Human Survivin Is a Kinetochore-associated Passenger Protein

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Abstract. Survivin, a dimeric baculovirus inhibitor of apoptosis repeat (BIR) motif protein that is principally expressed in G2 and mitosis, has been associated with protection against apoptosis of cells that exit mitosis aberrantly. Mammalian survivin has been reported to associate with centrosomes and with the mitotic spindle. We have expressed a human hemagglutinin-tagged survivin plasmid to determine its localization, and find instead that it clearly acts as a passenger protein. In HeLa cells, survivin first associates with the kinetochores, and then translocates to the spindle midzone during anaphase and, finally, to the midbody during cell cleavage. Its localization is similar to that of TD-60, a known passenger protein. Both a point mutation in the baculovirus IAP repeat motif (C84A) and a COOH-termi-nal deletion mutant (Δ106) of survivin fail to localize to either kinetochores or midbodies, but neither interferes with cell cleavage. The interphase localization of survivin is cell cycle regulated since in permanently transfected NIH3T3 cells it is excluded from the nuclei until G2, where it localizes with centromeres. Survivin remains associated with mitotic kinetochores when microtubule assembly is disrupted and its localization is thus independent of microtubules. We conclude that human survivin is positioned to have an important function in the mechanism of cell cleavage.

Key words: survivin • mitosis • kinetochore • passenger protein • cytokinesis

Introduction

Survivin, a baculovirus inhibitor of apoptosis (IAP)[1] repeat (BIR) motif protein (Ambrosini et al., 1997), has widespread tissue distribution and is expressed in many solid tumors (Ambrosini et al., 1997; Lu et al., 1998). Increased levels of survivin expression correlate strongly with cell survival after mitotic arrest by taxol, an inhibitor of spindle function (Li et al., 1998), while repression of expression by an antisense leads to induction of apoptosis (Ambrosini et al., 1998; Li et al., 1998). Survivin has been also reported to associate with the centrosome (Li et al., 1999), mitotic spindle, and midbody (Li et al., 1998). Based on these features, survivin is thought to be a key player in the pathway that links failure of mitotic checkpoint controls to apoptotic activation.

Because of its BIR motif, survivin is a potential member of the IAP family of proteins, which act at discrete steps to regulate the apoptotic pathway of cell death (Deveraux and Reed, 1999). The BIR motifs are essential for interaction of the IAP proteins with proapoptotic proteins, including the caspase family of death proteases. Not all BIR motif proteins act as inhibitors of apoptosis, indicating that they also have other functions. For example, the BIR motif proteins most closely related to survivin, Bir1p in budding yeast (Yoon and Carbon, 1999; Li et al., 2000), bir1 in fission yeast (Uren et al., 1999), and BIR-1 in *Caenorhabditis elegans* (Fraser et al., 1999) are not involved in suppression of apoptosis, but are implicated in spindle function and cell cleavage. Further, recent evidence supports a role in mitosis for mammalian survivin, as its suppression by antisense RNA expression generates failure in cell cleavage (Chen et al., 2000) and leads to a multinucleate polyploid phenotype (Li et al., 1999).

In *C. elegans*, the survivin ortholog BIR-1 acts as a passenger protein, first localizing to the chromosomes, and then migrating to the midbody during cleavage (Speliotes et al., 2000). Proteins of this class may play a role in induction of cleavage that coordinates with the position of the spindle midzone (Earnshaw and Bernat, 1991; Martineau et al., 1995).

In contrast to expectation from the above analyses, immunofluorescence microscopy suggested that survivin associates with the mitotic spindle (Li et al., 1998) and with the centrosomes in mammalian cells (Li et al., 1999). We have addressed the localization of survivin in mammalian cells by transfection of human hemagglutinin (HA)-tagged survivin and have obtained clear evidence that survivin localizes strongly to kinetochores until metaphase, migrating to the spindle midzone in early anaphase and to the cleavage furrow during telophase and cytokinesis. We find no evidence that survivin directly associates with either microtubules or with centrosomes. Its mitotic behavior closely duplicates that of TD-60, a known passenger protein (Andreassen et al., 1991).

We have also analyzed the localization of two HA-tagged survivin mutants. The first mutant substitutes Ala for a key cysteine (C84), disrupting the zinc finger in the BIR motif (Chantalat et al., 2000). This mutant has been reported to act as a dominant negative for apoptotic response in mammalian cells (Li et al., 1998). The second mutant is a truncation of the COOH-terminal α-helical extensions of survivin. We find that both mutants fail to localize to either kinetochores or to the spindle midzone, but do not interfere with cleavage. We conclude that survivin is a genuine passenger protein and that the majority of its structure is involved in its capacity for kinetochore and spindle midzone localization.

Materials and Methods

Cloning and Mutagenesis

The full-length cDNA coding human survivin (Chantalat et al., 2000) was introduced into the EcoRI site and in frame with the HA epitope in the mammalian expression vector pJF-HA (Rousseau et al., 1999). The full-length HA-chimeric survivin cDNA was subcloned into the XhoI-BamHI sites of the pCDNA3.1(−) expression vector (Invitrogen).

The C84A mutant was obtained by site-directed mutagenesis using the Quick Change Site-directed Mutagenesis kit (Stratagene). The oligonucleotide primer 5′ ATTCGTCCGGCGCCGCTTTCCTTTCTG 3′ and its complementary were designed to substitute the Cys 84 to an Ala along with a silent mutation generating a NarI restriction site. The EcoRI fragment containing the mutant survivin was subcloned in pJF-HA vector. The Δ106 mutant was obtained by PCR introducing a stop codon (UAG) in amino acid 107, using the construct pJF-HA-survivin as template. An oligonucleotide 5′ CCGGAATTCTATCTGTCCAGTTTCAAAAATTC 3′ annealing at position 299–318 of survivin coding region and an oligonucleotide 5′ TTTACTTCTAGGCCTGTA 3′ within the pJF-HA vector were used for the amplification. 19 cycles of amplification were performed using the Vent DNA Polymerase (Biolabs). The amplified fragment was gel purified, subcloned in the EcoRI site of the pJF-HA vector, and its orientation was confirmed by PCR. All constructs expressing survivin and its mutants were confirmed by sequencing (Genome Express; Grenoble).

Cell Culture and Transfection

HeLa and NIH3T3 cells were grown as monolayers in Dulbecco's Modified Eagle's Medium (GIBCO BRL) supplemented with 10% fetal bovine serum (Hyclone) and 10% bovine calf serum (Biological Industries), respectively, and maintained in a humid incubator at 37°C in a 5% CO_2 environment. Nocodazole, Taxol, and VP16 (Sigma-Aldrich) were dissolved in DMSO.

HeLa cells (3×10^6) were transfected by electroporation with 20 μg/ml JF-HA-survivin plasmid with an EMC600 apparatus (BTX) according to the manufacturer's instructions and replated onto 10-mm dishes. Cells adhered to coverslips in 35-mm dishes were also transfected by Exgen (Euromedex) with 3 μg of JF-HA-survivin or JF-HA-C84A or JF-HA-Δ106 plasmids according to the manufacturer's instructions. NIH3T3 cells transfected with the pCDNA 3.1(−) carrying the HA-survivin were selected in 1.2 mg/ml geneticin G418 (GIBCO BRL).

Immunofluorescence Microscopy

Cells, grown on poly-D-lysine–coated glass coverslips for immunofluorescence microscopy, were fixed with 2% paraformaldehyde-PBS for 20 min at 37°C, washed 5 min with PBS, permeabilized with 0.2% Triton X-100 in PBS for 3 min, and washed three times for 5 min with PBS. Cells were then processed with primary and secondary antibodies and counterstained with propidium iodide, as described previously (Martineau-Thuillier et al., 1998). HA monoclonal antiserum (BabCo) was diluted 1,500-fold. CREST GD and JH human autoimmune sera used to detect centromere antigens and TD-60 (Andreassen et al., 1991), respectively, were diluted 500×. Secondary antibodies, including FITC-conjugated affinity purified goat anti–mouse IgG (Jackson ImmunoResearch Laboratories), and rhodamine-conjugated anti–human IgG, were used at 2.5 μg/ml. Images were collected with a MRC-600 Laser Scanning Confocal Apparatus (Bio-Rad Laboratories) coupled to a Nikon Optiphot microscope.

Cell Extracts and Immunoblotting

For the preparation of extracts, cells were centrifuged, washed with PBS at 4°C, and lysed in 50 mM Tris-HCl, pH 7.5, 250 mM NaCl, 0.1% NP-40, and 5 mM EGTA containing 50 mM NaF, 60 mM β-glycerolphosphate, 0.5 mM Na-vanadate, 0.1 mM PMSF, 10 μg/ml aprotinin, and 10 μg/ml leupeptin. Lysate supernatants were then collected by centrifugation at 13,000 g. For each sample, 10 μg of cell extracts were resolved on 15% polyacrylamide gels using a mini-gel apparatus (Bio-Rad Laboratories) and transferred to nitrocellulose. A monoclonal antiserum to HA, diluted 1,000-fold, and a peptide antibody recognizing the COOH terminus (amino acids 110–123) of human survivin (Alpha Diagnostics International), diluted 2,000-fold, were used to detect expressed and endogenous protein. Blots were exposed to HRP-conjugated goat anti–mouse antibodies (KPL) and goat anti–rabbit IgG secondary diluted 5,000-fold (TAGO) for 1 h, and then developed by enhanced chemiluminescence (Pierce Chemical Co.).

Results

Transiently Expressed HA-Survivin Localizes as a Kinetochore Passenger Protein

We have transiently transfected HeLa cells with plasmid expressing human HA-tagged survivin by either electroporation or with exgen, and have examined the protein's localization through the cell cycle. With either method the results are equivalent. The HA antibody recognized one strong band in Western blots of whole cell extracts, and reacted with no protein in control cells (Fig. 1 A). Western blots with a survivin antibody confirmed the expression of HA-tagged survivin at levels higher than the endogenous protein (Fig. 1 A). The HA tag has been introduced into the NH_2 terminus of the protein, where structural analysis had previously shown modification did not interfere with native folding of the protein dimer (Chantalat et al., 2000).

The majority of interphase cells showed a disperse, but particulate, distribution of survivin in the cytosol, and in these cells survivin was specifically excluded from the nucleus (Fig. 1 B, a). By contrast, some interphase cells had nuclear staining with local punctate accumulations (Fig. 1 B, b). On entry into prophase, localization was entirely punctate. As cells proceeded to metaphase, localization was apparently associated with the centromeric elements of the chromatin (Fig. 1 B, c–e).

During anaphase and telophase, survivin disassociated from the chromatin and concentrated at the spindle equator as the chromosomes separated (Fig. 1 B, f–i), apparently participating as an element of the telophase disc, a structure that forms in late anaphase and contacts the cell cortex at the position of the cleavage furrow (Cooke et al., 1987; Martineau et al., 1995).
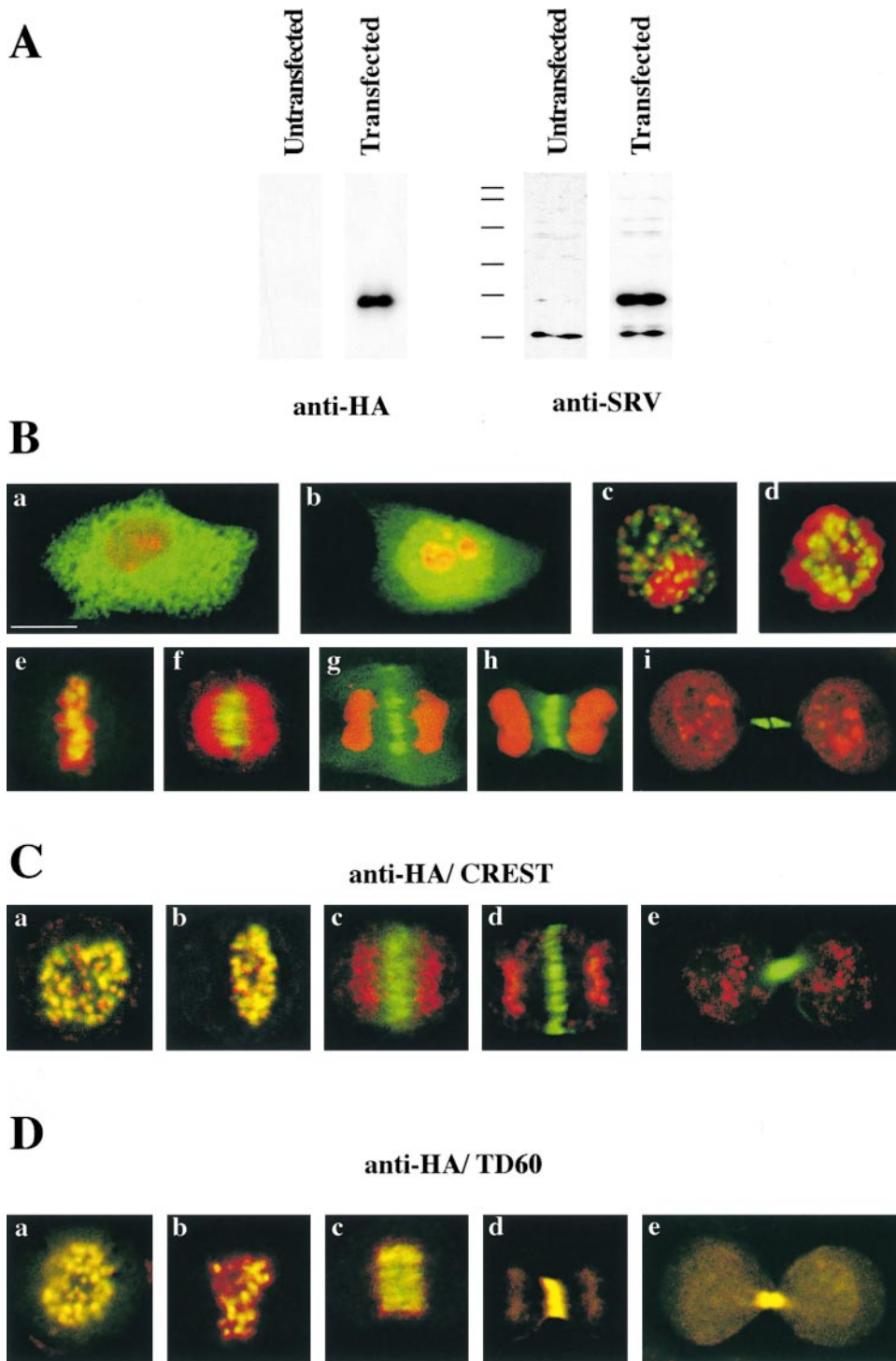
Figure 1. Transient overexpression of human survivin reveals kinetochore and cleavage furrow association. (A) Immunoblot analysis of HeLa cell extracts using antibodies specific to HA and to survivin from cells transiently expressing HA-tagged survivin. (B) Immunofluorescence microscopy of transfected cells stained with anti–HA (green) and propidium iodide (red) for chromatin. In different interphase cells, survivin localizes predominantly either in the cytoplasm (a) or the nucleus (b). In prophase through metaphase (c–e), survivin localizes in distinct spots on the chromatin. In anaphase, leaves the chromatin, associates with the spindle midzone (f), extending to the cortex (g), and remains localized in telophase (h) and the midbody (i). (C) Survivin (green) colocalizes with kinetochores stained with a human CREST serum (red) during mitosis until metaphase (a and b), and then dissociates from kinetochores and accumulates in the cleavage furrow and midbody (c–e). (D) Survivin (green) colocalizes with the kinetochore passenger protein TD-60 stained with a human autoimmune serum (red) through all the stages of mitosis: (a) prometaphase, (b) metaphase, (c) anaphase, (d) telophase, and (e) late telophase. Bar: 10 μm.

The association of survivin with the kinetochores in mitosis was confirmed (Fig. 1 C) by counterstaining with a CREST scleroderma autoimmune serum, which specifically reacts with centromere proteins and marks mitotic kinetochores. Survivin staining overlapped (Fig. 1 C, yellow) with the centromere staining up to metaphase (Fig. 1 C, a and b). Entry into anaphase led to a redistribution of survivin to the spindle midzone (Fig. 1 C, c and d) and later to the midbody (e).

Comparison of the distribution of survivin with that of TD-60, a passenger protein that we have previously described (Andreassen et al., 1991), showed that the overlap of stain (Fig. 1 C, yellow) between the two antigens was essentially complete throughout mitosis (Fig. 1 D). Therefore, survivin behaves as a passenger protein whose localization changes dynamically with progression of mitosis.

Distribution of Survivin in a Permanently Expressing Clonal NIH3T3 Cell Line

We established an NIH3T3 cell line that permanently expressed human HA-tagged survivin, and found that the distribution of survivin in this cell line was essentially identical to that described above for the transiently transfected
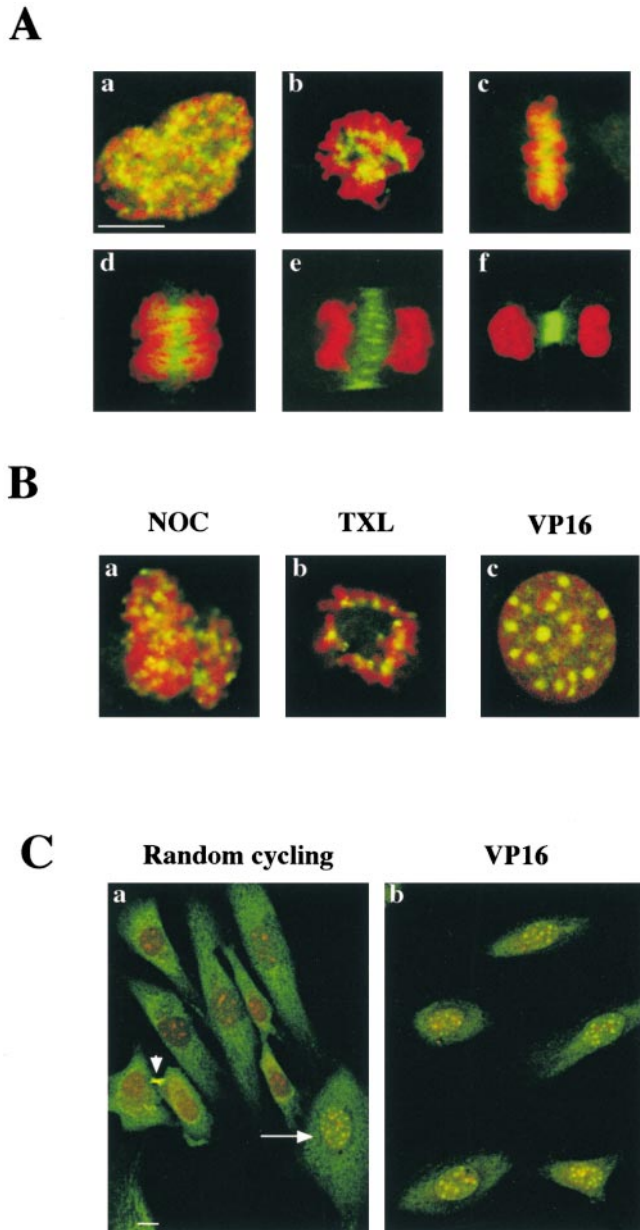
A

a b c

d e f

B

NOC | TXL | VP16

a | b | c

C

Random cycling | VP16

a | b

Figure 2. Cell-cycle distribution of survivin in permanently over-expressing HA-tagged survivin NIH3T3 cells. Immunofluorescence microscopy of expressing cells stained with an anti–HA (green) and propidium iodide (red). (A) During mitosis, survivin localizes to kinetochores in late G2/prophase (a), prometaphase (b), and metaphase (c). In late mitosis, survivin localizes to the spindle midzone (d), and then the cleavage furrow (e) and midbody (f). (B) Survivin retains kinetochore localization after disruption of the mitotic spindle by nocodazole (0.1 μg/ml, a) or taxol (20 μM, b). (C) Low-magnification fields of random cycling cells and cells blocked for 16 h in G2 with the topoisomerase II inhibitor VP16 (2 μg/ml). In untreated cells, survivin exhibits a cytoplasmic staining, nuclear punctate staining (arrow) and midbody staining (arrowhead). In 90% of G2 arrested cells, (VP16) survivin has a nuclear punctate distribution (right; see also B, c). Bar: 10 μm.

HeLa cells. In long term culture, we found no sign that overexpression of HA-survivin influenced cell cycle distribution or ploidy, as determined by FACscan® analysis, nor cell viability (data not shown) as previously reported for murine survivin (TIAP) (Kobayashi et al., 1999).

As in HeLa, mitotic NIH3T3 cells showed that survivin followed the typical distribution pattern of a passenger protein (Fig. 2 A, a–f). The association of survivin with kinetochores did not depend on microtubules since it was retained in the presence of the nocodazole and taxol, inhibitors of microtubule dynamics (Fig. 2 B, a and b).

We note that transient and constitutive overexpression of survivin in HeLa and NIH3T3 cells, respectively, showed no evidence of association with the metaphase mitotic spindle, nor with the centrosomes, in contrast to previous reports (Li et al., 1998, 1999). The midbody stain is the only previously reported localization consistent with our results and with those in *C. elegans* (Speliotes et al., 2000). Using a COOH-terminal–specific commercially available antibody to survivin, we were unable to localize survivin to any structure in the cell. Further, on repeated attempts to demonstrate survivin binding to microtubules in vitro, we found no evidence for specific association (data not shown).

The ectopically expressed survivin is present throughout the cell cycle, whereas the endogenous protein is expressed exclusively during G2/M (Li et al., 1998). However, the intracellular distribution of the constitutively expressed survivin appeared to be cell-cycle regulated. Most randomly cycling interphase cells showed survivin excluded from the nuclei (Fig. 2 C, a). Recently divided cells identified by midbodies were always negative for nuclear stain (Fig. 2 C, a, arrowhead). A minority of cells showed nuclear stain and this was strongly localized to centromeres (Fig. 2 C, a, arrow). By contrast, upon arrest in G2 by VP16, an inhibitor of topoisomerase II, 90% of the cells showed a nuclear punctate survivin distribution (Fig. 2, C, b, and B, c).

The data shown above suggest that entry of survivin into the nucleus is cell-cycle regulated, and there might be a mechanism of excluding survivin from the nucleus through most of interphase. A mechanism for nuclear exclusion has been elucidated for cyclin B, whose localization is controlled by a nuclear export signal (NES) (Yang et al., 1998). If a similar control regulates survivin nuclear export, the NES must be present in an associated protein, as survivin has no apparent NES. The control of survivin localization and its function in interphase remain to be determined.

Both the BIR Motif and the COOH-Terminal Alpha Helical Extensions of Survivin Are Important to Its Mitotic Localization

Structural analysis shows survivin is a bow-tie–shaped dimeric protein, with an NH$_2$-terminal BIR motif and a COOH-terminal α-helical extension from each monomer (Chantalat et al., 2000). The BIR motif contains a zinc finger stabilized by residues C57, C60, H77, and C84. C84 is predicted to be essential to maintenance of the BIR motif. Fig. 3 A (red arrow) shows the structural position of this residue. An earlier report gave evidence that mutation of cysteine 84 to alanine created a dominant-negative phenotype with respect to suppression of apoptosis (Li et al., 1998). Furthermore, the survivin dimer has two unusual COOH-terminal α-helical extensions that appear to have an adaptor or docking function. We have also expressed a mutant form of survivin in which the COOH-terminal extensions were truncated at residue 106 (Fig. 3 A, black arrow).
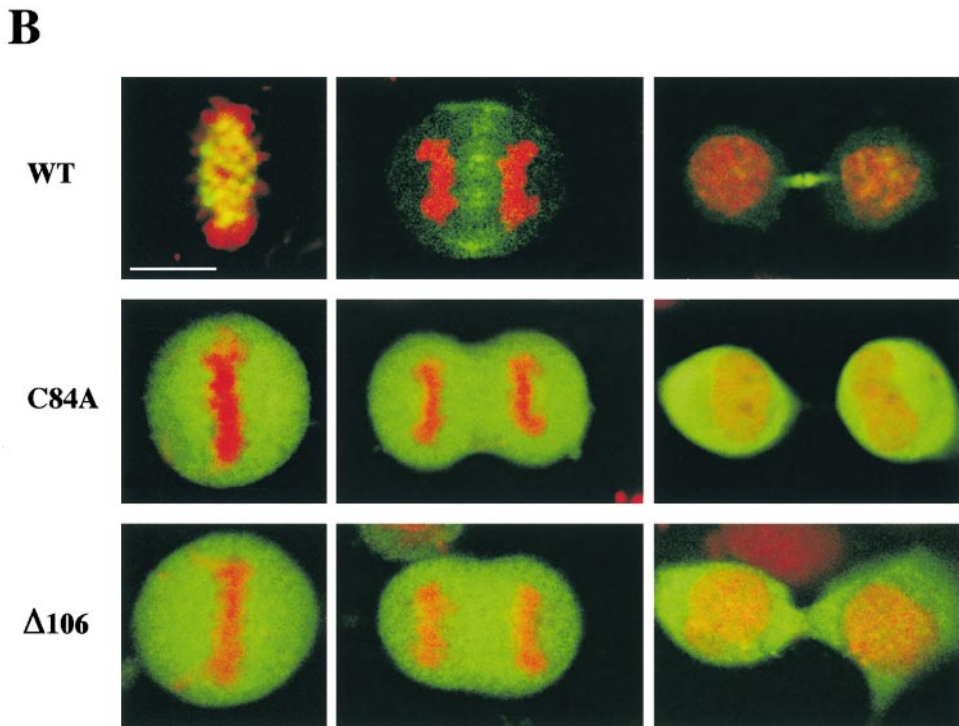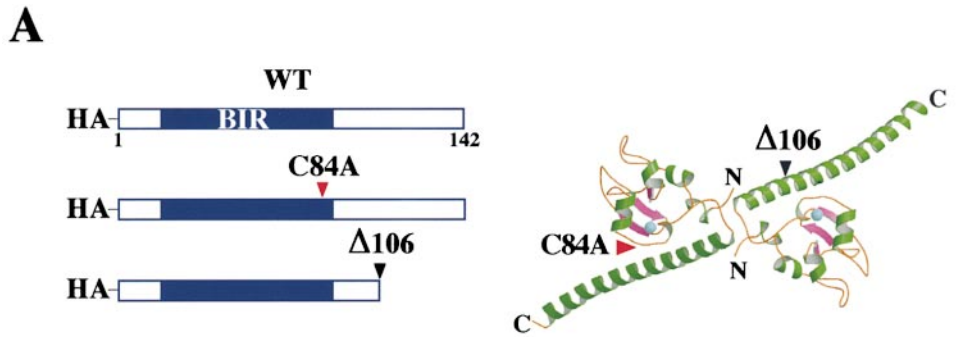
A

B

Figure 3. A BIR-motif C84A mutation and a Δ106 COOH-terminal truncation on survivin abolish kinetochore and cleavage furrow association. (A) Schematic representation of survivin and the introduced C84A point mutation (red arrowhead) and Δ106 deletion (black arrowhead). The locations of the introduced mutations are also depicted on the survivin dimer crystal structure (Chantalat et al., 2000). (B) Immunofluorescence microscopy of HeLa cells transiently overexpressing wild-type (WT) HA-survivin, HA-C84A, and HA-Δ106 survivin 48 h after transfection. Cells stained with anti–HA (green) and propidium iodide (red). As opposed to wild-type survivin, neither mutant protein localizes to kinetochores (left), the cleavage furrow (middle), or midbody (right). Bar: 10 μm.

We transfected HeLa cells with C84A and Δ106 mutants of HA-tagged survivin, and observed that both mutants (Fig. 3 B, green) distributed uniformly throughout the cytoplasm and did not localize to either kinetochores at metaphase, nor the spindle midzone or midbody in telophase, in contrast to the discrete localization observed with wild-type transfection done under the same conditions (Fig. 3 B). We conclude that both the COOH-terminal extension and intact BIR motif are important to survivin localization.

It is worth noting that the C84A survivin remained dimeric, as revealed by FPLC chromatography, despite extensive structural disruption (data not shown). The failure of C84A and Δ106 survivin to distribute as a passenger protein did not give a dominant-negative phenotype. After 48 h of expression, cells carrying wild-type and C84A or Δ106 mutants showed ~2% binucleate and no micronucleated cells. These results are compatible with the completed cleavage that we routinely saw in expressing cells (Fig. 3 B, right).

Since C84A and Δ106 mutants of survivin do not localize to the kinetochore and cleavage furrow, we asked whether overexpression of the two mutants altered TD-60 distribution during mitosis. Neither survivin mutant interfered with the normal distribution of TD-60 to the kinetochores or to the telophase disc (Fig. 4, A–C).

As survivin and BIR1 depletion cause gross defects in cleavage (Chen et al., 2000; Speliotes et al., 2000), we assume that the native survivin in the transfected cells was functioning normally and that the mutants we expressed did not have a dominant-negative effect. Therefore, binding partners that may require survivin to function at the kinetochore and spindle midzone are probably in large excess over survivin, and insensitive to the presence of nonfunctional protein.

Conclusions

Our results show that, during mitosis, human survivin localizes to the kinetochores until metaphase, and then redistributes to the spindle midzone and to the telophase
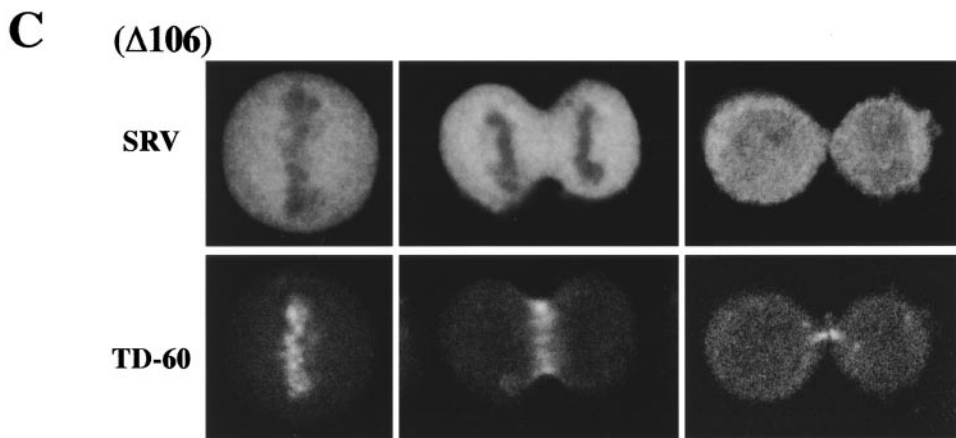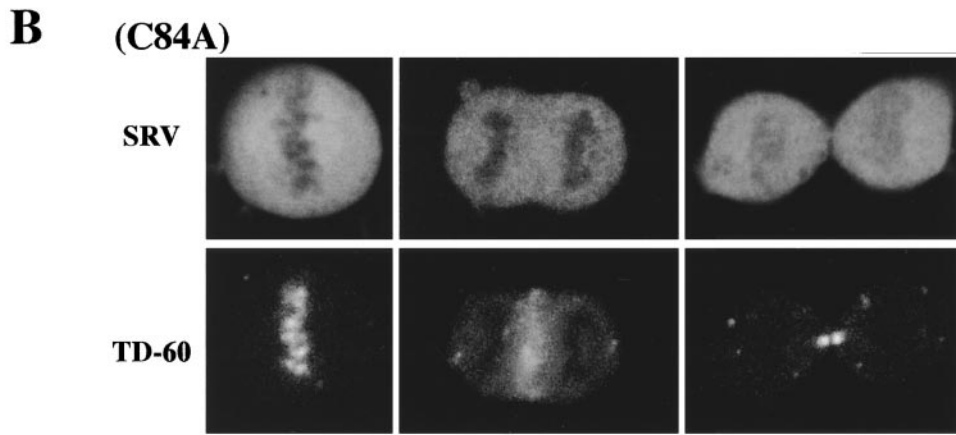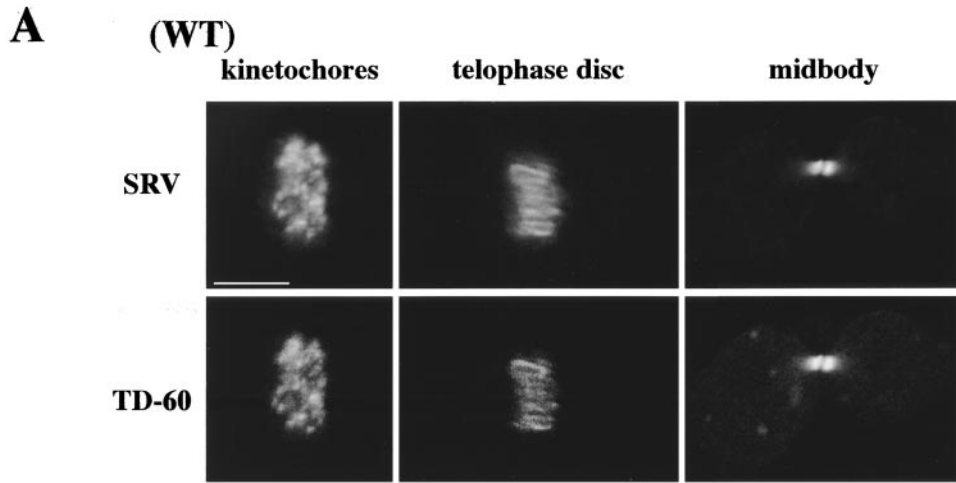
A (WT)

B (C84A)

C (Δ106)

Figure 4. The BIR-motif C84A mutation and the Δ106 COOH-terminal truncation on survivin do not alter the distribution of the kinetochore passenger protein TD-60. Double immunofluorescence of HeLa cells transiently transfected with wild type (A), C84A (B), and Δ106 (C) survivin stained with anti–HA (SRV) and with a human autoimmune serum recognizing TD-60. Both mutants of survivin do not alter the TD-60 localization to kinetochores (left) and neither mutant alters the distribution of TD-60 to the cleavage furrow (middle) or to midbody (left). Bar: 10 μm.

disc during anaphase, ending in the midbody during cell cleavage. The survivin localization to kinetochores is independent of microtubules. On the basis of its localization, survivin can be considered as one of the kinetochore passenger proteins that extend to the cell cortex, sharing this property with TD-60, INCENPs, and AIM-1 (Martineau-Thuillier et al., 1998). This class of proteins has been previously shown to play a key role in the proper completion of cytokinesis in mammalian cells (Martineau et al., 1995; Mackay et al., 1998; Terada et al., 1998), and they may be

targeted as complexes to the central spindle and cleavage furrow (Adams et al., 2000).

In contrast to the results in *C. elegans* (Speliotes et al., 2000), we find human survivin specifically localizes to early mitotic kinetochores rather than whole chromosomes. This discrepancy probably results from the holo-centric nature of the nematode chromosomes during mitosis (Albertson and Thomson, 1982). In *C. elegans*, BIR-1 is also required for the localization of the aurora-like kinase AIR-2 at the metaphase chromosome (Speliotes et al.,

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2000). AIR-2 also appears to be a passenger protein, and lack of either AIR-2 or BIR-1 causes failure of cell cleavage. Furthermore, the observed kinetochore association of survivin is in agreement with data in budding yeast, where Bir1p, through its COOH terminus, associates with members of the CBF3 centromeric protein complex (Yoon and Carbon, 1999).

In addition, we show that neither a COOH-terminal truncation mutant nor a BIR motif point mutant of survivin could localize to kinetochores or the spindle midzone in mitosis, but were instead dispersed throughout the cell. These results suggest that the BIR domain and the COOH-terminal α-helices both contribute to the proper dynamic redistribution of survivin during mitosis. However, neither mutant interferes with any aspect of chromosome separation or cell cleavage, at least at the expression levels attained.

Survivin, like other BIR motif proteins, has the potential to inhibit apoptosis by directly suppressing the activity of caspases; however, suppression of caspase activity by survivin is controversial (Verdecia et al., 2000). The survivin orthologs in lower eukaryotes appear to have no function in protection from cell death, but instead are directly involved in chromosome segregation, spindle elongation, and cell cleavage events (Fraser et al., 1999; Uren et al., 1999; Yoon and Carbon, 1999; Speliotes et al., 2000). It remains possible that human survivin has acquired IAP functions that its orthologs in lower eukaryotes do not have, and thus has roles to play in both the completion of late mitosis and in the inhibition of apoptosis.

Note added in proof: After these results were obtained, similar results regarding the passenger protein behavior of mammalian survivin were reported. Uren, A.G., L. Wong, M. Pakusch, K.J. Fowler, F.J. Burrows, D.L. Vaux, and K.H. Andy Choo. 2000. *Curr. Biol.* 10:1319–1328.

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