View metadata, citation and similar papers at core.ac.uk

brought to you by CORE

provided by Elsevier - Publisher Connector

Upregulation of Survivin in G2/M Cells and Inhibition of Caspase 9 Activity Enhances Resistance in Staurosporine-Induced Apoptosis

Anmol Chandele, Vandna Prasad, Jayashree C. Jagtap, Ravi Shukla and Padma R. Shastry

National Centre for Cell Science, NCCS Complex, Ganeshkhind, Pune 411 007, India

Abstract

Survivin, a member of the inhibitor of apoptosis (IAP) gene family, plays an important role in both the regulation of cell cycle and the inhibition of apoptosis, and is frequently overexpressed in many tumor types. In neuroblastomas, the expression of survivin correlates with a more aggressive and histologically unfavorable disease. Survivin is predominantly a cytoplasmic protein that is expressed in a cell cycle-dependent manner, increasing in the G2/M phase of the cell cycle followed by a rapid decline in the G1 phase. Recently, the role of survivin in resistance to chemotherapy has become an area of intensive investigation. In this study, we demonstrate a phase-specific resistance due to survivin in staurosporine (STS)–induced apoptosis in the human neuroblastoma cell line SK-N-MC. G2/M-arrested cultures show an upregulation of survivin expression and are more resistant, whereas G1-phase cells that show decreased levels of survivin are more sensitive to apoptosis. Localization studies revealed differences in the distribution of survivin in two synchronized populations, with G1 cells having weakly positive staining confined to the nucleus, in contrast to G2/M cells that depicted a more uniform and intense expression of survivin throughout the cell. In our experimental system, STS induced apoptosis through the mitochondrial–caspase 9–mediated pathway. Retention of survivin in G1 cells by inhibition of the ubiquitin–proteosome pathway or inhibition of caspase 9 protected the cells against apoptosis. Our data suggest that survivin exerts its antiapoptotic effect by inhibiting caspase 9 activity, an important event in STS-mediated apoptosis. In context with cell cycle–dependent responses to chemotherapy, the data from this study suggest the possibility of exploiting the survivin pathway for inducing apoptosis in tumor cells.

Neoplasia (2004) **6**, 29–40

Keywords: Survivin, caspase 9, G2/M, staurosporine, neuroblastoma.

Introduction

Apoptosis is a "default" process intrinsic to all cells, which is abrogated by the provision of survival signals within cells [1]. Chemotherapeutic drugs induce damage at a number of different loci, and the balance between the proapoptotic and survival signals determines cellular fate [2]. Intensive research has led to the identification of several proteins such as the Bcl-2 family, HSP27 and HSP70, and inhibitor of apoptosis (IAP) family proteins, which not only render tumor cells resistant to apoptosis but also promote tumorigenesis [3].

Survivin, a 16.5-kDa protein, is present as a homodimer *in vivo* [4]. It is one of the eight proteins of the human IAPs that contain at least one copy of the baculovirus inhibitor of apoptosis repeats (BIR) domain, expressed only in dividing cells and therefore is predicted to be a bifunctional protein that both suppresses apoptosis as well as regulates cell division [5,6]. Survivin expression is considered to be the most tumor-specific of all human gene products [7] and is reported to be highly expressed in a number of tumor types especially neuroblastoma, colorectal carcinomas, and gastric carcinomas, and correlates with poor prognosis of the disease [7–9]. Survivin contains a G1 transcriptional repressor element within its promoter. The survivin promoter also exhibits typical M-phase–inducible transactivation, suggesting that survivin is a cell cycle–regulated molecule, which is repressed in the G1 phase and is highly expressed in the G2/M phase. It associates with the mitotic spindle through its C-terminal domain [10] and the role of survivin during mitosis involves the regulation of microtubule assembly and the functional formation of a normal bipolar apparatus [6].

The cell cycle–specific regulation of survivin in tumor cells suggests that the protein contributes to the regulation of apoptosis during cell proliferation. Reports on the interaction between survivin and caspases have been contradictory. Although few studies have suggested that survivin binds and suppresses caspases 3, 7, and 9, others have failed to demonstrate a direct effect on these proteases [11–14]. Phosphorylation of survivin at threonine 34 is required for the interaction of survivin with caspases 3, 7, and 9 *in vitro*. A mutation from this threonine to alanine (T34A) can induce the release of cytochrome *c* from the mitochondria, leading to

Abbreviations: FITC, Fluorescein isothiocyanate; MMP, mitochondrial membrane potential; PARP, poly (ADP ribose) polymerase; PBS, phosphate-buffered saline; RT, room temperature; STS, staurosporine; TRITC, Tetramethyl rhodamine isothiocyanate; TST, Tris saline Tween; TUNEL, terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling

Address all correspondence to: Padma Shastry, National Centre for Cell Science, NCCS Complex, Ganeshkhind, Pune 411 007, India. E-mail: padma@nccs.res.in

Received 4 August 2003; Revised 10 September 2003; Accepted 12 September 2003.

Copyright © 2004 Neoplasia Press, Inc. All rights reserved 1522-8002/04/$25.00

apoptosis. The only kinase reported to date that phoshorylates survivin is cdc-2/CDK1, a cyclin-dependent kinase that is active only at certain stages of the cell cycle. This kinase has been coimmunoprecipitated with survivin during mitosis, indicating a direct physical interaction between the two for phosphorylation of survivin [15]. One of the important features for anticancer drug treatment to be effective is that the cancer cells must be sensitive to the effects of the drug before resistance emerges. Cell cycle–specific chemotherapy drugs work at specific points in the cell cycle. For instance, paclitaxel (Taxol; Bristol-Myers Squibb Oncology/Immunology Division, Princeton, NJ) a common chemotherapeutic agent used in breast cancer, works at the G2/M stage. Cell cycle–dependent resistance is an emerging concept in combination sequential chemotherapy [16]. Although survivin is specifically present in G2/M cells, its role in protecting cell populations in this phase to chemotherapeutic agents has not been reported. In this study, we demonstrate the differential sensitivity of the G1- and G2/M-synchronized human neuroblastoma cell line SK-N-MC to Staurosposine (STS)-induced apoptosis, and suggest a critical role for survivin in imparting resistance in the G2/M population. We observed that STS induces apoptosis primarily through the mitochondrial pathway by activating caspase 9 as the prime initiator caspase. Survivin appears to exert its phase-specific antiapoptotic effect by inhibiting this caspase 9 activity.

Materials and Methods

Cell Line and Culture

The human neuroblastoma cell line SK-N-MC was procured from the American Type Culture Collection (ATCC; Rockville, MD) and maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% heat-inactivated fetal bovine serum (Gibco BRL, Carlsbad, CA) at 37°C in a humidified atmosphere of 5% CO_2 and 95% air.

Cell Synchronization and Treatment

SK-N-MC cells were seeded at a density of 0.5×10^6 cells/ml and cultured for 24 hours before synchronization with 5 μM aphidicolin (Sigma, St. Louis, MO) for 12 hours to arrest them in the G1 phase, or with 30 nM nocodazole (Calbiochem, La Jolla, CA) for 12 hours to arrest the cells in the G2/M phase. The cells were then treated for 12 hours with 100 nM STS (Sigma) to induce apoptosis in synchronized and unsynchronized cells.

Cell Cycle Analysis

Cell cycle analysis for synchronized cells was monitored as DNA profiles after staining with propidium iodide. Cells were harvested, washed with 1 × phosphate-buffered saline (PBS), fixed in 70% chilled ethanol at 4°C for 10 minutes, hydrolyzed with 5 mg/ml ribonuclease A for 20 minutes, and stained with 50 μg/ml propidium iodide. DNA content was determined on a flow cytometer (FACS Vantage BD, San Jose, CA) equipped with a 488-nm argon laser and populations in cell cycle phases were quantified using MODFIT software.

TUNEL Assay

Terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling (TUNEL) assay was used to assess the apoptosis induced by STS and was measured by FragEL DNA fragmentation detection kit (Oncogene Research) as per the manufacturer's instructions. Briefly, the cells were fixed with 70% chilled ethanol at 4°C for 10 minutes, washed with 1 × TBS, rehydrated for 30 minutes in 1 × TBS, equilibrated for 30 minutes with 1 × equilibration buffer, and further incubated at 37°C for 2 hours with the labeling reaction mixture containing labeling reaction buffer, fluorescein isothiocyanate (FITC)-tagged dUTP, and TdT enzyme. The cells were washed and acquired on a flow cytometer equipped with a 488-nm argon laser. The extent of FITC positivity was used as a parameter to quantify the apoptosis induced by STS.

Study of Mitochondrial Membrane Potential (MMP)

Changes in the MMP of SK-N-MC cells were studied by the MitoCapture apoptosis detection kit (Oncogene Research), as per the manufacturer's instructions. The assay uses a fluorescence-based method utilizing the ability of the MitoCapture dye to differentially stain healthy and apoptotic cells. SK-N-MC cells were washed in 1 × PBS and incubated at 37°C for 20 minutes in the MitoCapture reagent (1:1000 dilution). The cells were washed and resuspended in incubation buffer and acquired on the FL2 channel (585/590) of a flow cytometer equipped with a 488-nm argon laser. A decrease in red fluorescence intensity was considered as a function of the breakdown in membrane potential. The cell population in the region of decreased fluorescence was quantified as apoptotic cells in untreated and treated cells.

Caspase 9 Activity and Inhibition Assay

The activation of caspase 9 in SK-N-MC cells treated with STS was assessed using caspase 9 colorimetric assay kit (Oncogene Research). SK-N-MC cells were seeded at a density of 3×10^4 cells/100 μl in a 96-well plate for 24 hours. After the specified time of treatment, the spent medium was aspirated and 50 μl of assay buffer with fresh 1 mM DTT was added to the wells and incubated at 37°C for 30 minutes followed by the incubation with caspase 9 colorimetric substrate LEDH-pNa for 2 hours. The release of pNa due to the cleavage of LEDH by active caspase 9 was read at 405 nm using a microplate reader (Molecular Devices, Sunnyvale, CA). Similar experiments were performed with cells treated for 1 hour prior to STS treatment with Z-LEDH-FMK (20 μM), a cell-permeable inhibitor of caspase 9, and activity was determined.

Inhibition of Ubiquitin–Proteasome Pathway

SK-N-MC cells were treated with 10 μM proteasome inhibitor MG132 for 2 hours prior to G1 synchronization followed by STS treatment.

Western Blot Analysis

SK-N-MC cells (0.5×10^6 cells) were harvested, washed with 1 × PBS, and lysed in 50 μl of 1 × Lamelli's sample

buffer. The resultant lysate was clarified and centrifuged at 22,000*g* for 10 min. The supernatant was aspirated and 10 μl was resolved on a denaturating polyacrylamide gel [10% for poly (ADP ribose) polymerase (PARP); 15% for Bcl-2, bax, survivin, and cdc-2] followed by electroblotting onto PVDF membrane in 20 mM sodium phosphate buffer, pH 6.8, at 100 mA per gel for 1 hour. The membrane was blocked with 5% BSA in Tris saline Tween (TST) buffer (20 mM Tris, 0.5 M NaCl, and 0.05% Tween 20) for 1 hour at room temperature (RT). The blots were incubated for 2 hours at RT in primary antibodies: anti–Bcl-2, anti-Bcl_{XL}, anti-PARP (Santa Cruz Biotechnology, San Diego, CA), anti-cdc2 (Pharmingen BD, San Diego, CA), antisurvivin (Chemicon, Temecula, CA), and antiactin antibody (Sigma), and diluted in TST buffer as recommended by the manufacturers. The blots were washed with TST buffer followed by incubation with appropriate HRP-labeled secondary antibodies (BioRad, Hercules, CA) at 1:10,000 dilution for 1 hour at RT. The membrane was washed with TST buffer and the probed proteins were detected by the Enhanced Chemiluminescence System (Amersham, Buckinghamshire, UK) according to the manufacturer's instructions. The blots were stripped and reprobed for actin. The expression of the proteins was normalized with respect to actin.

Immunostaining for Flow Cytometry

Immunostaining was performed to monitor the cell cycle–dependent expression of survivin by flow cytometry. SK-N-MC cells, synchronized in G1 and G2/M phases, were fixed with 70% chilled ethanol for 10 minutes at 4°C. The ethanol was washed with 1 × PBS and the cells were incubated with 1 μg of antisurvivin antibody (Santa Cruz Biotechnology) diluted in 1 × PBS containing 0.5% BSA for 1 hour at 4°C followed by incubation with 1 μg of FITC-labeled secondary antibody (Pharmingen BD) at 4°C for 1 hour. The cells were washed and resuspended in PBS containing 5 mg/ml RNase A and 50 μg/ml propidium iodide. Bivariant analysis was performed to determine the survivin-positive cells with respect to the DNA content.

Immunostaining for Confocal Microscopy

SK-N-MC cells were seeded at a density of 0.35×10^4 cells/ml per coverslip (Sigma), grown for a period of 24 hours, and synchronized as described earlier. The cells were fixed with freshly prepared 3.7% chilled paraformaldehyde (ICN, Aurora, OH) for 10 minutes, washed with 1 × PBS (Life Technologies, Frederick, MD) and permeabilized with cold 0.02% Triton X-100 for 30 seconds. The cells were incubated overnight at 4°C with antisurvivin antibody (Santa Cruz Biotechnology) and antitubulin antibody (Sigma) diluted in 1 × PBS containing 0.5% BSA. The coverslips were washed with 1 × PBS and incubated with appropriate secondary antibodies (DAKO, Glostrup, Denmark) labeled with FITC and tetramethyl rhodamine isothiocyanate (TRITC; diluted 1:50) at 4°C for 1 hour. The expression of survivin with respect to the presence of tubulin was observed by a confocal laser scanning microscope fitted with a 488-nm/540-nm argon laser (Carl Zeiss, Jena, Germany) at an image magnification of × 63 objective.

Transfection with Antisense Oligonucleotides

SK-N-MC cells were transfected with phosphorothioate antisense oligonucleotides (GenoMechanix, Alachua, FL) against survivin with Lipofectamine 2000 (Life Technologies) as per the manufacturers' instructions. Briefly, 0.4×10^6 cells were transfected with 212 nM and 424 nM survivin antisense 5′ CCCAGCCTTCCAGCTCCTTG 3′ 2 μl of Lipofectamine 2000 for 6 and 9 hours, and the expression of survivin was assessed by Western blot analysis. Cells transfected with scrambled sequence 5′ GCACCTAGTCTCCCTGCACC 3′ served as controls.

Semiquantitative Reverse Transcription Polymerase Chain Reaction (RT-PCR)

Total RNA was extracted from control and treated SK-N-MC cells using the Trizol method. cDNA was prepared from 3 μg of total RNA by reverse transcription with MMLV-RT enzyme (Life Technologies) at 37°C for 60 minutes. The survivin transcript of 450 bp was amplified from the cDNA using recombinant Taq polymerase (Life Technologies). β-Actin was used as an internal control.

The primers used were:

Survivin: Forward—5′ AATAAATGGATCCATGGGTGCCCCGACGTTGCCCAGC 3′
Reverse—5′ CTAAGCTTTCAATCCATGGCAGCAAGC 3′
β-Actin: Forward—5′ GTGGGGCGCCCCAGGCACCA 3′
Reverse—5′ CTCCTTAATGTCACGCACGATTTC 3′

Statistical Analysis

The data were analyzed with Sigma Stat software (Jandel Scientific, San Rafael, CA). Treated cells and the corresponding controls were compared using one-way analysis of variance (ANOVA) followed by Student-Newman-Kuels test. $P < .05$ was considered significant.

Results

Differential Sensitivity of SK-N-MC Cells Arrested in G1 and G2/M Phases to STS-Induced Apoptosis

SK-N-MC cells were synchronized with aphidicolin in the G1 phase or with nocodazole in the G2/M phase. Unsynchronized cells had a cell cycle distribution pattern of 63.54% cells in the G1 phase, 24.72% cells in the S phase, and 11.83% cells in the G2/M phase. Arrest of SK-N-MC cells in the G1 phase with aphidicolin showed an accumulation of 84.86% cells in the G1 phase, 15.32% cells in the S phase, and no cells in the G2/M phase. Arrest in the G2/M phase with nocodazole resulted in an accumulation of 67.16% cells in the G2/M phase and 29.15% and 3.69% cells in the S and the G1 phases, respectively (Figure 1*A*). We then examined the induction of apoptosis in the enriched populations treated with 100 nM STS for 6 and 12 hours by TUNEL assay. It was

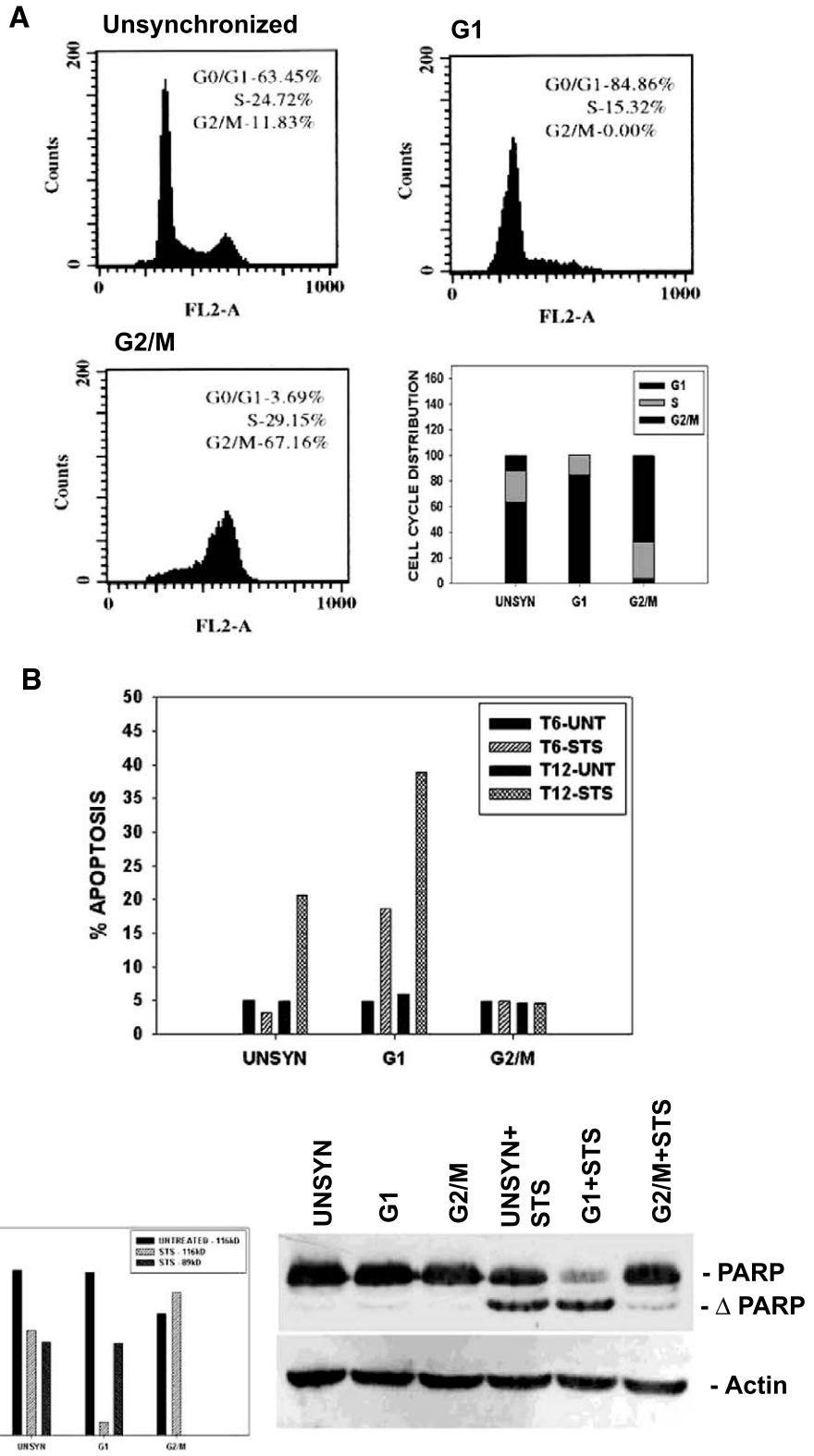

Figure 1. *Differential sensitivity of SK-N-MC cells arrested in G1 and G2/M phases to STS-induced apoptosis. (A) Cell cycle distribution of synchronized cells assessed by flow cytometry. SK-N-MC cells were synchronized with 5 μM aphidicolin and 30 nM nocodazole in the G1 and G2/M phases, respectively. A representative histogram of unsynchronized, G1- and G2/M-arrested populations is shown and the data from three similar experiments are presented as stacked bars. (B) Selective sensitivity of the arrested cell populations to undergo apoptosis induced by STS with 6 and 12 hours of treatment assessed by TUNEL assay. G1 cells were more susceptible to STS as compared to the G2/M population. The data are the means of three independent experiments. (C) Expression of PARP by Western blotting in unsynchronized and synchronized cells treated with STS for 12 hours. Densitometric analysis of band intensities normalized with β-actin is shown in the histogram.*

observed that with 6 hours of STS treatment, G1-arrested cells showed 18.67% apoptosis that increased to 38.86% by 12 hours in comparison with unsynchronized controls, which had 3.18% apoptosis at 6 hours and 20.64% at 12 hours. The G2/M population treated with STS showed no significant apoptosis, with an apoptotic population of 3.18% at 6 hours and 4.93% at 12 hours of treatment. These results indicated that G2/M cells are more resistant to STS-mediated apoptosis as compared to the G1 phase cells and unsynchronized control populations (Figure 1*B*). Cleavage of poly(ADP ribose) polymerase (PARP) is considered a hallmark of the apoptotic process. We therefore examined PARP cleavage in STS-treated cell populations. The full-length and cleaved PARP was normalized to the loading control, actin. We observed that the native 116-kDa PARP was cleaved to its 89-kDa signature cleavage product in the G1-arrested and the unsynchronized cell populations. The cleavage of PARP was more pronounced in G1 cells with increased intensity of the cleaved band and a corresponding decrease in the native protein. No significant PARP cleavage was detected in G2/M-arrested cells (Figure 1*C*).

Antiapoptotic Molecule Survivin Is Expressed in a Cell Cycle–Dependent Manner

Because a significant difference in response to STS between the G1 and G2/M phase cells was observed, we monitored the expression of Bcl-2 and Bcl_{XL}, antiapoptotic molecules known to play an important role in protecting cells against mitochondrial-dependent apoptosis induced by STS. The expression of survivin was also monitored, as it is an antiapoptotic molecule that is regulated in a cell cycle–dependent manner. We observed no significant difference in the expression of Bcl-2 and Bcl_{XL} in the STS-treated synchronized and unsynchronized cells compared to their respective untreated controls. However, a remarkable difference in the expression of survivin was observed. In comparison with unsynchronized cells, G1-arrested populations showed a 93.18% decrease in survivin expression, whereas G2/M-arrested cells showed a 52.75% increase that was sustained with STS treatment (Figure 2*A*). Cell cycle–dependent expression of survivin was confirmed in SK-N-MC cells by dual staining with antibody specific for survivin and propidium iodide for DNA, and analyzed by flow cytometry. Percent positivity was scored in comparison to the isotype control and 5% positivity was set as cutoff. The expression of survivin was observed to be restricted to the G2/M phase (Figure 2*B*), corroborating the results of our immunoblot analysis. Next we analyzed the subcellular localization of survivin with respect to tubulin by confocal microscopy. We observed a moderate amount of survivin in unsynchronized cultures that was localized in the nucleus, corresponding to green fluorescence. Cells arrested in G1 phase exhibited a low expression of survivin that was localized in the nucleus. In a striking contrast, survivin was distributed uniformly in G2/M cells and colocalized with tubulin, as evident from the yellow fluorescence in the cytosol (Figure 2*C*), which may be necessary for function as an antiapoptotic molecule.

Expression of cdc-2 Occurs in a Cell Cycle–Dependent Manner and Correlates with the Expression of Survivin

We observed that survivin was differentially expressed in the two phases of the cell cycle. The key requirement for the function of survivin is its phosphorylation at threonine 34 by CDK1. Therefore, the expression of cdc-2 was studied by Western blotting. We observed a 3.14-fold increase in the levels of cdc-2 in G2/M-arrested cells (Figure 2*D*). This expression pattern correlated with the expression of survivin. Thus, in accordance with previous reports, it is possible that cdc-2 interacts with survivin during the G2/M phase to phosphorylate and impart antiapoptotic property.

Downregulation of Survivin Sensitizes Cells to STS-Mediated Apoptosis

We reasoned that if survivin was responsible for the resistance to apoptosis induced by STS in G2/M-synchronized cells, then downregulation of survivin should affect the sensitivity of these cells to STS. SK-N-MC cells were transfected with 212 and 424 nM phosphorothioate-modified antisense survivin oligonucleotides for 6 and 9 hours followed by treatment with STS for 3 hours. The expression of survivin was monitored by Western blot analysis and the extent of PARP cleavage was evaluated as an indicator of apoptosis. We observed a 76.47% and 77.62% decrease in survivin expression in cells transfected with 212 nM survivin antisense oligonucleotide for 6 and 9 hours, respectively. A near-complete inhibition of survivin expression with 424 nM survivin antisense was observed (Figure 3*A*). Cleavage of PARP was observed within 3 hours of STS treatment in transfected cells with a four-fold increase in the sensitivity of cells to STS (Figure 3*B*). Cells transfected with scrambled survivin sequence did not show any apoptosis (data not shown). These data highlight the importance of survivin as a molecule mandatory for the survival of cells and enables them to evade the apoptotic insult. The antisense-transfected control cells not treated with STS did not show any inherent apoptosis as evaluated by trypan blue staining (data not shown) and propidium iodide staining (Figure 3*C*), indicating that a decrease in survivin expression alone does not lead to the apoptosis of cells.

Induction of Apoptosis by STS in SK-N-MC Cells Is Primarily through the Mitochondrial–Caspase 9 Pathway

During apoptosis, the mitochondrial inner transmembrane potential ($\Delta\psi_M$) is frequently disrupted due to the formation of permeability transition pores, or the insertion of proapoptotic proteins such as Bid or Bax in the mitochondrial membrane. We therefore examined the effect of STS on the membrane potential of SK-N-MC cells by staining with a cationic dye using the MitoCapture apoptosis detection kit. This cationic dye accumulates and aggregates in the mitochondria of healthy cells and emits an orange red fluorescence that is analyzed by flow cytometry. A decrease in the intensity of red fluorescence

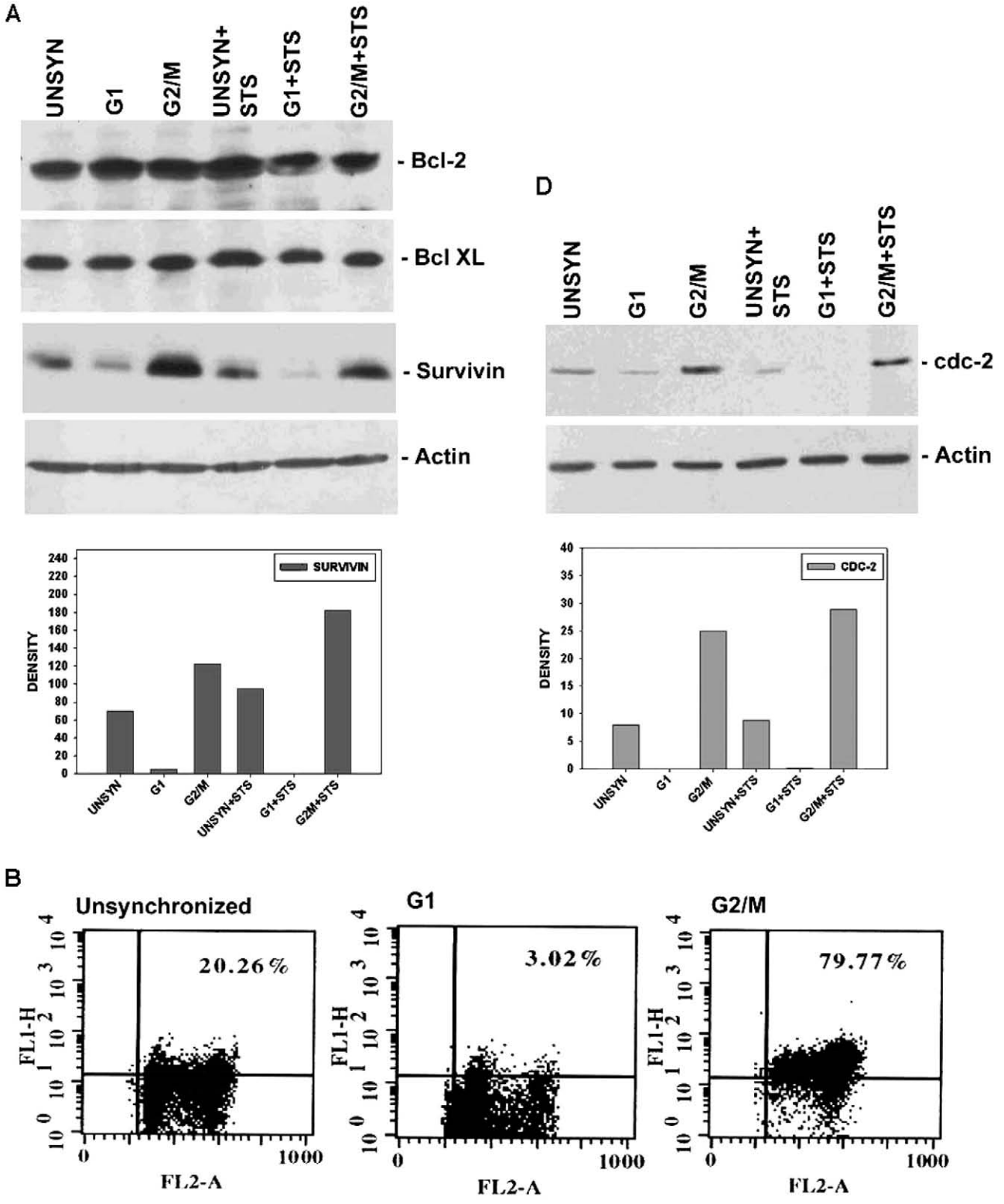

Figure 2. *Expression of survivin in a cell cycle–dependent manner. (A) Expression of antiapoptotic molecules, Bcl-2, Bcl_{XL}, and survivin in untreated and STS-treated synchronized cell populations studied by immunoblotting. (B) Expression of survivin in unsynchronized and synchronized cell populations with respect to the cell cycle analyzed by flow cytometry. FL2-A on the X-axis represents DNA content, and FL1-H on the Y-axis denotes fluorescence intensity for survivin staining. (C) Localization of survivin in synchronized cells studied by immunostaining and visualized under a confocal laser scanning microscope. Cells were stained for survivin (green) and tubulin (red), and colocalization of survivin and tubulin is seen as yellow fluorescence in merged images. (D) Immunoblot analysis for cdc-2 expression performed in synchronized cells. Western blots were normalized against actin used as loading control. The bar graphs are representatives for the densitometric analysis of survivin (a) and cdc-2 (b).*

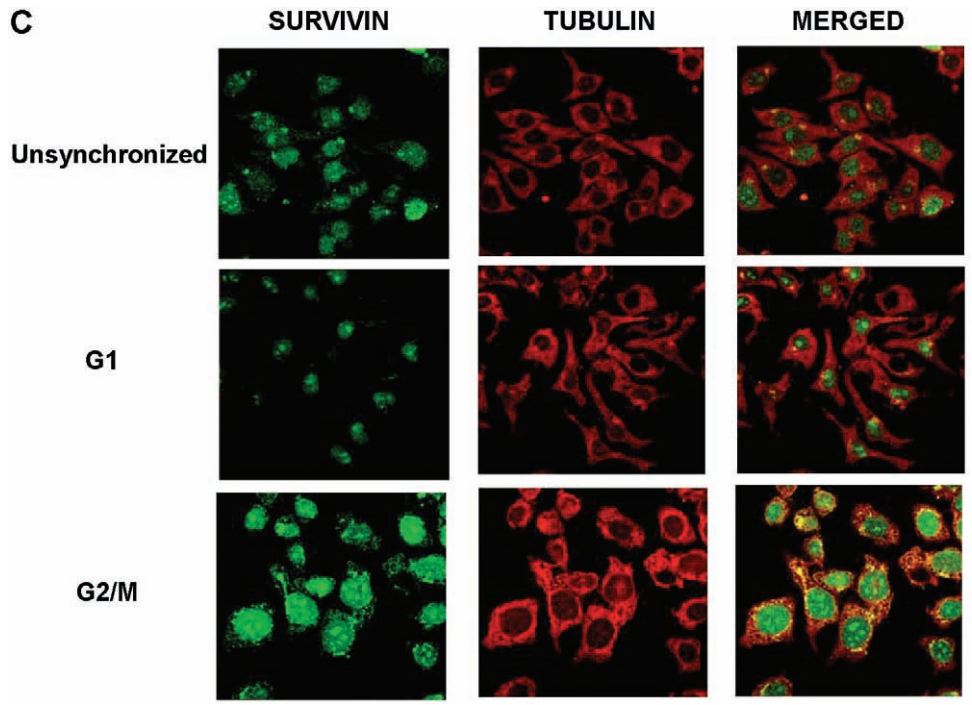

Figure 2. *(Continued)*

is considered a parameter for breakdown in membrane potential and hence a measure of apoptotic population. A 3.1-fold and 6.9-fold decrease in membrane potential was observed in unsynchronized and G1-arrested cells, respectively, compared to G2/M-arrested cells, which showed no breakdown of membrane potential with STS treatment. On this criterion, 69.22% cells were observed to undergo apoptosis with STS treatment in unsynchronized

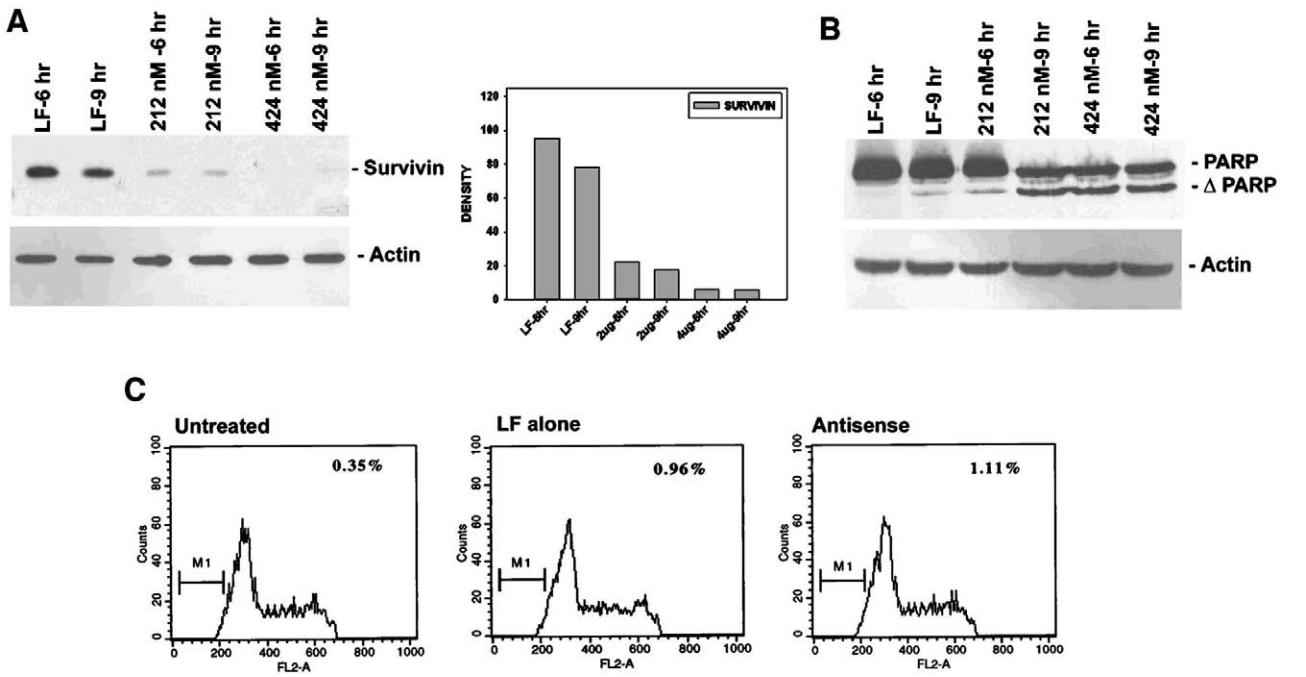

Figure 3. *Downregulation of survivin sensitizes cells to STS-mediated apoptosis. (A) SK-N-MC cells were transfected with phosphorothioate antisense oligonucleotides to survivin (212 and 424 μg) for 6 and 9 hours and the expression of survivin was analyzed by Western blot analysis using antisurvivin antibody. The bar graph represents densitometric analysis of the expression of survivin normalized against the loading control β-actin. (B) Western blot analysis of full-length PARP (116 kDa) and cleaved PARP (ΔPARP) in untreated and STS-treated (3 hours) SK-N-MC cells transfected with survivin antisense oligonucleotides. (C) Flowcytometry analysis of untreated SK-N-MC cells transfected with survivin antisense stained with propidium iodide to study the DNA profile. Marker denotes hypodiploidy region for apoptosis population.*

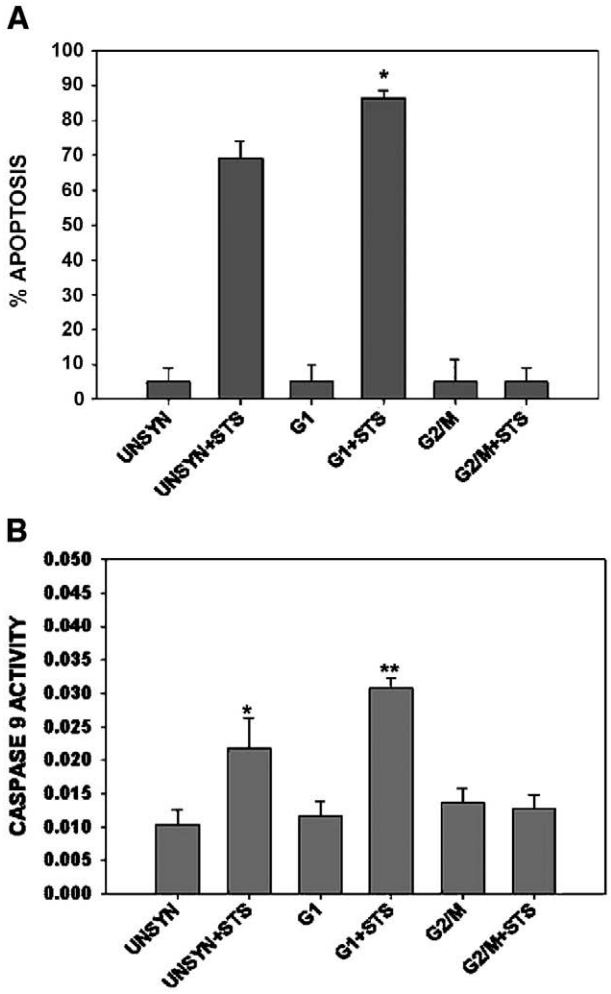

Figure 4. *Breakdown in MMP and caspase 9 activity. (A) Analysis of mitochondrial permeability transition after treatment of synchronized SK-N-MC cells with STS for 6 hours. Cells were stained with mitotracker dye and analyzed by flowcytometry. The data were acquired with the fluorescence median at 10^2 on FL2-H channel and the percentage of cells with low fluorescence intensity (considered as apoptotic population) was quantified using the same marker for control and STS-treated cells. The values indicated on the Y-axis are the percent apoptotic cells. The data are mean ± SD of three independent experiments. *P < .05 in comparison between G1-arrested and unsynchronized cells treated with STS. (B) The activation of caspase 9 in synchronized SK-N-MC cells treated with STS for 6 hours was monitored by caspase 9 colorimetric assay. The values on the Y-axis represent absorbance obtained with the release of pNa by active caspase 9 from its substrate LEDH-pNa. The data are represented as mean ± SD of three independent experiments. Activity was compared between STS and untreated corresponding controls. *P < .05; **P < .01.*

populations, which increased to 86.22% in G1-arrested cells. There was no difference in the apoptosis between control and STS-treated G2/M-arrested cultures (Figure 4*A*). Caspase 9 is the key initiator caspase that is activated during mitochondrial-dependent apoptosis. We therefore determined caspase 9 activity in SK-N-MC cells treated with STS for 6 hours by caspase 9 colorimetric assay that reads the absorbance of pNa released on cleavage of the substrate LEDH-pNa by active caspase 9. There was no difference in the basal levels of caspase 9 activity between the unsynchronized population and cells arrested in different phases, but on treatment with STS, caspase 9 activity increased with fold increases of 2.1 and 2.6 in unsynchronized and G1-arrested cells, respectively. However, no increase in caspase 9 activity was detectable in the G2/M-arrested population treated with STS (Figure 4*B*).

Inhibition of Caspase 9 Activity Abrogates Apoptosis in STS-Treated SK-N-MC Cells

As treatment of SK-N-MC cells with STS resulted in breakdown of membrane potential and activation of caspase 9, further experiments were performed to investigate the role of caspase 9 in STS-induced apoptosis in SK-N-MC cells. Cells were treated with the irreversible cell-permeable caspase 9 inhibitor Z-LEDH-FMK (20 μM) an hour prior to treatment with STS for 6 hours and caspase 9 activity was determined by a colorimetric assay. A 60.1% and 44.0% decrease in caspase 9 activity was observed in G1-arrested and unsynchronized cells, respectively, when treated with STS in the presence of caspase 9 inhibitor (Figure 5*A*). We further studied the effect of the inhibition of caspase 9 activity on the extent of apoptosis induced by STS. The cleavage of PARP was monitored for the apoptosis induced in SK-N-MC cells treated with STS in the presence of 20 μM caspase 9 inhibitor. We observed that the inhibition of caspase 9 activity

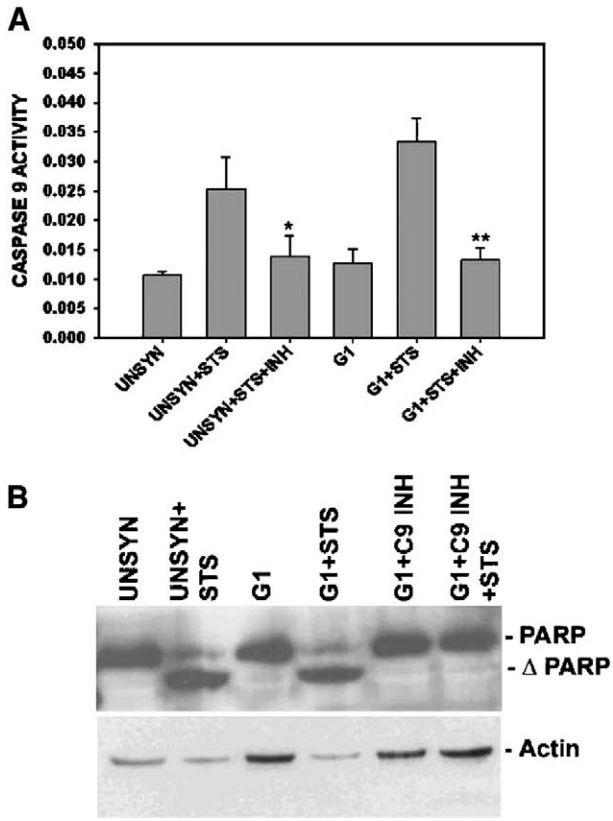

Figure 5. *Inhibition of caspase 9 activity in STS-treated SK-N-MC cells. (A) Inhibition of caspase 9 activity in unsynchronized and G1-arrested cells treated with STS for 12 hours in the presence and absence of the caspase 9 inhibitor, Z-LEDH-FMK, assessed by caspase 9 colorimetric assay. Data are mean ± SD of three experiments. Caspase 9 activity was compared between cells treated with STS in the presence or absence of inhibitor (*P < .05, **P < .01). (B) Western blot analysis for expression of PARP (116 kDa) and cleaved PARP (ΔPARP) in untreated and STS-treated cells in the presence or absence of caspase 9 inhibitor. Equal protein loading was confirmed with actin.*

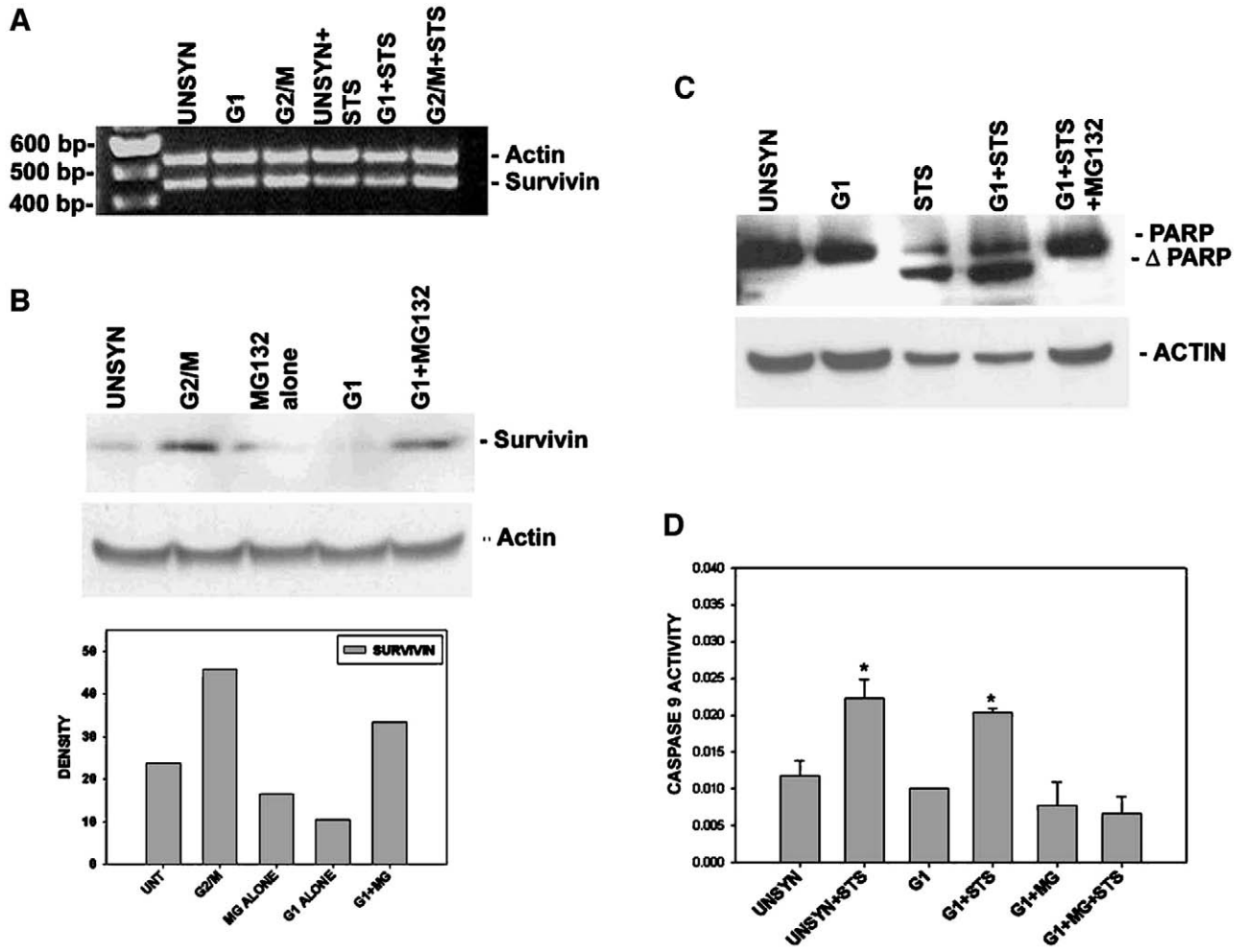

Figure 6. *Inhibition of the ubiquitin–proteasome pathway and survivin expression in G1-arrested SK-N-MC cells. (A) Expression of survivin RNA in unsynchronized, G1-arrested, and G2/M-arrested populations by semiquantitative RT-PCR. (B) Expression of survivin by Western blotting in SK-N-MC cells pretreated with the proteasome inhibitor MG132, 2 hours prior to G1 arrest. Histogram plots represent the density of band intensities of survivin normalized with β-actin controls. (C) Western blot analysis for expression of PARP (116 kDa) and cleaved PARP (ΔPARP) in untreated and STS (12 hours)–treated G1 population pretreated with MG132. (D) Caspase 9 activity in control and STS-treated unsynchronized and G1-arrested populations. Cells were incubated with MG132 prior G1 arrest followed by STS treatment. The data are mean ± SD of three independent experiments. *P < .05 compared to untreated controls.*

led to the complete abrogation of PARP cleavage (Figure 5*B*). These results suggest that STS-induced apoptosis in SK-N-MC cells is through this mitochondrial–caspase 9–mediated pathway and that caspase 9 is the sole initiator of this apoptotic cascade. Therefore, inhibition of caspase 9 protects cells from the insult imparted by STS.

Inhibition of the Ubiquitin–Proteasome Pathway Retains Survivin Expression in G1-Arrested SK-N-MC Cells

Our initial experiments demonstrated differences in expression of survivin in G1 and G2/M phases and a differential sensitivity of these arrested populations to STS-induced apoptosis. We therefore investigated whether retention of survivin in G1-arrested cultures could protect cells against STS-mediated apoptosis. The expression of survivin was monitored by semiquantitative RT-PCR. There was no significant difference in survivin expression in the G1 and G2/M phases at the RNA level (Figure 6*A*), suggesting that the differential expression of survivin was a phenomenon that was regulated posttranscriptionally, either during translation or degradation of protein. It is known that degradation of survivin is mediated through the ubiquitin–proteasome pathway. We studied survivin expression in cells arrested in G1 phase in the presence of a proteasome inhibitor, MG132. SK-N-MC cells were treated with 10 μM proteasome inhibitor MG132 2 hours prior to the G1 arrest, and the expression of survivin in the G1 phase with and without MG132 treatment was studied by Western blot analysis. G2/M-arrested cells were used as a control. We observed that the addition of MG132 led to the retention of survivin with 72.88% positivity in the G1 phase as compared to the cells not treated with MG132 that showed undetectable expression of survivin (Figure 6*B*). Thus, the differential expression of survivin observed in the two arrested cell populations is a posttranscriptionally regulated phenomenon and the degradation of survivin during the G1 phase is mediated through the ubiquitin–proteasome pathway. To emphasize the importance of survivin in STS-induced apoptosis, we subjected

G1-arrested cultures pretreated with MG132 to STS. We observed no cleavage of PARP in G1-arrested cells wherein the degradation of survivin was also inhibited (Figure 6*C*). Furthermore, caspase 9 activity was also not observed in these cells (Figure 6*D*), thus confirming that survivin is a vital antiapoptotic protein that protects SK-N-MC cells against STS-induced apoptosis and that the abrogation of apoptosis is due to the inhibition of caspase 9 activity.

Discussion

Major research efforts are aimed at the discovery of molecular targets that are specifically toxic to tumor cells and at devising their synergistic combinations. However, with a few exceptions, tumor cells lack sufficiently specific targets that would allow them to be killed selectively, and hence mechanism-based toxicity is an approach of choice. Cell cycle–mediated drug resistance is best described as the relative sensitivity of tumor cells to a chemotherapeutic agent because of its position in the cell cycle. Therefore, a better understanding of the molecular mechanisms of action of anticancer drugs or compounds in context with cell cycle regulation may contribute to the development of newer strategies for rationally and selectively manipulating the sensitivity of cancer cells to therapy [17].

In this study, we have demonstrated the differential responses of the G1- and G2/M-synchronized human neuroblastoma cell line SK-N-MC to STS. Although STS, a broad-spectrum inhibitor of protein kinases, has been used widely to induce apoptosis in a variety of cell types, the molecular mechanism(s) is still not clear and is considered more complex than that of other anticancer drugs [18]. Our data suggest that STS induces apoptosis through the mitochondrial-dependent pathway in the human neuroblastoma cell line SK-N-MC and that the apoptosis induced is inhibitable by the presence of the antiapoptotic protein survivin.

To understand the mechanism by which G2/M cells could evade the apoptotic insult caused by STS, we assessed the expression of antiapoptotic proteins Bcl-2 and Bcl_{XL} because both these proteins are known to play a crucial role in protecting cells by blocking apoptosis triggered through the mitochondrial pathway [19–21]. We observed that the expression of these molecules was not cell cycle–dependent and the expression levels were not altered with STS treatment, suggesting that they probably did not play an important role in the differential response of cells to apoptosis in this system. A direct link between survivin expression and tumor cell susceptibility to anticancer drugs such as Taxol has been reported [22]. As survivin is expressed in a cell cycle–dependent manner and particularly overexpressed when the cells transit through the G2/M phase, and our data revealed specific sensitivity of G1 and relative resistance to STS of the G2/M population, it was therefore of interest to examine the expression of survivin in our system. Low levels of survivin expressed during the G1 phase, but an increased expression of this protein, were observed in the G2/M population. Interestingly, localization studies revealed differences in the expression and distribution of survivin, with G2/M cells showing more intense nuclear staining for survivin and a yellow cytoplasm indicating colocalization with tubulin. G1-arrested cells, however, showed weakly positive staining that was confined to the nucleus. These data assume importance as expression patterns, and the levels of survivin play a crucial role in modulating apoptosis. Studies have reported that survivin plays an important role in microtubule stability and assembly of normal mitotic spindle. This may facilitate checkpoint evasion and promote resistance to chemotherapy in cancer [23]. However, subcellular distribution of survivin has been controversial and variously described as a microtubule-associated protein or chromosomal passenger protein [24,25]. Also there have been reports that survivin may sequester caspases such as 3 and 7 in an inactive state on the microtubules [12].

To perform its antiapoptotic function, survivin requires phosphorylation on threonine 34 by physically interacting with p34 cdc-2 [15]. An inhibition of p34 kinase by cdk inhibitors in mitotically arrested cells results in a loss of survivin levels with dramatic enhancement in chemotherapy-induced apoptosis [26]. It is also known that loss of phosphorylation of survivin results in dissociation of the survivin–caspase 9 complex on the mitotic apparatus leading to apoptosis [15]. On these lines, we examined the expression of cdc-2 in synchronized SK-N-MC cells. We observed an elevated level of this protein in the G2/M-arrested cultures, whereas no expression was detected in G1, suggesting that survivin was phosphorylated by cdc-2 in SK-N-MC cells in a cell cycle–dependent manner to perform its antiapoptotic function.

We further reasoned that if survivin was responsible for protecting G2/M cells against apoptosis by STS, then downregulation of survivin should effectively induce cell death. A dose- and time-dependent decrease in the expression of survivin was observed with antisense oligonucleotide transfection and treatment of these cells with STS resulted in apoptosis, suggesting the role of survivin in protecting cells against apoptosis. The loss of survivin itself in transfected cells did not affect cellular viability, or induce apoptosis as previously reported [27–29]. If the mere presence of survivin was the critical factor in promoting cell survival, then cells arrested in the G1 phase, which showed lower expression of survivin, should have undergone spontaneous apoptosis. In this context, it is noteworthy that only when the cells are challenged with an apoptotic insult does the antiapoptotic role of survivin to sequester the enzymes in an inactive state become important. The findings on the precise mechanism and interaction of survivin with caspases have been contradictory. Studies have suggested that a direct interaction of caspases with survivin is essential to inhibit their activity [12]. A recent report, however, contradicts this theory, wherein survivin does not bind to caspases but exerts its effect by physically interacting with other upstream molecules such as smac/DIABLO to form a complex that leads to the inhibition of downstream caspases in apoptosis induced by Taxol [30]. Marusawa et al. [11] have described that survivin forms complexes with a cellular

protein, hepatitis B X-interacting protein (HBXIP), which binds to procaspase 9, preventing its recruitment to Apaf-1 and suppressing the apoptosis initiated by the mitochondrial pathway. It is therefore not unreasonable to hypothesize that survivin plays a similar role in our system, with higher levels of survivin in the G2/M phase rendering the cells resistant to STS-induced apoptosis. However, the precise mechanism by which survivin enhances resistance in G2/M and the presence of HBXIP-like factor(s) in neuroblastomas remain to be deciphered.

We further studied the pathway that led to STS-induced apoptosis in SK-N-MC cells. In accordance with previous reports in different cell lines [31], we observed that STS induced a mitochondria-dependent pathway for apoptosis. Treatment of SK-N-MC cells with STS led to a sequential breakdown in the membrane potential by 3 hours, followed by caspase 9 activation that peaked at 6 hours, and, finally, the cleavage of PARP by 12 hours. However, studies have reported that apart from caspase 9, other caspases such as caspases 8 and 2, and proteases such as calpain are also involved in apoptosis induced by STS [32–34]. Our data also indicated that activation of caspase 9 was crucial for the induction of apoptosis, as addition of caspase 9 inhibitor in unsynchronized and G1-arrested cells completely ablated the apoptosis induced by STS. Furthermore, there was no increase in caspase 9 activity in STS-treated G2/M populations. These data point to the fact that the activation of caspase 9 alone might be critical for the initiation of the apoptotic process mediated by STS in SK-N-MC cells. In a recent study, enhanced sensitivity of G1 cells and the relative resistance of G2/M cells to a combination therapy of simvastatin and TRAIL were reported in SW480 and H460 cell lines [35], and the addition of caspase 9 inhibitor could only partially inhibit apoptosis. One possible explanation for differences between the findings in this report and our study could be that TRAIL-induced apoptosis involves the activation of caspase 8 besides caspase 9, whereas apoptosis induced by STS in SK-N-MC cells predominantly triggers the apoptotic pathway involving caspase 9 as the primary initiator caspase. Survivin expression is regulated at the transcriptional level with upregulation at G2/M and downregulation at the G1 phase [36], and G1 transcriptional repressor elements within the survivin promoter contribute to the cell cycle–dependent expression of the gene [10]. Interestingly, we found no change in the expression of the survivin transcript in the different phases of cell cycle in SK-N-MC cells.

The ubiquitin–proteosome pathway is important in spatial and temporal regulation of the cell cycle through elimination of proteins regulating cell cycle progression through mitosis. The involvement of this pathway in the regulation of survivin degradation resulting in the decline in G1 phase has been reported using specific inhibitors such as MG132 and AllN [37]. We reasoned that if the role of survivin is restricted to protecting cells on induction of apoptosis, then the retention of survivin by preventing degradation in G1 cells should protect the cells from apoptosis. We investigated the role of the ubiquitin–proteosome pathway in survivin degradation. Our data using proteosome inhibitor MG132 indicated that downregulation of survivin in the G1 phase was indeed due to the degradation of the protein. In the presence of MG132, the G1-phase cells expressed survivin and were resistant to STS treatment and also showed no increase in caspase 9 activity, thereby confirming a direct correlation between the expression of survivin and the activation of caspase 9 in SK-N-MC cells treated with STS.

Taken together, our study demonstrates the differential responses of G1 and G2/M populations in SK-N-MC cells to STS-induced apoptosis. The antiapoptotic protein survivin plays a critical role in imparting resistance to G2/M cells toward STS that induces apoptosis primarily through the mitochondrial–caspase 9–mediated pathway. Therefore, the retention of survivin or the inhibition of caspase 9 led to a complete abrogation of apoptosis induced by STS in G1 cells. The study also shows that survivin appears to exert its antiapoptotic effect by inhibiting caspase 9 activity. With cell cycle–dependent resistance emerging as a new concept in combination sequential chemotherapy, it is envisaged that the results from this study will provide information for the development of tools to facilitate the rational design of chemotherapy combinations.

Acknowledgements

The authors thank Sanjeev Galande for helpful suggestions, Ashwini Atre for providing technical assistance in confocal microscopy, and the late Atul Suple for help with flowcytometric analysis.

References

[1] Raff MC (1992). Social controls on cell survival and cell death. *Nature* **356,** 397–400.

[2] Hickman JA (1996). Apoptosis and chemotherapy resistance. *Eur J Cancer* **32,** 921–926.

[3] Jaattela M (1999). Escaping cell death: survival proteins in cancer. *Exp Cell Res* **248,** 30–43.

[4] Deveraux QL and Reed JC (1999). IAP family proteins—suppressors of apoptosis. *Genes Dev* **13,** 239–252.

[5] Miller LK (1999). An exegesis of IAPs: salvation and surprises from BIR motifs. *Trends Cell Biol* **9,** 323–328.

[6] Li F, Ambrosini G, Chu EY, Plescia J, Tognin S, Marchisio PC, and Altieri DC (1998). Control of apoptosis and mitotic spindle checkpoint by survivin. *Nature* **396,** 580–583.

[7] Islam A, Kageyama H, Takada N, Kawamoto T, Takayasu H, Isogai E, Ohira M, Hashizume K, Kobayashi H, Kaneko Y, and Nakagawara A (2000). High expression of Survivin, mapped to 17q25, is significantly associated with poor prognostic factors and promotes cell survival in human neuroblastoma. *Oncogene* **19,** 617–623.

[8] Adida C, Berrebi D, Peuchmaur M, Reyes-Mugica M, and Altieri DC (1998). Anti-apoptosis gene, *survivin*, and prognosis of neuroblastoma. *Lancet* **351,** 882–883.

[9] Kawasaki H, Altieri DC, Lu CD, Toyoda M, Tenjo T, and Tanigawa N (1998). Inhibition of apoptosis by survivin predicts shorter survival rates in colorectal cancer. *Cancer Res* **58,** 5071–5074.

[10] Altieri DC and Li F (1999). The cancer antiapoptosis mouse *survivin* gene: characterization of locus and transcriptional requirements of basal and cell cycle–dependent expression. *Cancer Res* **59,** 3134–3151.

[11] Marusawa H, Matsuzawa S, Welsh K, Zou H, Armstrong R, Tamm I, and Reed JC (2003). HBXIP functions as a cofactor of survivin in apoptosis suppression. *EMBO J* **22,** 2729–2740.

[12] Shin S, Sung BJ, Cho YS, Kim HJ, Ha NC, Hwang JI, Chung CW, Jung YK, and Oh BH (2001). An anti-apoptotic protein human survivin is a direct inhibitor of caspase-3 and-7. *Biochemistry* **40,** 1117–1123.

[13] Verdecia MA, Huang H, Dutil E, Kaiser DA, Hunter T, and Noel JP (2000). Structure of the human anti-apoptotic protein survivin reveals a dimeric arrangement. *Nat Struct Biol* **7,** 602–608.

[14] Banks DP, Plescia J, Altieri DC, Chen J, Rosenberg SH, Zhang H, and Ng SC (2000). Survivin does not inhibit caspase-3 activity. *Blood* **96,** 4002.

[15] O'Connor DS, Grossman D, Plescia J, Li F, Zhang H, Villa A, Tognin S, Marchisio PC, and Altieri DC (2000). Regulation of apoptosis at cell division by p34cdc2 phosphorylation of survivin. *Proc Natl Acad Sci USA* **97,** 13103–13107.

[16] Abal M, Andreu JM, and Barasoain I (2003). Taxanes: microtubule and centrosome targets, and cell cycle dependent mechanisms of action. *Curr Cancer Drug Targets* **3,** 193–203.

[17] Blagosklonny MV, and Pardee AB (2001). Exploiting cancer cell cycling for selective protection of normal cells. *Cancer Res* **61,** 4301–4305.

[18] Stepczynska A, Lauber K, Engels IH, Janssen O, Kabelizt D, Wesselborg K, and Schulze-Osthoff K (2001). Staurosporine and conventional anticancer drugs induce overlapping, yet distinct pathways of apoptosis and caspase activation. *Oncogene* **20,** 1193–1202.

[19] Shimizu S, Narita M, and Tsujimoto Y (1999). Bcl-2 family proteins regulate the release of apoptogenic cytochrome *c* by the mitochondrial channel VDAC. *Nature* **399,** 483–487.

[20] Shimizu S, Konishi A, Kodama T, and Tsujimoto Y (2000). BH4 domain of antiapoptotic Bcl-2 family members closes voltage-dependent anion channel and inhibits apoptotic mitochondrial changes and cell death. *Proc Natl Acad Sci USA* **97,** 3100–3105.

[21] Li X, Marani M, Mannucci R, Kinsey B, Andriani F, Nicoletti I, Denner L, and Marcelli M (2001). Adenovirus-mediated Bax overexpression for the induction of therapeutic apoptosis in prostate cancer. *Cancer Res* **61,** 1699–1706.

[22] Zaffaroni N, Pennati M, Colella G, Perego P, Supino R, Gatti L, Pilotti S, and Zunino F (2002). Expression of the anti-apoptotic gene *survivin* correlates with Taxol resistance in human ovarian cancer. *Cell Mol Life Sci* **59,** 1406–1412.

[23] Fortugno P, Wall NR, Giodini A, O'Connor DS, Plescia J, Padgett KM, Tognin S, Marchisio PC, and Altieri DC (2002). Survivin exists in immunochemically distinct subcellular pools and is involved in spindle microtubule function. *J Cell Sci* **115,** 575–585.

[24] Skoufias DA, Mollinari C, Lacroix FB, and Margolis RL (2000). Human survivin is a kinetochore-associated passenger protein. *J Cell Biol* **151,** 1575–1581.

[25] Giodini A, Kallio MJ, Wall NR, Gorbsky GJ, Tognin S, Marchisio PC, Altieri DC, and Altieri DC (2002). Regulation of microtubule stability and mitotic progression by survivin. *Cancer Res* **62,** 2462–2467.

[26] Wall NR, O'Connor DS, Plescia J, Pommier Y, and Altieri DC (2003). Suppression of survivin phosphorylation on Thr34 by flavopiridol enhances tumor cell apoptosis. *Cancer Res* **63,** 230–235.

[27] Ambrosini G, Adida C, Sirugo G, and Altieri DC (1998). Induction of apoptosis and inhibition of cell proliferation by *survivin* gene targeting. *J Biol Chem* **273,** 11177–11182.

[28] Shankar SL, Mani S, O'Guin KN, Kandimalla ER, Agrawal S, and Shafit-Zagardo B (2001). Survivin inhibition induces human neural tumor cell death through caspase-independent and -dependent pathways. *J Neurochem* **79,** 426–436.

[29] Chen J, Wu W, Tahir SK, Kroeger PE, Rosenberg SH, Cowsert LM, Bennett F, Krajewski S, Krajewska M, Welsh K, Reed JC, and Ng SC (2000). Down-regulation of survivin by antisense oligonucleotides increases apoptosis, inhibits cytokinesis and anchorage-independent growth. *Neoplasia* **2,** 235–241.

[30] Song Z, Yoa X, and Wu M (2003). Direct interaction between survivin and Smac/DIABLO is essential for the anti-apoptotic activity of survivin during Taxol-induced apoptosis. *J Biol Chem* **278,** 23130–23140.

[31] Tafani M, Minchenko DA, Serroni A, and Farber JL (2001). Induction of the mitochondrial permeability transition mediates the killing of HeLa cells by staurosporine. *Cancer Res* **61,** 2459–2466.

[32] Tang D, Lahti JM, and Kidd VJ (2000). Caspase-8 activation and bid cleavage contribute to MCF7 cellular execution in a caspase-3–dependent manner during staurosporine-mediated apoptosis. *J Biol Chem* **275,** 9303–9307.

[33] Lopez E and Ferrer I (2000). Staurosporine- and H-7–induced cell death in SH-SY5Y neuroblastoma cells is associated with caspase-2 and caspase-3 activation, but not with activation of the FAS/FAS-L–caspase-8 signaling pathway. *Brain Res Mol Brain Res* **85,** 61–67.

[34] Neumar RW, Xu YA, Gada H, Guttmann RP, and Siman R (2003). Cross-talk between calpain and caspase proteolytic systems during neuronal apoptosis. *J Biol Chem* **278,** 14162–14167.

[35] Jin Z, Dicker DT, and Deiry WS (2002). Enhanced sensitivity of G1 arrested human cancer cells suggests a novel therapeutic strategy using a combination of simvastatin and TRAIL. *Cell Cycle* **1,** 82–89.

[36] Li F and Altieri DC (1999). Transcriptional analysis of human *survivin* gene expression. *Biochem J* **344,** 305–311.

[37] Zhao J, Tenev T, Martins LM, Downward J, and Lemoine NR (2000). The ubiquitin–proteasome pathway regulates survivin degradation in a cell cycle–dependent manner. *J Cell Sci* **113,** 4363–4371.