function has also been linked to axenic growth and our results do not rule out or support the co-function of Cdk1 in cell proliferation [Luo et al., 1995].

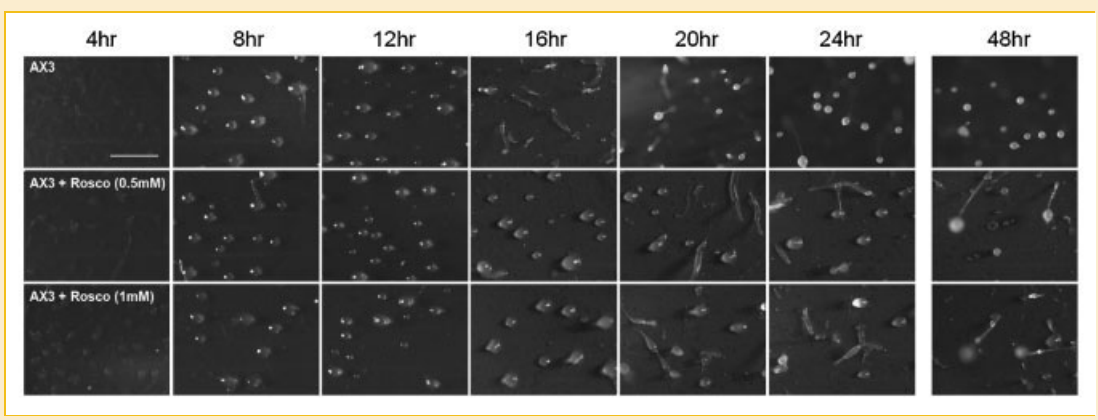

Fig. 5. Effect of roscovitine on asexual development. AX3 cells (6×10^7 cells/ml) were allowed to develop in a humidity chamber on filters pre-soaked in DB ± roscovitine. Development was monitored every 4 h over a 24-h period and after 48 h. Scale bar = 1 mm.

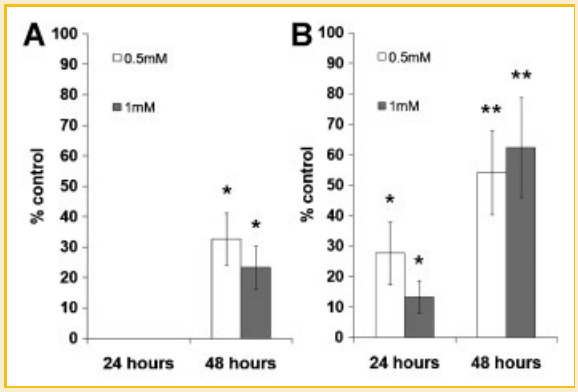

Fig. 6. Effect of roscovitine on fruiting body formation. The total number of fruiting bodies on filters pre-soaked with DB ± roscovitine was counted after 24 and 48 h of development. A: Fruiting body formation in the presence of roscovitine from the onset of development. Data presented as mean % control ± SEM (n = 11). *P-value <0.00003 versus 48-h control. B: Fruiting body formation in the presence of roscovitine from 12 h into development. Data presented as mean % control ± SEM (n = 5). *P-value <0.008 versus 24-h control. **P-value <0.03 versus 48-h control.

Our data shows that roscovitine can significantly inhibit kinase activity in assays using *Dictyostelium* AX3 whole cell lysates, but not in assays using AX3/[act15]:cdk5:GFP lysates suggesting that Cdk5-GFP over-expression rescues the reduced kinase activity. However, we were unable to inhibit the activity of immunoprecipitated Cdk5 and Cdk5-GFP possibly due to the steric hindrance imposed by the antibody or loss of essential co-factors for enzyme activity during the immunoprecipitation process. Despite this, the sequence similarity between mammalian and *Dictyostelium* Cdk5 [Michaelis and Weeks, 1993; Sharma et al., 2002; Huber and O'Day, 2011a], the effect of roscovitine on established Cdk5-dependent cellular processes in *Dictyostelium*, the novel effect on Cdk5 nuclear translocation, and the rescue of kinase activity by Cdk5-GFP overexpression, suggests that Cdk5 is a primary target of the drug. In addition, since the whole cell lysate kinase assays utilized the well-established Cdk substrate histone H1, this provides further support that the inhibited kinase(s) were Cdks.

In a previous study, Cdk5 was shown to localize to both the nucleus and cytoplasm of *Dictyostelium* amoebae [Huber and O'Day, 2011a]. Here, a small amount of Cdk5 was also detected in the cytoskeleton, fitting with the established role of Cdk5 in regulating fluid-phase endocytosis and phagocytosis in *Dictyostelium* [Sharma et al., 2002]. In mammalian cells, the Cdk5 activator p39 has been shown to localize to the actin cytoskeleton and the Cdk5/p39 and Cdk5/p35 complexes have been suggested to play a role in regulating actin cytoskeletal dynamics [Nikolic et al., 1996; Pant et al., 1997; Humbert et al., 2000; Veeranna et al., 2000]. Cdk5 leaves the nucleus during mitosis and returns during cytokinesis [Huber and O'Day, 2011a]. In this study, roscovitine significantly inhibited axenic growth and decreased the amount of nuclear Cdk5 indicating that Cdk5 nuclear localization is involved during cell division. This is the first evidence for roscovitine affecting the nuclear localization of Cdk5. The association of Cdk5 with the cytoskeleton and the ability of roscovitine to increase the amount of cytoskeletal associated Cdk5 does indicate that roscovitine has additional effects on Cdk5.

The effect of roscovitine on multicellular development and spore cell differentiation is not so clear-cut. *Dictyostelium* possesses a number of putative Cdks (i.e., Cdk7, Cdk9, Cdk10, and Cdk11) but none of these have been characterized. Although Cdk8 function has been linked to development in *Dictyostelium*, roscovitine has been

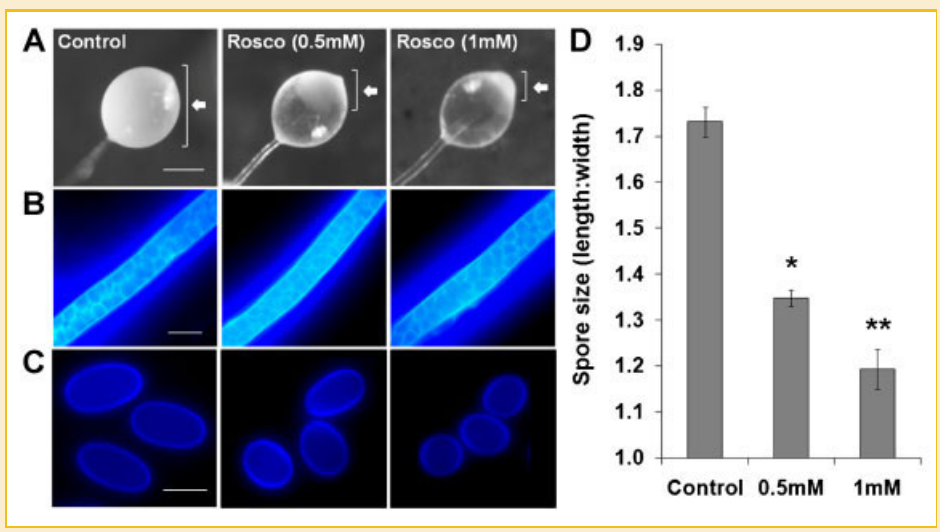

Fig. 7. Effect of roscovitine on fruiting body morphology. Cells were allowed to develop on filters pre-soaked with DB ± roscovitine for 48 h. A: Representative spore masses from untreated and roscovitine-treated fruiting bodies. Arrows indicate location of spores within the spore mass. Scale bar = 100 μm. B: Stalks stained with calcofluor. Scale bar = 15 μm. C: Spores stained with calcofluor. Scale bar = 5 μm. D: Effect of roscovitine on spore morphology. The length and width of at least 50 spores was measured for each experimental replicate and then expressed as a ratio. Data presented as mean spore size (length:width) ± SEM (n = 6–8). *P-value <0.0007 versus control. **P-value <0.02 versus control and 0.5 mM. [Color figure can be seen in the online version of this article, available at http://wileyonlinelibrary.com/journal/jcb]

shown to be a poor inhibitor of mammalian Cdk8 [Takeda et al., 2002; Lin et al., 2004; Pinhero et al., 2004; Bach et al., 2005; Greene et al., 2011]. Previous studies report that Cdk1 is not involved in regulating development, including the mitotic events that occur during later developmental stages, indicating that Cdk1 is likely not a target of roscovitine during development [Zada-Hames and Ashworth, 1978; Michaelis and Weeks, 1992; Luo et al., 1995; Sharma et al., 1999; Rot et al., 2009]. In contrast, *cdk5* mRNA and protein expression increases significantly during development reaching peak levels after 16 h and remaining relatively high during terminal differentiation [Michaelis and Weeks, 1993; Sharma et al., 1999; Rot et al., 2009]. The observations described in this study fit with those previously described by Sharma et al. [2002] who reported a 24-h delay in fruiting body formation for cells over-expressing a dominant negative form of Cdk5. Also, the number of fruiting bodies and spores that did form was significantly reduced compared to parental cells [Sharma et al., 2002]. These similarities, in addition to the expression profile of Cdk5 during development, the function of Cdk5 during cell proliferation, and the ability of roscovitine to inhibit Cdk5 activity during axenic growth, suggest that roscovitine, at least in part, inhibits Cdk5 during development possibly during the mitotic events that occur during the later stages of development. Sharma et al. [2002] also reported an aggregation defect. Roscovitine did not affect early developmental events likely because developing cells are much less permeable to drugs than growing cells. The cause of the round spore phenotype remains to be elucidated, however, previous studies have reported a similar phenotype for mutants affecting microtubules, the catalytic subunit of cellulose synthase (*dcsA*), and the clathrin associated proteins Hip1r and epsin [Welker and Williams, 1983; Zhang et al., 2000; Repass et al., 2007]. Roscovitine-treated spores stained brightly with calcofluor (i.e., cellulose stain) indicating that the aberrant phenotype was not due to altered cellulose accumulation.

Together, this data suggests roscovitine can be used to further clarify the function of Cdk5 during cell proliferation and development by identifying signaling pathways that mediate Cdk5 activity in *Dictyostelium* and the downstream effects of Cdk5 inactivation by this inhibitor. *Dictyostelium* could also be used as a model system to understand how roscovitine works in other organisms.

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