Separating the Anti-apoptotic and Mitotic Roles of Survivin*

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Survivin is a bifunctional protein that acts as a suppressor of apoptosis and has an essential role in mitosis. To date whether these two functions can be divorced has not been addressed. Here we show that the linker region between the BIR (baculovirus inhibitor of apoptosis repeat) domain of survivin and COOH-terminal α helix may be the key to separating its roles. When overexpressed survivin is present in interphase cells and shuttles between the cytoplasm and nucleus. Here we identify a REV-like nuclear export signal (NES) in the central domain of survivin and demonstrate that point mutations within this region cause accumulation of survivin in the nucleus. Interestingly cells expressing NES mutants exhibit reduced survival after X-irradiation. Moreover, cells expressing survivinL98A-green fluorescent protein (GFP) showed increased poly(ADP-ribose) polymerase-cleavage and caspase-3 activity after tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) treatment compared with cells expressing full-length survivin-green fluorescent protein. These data suggest a direct link between the interphase localization of survivin and cellular responsiveness to apoptotic stimuli. Using a cell proliferation assay, we also found that ectopic expression of NES mutants can complement for depletion of endogenous survivin, indicating that they can execute the mitotic duties of survivin. Thus we demonstrate for the first time that 1) survivin has a functional NES; 2) nuclear accumulation of overexpressed survivin correlates with increased sensitivity of cells to ionising radiation; and 3) the anti-apoptotic and mitotic roles of survivin can be separated through mutation of its NES. Separating these two functions of survivin could open up new possibilities for therapeutic strategies aimed at eliminating cancer cells yet preserving normal cell viability.

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**The abbreviations used are: GFP, green fluorescent protein; BIR, baculovirus inhibitor of apoptosis repeat; CRM1, chromosome region maintenance-1; DAPI, 4',6-diamidino-2-phenylindole; LMB, leptomycin B; NES, nuclear export signal; PARP, poly(ADP-ribose) polymerase; AMC, aminomethylcoumarin; GST, glutathione S-transferase; RNAl, RNA interference; dsRNA, double-stranded RNA; Gy, gray; TRAIL, tumor necrosis factor-related apoptosis-inducing ligand.

EXPERIMENTAL PROCEDURES

Cloning and Cell Culture—Site-directed mutagenesis was performed using Quikchange (Stratagene) on full-length human survivin with a COOH-terminal GFP tag in pBluescript, then constructs were subcloned into pcDNA3.1 for expression in mammalian cells.

HeLa and U2OS cells were maintained at 37°C with 5% CO2 in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal calf serum, penicillin/streptomycin, and fungizome. pcDNA3.1 constructs were transfected into HeLa cells using FuGENE 6 (Roche Diagnostics). To create cell lines stably expressing the desired constructs, transfected cells were treated with 500 μg/mL G418, and GFP-positive colonies were selected 7–10 days post-transfection. To ensure homogeneity prior to analysis, clones were sorted by GFP-positive expression using an LSRII fluorescence activated cell sorter (BD Bioscience; see Ref. 1). All tissue culture reagents were supplied by Invitrogen.

Fluorescence Microscopy—Cells were grown on poly-L-lysine coated coverslips then fixed with 4% formaldehyde and permeabilized with 0.15% Triton as described previously (3). Cells were viewed using an inverted Olympus microscope fitted with a ×63 (NA 1.35) oil immersion objective. Images were captured using a Hamamatsu CCD camera and DeltaVision Spectris software (Applied Precision), and snap shot jpeg images were prepared from three-dimensional projections of deconvolved stacks.

Leptomycin B and TRAIL Treatments—To inhibit CRM1-mediated export from the nucleus, exponentially growing cul-
FIGURE 1. Ectopically expressed full-length survivin is cytoplasmic due to a CRM1-dependent NES in its central domain. HeLa cells stably expressing full-length survivin-GFP (A and B), survivin_{L98A}-GFP (D and E), or survivin_{T97E}-GFP (G and I) were incubated for 2 h in the absence (A, D, G) or presence (B, E, H) of 6 ng/ml LMB, then fixed and stained with DAPI (blue). Bar represents 5 μm. Subcellular localization of the GFP fusion proteins was scored (C, F, I) as cytoplasmic greater than nuclear (C > N), equivalent in each compartment (C = N), or nuclear greater than cytoplasmic (N > C). These data represent the average of three independent experiments (n > 250 cells); error bars indicate standard deviation. Full-length survivin-GFP was predominantly cytoplasmic, and this localization was LMB-sensitive (striped bars). Survivin_{L98A}-GFP was predominantly nuclear prior to and after LMB treatment, while Thr^{97} point mutants were equally distributed between the nucleus and cytoplasm and showed complete nuclear sequestration upon LMB treatment. J, GFP and survivin-GFP were immunoprecipitated using anti-GFP antibodies from asynchronous U2OS cells, and immunoprecipitants were probed with anti-CRM1 antibodies. CRM1 co-immunoprecipitated with survivin-GFP but not with GFP. This interaction was abolished by pretreatment with 6 ng/ml LMB. The lower panel was probed with anti-GFP antibodies. K, a GST pulldown assay was performed with in vitro translated CRM1. The interaction between GST-survivin and CRM1 was significantly stronger than that of GST and CRM1 indicating a direct interaction. NES point mutants were also able to pull down CRM1. Lower panel, Coomassie-stained gel showing equal loading.
Separating the Dual Roles of Survivin

(A) Irradiation dose (Gy)

Surviving Fraction

○ GFP ■ survivin-GFP ▲ L98A-GFP ▲ T97E-GFP

(B) Irradiation dose (Gy)

(C) Surviving Fraction

2.5 Gy 5 Gy

○ GFP ■ Svn-GFP ▲ L98A-GFP

(D) Cell Number

0 Gy 5 Gy

(E) TRAIL

PARP Cleaved PARP Survivin-GFP

GFP Svn-GFP L98A-GFP T97A-GFP T97E-GFP

(F) Relative Fluorescence Units

30 minutes 60 minutes 90 minutes

cfil cfil/TRAIL cfil/CHO sfil sfil/CHO L98A L98A/CHO T97A T97A/CHO T97E T97E/CHO
Separating the Dual Roles of Survivin

**FIGURE 2.** NES mutants abrogate the anti-apoptotic activity of survivin. A, pooled clones of HeLa cells expressing the GFP-tagged proteins indicated were exposed to X-irradiation as indicated. Expression of full-length survivin-GFP conferred a survival advantage over cells expressing GFP alone. This advantage was abrogated in cells expressing survivinΔA-GFP. Data shown are the average of three independent experiments; error bars represent the standard deviation. B, same as for A but using individual cell lines including one expressing survivinΔNE-GFP. C, a representative experiment indicating the difference in X-irradiation response of cells expressing GFP, survivin-GFP, or survivinΔNE-GFP. The survival of cells expressing survivin-GFP versus GFP alone was significantly different at 2.5 and 5 Gy (p values 0.0197 (*) and 0.0365 (*), respectively), as was the case for survivin-GFP versus survivinΔNE-GFP (p values 0.0138 (*) and 0.0139 (*), respectively). D, average number of cells per colony was counted at 0 and 5 Gy for cells expressing the various constructs and found to be higher for cells expressing survivin-GFP compared with GFP or survivinΔNE-GFP. E, apoptosis was induced in HeLa cells stably expressing GFP, survivin-GFP, or survivinΔNE-GFP using 250 μg/ml TRAIL and cell lysates prepared 75 min later. Samples were separated by SDS-PAGE transferred to nitrocellulose, and blots were probed with anti-PARP antibodies to reveal full-length PARP and its 80-kDa apoptosis-related cleavage product (upper panel). Lower panel, probed with anti-GFP antibodies to demonstrate equal loading. Cells expressing GFP and survivin-GFP show some residual full-length PARP in the TRAIL-treated population, while PARP is fully cleaved in the cells expressing survivinΔNE-GFP and survivinΔNE-GFP. F, a quantitative fluorogenic caspase-3 activity assay was performed in triplicate using cell lysates prepared from cells incubated in the presence or absence of TRAIL. The assay was carried out at 37 °C in a 96-well microtiter plate using Ac-DEVD-AMC (Sigma) as substrate and fluorescence release measured at 30, 60, and 90 min using a SpectraMax Gemini Spectrofluorometer (Molecular Devices) with excitation and emission wavelengths set at 380 and 440 nm, respectively.

**RESULTS AND DISCUSSION**

**Full-length Survivin Has a Functional NES and Binds Directly to CRM1**—Ectopically expressed full-length survivin-GFP is predominantly cytoplasmic (6–8) and this localization has been shown to be CRM1/exportin-dependent (9, 10). Using the web-based site NetNES 1.1 (11), we found a sequence between the BIR domain and the COOH-terminal α helical coil of survivin that closely follows the rev-like NES consensus of LXXL (where X is any residue and L = L, I, V, F, or M) and has high NES predicted scoring. This sequence LTLGEFLK104 is also preceded by an acidic residue, Glu95, a feature that is common in rev-like NES consensus. Moreover, NES motifs are frequently positioned at transition points between different structural elements or sites of protein-protein interactions were treated with 6 ng/ml leptomycin B (LMB; LC Laboratories) for 2 h at 37 °C. To induce apoptosis exponentially growing cells were treated for 75 min with 250 μg/ml recombinant human TRAIL (PeproTech EC Ltd.).

Immunoblotting and Immunoprecipitation—Immunoblot analysis was performed as described in Noton et al. (1). Membranes were probed with anti-survivin (1/1000, Ab469, www.abcam.com) to reveal both exogenous survivin-GFP and endogenous survivin, and with anti-tubulin (B512, 1/2000, Sigma) as a loading control. Secondary antibodies were horseradish peroxidase-conjugated (Dakocytomation), and bands were detected using ECL and x-ray film (GE Healthcare). Polyclonal anti-Crm1 (1/3000), was a kind gift of D. Gorlich; see Ref. 2), anti-PARP was from Abcam (clone C2–10; 1/200); and anti-GFP (3E1; 1/1000) was from Cancer Research-UK.

X-irradiation and Clonogenic Survival Assay—Pooled clones expressing the constructs of interest were seeded at low density in 9-cm² Petri dishes and allowed to attach to the substrate before exposure to X-irradiation using an Hs-X-Ray System (A.G.O. Installations Ltd., Reading, UK). 7 days post-irradiation cultures were stained with methylene blue (1 h, room temperature), dried, then rinsed with water. Colonies of >50 cells were counted.

Quantitation of Caspase-3 Activity—Cell lysates were prepared by incubating 10⁶ cells/ml in mammalian protein extraction buffer (Perbio Science), containing 1 mM EDTA and protease inhibitors, 1 μg/ml pepstatin A, 1 mM phenylmethylsulfonyl fluoride, for 1 h at room temperature. Lysates were then centrifuged and the supernatant snap-frozen and stored at −80 °C. Caspase-3 activity assays were carried out in a 96-well microtiter plate using the fluorogenic tetrapeptide, Ac-DEVD-AMC (Sigma) as substrate. The substrate (5 ng) was incubated at 37 °C with 20 μl of cell lysate in 200 μl of reaction buffer (20 mM HEPES, 10% glycerol, 2 mM dithiothreitol, pH 7.5), in the presence or absence of the caspase-3 specific inhibitor, Ac-DEVD-CHO (0.5 μg; Biomol). Relative fluorescence produced by AMC release was measured at 30, 60, and 90 min using a SpectraMax Gemini Spectrofluorometer (Molecular Devices) with excitation and emission wavelengths set at 380 and 440 nm, respectively.

**GST Pulldown**—GST pulldown experiments were performed as described in Noton et al. (1). Crm1 was in vitro translated from a plasmid construct supplied by D. Gorlich (see Ref. 2).

RNAi and Cell Proliferation Index—RNAi was performed as described in Ref. 3 (see also Ref. 1). A silent mutation (C54G) was generated in the RNAi target region to confer resistance to RNAi-mediated depletion, using site-directed mutagenesis (described above). Cells were plated at 5 × 10⁵ per well of a 24-well dish and transfected with 60 pmol of dsRNA (Qiagen) using siPORT NeoFx (Ambion) or hyperfect (Qiagen). Cell proliferation was assessed by trypan blue exclusion.
Separating the Dual Roles of Survivin

A. Western blot analysis showing Survivin-GFP and endogenous Survivin.

B. Graph showing cell number over time for Survivin(NR)-GFP.

C. Graph showing cell number over time for Survivin_{L98A}-GFP.

D. Graph showing cell number over time for Survivin_{T97A}-GFP.

E. Graph showing cell number over time for Survivin_{T97E}-GFP.

F. Bar graph comparing the percentage of cell states for different Survivin variants.

G. Bar graph showing the distribution of cell states for different Survivin variants.
tein interaction e.g. BRCA1 (12, 13) and APC (14). Again, residues \(^{96}\)LTLGEFLKL\(^{104}\) of survivin conform to this trend as they link the fourth beta sheet from the NH\(_2\)-terminal half of the protein with the beginning of the COOH-terminal \(\alpha\) helix. Finally, NES sequences can be regulated by phosphorylation, as is the case for cyclin B1 (15). Within the identified sequence threonine 97 could be a phospho-target. Thus we propose that \(^{96}\)LTLGEFLKL\(^{104}\) is a rev-like NES in survivin. This sequence is present in the 26 isoform, which is also cytoplasmic, but not in survivin-DE3, which localizes to the nucleus (1).

As reported previously (9, 10), overexpressed full-length survivin-GFP was predominantly cytoplasmic in interphase cells (Fig. 1, A and C) and treatment for 2 h with the CRM1 inhibitor LMB caused nuclear accumulation (Fig. 1B). To test whether \(^{96}\)LTLGEFLKL\(^{104}\) was a functional NES, we mutated the conserved hydrophobic residue with highest predictive NES participation scoring, leucine 98, and its adjacent threonine, Thr97. Interestingly while full-length survivin-GFP was almost exclusively cytoplasmic (Fig. 1, A and C), survivin\(_{L98A}\)-GFP was predominantly nuclear (Fig. 1, D and F). Cells expressing survivin\(_{T97A/E}\)-GFP (data not shown) or survivin\(_{T97E}\)-GFP (Fig. 1, G and I) showed an intermediate localization with equal localization within the cytoplasmic and nuclear compartments and complete nuclear sequestration upon LMB treatment (Fig. 1H).

These data demonstrate that nucleo-cytoplasmic shuttling of full-length survivin is CRM1-dependent and that the predicted NES in the central linker region of survivin is functional.

As CRM1 recognizes the NES of proteins, we asked whether CRM1 could interact directly with full-length survivin. Using anti-GFP antibodies we immunoprecipitated GFP or survivin-GFP from asynchronous U2OS cells stably expressing these proteins. CRM1 co-immunoprecipitated with survivin-GFP but not with GFP (Fig. 1J), demonstrating that CRM1 interacts with full-length survivin in vivo. This interaction was disrupted by 2 h pretreatment with LMB. Next we performed a pull down assay using GST or GST-survivin and in vitro translated CRM1 (Fig. 1K). CRM1 bound to GST-survivin indicating that the interaction between full-length survivin and CRM1 is direct. In this assay, however, we found that all the NES point mutants were still able to interact with CRM1; however, this is not surprising as in the crystal structure Leu98 is embedded in the hydrophobic core of the dimer interface rather than being exposed. Thus although mutation of these residues is sufficient to prevent or reduce nuclear exportation of survivin, other residues within this region probably contribute to its binding to CRM1.

**Overexpression of Full-length Survivin-GFP Confers Resistance to Ionizing Radiation, Which Is Abrogated by Mutation of the NES**—As we now had cytoplasmic and nuclear versions of survivin, we next asked whether the subcellular localization of survivin affected the sensitivity of cells to X-irradiation. In Fig. 2, A and B, we used a clonogenic survival assay to demonstrate that HeLa cells expressing full-length survivin-GFP are more resistant to X-irradiation than cells expressing GFP. This cytoprotection was abolished in populations expressing the NES point mutations, survivin\(_{L98A}\)-GFP or survivin\(_{T97E}\)-GFP (Fig. 2B), rather than full-length survivin-GFP. Indeed, expression of survivin\(_{L98A}\)-GFP and survivin\(_{T97E}\)-GFP increased sensitivity to X-irradiation compared with the GFP control. The differences between the sensitivities of cell lines to X-irradiation was found to be significant at 2.5 and 5 Gy (Fig. 2C), but not at higher doses. In addition to showing greater clonogenic survival after irradiation we observed that the average number of cells per colony was greater for pooled populations of cells expressing survivin-GFP than for GFP or survivin\(_{L98A}\)-GFP, suggesting that cells overexpressing survivin-GFP may proliferate more rapidly (Fig. 2D). Together these data suggest a direct link between subcellular localization of survivin and sensitivity to ionising radiation.

To determine whether the localization of survivin was important for its anti-apoptotic function we induced apoptosis in HeLa cells expressing GFP, survivin-GFP, or survivin NES point mutants using TRAIL, and monitored PARP cleavage by immunoblotting (Fig. 2E). TRAIL caused the appearance of an 80 kDa PARP cleavage product and reduced the amount of full-length PARP. Quantification of these bands revealed that the apoptosis-related PARP cleavage product represented 82.2% of total PARP for GFP expressing cells, 73.7% for survivin-GFP cells, and 93.9% for survivin\(_{L98A}\)-GFP cells. PARP cleavage was less apparent in lines expressing Thr97 mutants. Next we prepared cell lysates from the lines indicated, in the presence and absence of TRAIL, and performed a quantitative caspase-3 activity assay using the fluorogenic substrate Ac-DEVD-AMC (Fig. 2F). As indicated by the release of fluorescence, in control cells caspase activity was induced by TRAIL, increased over time, and was inhibited by the addition of the caspase-3-specific inhibitor, DEVD-CHO. In contrast, this activity was substantially reduced in lysates prepared from samples expressing survivin-GFP and survivin\(_{T97A/E}\)-GFP but not survivin\(_{L98A}\)-GFP. Together these data suggest that expression of survivin confers resistance against caspase-3-mediated apoptosis, and this protection can be eliminated by a point...
mutation in the NES. We note that mutation of Thr97 did not abrogate the ability of survivin to inhibit caspase-3 activity after TRAIL-induced apoptosis, but cells expressing survivin97E-GFP did show increased sensitivity to X-irradiation. The reason for the differences in the two assays is unclear but may reflect the intermediate localization of Thr97 mutants (Fig. 1G). Nevertheless, these data demonstrate that residue Leu98 is critical for the anti-apoptotic activity of survivin and further suggest that survivin may need to be cytoplasmic to be cytoprotective.

Survivin NES Mutants Fulfil the Mitotic Role of Survivin—Next we asked whether the NES mutants that sensitize cells to X-irradiation and apoptosis affect survivin function during mitosis. Fluorescence analysis demonstrated that survivinL98A-GFP was distributed similarly to full-length survivin-GFP during mitosis, exhibiting a typical chromosomal passenger localization present at the centromeres, the midzone, and the midbody during prometaphase, anaphase, and telophase respectively (Fig. 3). However, binding of survivinL98A-GFP to the inner centromeres was reduced (pixel intensity: 308 ± 26.5, n = 36) and less variable than that of full-length survivin (pixel intensity: 791 ± 354, n = 36). In addition, survivinL98A-GFP accumulated at the spindle poles to a similar level to its accumulation at the centromeres (Fig. 3B). Previously survivin has been reported to localize to the centrosome in interphase cells; however, in our experience centrosomal localization is only seen with certain mutant forms of the protein, and with survivinL98A-GFP, recruitment to the centrosome is confined to mitosis. Localization of Thr97 point mutants was similar at all stages to survivinL98A-GFP (Fig. 3C and data not shown). From these data we conclude that NES point mutants localize as chromosomal passengers during mitosis and have additional affinity for the centrosomes.

Depletion of survivin from somatic human cells causes cessation of cell proliferation 48 h post-treatment (3, 21). As NES point mutants localized normally during mitosis we asked whether they could substitute for loss of endogenous survivin after RNAi-mediated depletion. Endogenous survivin was effectively eliminated from cells 72 h post-transfection with survivin-specific (S) oligonucleotides (Fig. 4A). By contrast exogenously expressed GFP-tagged versions of the protein rendered resistant (survivinR-GFP) to RNAi-mediated depletion were still present at similar levels to control (C) treated cells. Next we monitored cell proliferation over 72 h post-transfection. When transfected with survivin-specific oligonucleotides, cells expressing the non-resistant version of survivin-GFP did not grow (Fig. 2B), while those expressing the RNAi-resistant NES point mutants were able to proliferate, albeit less efficiently than controls. Comparison of cell number 72 h post-transfection with survivin-specific dsRNA indicated that NES mutants restored cell proliferation to a level similar to the wild type, survivinR-GFP control (Fig. 4F). Furthermore, cells complemented with NES mutants had predominantly mononucleated cells in the population 72 h post-RNAi transfection, as compared with the RNAi sensitive survivin-GFP line that became highly multinucleated (70%, n > 300, Fig. 4G).

Together these data demonstrate that the essential mitotic activity of survivin is largely preserved in NES mutants.

CONCLUSIONS

One considerable impediment in developing anti-cancer treatments is that many of the targets are essential and thus cannot be effectively down-regulated without affecting normal cells. Here we report that mutation of the NES of survivin can abrogate its cytoprotective activity while maintaining its essential mitotic role. Our data suggest that this may be a way in which survivin can be targeted with significant therapeutic potential.

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