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Inositol 1,4,5-Trisphosphate Receptor (Type 1) Phosphorylation and Modulation by Cdc2

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Abstract Calcium (Ca^{2+}) release from the endoplasmic reticulum (ER) controls numerous cellular functions including proliferation, and is regulated in part by inositol 1,4,5-trisphosphate receptors (IP3Rs). IP3Rs are ubiquitously expressed intracellular Ca^{2+}-release channels found in many cell types. Although IP3R-mediated Ca^{2+} release has been implicated in cellular proliferation, the biochemical pathways that modulate intracellular Ca^{2+} release during cell cycle progression are not known. Sequence analysis of IP3R1 reveals the presence of two putative phosphorylation sites for cyclin-dependent kinases (cdks). In the present study, we show that cdc2/CyB, a critical regulator of eukaryotic cell cycle progression, phosphorylates IP3R1 in vitro and in vivo at both Ser^{421} and Thr^{799} and that this phosphorylation increases IP3 binding. Taken together, these results indicate that IP3R1 may be a specific target for cdc2/CyB during cell cycle progression. J. Cell. Biochem. 90: 1186–1196, 2003.

Key words: proliferation; IP3R1; Cdc2; phosphorylation; calcium

IP3R-mediated Ca^{2+} signaling is involved in modulating cell growth and death pathways [Jayaraman and Marks, 1997; Marks, 1997], and IP3Rs are ubiquitously expressed intracellular Ca^{2+}-release channels in many cell types [Ehrlich et al., 1994; Marks, 1997]. In mammalian tissues, at least three forms of IP3R have been identified. The channel exists as homotetrameric and heterotetrameric structures [Joseph et al., 1995; Nucifora et al., 1996] with three functional domains: a transmembrane domain containing the Ca^{2+}-channel pore close to the carboxy-terminus, the amino-terminal IP3-binding domain, and a large cytosolic domain that connects the Ca^{2+} channel with the IP3-binding region [Mignery and Sudhof, 1990; Joseph, 1996]. Most of the IP3Rs, excluding a short transmembrane Ca^{2+}-channel region, are exposed to the cytoplasm and are targets for several accessory proteins as well as kinases. For instance, IP3R1 functions are modulated by several accessory proteins including the FK-506 binding protein, FKBP12, a member of the immunophilin family of *cis–trans* peptidyl-prolyl isomerases [Cameron et al., 1995b; Poirier et al., 2001], calcineurin [Cameron et al., 1995a], homer protein that binds to a proline-rich motif [Tu et al., 1998], the nonreceptor protein tyrosine kinase Fyn [Jayaraman et al., 1996], and inositol 1,4,5-trisphosphate receptor-associated cGMP substrate (IRAG) [Schlossmann et al., 2000]. A homer ligand-like motif is conserved in IP3R1 at amino acids 48–55; the binding regions for Fyn and PKG have not yet been determined.

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In addition to accessory proteins, five protein kinases, Fyn [Jayaraman et al., 1996], calmodulin-dependent kinase II (CaMKII) [Zhu et al., 1996], protein kinase G (PKG) [Schlossmann et al., 2000], protein kinase A (PKA) [Nakade et al., 1994], and protein kinase C (PKC) [Matter et al., 1993] modulate IP3R1 via phosphorylation. Although the precise details of how phosphorylation by specific kinases modulates IP3R1 function, and the significance of these signaling events in the context of cellular function, remain to be elucidated, there are well-documented functional effects that are observed upon phosphorylation of IP3R1 [Matter et al., 1993; Komalavilas and Lincoln, 1994; Nakade et al., 1994].

Ca^{2+} transients occur when cells progress from quiescence, at the G1/S transition, during S-phase, and at the exit from mitosis [Berridge, 1995], as the resulting Ca^{2+} signals are required to initiate many types of transcriptional events during cellular proliferation. Ca^{2+} transients are elicited in the form of oscillations during the cell cycle in dividing *Xenopus* embryos and in cycling egg extracts [Poenie et al., 1985; Grandin and Charbonneau, 1991; Kubota et al., 1993; Swanson et al., 1997; Tokmakov et al., 2001]. The finding that injection of an antibody specific to IP3R1 blocks Ca^{2+} oscillations in fertilized hamster eggs suggests the involvement of IP3R1 [Miyazaki et al., 1992]. Injection of Ca^{2+} chelators into fertilized eggs blocks cell cycle progression, whereas Ca^{2+} ionophores induce its resumption. Microinjection of BAPTA, an intracellular Ca^{2+} chelator, prevents mitosis in *Xenopus* embryos (which express only IP3R1) and inhibits proliferation of mesangial cells in response to PDGF, endothelin-1, and FBS [Whiteside et al., 1998]. Moreover, an IP3R1 deletion mutant lacking the IP3-binding region and IP3R1-deficient T cells (generated using 2.9 kb of 5′ antisense DNA) display reduced cell growth in response to serum [Fischer et al., 1994; Jayaraman and Marks, 1997]. In addition, a recent report demonstrates a crucial role of IP3R1, but not IP3R3, in IP3-induced Ca^{2+} release and proliferation of vascular smooth muscle cells [Wang et al., 2001]. These studies suggest that IP3R1-mediated Ca^{2+} release is important for cellular proliferation; however, the molecular mechanism by which IP3R1 functions are modulated during cell cycle progression is not known.

Orderly progression through the cell cycle depends on the activation and inactivation of cdks. While cdc2/CyB (also known as the cdk1/CyB complex) is necessary for G2/M transition, cdk4/CyD, cdk2/CyE, and cdk2/CyA play major roles in the G1, G1/S, and S-phase transitions of the cell cycle, respectively [Morgan, 1997]. Each of these cdks is active for only a short period of the cell cycle, during which time it phosphorylates a number of substrates required for entry into the next phase. In this study, we examined IP3R1 phosphorylation by the cdc2/CyB complex in vitro and determined the effect of phosphorylation on IP3 binding.

MATERIALS AND METHODS

Cell Culture and Reagents

The human leukemic T cell line, Jurkat cells (Clone E6.1 from the American Type Culture Collection, Rockville, MD), was cultured in RPMI medium containing 10% FBS and 100 U/ml penicillin and streptomycin. The cells were split every 2 days to maintain log phase cultures. Antiserum to IP3R1 was kindly provided by Dr. Greg Mignery (Loyola University, Chicago, IL). Human recombinant cdc2/CyB and nocodazole were obtained from CalBiochem (La Jolla, CA).

Generation of Phosphospecific Antibodies to IP3R1

Polyclonal antibodies were raised against two phosphopeptides (MLKIGTS*PVKEDKEA and DPQEQVT*PVKYARL) that encode the putative phosphorylation residues at Ser^{421} and Thr^{799}, respectively. The polyclonal antibodies were affinity-purified with two cycles of purification by initially passing through nonphoscphorylated peptides and finally through the respective phosphorylated peptides. Titration and specificity of phosphospecific antibodies were determined by ELISA and immunoblotting.

Western Blotting, Immunoprecipitation, and In Vitro Kinase Reactions

Cells were equalized for number and lysed in ice-cold lysis buffer containing 0.5% Nonidet P-40, 25 mM HEPES, pH 7.4, 150 mM NaCl, 1 mM EDTA, 1 mM sodium orthovanadate, and protease inhibitors. Cell lysates were centrifuged at 13,000*g* in a microcentrifuge, and the supernatants were subjected to immunoblotting and immunoprecipitation [Frangioni and

Neel, 1993; Matter et al., 1993]. The membranes were blocked in TBST (20 mM Tris/HCl, pH 7.4, 0.9% NaCl, and 0.05% Tween 20) containing 5% nonfat dried milk for 1 h followed by incubation with primary antibodies. After extensive washings, the membranes were incubated with secondary antibody conjugated to horseradish peroxidase (goat anti-rabbit IgG; Pharmingen, San Diego, CA) in TBST containing 5% nonfat dried milk. The immunoblots were analyzed using an ECL detection system (Amersham, Piscataway, NJ). For detecting in vivo phosphorylated IP3R1, DT40 cells were cultured in 10% FBS–RPMI medium with and without 1 μM nocodazole. Immunoprecipitations were performed with αIP3R1 antibody as described [Frangioni and Neel, 1993] and the immune complexes were washed three times with ice-cold buffer containing 25 mM HEPES, pH 7.4, 150 mM NaCl, 1 μM sodium orthovanadate, 0.5% Nonidet P-40, and a cocktail of protease inhibitors containing 4-(2 aminoethyl) benzenesulfonyl fluoride, pepstatin A, E64, bestatin, leupeptin, and aprotinin (Sigma Biochemicals, St. Louis, MO). The kinase assays were performed at 30°C for 10 min in a 25-μl volume containing 50 mM Tris, pH 7.4, 10 mM $MgCl_2$, 1 mM DTT, and 10 μCi of [γ-^{32}P] ATP with and without exogenous cdc2/CyB. Phosphoproteins were separated by SDS–PAGE and detected by autoradiography as described [Frangioni and Neel, 1993; Means, 1994].

Generation of Wild-Type and Cdc2 Phosphorylation-Deficient Mutant GST Proteins

The generation of pGEX vectors that encode glutathione S-transferase (GST) fusion proteins and the purification of the expressed proteins have been described previously [Frangioni and Neel, 1993]. The regions corresponding to residues 375–473, 753–886, and 1–900 of mouse IP3R1 were amplified by PCR and cloned into pGEX2T into BamH1 and EcoR1 sites. S421A and T799A mutations were introduced using the Quik Change mutagenesis kit (Strategene, La Jolla, CA) and mutations were confirmed by sequencing. GST fusion constructs containing residues 375–473 (IP3R1/375–473) and 753–886 (IP3R1/775–886) as well as the mutant constructs (S421A and T799A) were expressed in JM101. Fifteen milliliters of cell culture ($A_{600} = 0.5$) was induced with 0.1 mM isopropyl-β-D-thiogalactopyronoside (IPTG) at 37°C for IP3R1/375–473 and at 12°C for IP3R1/775–886 fusion proteins. Fusion proteins were purified on glutathione-agarose beads as per manufacturer's instructions (Amersham Pharmacia Biotech) and washed three times in PBS-1% Triton X-100 to remove nonspecific bound proteins.

[^{3}IP3]IP3-Binding Assay

IP3-binding assay was performed using the IP3R1(1–900) fragment essentially as described [Zhu et al., 1996]. Soluble protein (30 μg) was incubated with 9.6 nM [^{3}IP3] in 100 μl of binding buffer for 10 min at 4°C. The mixture was then added to 4 μl of γ-globulin (50 mg/ml) and 100 μl of solution containing 30% (w/v) polyethylene glycol 6000, 50 mM Tris-HCl (pH 8.0 at 4°C), 1 mM 2-mercaptoethanol, and 1 mM EDTA. After incubation at 4°C for 5 min, the protein–polyethylene glycol complex was collected by centrifugation at 10,000*g* for 5 min at 2°C. The pellets were dissolved in 180 μl of Solvable (DuPont NEN). After neutralization with 18 μl of acetic acid, radioactivity was measured in 5 ml of Atomlight (Dupont NEN) in a liquid scintillation counter. The specific binding was determined by subtracting the nonspecific binding in the presence of 2 μM IP3 from the total binding.

RESULTS

IP3R1 is a Target for Cdc2/CyB In Vitro

To understand the decreased proliferation of IP3R-deficient cells at a molecular level, we analyzed the IP3R1 primary sequence for motifs that could influence cellular proliferation. We specifically looked for a consensus cdk-phosphorylation motif, (S/T)PX(K/R). IP3R1 is a 308 kDa polypeptide that contains two putative cdk-phosphorylation sites, at residues 421 (Ser^{421}) and 799 (Thr^{799}). In IP3R1, Ser^{421} is within the IP3-binding domain and Thr^{799} is proximal to the IP3-binding domain (based on primary structure). Interestingly, the phosphorylation sites in IP3R1 are conserved from *Xenopus* to human (Fig. 1A). To determine if IP3R1 may function as a cdc2/CyB substrate, we performed in vitro kinase reactions on immunoprecipitates of IP3R1 from brain microsomes with human cdc2/CyB complex. The results show that cdc2/CyB phosphorylates IP3R1 in vitro and that this phosphorylation is completely inhibited by the inclusion of roscovitine, a potent cdc2 inhibitor

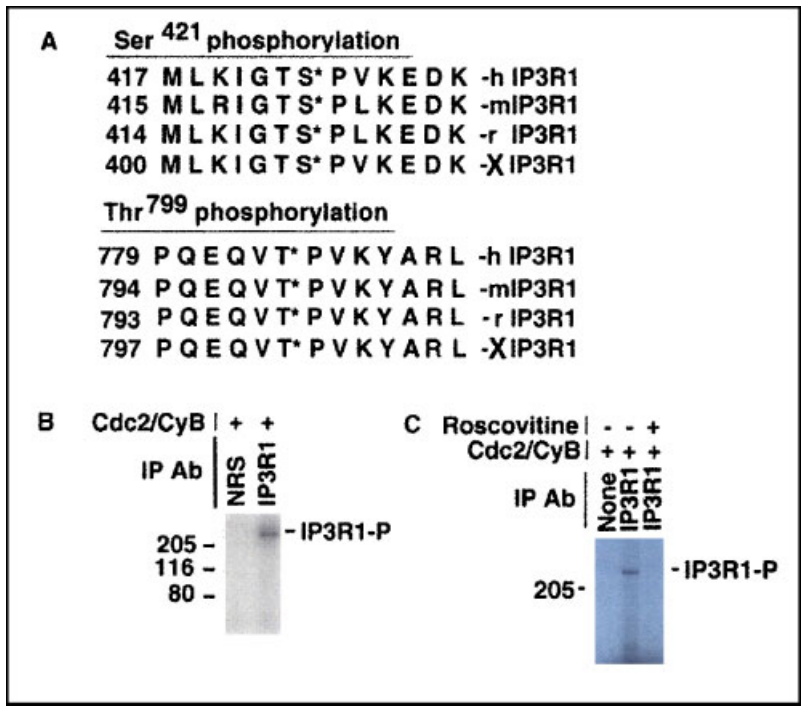

Fig. 1. Cdks phosphorylate IP3R1 in vitro. **A**: Sequence alignment of cdk phosphorylation motifs in IP3Rs and species. The letters denote: m (mouse), r (rat), h (human), and x (*Xenopus*). GenBank accession numbers are: mIP3R1 (X15373); rIP3R1 (J005510); hIP3R1 (L38019); and xIP3R1 (D14400). **B**: In vitro kinase reactions with ^{32}P γ[ATP] were performed in the presence of cdc2/CyB kinase with αIP3R1 immunoprecipitates from brain microsomes made using either normal rabbit serum (NRS) or αIP3R1 antibody. **C**: The cdk inhibitor roscovitine (100 nM) inhibited phosphorylation of IP3R1. The negative control was immunoprecipitation without αIP3R1 antibody. [Color figure can be viewed in the online issue which is available at www.interscience.wiley.com.]

(Fig. 1C). We did not detect any false-positive phosphorylation signals in our kinase assays, either from beads or from immunoprecipitates made with normal rabbit serum (NRS) (Figs. 1B,C).

Identification of Cdc2 Phosphorylation Sites

To determine the site(s) of IP3R1 phosphorylation by cdc2/CyB and their specificity, we generated GST fusion proteins containing the putative cdk phosphorylation sites [site 1 (IP3R1/375–473) and 2 (IP3R1/753–886), and their mutants ($421^{S\rightarrow A}$ and $799^{T\rightarrow A}$)] (Fig. 2A). Both wild-type IP3R1/375–473 (Fig. 2B) and IP3R1/753–886 (Fig. 2C) and their mutants expressed equally well. Purified GST was included as a negative control. In vitro kinase reactions were performed with 200 ng of GST fusion proteins, gamma ATP, and 2 U of cdc2/CyB. The phosphorylated proteins were size-fractionated on 12% SDS–PAGE gels and detected by autoradiography. Both GST fragments containing Ser^{421} and Thr^{799} were phosphorylated by cdc2/CyB (Fig. 2D). These results suggest that cdc2/CyB phosphorylates both residues (S^{421} and T^{799}) in vitro and that the mutation of these residues to alanine abrogates phosphorylation (Fig. 2D, lanes 2 and 4).

Generation of Phosphospecific IP3R1 Antibodies

To study cdk-mediated phosphorylation of IP3Rs, we generated two phosphospecific polyclonal antibodies that recognize the phosphorylated forms of Ser^{421} and Thr^{799} in IP3R1. The affinity-purified antibodies were tested by dot-blot analysis using nonphosphorylated and phosphorylated peptides. The results show that both antibodies react only with their respective antigenic phosphopeptides (Fig. 3A,B). There was no reactivity with nonphosphorylated peptides. To further characterize these antibodies, we generated GST fusion proteins that contained one of the Ser^{421} and Thr^{799} phosphorylation sites for cdks and mutant GST fusion proteins in which the Ser^{421} and Thr^{799} were replaced with alanine. These fusion proteins were immunoblotted with αSer^{421} (Fig. 3C) and

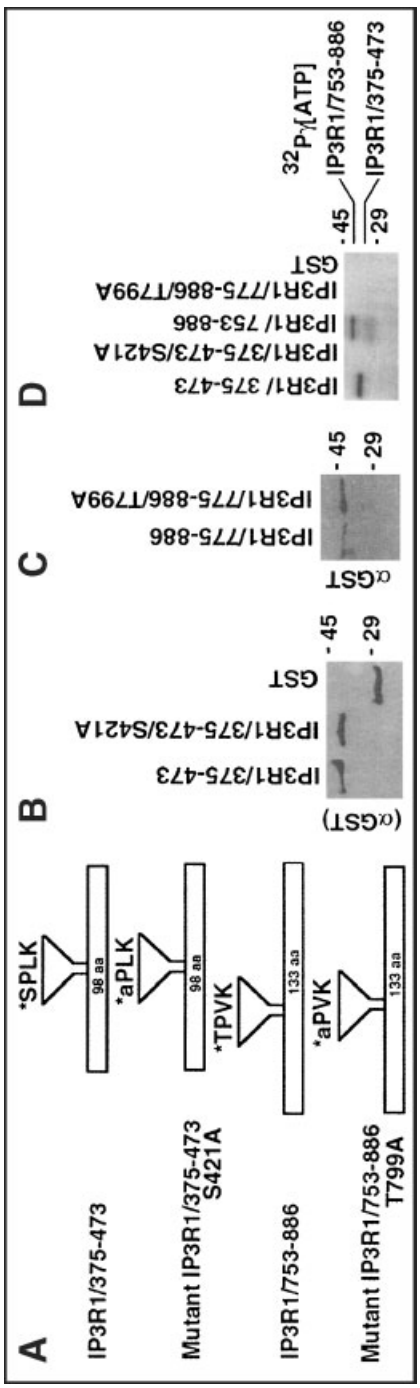

Fig. 2. Cdc2/CyB phosphorylates IP3R1 at Ser^{421} and Thr^{799} in vitro. WT and mutant (S421A and T799A) GST-IP3R1 fragments were expressed. **A**: Schematic representation of WT and mutant constructs used for the experiments. **B**: Immunoblot showing the input GST-fusion proteins for IP3R1/375–473 and the mutant IP3R1/375–473/S421A. **C**: Immunoblot showing the input GST-fusion proteins for IP3R1/753–886, and the mutant IP3R1/753–886/T799A. D: In vitro kinase reactions performed with [γ^{32}P] ATP and cdc2/CyB.

αThr^{799} (Fig. 3D) antibodies. Again, αSer^{421} strongly reacted only with the wild-type IP3R1/375–473 protein phosphorylated by cdc2/CyB, and αThr^{799} recognized only the wild-type fusion protein IP3R1/753–886 phosphorylated by cdc2/CyB. S421A and T799A mutations completely abolished reactivity with the respective antibodies. Importantly, these antibodies recognized only their respective phosphorylated proteins. These results clearly indicate that the antibodies are specific and recognize the phosphorylated Ser^{421} and Thr^{799} residues in IP3R1.

To determine whether these phosphospecific antibodies react with the native IP3Rs, we immunoprecipitated IP3R1 from Jurkat cells, performed kinase reactions in the absence and presence of cdc2/CyB, and immunoblotted with αSer^{421} (Fig. 3E), αThr^{799} (Fig. 3F), and αIP3R1 antibodies (Fig. 3G). Interestingly, both αSer^{421} and αThr^{799} recognized phosphorylated native IP3R1. A very faint band seen with the IP3R1 immunoprecipitate suggests a basal level of endogenous phosphorylation. Exogenous addition of cdc2/CyB increased the steady-state phosphorylation of IP3R1 with increased antibody reactivity/signals (Fig. 3E,F) without any change in IP3R1 levels (Fig. 3G).

IP3R1 Phosphorylation Occurs at Ser^{421} and Thr^{799} In Vivo

To determine whether IP3R1 phosphorylation occurs in vivo, we serum-starved DT40 B cells for 24 h, then cultured them with and without 1 μM nocodazole in 10% FBS–RPMI medium for 16 h, lysed the cells, and immunoblotted cell lysates with αSer^{421} (Fig. 4A), αThr^{799} (Fig. 4B), or αIP3R1 (Fig. 4C) antibodies. Nocodazole treatment, which arrests cells at G2/M phase with increased cdc2 activity, greatly enhanced IP3R1 phosphorylation at both Ser^{421} and Thr^{799} residues without affecting total IP3R1 protein expression as compared to control cells (Fig. 4C). These data suggest that cdc2-mediated IP3R1 phosphorylation occurs in vivo.

Cdc2/CyB Phosphorylation Increases IP3R1's Affinity for IP3

Previous study has shown that IP3 binding to purified IP3R1 from cerebellum is stoichiometric, and that affinity residues 1–604 of mIP3R1 for IP3 were comparable to that of the native cerebellar IP3R (83 nM) [Yoshikawa et al., 1999]. Since the IP3-binding specificity

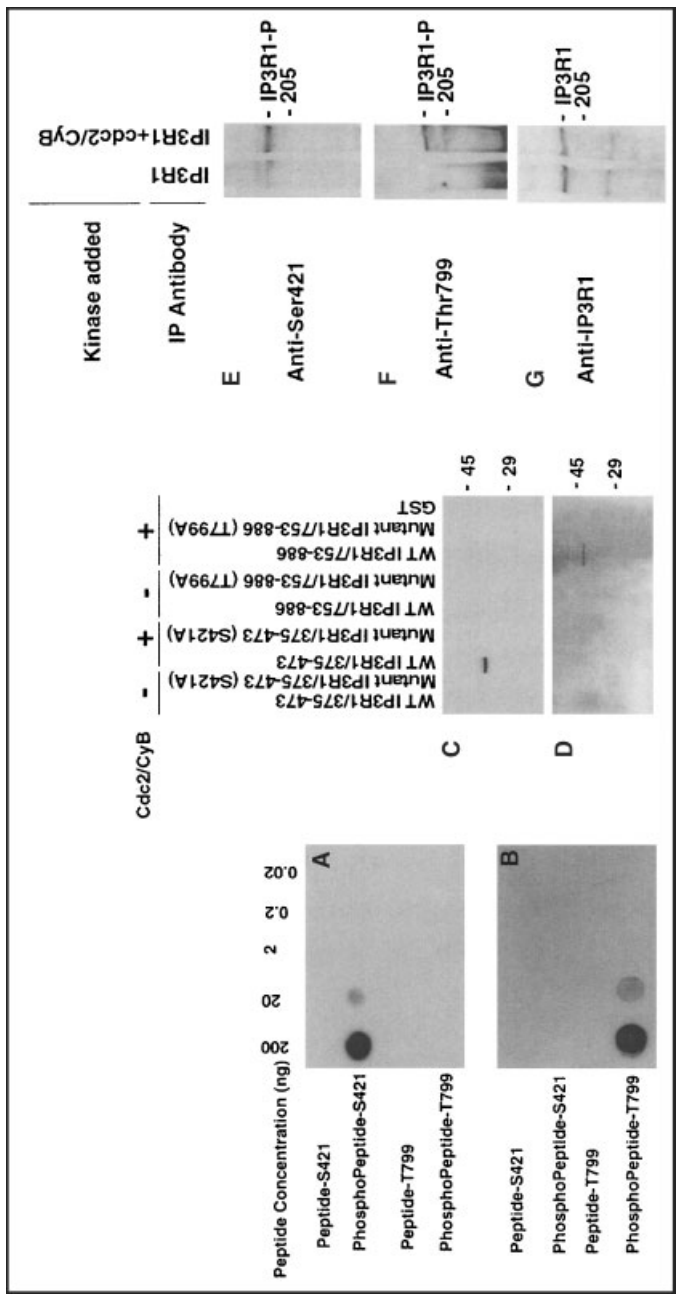

Fig. 3. αS421 and αT799 specifically recognize phosphorylated IP3R1 peptides and native protein. Nonphosphorylated and phosphorylated IP3R1 peptides were blotted at the indicated concentration and immunoblotted with 1:1,000 dilution of αSer^{421} (**A**) and αThr^{799} antibody (**B**). The in vitro kinase reactions were performed with 200 ng of fusion proteins with 2 U of cdc2/CyB complex with and without cdc2/CyB in kinase buffer at 30°C for 10 min. The reaction was terminated by the addition of 3× sample buffer and heating to 95°C for 5 min. The proteins were transferred to nitrocellulose membrane and probed with 1:1,000 dilution of αSer^{421} antibody (**C**) and developed with ECL reagents. The blot was then stripped and probed with 1:1,000 dilution of αThr^{799} antibody (**D**). In vitro kinase reactions were performed from IP3R1 immunoprecipitates with and without cdc2/CyB and immunoblotted with αSer^{421} antibody (**E**), αThr^{799} antibody (**F**), and αIP3R1 (**G**).

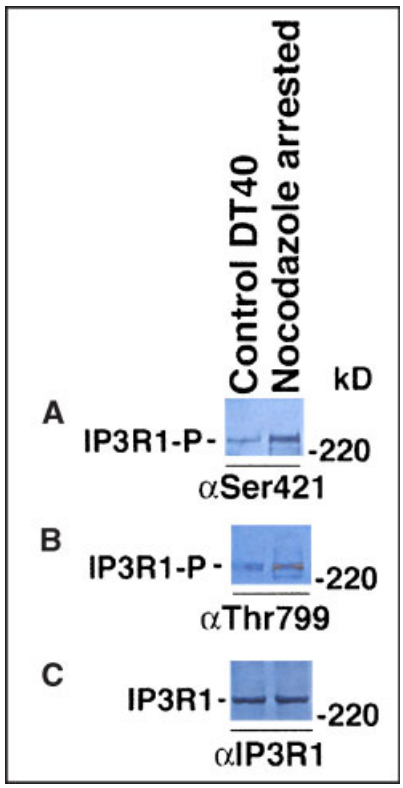

Fig. 4. IP3R1 phosphorylation at Ser421 and Thr799 occurs in vivo. DT40 cells were serum-starved for 24 h and cultured with and without 1 αM nocodazole (control) for 16 h in RPMI-1640 medium containing 10% FBS. After 16 h in culture, cells were washed two times with PBS, and cell lysates were resolved on 6% SDS–PAGE gels and immunoblotted with αSer^{421} (**A**), αThr^{799} (**B**), or αIP3R1 (**C**). [Color figure can be viewed in the online issue which is available at www.interscience.wiley.com.]

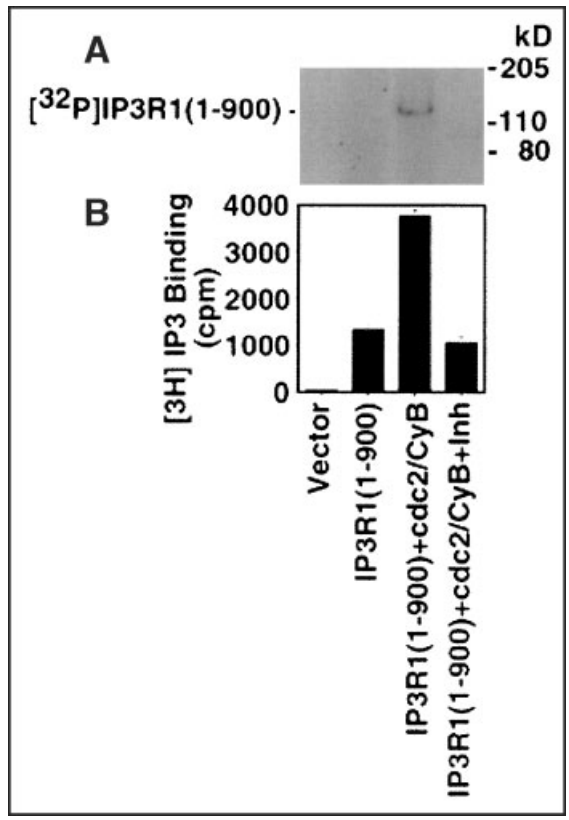

Fig. 5. Cdc2/CyB phosphorylation of IP3R1 increases IP3 binding. **A**: The soluble proteins (30 μg) from *E. coli* expressing IP3R1 (1–900) were either left alone or phosphorylated with cdc2/CyB complex. Controls included *E. coli* proteins from vector alone and the inclusion of roscovitine, a cdc2 inhibitor. The inclusion of roscovitine completely blocked the phosphorylation by cdc2/CyB complex. **B**: [^{3}H]IP3 binding to soluble proteins (30 μg) from *E. coli* transformed with pGEX-IP3R1 (1–900), phosphorylated with cdc2/CyB, and phosphorylated with cdc2 in the presence of roscovitine (cdc2 inhibitor) and pGEX-2T (vector). Nonspecific binding was measured in the presence of 2 μM IP3. Values are the means ± SD of three separate experiments. Identical parallel phosphorylation reactions were set up for detecting phosphorylation (A) and IP3 binding (B).

of the N-terminal IP3R1 fragment expressed in *E. coli* was very similar to that of the native IP3R from mouse cerebellum, we employed this approach to investigate the effect of cdc2 phosphorylation on IP3 binding. We generated a GST fragment containing the first 900 N-terminal amino acids of mIP3R1 [IP3R1(1–900)], which encode the IP3 binding pocket and the two cdc2/CyB phosphorylation sites. To directly determine whether cdc2/CyB phosphorylation modulates IP3 binding, we performed an in vitro kinase reaction with purified cdc2/CyB (Fig. 4A) followed by IP3 binding as described [Yoshikawa et al., 1996].

Phosphorylation of this ~126-kDa protein was observed only with the addition of cdc2/CyB and was completely inhibited by roscovitine, a cdc2 inhibitor (Fig. 5A). The phosphorylated protein fraction had approximately threefold higher IP3 binding as compared with nonphosphorylated and cdc2-inhibitor treated fractions. The vector-alone fraction showed very little IP3 binding (Fig. 5B). These experiments indicate that cdc2 phosphorylation increases IP3 binding to IP3R1.

DISCUSSION

In this study, we have demonstrated for the first time that IP3R1 is a target for cdc2/CyB complex in vitro and in vivo and that cdc2 phosphorylation of IP3R1 leads to increased IP3 binding in vitro.

Initial in vitro phosphorylation studies were carried out using dog cerebellum because: (1) IP3R1 is expressed at a high level (>99%) with

very little IP3R2 and IP3R3; and (2) the near-exclusive expression of IP3R1 in cerebellum permits easy detection of IP3R1 phosphorylation unambiguously, as the presence of other isoforms would confound the phosphorylation analysis due to heterotetramer formation [Joseph et al., 1995; Nucifora et al., 1996]. While these results clearly demonstrate IP3R1 phosphorylation by cdc2/CyB in vitro, the blots from DT40 cells arrested with nocodazole demonstrate that IP3R1 phosphorylation occurs in vivo (Fig. 4A,B). Nocodazole is commonly used in cell cycle research to arrest cells at mitosis and to study mitotic events [Pathan et al., 1996; Lampe et al., 1998; Pepst et al., 1998]. Although untested, based on our results it is conceivable that cdk2 also could phosphorylate IP3R1, because both cdk1 and 2 demonstrate broad specificity over substrates containing Ser/Pro or Thr/Pro sequence motifs [Holmes and Solomon, 1996] and are blocked by roscovitine.

Analysis of the sequences of other IP3Rs suggests that the Thr^{799} phosphorylation site is conserved in IP3R3, while neither the Ser^{421} or Thr^{799} phosphorylation site is conserved in IP3R2. In IP3R1, both cdk-phosphorylation motifs are remarkably conserved from *Xenopus* to human. The fact that the clustered cdk-phosphorylation sites are conserved in other species is consistent with cdk-mediated IP3R phosphorylation potentially being a conserved mechanism for modulating intracellular Ca^{2+} release during cell cycle progression.

A complete analysis of the physiological roles of cdc2-mediated IP3R1 phosphorylation has been hampered by the lack of reagents that specifically recognize the phosphorylated state of the protein. We have generated phosphospecific antibodies that recognize phosphorylated Ser^{421} and Thr^{799} in IP3R1, and demonstrated that the protein's antigenicity is abolished by mutations of Ser and Thr residues to alanine in IP3R1. The generation of these antibodies facilitated our detection of native IP3R1 protein phosphorylated by the cdc2/CyB complex in vitro (Fig. 3E,F) and in vivo. These results suggest that the antibodies recognize site-specific phosphorylation of IP3R1, and can thus be used to further investigate the implications of this phenomenon.

IP3 mediates Ca^{2+} release from ER after binding to its receptor, IP3Rs. In IP3Rs, the IP3-binding core consists of amino acid regions 1–225 and 226–604 in the N-terminal portion of the IP3R channel [Yoshikawa et al., 1996]. Previous studies have shown that the N-terminal (amino acids 1–225) portion of the molecule acts as a suppressor of IP3 binding. The binding of at least two IP3 molecules to a single tetrameric IP3R channel is required for channel opening, and IP3 binding elicits a large conformational change in the N-terminal portion of IP3R1 [Kaftan et al., 1997; Moraru et al., 1999]. Our results show a threefold increase in IP3 binding to IP3R1(1–900) upon phosphorylation by cdc2 (Fig. 4). This increase may be due to conformational changes in IP3R1 upon phosphorylation, or because phosphorylated IP3R1 may no longer be susceptible to suppression elicited by the N-terminal 225 amino acids. Additional experiments are required to test this possibility.

Independent studies have shown that: (1) cytosolic Ca^{2+} is increased during the G2/M phase or just after exit from mitosis; and (2) cdc2/CyB activity controls the generation of sperm-triggered Ca^{2+} oscillations in oocytes during the cell cycle [Deng and Shen, 2000; Levasseur and McDougal, 2000; Tokmakov et al., 2001]. Although these studies support the fact that Ca^{2+} oscillations occur during cell cycle transitions, the importance of IP3-gated Ca^{2+} release for cellular proliferation remains controversial. For instance, while IP3R1-deficient Jurkat cells display reduced cell growth [Jayaraman and Marks, 1997], T cells from an IP3R1-deficient mouse proliferate equally well as compared to T cells from the wild-type [Hirota et al., 1998]. The discrepancy between the results reported for primary T cells from IP3R1-deficient mice and for transformed Jurkat T cell lines might be due to the presence of other IP3R subtypes, which could compensate for the loss of IP3R1 function. IP3R1-deficient Jurkat lymphocytes express other IP3R subtypes at greatly reduced levels [Jayaraman and Marks, 1997; Lee et al., 2003], while IP3R1-deficient mouse T cells express other IP3R subtypes to the same levels as in the wild-type. In addition, it is conceivable that primary and transformed cells differ in their cellular effector responses upon activation. Upon activation by TCR and mitogens, primary T cells proliferate while Jurkat cells undergo apoptosis. Thus, it is likely that additional Ca^{2+}-dependent and Ca^{2+}-independent events might be activated differently in primary and transformed cells. Alternatively, other Ca^{2+}-channels and/or

pumps that maintain Ca^{2+} homeostasis could compensate for the lack of IP3R1 in IP3R1-deficient T cells to sustain cellular proliferation.

Cdc2 is inactivated in normally progressing cells to allow mitosis to proceed via CyB degradation [Means, 1994; Morgan, 1997]. However, cdc2 activation is also linked to some forms of cellular apoptosis in several pathological conditions. For instance, premature or inappropriate activation of cdc2 and increased IP3-gated intracellular Ca^{2+} release have been causally linked to pathogenesis of HIV and Alzheimer's disease [Vincent et al., 1997; Haughey et al., 1999; Castedo et al., 2002]. Cdc2 activity is also increased in peripheral blood mononuclear cells (PBMCs) from HIV-1 infected patients, a phenomenon that has been attributed to T cell activation, and therapeutic inhibition of HIV-1 replication decreases the expression of CyB in PBMCs [Fotedar et al., 1995; Piedimonte et al., 1999; Cannavo et al., 2001]. Inhibition of cdc2 activity by roscovitine or olomoucine also prevented syncytial cell death induced by HIV infection [Castedo et al., 2002]. If IP3R phosphorylation and modulation occurs in vivo in a manner similar to our in vitro results, it is possible that this regulation could play a substantial role in many pathophysiological conditions. Following this hypothesis, it is further tempting to speculate that the kinetics, magnitude, and spatial and temporal localization of phosphorylated IP3Rs could lead to different cellular functions [Dolmetsch et al., 1997]. In this regard, the availability of the present phosphospecific antibodies may help in investigating the IP3R1-mediated Ca^{2+}-signaling events during cell cycle progression.

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