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Evidence for Phosphatase Activity of p27SJ and its Impact on the Cell Cycle

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ABSTRACT

p27SJ, a novel protein isolated from St John's wort (*Hypericum perforatum*), belongs to an emerging family of DING proteins that are related to a prokaryotic phosphate-binding protein superfamily. Here we demonstrate that p27SJ exhibits phosphatase activity and that its expression in cells decreases the level of phosphorylated Erk1/2, a key protein of several signaling pathways. Treatment of p27SJ-expressing cells with phosphatase inhibitors including okadaic acid, maintained Erk1/2 in its phosphorylated form, suggesting that dephosphorylation of Erk1/2 is mediated by p27SJ. Further, expression of p27SJ affects Erk1/2 downstream regulatory targets such as STAT3 and CREB. Moreover, the level of expression of cyclin A that associates with active ERK1/2 and is regulated by CREB, was modestly reduced in p27SJ-expressing cells. Accordingly, results from in vitro kinase assays revealed a noticeable decrease in the activity of cyclin A in cells expressing p27SJ. Cell cycle analysis demonstrated dysregulation at S and G2/M phases in cells expressing p27SJ, supporting the notion that a decline in cyclin A activity by p27SJ has a biological impact on cell growth. These observations provide evidence that p27SJ alters the state of Erk1/2 phosphorylation, and impacts several biological events associated with cell growth and function. J. Cell. Biochem. 107: 400–407, 2009.

KEY WORDS: DING FAMILY; PHOSPHATASE ACTIVITY; p27SJ

In recent years, *Hypericum perforatum*, also known as St John's Wort, has received special attention due to its pharmacological properties [Wagner and Bladt, 1994; Diwu, 1995; Roth et al., 2004]. Extracts from this plant contain active secondary metabolites including hypericin, a photosensitive red colored naphthodianthron which is a bioactive compound that can act as a kinase inhibitor [Agostinis et al., 2002; Ursaciuc et al., 1997]. Furthermore, *Hypericum perforatum* extracts contain other flavonoids such as rutin, with a free radical scavenging activity and a potential antioxidant activity [Saija et al., 1995].

Recently, we have purified a novel 38 kDa protein (p38SJ) and cloned a DNA fragment expressing a large segment of the protein, p27SJ, from an in vitro cultivated callus culture of *Hypericum perforatum* [Darbinian-Sarkissian et al., 2006]. Sequence analysis of the DNA expressing p27SJ showed that this protein belongs to the DING family of proteins characterized by the N-terminal amino acid sequences DINGGG [Diemer et al., 2008]. While the presence of the DING family in plant species has previously been reported, little is known about the biochemical properties of these proteins [Perera et al., 2008]. DING proteins have been identified by affinity chromatography through binding to ligands such as the plant metabolites, oxalate, genistein, and cotinine [Bush et al., 1998; Mehta et al., 2001; Weebadda et al., 2001; Berna et al., 2002, 2008; Belenky et al., 2003; Kumar et al., 2004; Renault et al., 2006; Du et al., 2007]. In humans, the DING family has been associated with various diseases such as rheumatoid arthritis, cancer, infections, and atherosclerosis [Hain et al., 1996; Mehta et al., 2001; Weebadda et al., 2001; Belenky et al., 2003; Kumar et al., 2004; Renault et al., 2006]. A peptide containing DING was first identified in synovial fluid that was part of a larger protein of p205 synovial T-cell stimulating protein [Hain et al., 1996; Blass et al., 1999]. Subsequent studies led to the identification of another member of the human DING family with growth-promoting effects in normal and tumor cells [Adams et al., 2002; Belenky et al., 2003; Morales et al., 2001].

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In addition to human tissue, DING proteins have been isolated from various fungi, animal and plant tissues, and exhibit close homology with Pseudomonas proteins [for review see Riah et al., 2000; Berna et al., 2002, 2008; Lewis and Crowther, 2005; Scott and Wu, 2005; Ahn et al., 2007; Chen et al., 2007; Moniot et al., 2007; Pantazaki et al., 2007]. It has also been shown that in rat neurons, a 38 kDa DING protein can bind to cotinine, and mediate the activities of nicotine, where cotinine is the major metabolic oxidation product [Riah et al., 2000].

Earlier studies demonstrated that p27SJ derived from callus cultures of *Hypericum perforatum*, exhibits the capacity to interact with several important regulatory proteins and modulate expression of viral and cellular genes including the HIV-1 LTR and MCP-1 [Darbinian-Sarkissian et al., 2006; Mukerjee et al., 2008]. Here we demonstrate that p27SJ has phosphatase activity and its expression in human cells impacts on the state of Erk1/2 phosphorylation and several other important cellular regulatory proteins.

MATERIALS AND METHODS

PLASMIDS

GST-p27SJ recombinant plasmid and the GST-p27SJ deletion mutants (GST-p20, GST-p15, GST-p10, and GST-p5) were described previously [Darbinian-Sarkissian et al., 2006]. The p27SJ deletion mutant, GST-p7c, was created by PCR amplification of a 169 base pair DNA fragment containing C-terminal region of p27SJ encompassing amino acids 200-263, cloned into *Eco*RI-*Xho*I-digested pGEX-4T1. The nucleotides comprising all the plasmids were verified by DNA sequencing using an ABI automatic sequencer. Oligonucleotides were obtained from Oligos Etc, Inc. (Wilsonville OR).

ANTIBODIES

Antibody specific for p27SJ (anti-p27SJ rabbit polyclonal antibody) was generated by Lampire Biological Laboratories, Inc. (Pipersville, PA). Anti-α-tubulin clone B512 was obtained from Sigma–Aldrich Co (St. Louis, MO). Anti-myc antibody was purchased from Invitrogen (Carlsbad, CA). Anti-phospho-p44/42 mitogen-activated protein kinase (MAPK/Erk1/2), anti-p44/42 MAPK, rabbit polyclonal, and anti-GRB2 rabbit polyclonal antibodies were purchased from Cell Signaling (Danvers, MA). Anti-cyclin A and anti-CREB antibodies were from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA).

CELL CULTURE

U-87MG is a human glioblastoma cell line that was obtained from the American Type Culture Collection (ATCC, Manassas, VA). Cells were maintained in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% fetal bovine serum (Life Technologies, Inc.) and antibiotics (100 U/ml penicillin and 10 μg/ml streptomycin) at 37°C in a humidified atmosphere containing 7% CO_2. Creation of a p27SJ inducible stable cell line was based on the pTet-On Gene Expression System (BD Biosciences Clontech, Palo Alto, CA), and has been described previously [Darbinian-Sarkissian et al., 2006]. Treatment of cells with doxycycline was done for 48 h and with okadaic acid (OA) at 20 nM for 12 h as described [Rami et al., 2003].

PREPARATION OF PROTEIN EXTRACTS AND IMMUNOBLOT ANALYSIS

For preparation of whole-cell extracts, cells were washed with cold phosphate-buffered saline (PBS) and solubilized in lysis buffer containing 50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 0.1% Nonidet P-40 and 1% protease inhibitor (PI) cocktail (Sigma). Cell debris was removed by centrifugation for 5 min at 4°C. Fifty micrograms of proteins in Laemmli sample buffer were heated at 95°C for 10 min and separated by 10% SDS–PAGE. For Western blot analysis, after gel electrophoresis, proteins were transferred to supported nitrocellulose membranes, and after incubation with specific antibodies, the proteins were visualized with the enhanced chemiluminescence detection system, ECL+ according to manufacturer's instructions (GE Healthcare, Piscataway NJ), and exposed to X-ray film.

PURIFICATION OF RECOMBINANT PROTEINS

GST-p27SJ fusion protein and GST-p27SJ deletion mutants were produced and purified as described previously [Darbinian-Sarkissian et al., 2006]. Bacteria harboring the plasmids pGST, pGST-p27SJ, pGST-p20, pGST-p25, pGST-p10, pGST-p5, or pGST-p7 were grown overnight at 37°C in LB supplemented with 100 mg/L ampicillin. The following morning, cells were diluted 1:10 with fresh medium, were grown to an optical density at 600 nm of 0.6, and were induced for 90 min at 37°C with 0.35 mM isopropyl-β-D-thiogalactopyranoside (IPTG). Cells were pelleted at 4°C, resuspended in NETN buffer with PI and sonicated on ice. The bacterial lysate was centrifuged at 4°C to remove insoluble material. Glutathione-Sepharose beads (Amersham Pharmacia) were added to the lysate and binding of GST fusion proteins was allowed to occur at 4°C for 3 h. Beads were pelleted and washed three times with 100 volumes of NETN buffer, and the integrity and purity of the GST fusion proteins were analyzed by SDS–PAGE followed by Coomassie blue staining.

In Vitro *p*NPP Phosphatase Assay. Phospatase assays were performed using the EnzoLyte *p*NPP protein phosphatase assay kit (colorimetric) according to manufacture's recommendation (AnaSpec Corporate, San Jose, CA). *p*NPP is a colorimetric substrate for measuring the activity of tyrosine and serine/threonine protein phosphatases, and ATP-ases. Upon dephosphorylation by phosphatases, *p*NPP turns yellow and can be detected by its absorbance at 405 nm. The activities of recombinant p27SJ (20 nM) and its deletion mutants was tested in *p*NPP phosphatase assay upon incubation with the substrate at 37°C during 1 h as described by the manufacturer. Results are presented as histograms showing absorbance at 405 nm (phosphatase activity).

In Vitro cyclin A Kinase Assay. The kinase assay was performed using extract from U-87MG cells containing the inducible p27SJ gene. Total protein extracts from cells grown for 48 h in media containing doxycycline were incubated with anti-cyclin A antibody. The immunoprecipitated cyclin A complexes were washed three times with kinase buffer (25 mM Tris–HCl (pH 7.4), 10 mM $MgCl_2$, 2 mM dithiothreitol) and used to assay Histone-1 (H1) phosphorylation by incubating with 5 μCi [γ-^{32}P]ATP (PerkinElmer, Shelton, CT) in 50 μl of kinase buffer at 30°C for 45 min. The phosphorylated

H1 was analyzed by 10% SDS–PAGE, and cyclin A activation was assayed by autoradiography.

Cell Cycle Analysis. Cells containing inducible p27SJ were maintained in serum-free medium for 48 h, and then changed to medium containing 10% FBS and 2 mg/ml doxycycline. At different time points ranging from 16 to 32 h, cells were fixed in 88% ethanol at −20°C, pelleted and stained with propidium iodide (PI)-RNase A solution for 30 min at 37°C. FACS analysis to determine cell cycle distribution was performed with a FACSORT Flow cytometer (Becton Dickson) using Cell Fit Software, versus 2.01.2 (Becton Dickson). FACS analysis data were derived from counting at least 20,000 events in each sample.

Statistical analysis was performed on data imported from Microsoft Excel software.

p27SJ modelization. The sequence of p27SJ was obtained from the sequence database Uniprot (Q5G1J7). Since no p27SJ experimental structure is available in the Protein Data Bank, we performed 3D modeling. p27 is 263 amino acids in length with a molecular weight of 26,225 Da. Template protein was searched using blastp [Altschul et al., 1997] against the protein data bank and two sequences with high identity with p27SJ were identified. The identified seqeunces of PfluDING and HPBP share 87.9% and 70.8% sequence identity with p27SJ over 263 amino acids, respectively. The sequence of PfluDING was chosen as a template. The sequence alignment of p27SJ and PfluDING was made using align [Lassmann and Sonnhammer, 2005] and default parameters. No gaps were present in the alignment. The model of p27SJ was calculated using the program MODELLER 8.2 [Fiser et al., 2000] with the model-default options and using the X-ray structure of PfluDING as a template (2q9t). The resulting model of p27SJ is 260 residues in length and includes all residues of the protein, except the first two and the last one. The model validation was performed using PROCHECK [Collaborative Computational Project Number 4, 1994]. The ramachandran plot shows a good geometry with 96.7% of residues in most favored regions and 3.3% in additionally allowed regions of the plot. Structural representations of p27SJ were performed using PyMol [DeLano, 2002].

RESULTS AND DISCUSSION

To evaluate the phosphatase activity of p27SJ, bacterially produced GST-fusion p27SJ (GST-p27SJ) and GST proteins were tested using the EnzoLyte pNpp protein phosphatase assay [Coyne et al., 2007]. As shown in Figure 1A, incubation of GST-p27SJ, but not GST, with pNpp substrate resulted in the dephosphorylation of pNpp (yellow color), which was detected by its absorbance at 405 nm. As expected, purified alkaline phosphatase, which served as a positive control, exhibited very high phosphatase activity. Results from the time course studies revealed that the phosphatase activity of p27SJ peaked at 30–60 min incubation with a gradual decline thereafter (Fig. 1B). The linear structure of p27SJ exhibits several interesting features including DINGGG domains at the N-terminus between residues 1 and 8, followed by two clusters of tyrosine-based sorting signals that are responsible for the interaction with the μ subunit of the AP-1 adaptor protein at positions 29–32 (YIGV) and 46–49 (YTKF) [Boll et al., 2002]. At position 54–61, there exists a cluster of eight amino acids (TNKNVHWA) that is a conserved PP1C binding motif specific for protein phosphatase type 1 complex. Also, there are three sites for phosphorylation by PKC at the central region and C-terminus of the protein. Computer-assisted evaluation of the secondary structure of the protein revealed a distinct cleft for binding of the phosphate at four distinct areas including residues 9, 10, 11, 34, 64; and 143, 148, 149 (shown in Fig. 1C). To assess the importance of these phosphate binding domains in the phosphatase activity of p27SJ, a series of deletion mutant proteins encompassing the various regions of p27SJ was generated in bacteria using the GST fusion system. Results from the phosphatase assay revealed that GST-p15, which contains amino acids 1–150 and spans the phosphate binding cleft possesses significant phosphatase activity that is equal to approximately 75% of the full-length protein (Fig. 1D). Removal of the regions containing residues 143 and 147–149 (GST-p10) decreased the phosphatase activity of the protein, pointing to the importance of these phosphate binding sites in the phosphatase activity of p27SJ. Extended deletions of the p27SJ gene that removes the site at residue 64, further reduced the phosphatase activity of the truncated p27SJ protein. The smallest peptide that contains the 50 amino acid residue of the protein (GST-p5) and includes the phosphate binding residues 9–11 and 34 still showed noticeable phosphatase activity (approximately 35% of the full-length protein). Unlike GST-p5, a truncated peptide (GST-p7c) that contains 63 amino acids of the C-terminus of p27SJ with no phosphate binding site, exhibited no phosphatase activity. Interestingly, our results show that inclusion of 50 amino acids from the C-terminus of the protein from residues 150 to 200, which has no phosphate binding site, improves phosphatase activity of GST-p15 (Fig. 1D, compare GST-p15 to GST-p20).

The linear structure of p27SJ revealed a significant identity (>85%) with the bacterial DING protein, PfluD, and the human phosphate-binding protein, HPBP [Ahn et al., 2007]. The predicted three dimensional structure of PfluDING showed a unique feature where two adjacent globular domains constitute a deep cleft in the phosphate binding site [Morales et al., 2001; Ahn et al., 2007]. By using an X-ray structure of PfluDING as a model, we obtained a three dimensional model for p27SJ (Fig. 2). Although p27SJ (27 kDa) is shorter than other DING proteins (e.g., HPBP and PfluDING, 38 kDa), the predicted p27SJ structure shows that the overall structure is still globular, folded and also contains two domains linked by a hinge, the phosphate binding cavity being a central cleft between the two domains. All residues previously described as involved in phosphate binding in PfluDING are conserved in p27SJ. The model shows that all residues involved in phosphate binding are conserved and are superposed with those of DING proteins. This clearly demonstrates that p27SJ is a phosphate binding protein like PfluDING. The importance of this configuration in phosphatase activity of p27SJ remains to be determined. Further, it is noteworthy to mention that the observed differences in the activity of full-length p27SJ and GST-p15, both of which contain the phosphate binding cleft, suggest that the C-terminal portion of the protein may help with protein stability that is important for phosphatase activity of the protein by its N-terminus.

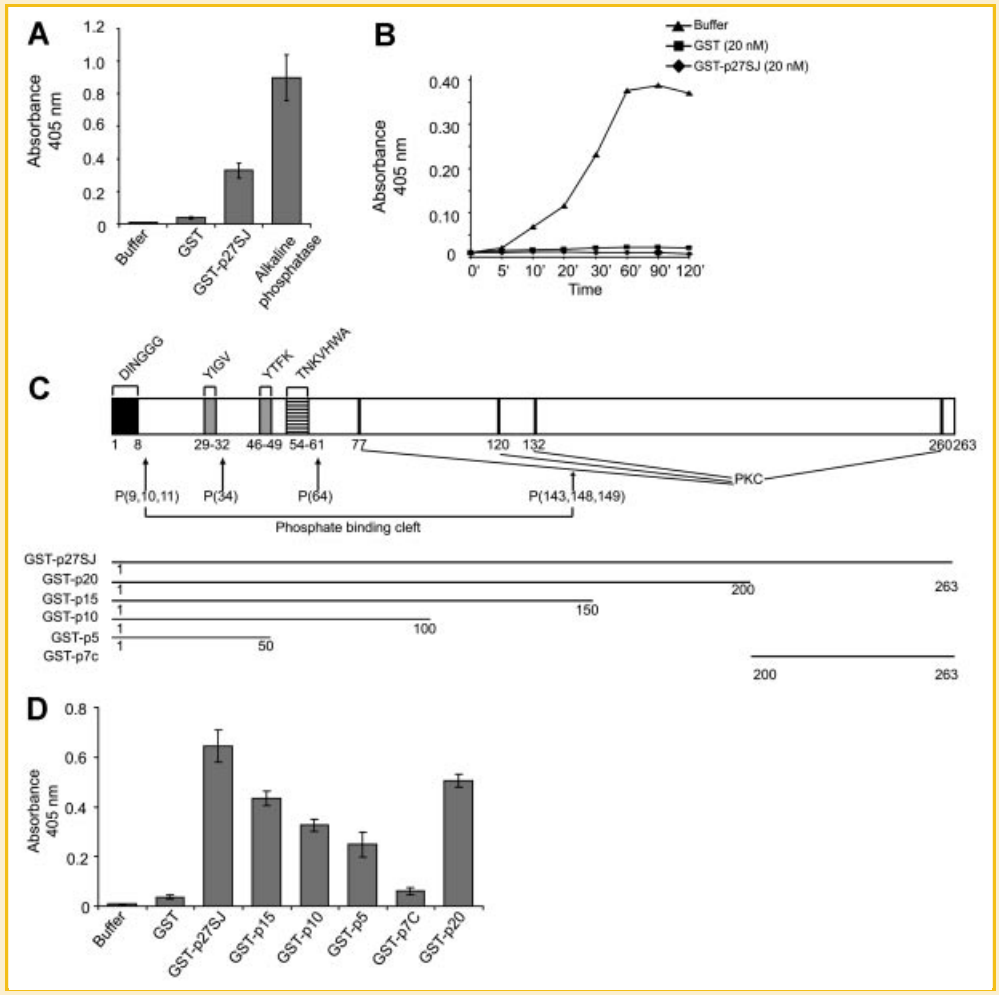

Fig. 1. p27SJ exhibits phosphatase activity. A: In vitro assay to demonstrate phosphatase activity of bacterially produced p27SJ (GST-p27SJ), GST, and control samples including buffer and alkaline phosphatase. Error bars represent the standard deviation from three independent readings. B: Time course experiments demonstrating phosphatase activity of GST and GST-p27SJ at the indicated times. C: Schematic presentation of p27SJ proteins highlighting the various regions of the protein including phosphate binding sites as depicted by P(9,10,11), P(34), P(64), and P(143,148,149), which form a phosphate binding cleft, and the various mutants that were used to demonstrate the region of the protein with phosphatase activity. D: Phosphatase activity of full-length and various mutants of p27SJ assayed as described in Materials and Methods Section.

To investigate the effect of p27SJ on the overall phosphatase activity of eukaryotic cells, we utilized protein extracts from the human astrocytic cell line, U-87MG, that conditionally expresses p27SJ, in the phosphatase assay. The creation of the p27SJ inducible U-87MG cell line was based on the pTet-on gene expression system that we have previously described [Darbinian-Sarkissian et al., 2006]. Cells were treated with doxycycline for 48 h prior to harvest and protein extraction. As shown in Figure 3A, treatment of cells with doxycycline induces expression of p27SJ and increased overall phosphatase activity of the extracts. Examination of ERK1/2, a central regulatory protein kinase that, upon phosphorylation, regulates several signaling pathways showed a decrease in the level of its phosphorylated form, but not its total level (Fig. 3B). Several of ERK1/2 downstream proteins including CREB and Stat3 whose state of phosphorylation is partly regulated by ERK1/2 [Dalle et al., 2004], showed reduced levels of phosphorylated forms upon expression of p27SJ in the cells (Fig. 3C). The total level of Stat3 as well as the housekeeping protein, Grb2, remained unchanged. Treatment of the cells expressing p27SJ with okadaic acid, a serine/threonine phosphatase inhibitor [Dounay and Forsyth, 2002; Brenchley et al., 2007] rescued ERK1/2 from dephosphorylation upon expression of p27SJ (Fig. 3D).

In light of earlier observations linking Erk1/2 to cyclin A, and a potential role for CREB in the regulation of cyclin A [Desdouets et al., 1995; Jørgensen et al., 2003; Ko et al., 2004], we examined the level of cyclin A expression and its kinase activity in cells that conditionally express p27SJ. As seen in Figure 4A, expression of p27SJ in U-87MG cells is concurrent with a noticeable decrease in the level of cyclin A, but not Cdk2 and the control protein, α-tubulin. This observation suggests that a decrease in the activities of Erk1/2, upon its phosphorylation, and its downstream targets including CREB and possibly Stat3 may impact on the level of cyclin A. Further, results from the H1 kinase assay demonstrated a reduced level of cyclin A activity in cells expressing p27SJ (Fig. 4B). As dysregulation of cyclin A can affect cell cycle progression, in the next series of experiments we compared the

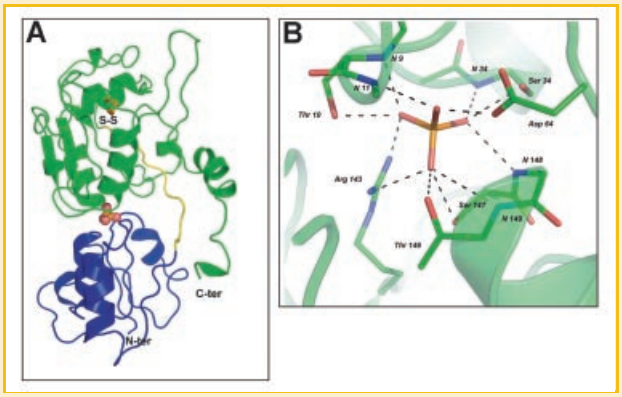

Fig. 2. Predicted structure of p27SJ. A: Model of p27SJ. The predicted structure is constituted of two globular domains, N-terminus (blue) and C-terminus (green) linked by a hinge (yellow). The phosphate ion is shown in sphere. The disulfide bridge in the C-terminal domain is indicated. B: Close view into the phosphate binding cleft of p27SJ model. The protein residues that form the phosphate binding cleft are indicated and the hydrogen bonds that they form with the phosphate ion are shown by black dashes.

status of p27SJ-expressing cells with the control p27SJ negative cells at various stages of the cell cycle. To this end, U-87MG cells (with p27SJ Tet-On system) kept in media with or without doxycycline were synchronized by serum starvation for 48 h. At 16, 24, 28, and 32 h after serum starvation, cells were harvested and their DNA content was determined by flow cytometry upon labeling with propidium iodide. As seen in Figure 4C, in the absence of p27SJ (no doxycycline treatment), cells progressed from G0/G1 to S at 24 h and to G2/M at 28 h after release from serum starvation. In cells expressing p27SJ, cells also progressed from G0/G1 to S phase at 24 h with approximately 8% decreased levels compared to those from p27SJ-negative cells. Also, progression of the cells from S to G2/M was reduced in p27SJ cells. Unlike the control cells, which had 27.4% of the cells at G2/M stage, only 16.5% of the cells expressing p27SJ at 28 h were detected at the G2/M phase. At 32 h, 30% of the control cells were found at S phase, while at the same time period, more than 28% of the p27SJ positive cells were detected at this stage. These results taken together show that dysregulation of cell cycle factors including cyclin A, which was shown to impact S and G2 phases [Pagano et al., 1992], has a biological consequence on cell cycle progression of the p27SJ-expressing cells. While our results do not detail the impact of p27SJ on specific cell cycle stages, our

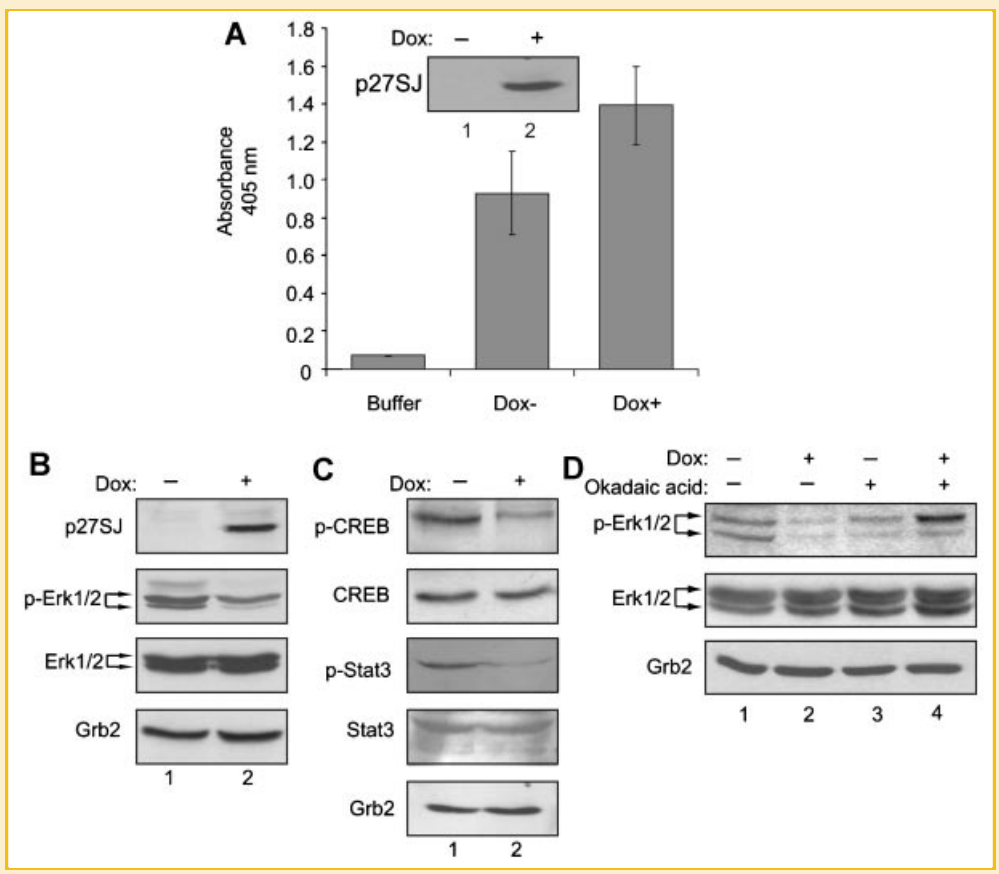

Fig. 3. Effect of p27SJ on Erk1/2. A: Phosphatase activity of protein extract from U-87MG cells before and after induction of p27SJ expression by doxycycline (Dox) treatment. The insert shows Western blot analysis for the expression of p27SJ upon Dox treatment. B,C: Western blot analysis of U-87MG protein extracts showing production of phosphorylated forms of Erk1/2 (pErk1/2) as well as total Erk1/2, phosphorylated CREB (pCREB) and Stat3 (pStat3), total Stat3 and the housekeeping protein, Grb2, upon Dox treatment. D: Western blot assay illustrating the level of pErk1/2, Erk1/2, and Grb2 in cells with okadaic acid ± Dox.

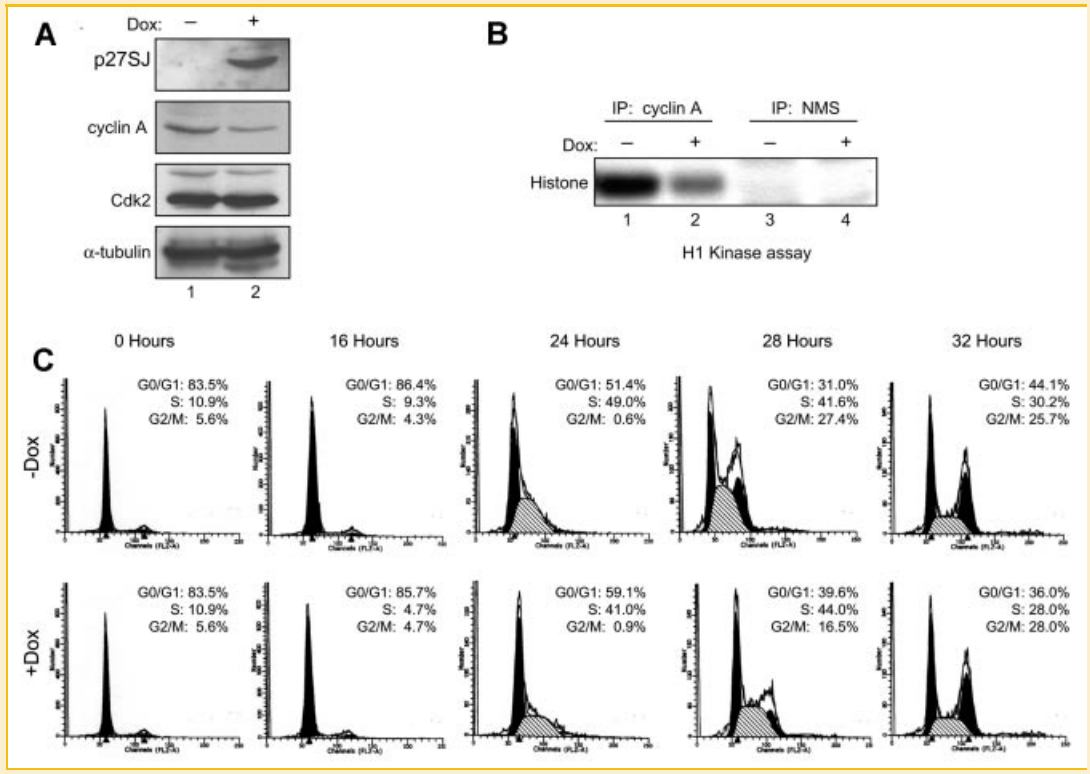

Fig. 4. Effect of p27SJ on cyclin A and cell cycle progression. A: Western blot showing the level of cyclin A, Cdk2, and tubulin in the absence (Dox−) or presence (Dox+) of p27SJ. B: H1 kinase assay of immunocomplex containing cyclin A (lanes 1 and 2) or the complex pulled down by control normal serum (lanes 3 and 4) from U-87MG extract before (lanes 1 and 3) and after (lanes 2 and 4) treatment with Dox. C: Cell cycle analysis of U-87MG before (−p27SJ) and after (+p27SJ) treatment with Dox at various times after release from serum starvation. The numbers represent the average of two experiments.

recent observations demonstrate that p27SJ impacts the overall progression of the cell cycle [Darbinian et al., data not shown].

A partial cDNA isolated from a callus culture of *Hypericum perforatum* has the capacity to express a 27 kDa protein named p27SJ. The presence of a characteristic DINGGG amino acid domain at the N-terminus of p27SJ classifies this protein as a member of a highly evolutionary conserved DING family [Berna et al., 2008; Perera et al., 2008]. While p27SJ represents the first eukaryotic member of this family whose gene has been isolated, proteins belonging to the family of DING proteins have been purified from a variety of eukaryotic cells, tissues and fluids. Of its thirty members, twelve have originated from animals, ten members including p27SJ were derived from plants, four from prokaryotic cells, and three were identified in fungi [Berna et al., 2008]. The prokaryotic DING members exhibit structural homology to phosphatases or phosphate-binding proteins [Ahn et al., 2007]. Here we demonstrate that p27SJ possesses phosphatase activity that seems to be mediated by the N-terminal portion of p27. Further, our results show that the C-terminal region of the protein, which has no phosphate binding activity, contributes to the phosphatase activity of the protein, perhaps by altering the tertiary structure of the protein. Of note, earlier studies on bacterial DING proteins unraveled the three dimensional structure of the protein that features Venus flytrap configuration in the phosphate binding sites [Ahn et al., 2007]. Our results also show that expression of p27SJ in cells results in hypophosphorylation of Erk1/2, which is a critical modulator of the MAP kinase pathway controlling cell proliferation, differentiation, and apoptosis. Furthermore, several downstream effectors of Erk1/2 such as CREB and Stat3, which by altering several cell cycle regulators including cyclin A, are dysregulated in cells with p27SJ expression can lead to changes in cell cycle progression at S and G2 phases. This observation provides the first evidence for the biological importance of eukaryotic members of the DING family in the control of cell signaling and proliferation via phosphatase activity.

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