Nuclear Survivin Abrogates Multiple Cell Cycle Checkpoints and Enhances Viral Oncolyis

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Abstract
Survivin (BIRC5) promotes cell division and survival with roles as chromosomal passenger protein and inhibitor of apoptosis protein (IAP). It is overexpressed in many cancers and is associated with resistance to chemotherapy and radiation. Previously, we showed that expression of survivin within the nucleus of HeLa cells accelerates its degradation and blocks apoptosis inhibition without affecting localization during mitosis. Here, we have investigated the effects of survivin on cell cycle control and potential therapeutic consequences using HeLa and IGROV1 cells expressing wild-type and nuclear-targeted survivin. We show that overexpression of survivin, especially within the nucleus, increases control over G1-S checkpoint via increased nuclear accumulation of cyclin D and cyclin-dependent kinase 4 and subsequent pRb phosphorylation. We investigated the influence of survivin on the activity of the E1A CR2-deleted oncolytic adenovirus d922-947, which depends critically on an aberrant G1-S checkpoint. Nuclear expression of survivin augments virus-induced S-phase induction and increases viral protein expression and overall viral replication. There is a consequent increase in antitumor activity both in vitro and in vivo. The increased d922-947 activity is restricted to malignant cells and is not associated with induction of apoptosis, nor does it rely on the role of survivin as an IAP. In addition, we observe the appearance of a large ≥4N population coincident with multiple mitotic defects in d922-947-infected cells, both of which are significantly increased by nuclear survivin. This indicates that adenoviral activity is facilitated by abrogation of multiple cell cycle checkpoints and can be enhanced by expression of survivin within the nucleus. [Cancer Res 2008;68(19):7923–31]

Introduction
Survivin is a member of the BIR-containing inhibitor of apoptosis protein (IAP) family, as well as being a chromosomal passenger protein whose selective expression during late G2-M enables it to mediate mitotic and cytokinetic fidelity (reviewed in ref. 1). The importance of survivin in inhibiting cell death and promoting cell proliferation is emphasized by its overexpression in many human tumors, often accompanied by aberrant expression throughout interphase. Expression of survivin in G1-S phase is associated with enhanced pRb phosphorylation, an increase in the proportion of cells in S phase, and resistance to G1 arrest (2), indicating the multiple effects that survivin has throughout the cell cycle. We and others have recently shown that nuclear relocalization of survivin precludes any antiapoptotic activity during interphase (3–7). It has also been shown that survivin induces S-phase entry by interacting with cyclin-dependent kinase (cdk) 4, which leads to inactivation of pRb. This suggests a true nuclear function of survivin (2).

Selectively replicating adenoviruses have shown considerable promise as antitumor agents by means of their ability to infect and replicate specifically within malignant cells, leading to cell death and viral amplification at the tumor site. The adenoviral mutant d922-947 contains a 24-bp deletion within the E1A CR2 region, a region responsible for facilitating S-phase entry by disrupting the interaction between host cell pRb and E2F. As a consequence, d922-947 replicates only in cells with a deficient G1-S checkpoint (8, 9). We have shown its therapeutic potential in ovarian cancer (9), and a phase I trial in women with relapsed ovarian cancer is imminent. Efficient production of viral progeny necessitates profound modulation of host cell gene transcription (10, 11). The adenovirus must force the cell into S phase to ensure optimal conditions for viral replication while counteracting antiviral defense and cell death mechanisms. The necessity for this S-phase induction and subversion of normal cell cycle control led us to investigate the influence that survivin expression may exert over the activity of d922-947.

Here, we report that promoting nuclear import of survivin by addition of a nuclear localization signal (NLS) accentuates the control it exerts over the G1 checkpoint. This feature can be exploited to improve the efficacy of oncolytic adenoviruses by increasing S-phase entry, a prerequisite for viral replication, and by promoting subsequent progression through the later stages of infection. Such progression into aberrant mitoses results in a reconstitution of the interphase nucleus and reinitiation of DNA replication. This culminates in prolific viral replication and virion production, combined with enhanced oncolytic potency. This exposes a therapeutic opportunity by which overexpression of survivin in tumors may be exploited for oncolytic viral therapy. It also suggests that multiple cell cycle checkpoints need to be subverted for optimal viral activity.

Materials and Methods
Cell culture, cell viability, and viral replication assays. HeLa and IGROV1 cells were maintained at 37°C with 5% CO2 in DMEM, supplemented with 10% FCS, penicillin/streptomycin, and fungizone. MRC5 cells were maintained in DME supplemented with 15% FCS. The generation of stable HeLa cell lines expressing green fluorescent protein (GFP), survivin-GFP, and nuclear-localized survivin has been described previously (3, 5, 12, 13). Cells were sorted by GFP positivity using an LSRII fluorescence-activated cell sorter (BD Biosciences) to ensure homogeneity
before use. IGROV1 and MRC5 cells were transfected with pcDNA3.1 constructs using FuGene 6 and selected with 1 mg/ml (IGROV1) or 0.3 mg/ml (MRC5) of G418 for one passage only before use. For cell viability assays, 2 × 10^5 cells were infected with d922-947 in serum-free medium at multiplicities of infection (MOI) of 0.01 to 1,000 plaque-forming units (pfu)/cell. After 2 h, cells were re-fed with medium containing 5% FCS. Cell viability was assayed up to 120 h later by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay using a SpectraMax Gemini fluorimeter (Molecular Devices). All viability assays were done in triplicate and experiments were repeated at least twice. Representative results are shown unless otherwise stated. For small interfering RNA (siRNA) experiments, cells were transfected with 60 pmol of double-stranded RNA (Qiagen) using siPORT NeoFX (Ambion). Cells were infected with virus 4 h after addition of RNA interference in serum-free medium and re-fed 2 h later, at which point knockdown was confirmed by immunoblotting with anti-survivin antibodies (R&D Systems). Viability was assessed by MTT after 96 h.

**Immunoblotting.** Protein lysates were electrophoresed on SDS-polyacrylamide gels and transferred onto nitrocellulose membranes with a semidyblotting system. Antibody binding was visualized using enhanced chemiluminescence (GE Healthcare). Antibodies used were anti-survivin (R&D Systems), anti-tubulin (BS12 Sigma-Aldrich), anti-E1A (Santa Cruz Biotechnology), anti-cyclin E (BD Biosciences), anti-cyclin D (BD Biosciences), anti-cdk4 (BD Biosciences), anti-pIκBα (H1; Cell Signaling Technology), anti-phosphorylated pRb (Ser807/811; Cell Signaling Technology), anti-adenovirus (Abcam), and anti-actin (Santa Cruz Biotechnology).

**Fluorescence microscopy and live cell imaging.** Cells were grown on poly-l-lysin–coated coverslips and then fixed with 5% formaldehyde (Service for 5 min and permeabilized with 0.15% Triton for 2 min. Primary antibody binding was visualized using Texas red–conjugated secondary antibodies (Vector Laboratories). Coverslips were mounted in 4',6-diamidino-2-phenylindole (DAPI)-containing Vectashield and viewed using an inverted Olympus microscope (numerical aperture, 1.35). Images were captured using a Hamamatsu charge-coupled device camera and Delta Vision Spectris software (Applied Precision). Jpeg images were prepared as three-dimensional projections of deconvoluted z-stacks. For live cell imaging, cells were infected (MOI, 10) and both phase-contrast and GFP images were taken every 40 min using an inverted Leica DMIRB microscope heated at 37°C and a 20× objective operated with Simple PCI software. Live cell imaging movies were scored by quantifying the number of cells dying during interphase, the number entering mitosis, and the number becoming polyplody in five sequences from 0 to 96 h post infection (pi) view on 20× fields.

**Flow cytometry.** Cells were infected with d922-947, trypsinized, washed twice in ice-cold PBS, and fixed in 70% ethanol. Cells were then washed with PBS and resuspended in 200 μL of 50 μg/mL propidium iodide and 100 μg/mL RNase A (MP Biomedicals). Cells were analyzed with a FACScan cytometer using CellQuest software (Becton Dickinson).

**In vivo analysis.** HeLa GFP, survivin-GFP, or survivin-NLS1 cells (5 × 10^4) were inoculated s.c. onto the right flank of female BALB/c nude mice on day 1. Once tumors reached ~ 200 mm^3 (day 25), d922-947 was injected intratumorally into size-matched tumors (3 × 10^5) particles per day for 3 consecutive days). Tumor size was measured weekly using calipers and the E1A region were as follows: sense primer, 5'-ATGGATTTAGACGTGACGGCC. PCR conditions were as follows: 50°C for 2 min, 95°C for 10 min, followed by 40 cycles of 95°C for 15 s and 60°C for 60 s. A standard curve using 10^6 to 10^9 viral DNA genomes was used for quantification. For TCID50 assays, 10^5 cells were infected with d922-947 (MOI, 10) and harvested up to 72 h later into 0.5 mL Tris (pH 8.0). Intracellular virus was released by three rounds of freeze/thaw (liquid nitrogen/37°C) and titrated on JH293 cells by serial dilution.

**Results**

Nuclear survivin is associated with increased phosphorylation of pRb. It has previously been shown that survivin promotes G1-S transition by interaction with cdk4, with subsequent activation of cdk2/cyclin E–mediated pRb phosphorylation (2). This transition is associated with a redistribution of survivin from the cytoplasm to the nucleus. Therefore, we hypothesized that promoting nuclear accumulation of survivin may enhance its influence over the G1 checkpoint. We have previously shown that fusion of survivin to either of two NLS sequences, the bipartite LANA sequence RHERPETTRIRHRKLRS or the monopartite SV40 T-antigen NLS sequence PKKRRKV (hereafter referred as survivin-NLS1 and survivin-NLS2, respectively), enhances the nuclear import of survivin and is sufficient to relocalize the steady-state pool from the cytoplasm to the nucleus (3). To assess what effect survivin localization may exert over the G1 checkpoint, we immunoblotted whole-cell extracts from asynchronous populations of HeLa cells expressing GFP, survivin-GFP, survivin-NLS1, or survivin-NLS2 with specific G1- and S-phase markers (Fig. 1). Cells expressing survivin-GFP exhibit increased levels of phosphorylated and total pRb compared with control cells expressing GFP alone (Fig. 1A). Interestingly, cells expressing survivin-NLS1 (Fig. 1A) or survivin-NLS2 (data not shown) exhibit a further increase in phosphorylated pRb. Likewise, elevated levels of cyclin D are found in survivin-GFP–expressing cells compared with control GFP cells, and this effect is enhanced in survivin-NLS1 and survivin-NLS2 cells. In contrast, levels of cyclin E are not discernibly different between the cell lines (Fig. 1A), indicating a specific up-regulation of the D-type G1 cyclin. To assess whether these changes are cell type specific, we immunoblotted pooled populations of IGROV1 cells expressing GFP, survivin-GFP, or survivin-NLS1 (Supplementary Fig. A). The amount of phosphorylated pRb is greatly increased in cells expressing survivin-GFP.

RNA extraction and relative quantitative PCR. RNA was extracted from 10^6 asynchronous log-phase HeLa cells using the RNAsque kit (Ambion). RNA samples were incubated for 1 h at 37°C with RNase-free DNase (Promega) to eliminate any contaminating DNA. After inactivation of the DNase (70°C for 10 min), RNA was precipitated with 1 volume of isopropanol and then resuspended in RNase-free water. Each sample (4 μg) was used for cDNA synthesis using a First-Strand cDNA Synthesis kit (GE Healthcare). The cyclin D primers used were 5'-AAGATCGTGGC-CACCTGG-3' (forward) and 5'-CCAGGGGAAAGCTCTTCT-5' (reverse). The 6-FAM probe was 6-TGCTGGAGGTGTGCGAGAAAGATG. Eukaryotic control 18S primers were used as per the manufacturer's instructions (Applied Biosystems). PCR conditions were as above. Transcript levels were normalized to 18S control.

**Apoptosis assay.** To determine apoptotic activity, tetrapedate cleavage assays were performed in a 96-well plate, as previously described (3). Briefly, cells were infected with d922-947 (MOI, 10) and lysed in mammalian protein extraction buffer (Piecer) supplemented with 1 mmol/L EDTA, 1 μg/mL pepstatin A, and 1 mmol/L 4-(2-aminoethyl)-benzenesulfonyl fluoride. Lysates were then incubated with 25 μg/mL caspase-3–specific tetrapeptide substrate (DEVD-AMC; Biomol) at 37°C for 1 h. Relative fluorescence was measured using a SpectraMax Gemini fluorimeter with excitation set at 380 nm and emission at 440 nm. As positive control, cells were treated with 250 μg/mL recombinant human tumor necrosis factor–related apoptosis-inducing ligand (rHTRAIL; PeproTech EC Ltd.) for 90 min and analyzed as above.

**Statistical analyses.** All graphs and statistical analyses were generated using Prism4 for Mac (GraphPad). Unless otherwise stated, all statistical analyses are unpaired, two-tailed Student's t test, where P < 0.05 is considered statistically significant.

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compared with control GFP cells. Furthermore, similar levels of phosphorylated pRb are seen in the survivin-GFP and survivin-NLS cells despite the lower levels of survivin expression, indicating that the effects of nuclear survivin on the G1-S checkpoint are not cell type specific.

Quantitative reverse transcription-PCR revealed no difference in the relative abundance of cyclin D mRNA between the different cell lines (data not shown), indicating that the up-regulation of cyclin D must be posttranscriptional. As cyclin D is degraded in the cytoplasm (14), we assessed whether the localization of cyclin D varies between the different cell lines. As shown in Fig. 1A, cyclin D is distributed throughout both the cytoplasm and nucleus in GFP and survivin-GFP cells. However, in survivin-NLS cells, cyclin D is predominantly nuclear, a localization that has previously been associated with cyclin D stabilization (14). We also detected a concomitant increase in the nuclear localization of cdk4 (Fig. 1C). Therefore, expression of nuclear survivin seems to induce an increase in nuclear localization of both cyclin D and its catalytic subunit cdk4.

Nuclear import of survivin facilitates virus-induced G1-S transition and subsequent progression through the cell cycle. The adenoviral mutant dl922-947 has shown therapeutic potential by its ability to infect and replicate specifically within malignant cells (9). S-phase induction is essential for viral replication, and we hypothesized that overexpression of survivin, with consequent up-regulation of cyclin D and phosphorylated pRb, may enhance viral efficacy by subversion of G1-S checkpoint control. Cells were infected with dl922-947 and harvested up to 72 h later for cell cycle analysis. When uninfected, the proportion of cells in S phase are similar in all three sublines (GFP, 12.8%; survivin-GFP, 13.0%; survivin-NLS, 15.0%) and survivin expression has no effect on cell proliferation (Supplementary Fig. B). Following dl922-947 infection, there is a progressive reduction in G1-phase cells with a concomitant increase in S and G2-M phases in all three lines, together with a notable increase in cells with >4N DNA at later time points. These effects are most pronounced in cells expressing survivin-NLS1; by 48 h pi, only 9.6% of NLS1 cells remain in G1 compared with 21.8% for survivin-GFP (P ≤ 0.005) and 19.0% (P ≤ 0.005) for GFP. At the same time point, 67.1% of survivin-NLS cells had a ≥4N DNA content compared with 48.8% (P ≤ 0.005) of survivin-GFP cells and 43.2% (P ≤ 0.005) of GFP cells (Fig. 2A). Expression of nuclear survivin can therefore greatly enhance S-phase induction and subsequent progression through the cell cycle following adenoviral infection.

To account for the accumulation of cells with ≥4N DNA, and to address concerns that viral DNA could confound the analysis of fluorescence-activated cell sorting (FACS) DNA profiling, we imaged cells by time-lapse microscopy following viral infection to monitor their fate. Figure 2B shows a mock-infected HeLa survivin-NLS1 cell as it transits through mitosis; the cell rounds up (0 min) and proficiently aligns its chromosomes in metaphase (40–80 min; survivin-NLS1 localizes to the centromeres) followed by initiation of the cleavage furrow (120 min), formation of the midbody (160 min; note survivin-NLS1 colocalization), and cytokinesis, with the daughter cells settling down by 200 min. In contrast, when infected with dl922-947, cells experience several mitotic defects, including a failure to congress their chromosomes, aberrant multipolar spindles, and a failure in midbody abscission and cleavage furrow regression. This may result in a single, polyploid micronucleated cell (Fig. 2C), or binucleation, often following a protracted metaphase arrest of up to 280 min (Supplementary Fig. C).
defects are observed in all cell lines but are most notable in the survivin-NLS lines, as assessed by FACS profiling (Fig. 2A). Similarly, quantification of live imaging movies of survivin-GFP and survivin-NLS1 cells taken following infection with dl922-947 showed that survivin-GFP cells were more likely to die during interphase, whereas the NLS1 cells had a significantly greater propensity to enter mitosis and become polyploid (Supplementary Fig. D). These mitotic defects partially resemble those reported for the E1B55K-deleted adenovirus dl1520 (ONYX-O15; ref. 15) and indicate that dl922-947 overrides multiple checkpoints, including G1-S, G2-M, and spindle. Cell cycle analysis and live cell imaging performed on parental IGROV1 cells yielded similar results, again indicating that these effects were not cell type specific (data not shown).

We next used immunofluorescence microscopy to characterize the multinucleation phenotype in more detail. Cytokinetic failure coincides with residual chromatin bridges linking daughter nuclei (Fig. 3A; arrows denote internuclear bridges between dividing cells; note also the persistent survivin-NLS1 staining at these nuclear bridges). Thus, both karyokinesis and cytokinesis are impaired following viral infection. Over time, this defective cell division leads

**Figure 2.** Nuclear survivin expression promotes cell cycle progression and mitotic entry following adenoviral infection. A, cells were infected with dl922-947 (MOI, 10 pfu/cell) and harvested up to 72 h later and stained with propidium iodide for cell cycle analysis. B and C, HeLa survivin-NLS1 cells were grown on glass-bottomed culture dishes and followed by time-lapse imaging. Images shown represent 40-min intervals. B, a mock-infected cell transiting through mitosis. C, typical aberrant mitoses observed following infection with dl922-947 (MOI, 10); images are shown from 9 h pi. Bar, 15 μm (C).
to the development of syncytia-like cells containing multiple nuclei (Fig. 3B). Following viral infection, GFP cells exhibit an increase in the levels of phosphorylated pRb, peaking at 24 h pi but plateauing thereafter (Fig. 3C). Similarly, cells expressing survivin-NLS also displayed an increase in phosphorylated pRb 24 h pi; however, levels were considerably greater than those within GFP cells throughout the time course (Fig. 3C). This raises the possibility that, in addition to initial S-phase entry, differential pRb

**Figure 3.** Nuclear survivin expression promotes multinucleation and sustained pRb phosphorylation. A and B, survivin-NLS1 cells were grown on poly-L-lysine–coated coverslips, fixed, and stained up to 72 h following infection with dl922-947 (MOI, 10). Cells were probed for tubulin and counterstained with DAPI to visualize the nuclei. A, arrows, failure in karyokinesis 24 h pi. Note persistent survivin-NLS staining at the remnant midbody. B, multinucleation at later stages of infection. Note also the karyokinetic failure in panel 2. Bar, 15 μm. C, HeLa survivin-NLS1 cells were infected with dl922-947 (MOI, 10). Protein lysates were harvested up to 72 h pi, separated by SDS-PAGE, and analyzed by immunoblot for expression of phosphorylated pRb.
phosphorylation may account for accelerated progression through the cell cycle at later stages of infection.

**Accelerated cell cycle progression enhances viral protein expression and viral replication.** Following infection with dl922-947, there is earlier expression of E1A, the key activator of viral transcription, in both survivin-GFP and survivin-NLS cells than in GFP cells. In addition, E1A expression continues for at least 48 h in survivin-NLS cells but declines rapidly in survivin-GFP cells (Fig. 4A). Quantitative PCR using both E1A and hexon region primers was used to analyze replication of the viral genome. Cells were infected with dl922-947 (MOI 10) and DNA was extracted 30 min and 24, 48, and 72 h pi. As viral replication is negligible before 1 h pi, intracellular E1A and hexon gene copy number 30 min pi is a measure of infectivity. There was no difference in infectivity at this early time point between the cell lines, showing that survivin does not affect viral uptake (data not shown). Conversely, from 24 h pi, the quantity of viral DNA increased to a greater extent in the survivin-NLS1 cells compared with the GFP control cells, with the survivin-GFP cells exhibiting an intermediate phenotype: at 48 h pi, E1A gene copy number was 3.03 \( \times \) 10^5 per cell in survivin-NLS1 cells compared with 1.42 \( \times \) 10^5 and 1.99 \( \times \) 10^5 for GFP and survivin-GFP, respectively \((P < 0.001, \text{NLS1 versus either GFP or survivin-GFP; Fig. 4B})\). Similarly, expression of the viral structural proteins is more pronounced at early time points in survivin-NLS1 cells compared with GFP controls (Fig. 4A). Production of infectious virion progeny was analyzed at 24, 48, and 72 h pi by TCID50 assay. Virus output exceeded the 10 pfu/cell input dose by 48 h in all cell lines, but production was significantly higher in survivin-NLS1 cells compared with both GFP \((P < 0.0001)\) and survivin-GFP \((P = 0.015)\) cells at both 48 and 72 h pi (Fig. 4C).

**Expression of survivin-NLS enhances cytopathic effect of adenovirus dl922-947 in tumor cells but not normal cells.** The cytopathic effect of dl922-947 was analyzed by MTT assay 120 h pi. The IC50 for HeLa-GFP cells is 31.5 pfu/cell compared with 18.8 pfu/cell for survivin-GFP–expressing cells, 2.3 pfu/cell for survivin-NLS1 cells, and 2.9 pfu/cell for survivin-NLS2 cells (Fig. 5A). A similar trend of sensitization to virus cytocytotoxicity is seen in pooled populations of transiently transfected IGROV1 cells expressing survivin-NLS1 (IC50 2.9 pfu/cell) compared with GFP (IC50 6.9 pfu/cell) and survivin-GFP (IC50 8.1 pfu/cell), although these differences are less marked than in the single-cell HeLa clones (Fig. 5B).

dl922-947 has been shown to replicate specifically in human cancer cells but not in epithelial cells that contain an intact retinoblastoma pathway (8, 9). Based on the ability of nuclear survivin to promote S-phase entry following infection of HeLa (human papillomavirus E6/7 transformed) and IGROV1 (mutant p53, deficient p16 expression) cancer cells, we assessed whether expression of survivin or survivin-NLS could exert the same effect on cells with a fully intact G1-S checkpoint. MRC5 embryonic lung fibroblasts were transfected with GFP, survivin-GFP, or survivin-NLS and subjected to an MTT cytotoxicity assay following a single passage of selection in G418. Despite expression of the constructs (Supplementary Fig. E), it was not possible to detect any phosphorylated pRbin any MRC5 population under the same immunoblotting conditions as used for HeLa and IGROV1 cells (data not shown) and the expression of survivin-GFP or survivin-NLS exerted no effect on sensitivity to dl922-947 (Fig. 5C).

To test whether the observed *in vitro* sensitization to adenovirus in tumor cells can be recapitulated *in vivo*, female BALB/c nude mice were injected s.c. with 5 \( \times \) 10^6 HeLa GFP, survivin-GFP, or survivin-NLS cells on day 1. On days 25 to 27 inclusive, dl922-947 infection was assessed up to 72 h pi by TCID50 assay, as detailed in Materials and Methods. Dotted line, input dose (10 pfu/cell).
(3 × 10^9 particles per day) was injected intratumorally into size-matched tumors. Whereas the HeLa GFP and survivin-GFP tumors continue to grow following dl922-947 injection, growth of survivin-NLS1 tumors is significantly impeded, an effect sustained for the duration of the experiment (Fig. 5D). Thus, expression of nuclear survivin can facilitate a favorable response to oncolytic viral therapy both in vitro and in vivo.

**Apoptosis is not the primary mode of cell death.** Our recent work argues that cytotoxicity induced by oncolytic adenoviruses does not involve the classic pathways of apoptosis, autophagy, and necrosis (16). Nuclear survivin is associated with p53 and Bax induction (17), and so, to assess whether nuclear survivin promotes apoptosis following viral infection, cells were infected with dl922-947 and caspase-3 activity measured in a fluorogenic tetrapeptide cleavage assay. As shown in Fig. 6A, there is no increase in caspase-3 activity in virus-infected cells up to 96 h pi compared with cells treated with rhTRAIL. Overexpression of survivin-NLS1 does not affect this. In addition, overexpression of survivin T34A, the proapoptotic mutant that cannot be phosphorylated by p34(cdc2) (18), has no effect on dl922-947 activity (data not shown). Therefore, these results indicate both that apoptosis does not contribute to the cytotoxicity of adenovirus dl922-947 in cells expressing nuclear survivin and that the sensitization to dl922-947 mediated by nuclear survivin does not involve its function as an IAP.

Reduced levels of survivin do not affect viral efficacy. We have previously shown that nuclear-localized survivin is subject to accelerated proteasomal degradation (3). This raises the possibility that the effects seen following expression of survivin-NLS1 result from enhanced degradation of endogenous survivin. To assess this possibility, we assessed the efficacy of dl922-947 following depletion of endogenous survivin in HeLa GFP cells by siRNA. There is no significant difference in IC50 value between cells pretreated with survivin siRNA and those pretreated with control siRNA (Fig. 6B). Therefore, the observed sensitivity to dl922-947 of cells expressing nuclear survivin does not result from depletion of the endogenous protein.

**Discussion**

Our recent work has shown that cytoprotection by survivin during interphase is dependent on cytoplasmic localization (3). Conversely, release from G1 arrest is associated with nuclear translocation of survivin, and strong expression of survivin has been shown to increase the proportion of cells in S phase by augmenting cdk4 release from its inhibitor p16ink4a (2). This suggests that nuclear localization is important for the proliferative effects of survivin. By promoting nuclear import, we confirm that survivin facilitates S-phase entry by subversion of G1-S checkpoint.
control. Cyclin D and its catalytic partners cdk4/6 promote G1-S transition through phosphorylation of pRb and through titration of cell cycle inhibitors p27KIP and p21CIP1. Cyclin D is degraded during S phase through glycogen synthase kinase 3β–mediated phosphorylation on Thr286, which promotes CRM1-mediated nuclear export and subsequent cytoplasmic proteasomal degradation (14). We show that nuclear survivin is associated with a posttranscriptional up-regulation of cyclin D levels and redistribution of both cyclin D and cdk4 to the nucleus, suggesting that cyclin D is stabilized through evasion of cytoplasmic proteasomal degradation. D-type cyclins are considered to be the mitogen sensors for G1 progression. Although structurally related, they are functionally heterogeneous and can promote both phosphorylation-dependent (19) and phosphorylation-independent (20) repressive effects on pRb. Nuclear survivin sensitizes cells to S-phase induction by promoting pRb inactivation, which allows elevated levels of free E2F to act as the potent transcriptional activators needed for the G1-S transition. These results complement the previous observation that survivin translocation to the nucleus correlates with tumor cell dedifferentiation (21), and are also wholly compatible with subsequent relocation to the cytoplasm, as seen in aging normal human lung fibroblasts, which provides resistance to apoptosis (17).

We have previously shown that the E1A CR2 adenoviral mutant dl922-947 has potential as an anticancer agent (9). The requirement for S-phase induction for efficient viral replication led us to investigate whether nuclear expression of survivin would affect viral efficacy. We found that nuclear survivin facilitates accelerated S-phase entry, which in turn promotes early viral replication, protein expression, and virion production. This is accompanied by increased cytotoxicity and significant antitumor activity in vivo.

The mechanisms triggering cell death following infection by adenovirus mutants have remained elusive. Our recent work argues against well-characterized modes of cell death, including apoptosis, autophagy, and necrosis (16). Based on the association of nuclear survivin with p53 and Bax expression (17), it was conceivable that nuclear survivin could predispose to apoptotic induction following infection. However, caspase activation was negligible in all cell lines, indicating that apoptotic induction was not augmenting or even causing cytotoxicity. Similarly, inhibition of survivin IAP function had no effect on viral activity. Nuclear survivin also did not affect viral efficacy through reducing the stability of survivin (3).

Our data show that following infection with dl922-947, cells are not only driven to enter S phase but continue to progress through the cell cycle and enter into aberrant mitosis leading to an accumulation of cells with ≥4N DNA. Recent gene expression analysis has confirmed that infection of normal human cells with group C adenoviruses causes changes in the expression of vast numbers of genes (at least 1,000–2,000) and that many of these genes are involved in the control of the cell cycle (10, 11). Genes related to S-phase induction (including cyclins D2 and E1/2, cdk4, and cdk2) were prominent, and our results are entirely in keeping with this, emphasizing the importance of the G1-S transition for viral function. However, significant changes were also observed in genes associated with mitosis, including AuroraB kinase and cyclin B2. Likewise, our data support an induction of mitosis following infection of both HeLa and IGROV1 cells, which is similarly augmented by nuclear expression of survivin. Defects were observed in chromosome congression and alignment leading to karyokinetik failure, which, together with incomplete midbody abscission, engendered multinucleation. We addressed the possibility of viral...
DNA accounting for the ≥4N DNA content by live cell imaging, which clearly shows mitotic entry following viral infection. We also acknowledge that the accumulation of viral DNA within cells may be a contributory factor at later time points; however, FACS analysis of HeLa cells 4 h pi with 10^5 particles of dl922-947 showed no ≥4N population, suggesting again that the changes seen at later time points of viral infection reflect virus-induced anomalous host cell mitosis (data not shown). The enhanced accumulation of cells with ≥4N in the presence of nuclear survivin is associated with sustained phosphorylated pRb throughout the time course of infection. Such pRb phosphorylation indicates an environment permissive for reinitiation of DNA replication such that, by altering cell cycle controls, nuclear survivin may maximize time spent in viral DNA replication during infection and also increase the number of cells able to replicate viral DNA; cells infected during G2, for example, will pass through the aberrant mitosis to enter a pseudo-G1 from which DNA replication can be initiated. Additionally, the formation of synctia has been shown to facilitate viral dispersion and virion release (22), possibly contributing to the observed viral efficacy in cells expressing nuclear survivin.

The specific replication of dl922-947 in cells with a defective pRb checkpoint is essential for maximizing the therapeutic index in vivo. Our data show that, although expression of survivin can facilitate G1-S transition following infection and increase cytotoxicity in cancer cells, survivin cannot exert such function in the context of fully intact G1-S checkpoint control, as found in normal, nontransformed cells. We believe that survivin exerts its effects via a protein that is aberrantly expressed in cancer; for example, the promotion of cyclin D/cdk4 nuclear import will have greater effect in tumor cells, which express these proteins at high levels. It is also likely that the action of survivin on promoting proliferation through the cdk4 pathway is counteracted in normal cells by the repertoire of redundant pathways to ensure G1-S arrest, pathways commonly lost during the process of transformation. This presents an exciting opportunity whereby the expression of nuclear survivin in combination with dl922-947 can be used, firstly, to increase the cytotoxicity of dl922-947 in tumor cells and, secondly, to improve the therapeutic index in vivo by further differentiating the sensitivity of tumor versus normal cells.

The complementary roles of survivin in cell proliferation (2), mitotic fidelity, and inhibition of apoptosis make it an attractive therapeutic target. Molecular antagonists of survivin are in phase I clinical trials (23). The results presented here indicate that the overexpression of survivin in tumor cells can also be exploited for oncolytic viral therapy. Nuclear survivin facilitates rapid S-phase induction, prolific viral replication, and virion production, combined with subsequent cell cycle progression and mitotic catastrophe. These culminate in superior oncolytic potency in vivo and translate into significant activity in vivo. The results reemphasize the importance of G1-S control in the biology of adenoviruses and also indicate that other stages of the cell cycle are effectively targeted. Through a greater understanding of adenoviral control of mitosis, further augmentation of their activity will be possible.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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References


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Claire M. Connell, Sally P. Wheatley and Iain A. McNeish