Survivin exists in immunochemically distinct subcellular pools and is involved in spindle microtubule function

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Summary

Survivin is a member of the inhibitor of apoptosis gene family that has been implicated in both apoptosis inhibition and regulation of mitosis. However, the subcellular distribution of survivin has been controversial and variously described as a microtubule-associated protein or chromosomal passenger protein. Here, we show that antibodies directed to the survivin sequence Ala^{3}-Ile^{19} exclusively recognized a nuclear pool of survivin that segregated with nucleoplasmic proteins, but not with outer nuclear matrix or nuclear matrix proteins. By immunofluorescence, nuclear survivin localized to kinetochores of metaphase chromosomes, and to the central spindle midzone at anaphase. However, antibodies to Cys^{57}-Trp^{67} identified a cytosolic pool of survivin, which associated with interphase microtubules, centrosomes, spindle poles and mitotic spindle microtubules at metaphase and anaphase. Polyclonal antibodies recognizing survivin epitopes Ala^{3}-Ile^{19}, Met^{38}-Thr^{48}, Pro^{47}-Phe^{58} and Cys^{57}-Trp^{67} identified both survivin pools within the same mitotic cell. A ratio of ~1:6 for nuclear versus cytosolic survivin was obtained by quantitative subcellular fractionation. In synchronized cultures, cytosolic survivin abruptly increased at mitosis, physically associated with p34^{cdc2}, and was phosphorylated by p34^{cdc2} on Thr^{34}, in vivo. By contrast, nuclear survivin began to accumulate in S phase, was not complexed with p34^{cdc2} and was not phosphorylated on Thr^{34}. Intracellular loading of a polyclonal antibody to survivin caused microtubule defects and resulted in formation of multipolar mitotic spindles, but did not interfere with cytokinesis. These data demonstrate that although both reported localizations of survivin exist in mitotic cells, the preponderant survivin pool is associated with microtubules and participates in the assembly of a bipolar mitotic spindle.

Key words: Survivin, Microtubules, Mitotic spindle, Kinetochore, p34^{cdc2}

Introduction

Among the regulators of apoptosis (Hengartner, 2000), considerable interest has been focused on the Inhibitor of Apoptosis (IAP) gene family (Deveraux and Reed, 1999). IAP proteins are identified by the presence of one to three copies of a ~70 amino acid Zn^{2+} finger baculovirus IAP repeat (Sun et al., 1999), and are present in many eukaryotic species (Deveraux and Reed, 1999). The mode of apoptosis inhibition by certain IAPs involves suppression of the executioner phase of cell death, thus preventing maturation and proteolytic activity of initiator and effector caspases (Deveraux and Reed, 1999). Adding to their functional complexity, certain IAPs have been recently implicated in processes other than apoptosis, particularly chromosome segregation and cytokinesis (Fraser et al., 1999; Speliotes et al., 2000; Uren et al., 1999). Deletion experiments of IAP genes in yeast (Uren et al., 1999), or RNA interference of a *C. elegans* IAP (Fraser et al., 1999; Speliotes et al., 2000), did not affect cell death pathways, but caused multiple meiotic and mitotic abnormalities with defective metaphase alignment, chromosome segregation and spindle midzone formation.

Mammalian cell survivin (Ambrosini et al., 1997) may share properties of IAPs involved in both apoptosis inhibition and cell division control (Reed and Bischoff, 2000; Reed and Reed, 1999). Previous studies demonstrated that survivin is expressed at G2/M in a cell cycle-dependent manner, and localizes to components of the mitotic apparatus, including centrosomes, and mitotic spindle microtubules (Li et al., 1998). In vitro, survivin bound polymerized microtubules with μM affinity (Li et al., 1998), and a putative tubulin-binding domain was identified by mutational analysis in the extended survivin C-terminal α-helix (Verdecia et al., 2000). Moreover, forced expression of survivin counteracted cell death induced by various apoptotic stimuli (Reed and Bischoff, 2000), whereas interference with survivin expression/function by antisense or dominant negative mutants caused spontaneous apoptosis and multiple cell division defects with supernumerary centrosomes, multipolar mitotic spindles and multinucleation (Chen et al., 2000; Li et al., 1999; Olie et al., 2000). This suggested that survivin may act both as a mitotic regulator and a cytoprotective factor at cell division, a pathway potentially

exploited in cancer where the *survivin* gene is broadly upregulated (Ambrosini et al., 1997; Velculescu et al., 1999). However, recent studies proposed a different subcellular localization of endogenous (Uren et al., 2000), or transfected (Skoufias et al., 2000; Wheatley et al., 2001) survivin, which was found associated with kinetochores of metaphase chromosomes and the central spindle midzone at anaphase. This pattern was reminiscent of 'chromosomal passenger proteins', molecules that participate in cleavage furrow formation (Giet and Glover, 2001; Kaitna et al., 2000), and suggested that survivin and IAPs in yeast and *C. elegans* (Speliotes et al., 2000; Uren et al., 1999) could participate in an evolutionary conserved pathway of cytokinesis (Uren et al., 2000).

To gain further insights into the structure-function of the survivin pathway, we have now revisited the topography and cell cycle regulation of endogenous survivin with a novel panel of monoclonal and polyclonal antibodies. Inconsistent with a proposed definition of 'chromosomal passenger protein' (Skoufias et al., 2000; Uren et al., 2000; Wheatley et al., 2001), we found that only 20% of cellular survivin is actually associated with kinetochores, whereas the majority of endogenous survivin is bound to microtubules, complexed with $p34^{cdc2}$-cyclin B1, and required for the assembly of a normal bipolar mitotic spindle.

Materials and Methods

Cells, cell-cycle synchronization and transfections

Human cervical carcinoma HeLa cells were obtained from American Type Culture Collection (Manassas, VA), and maintained in culture in DMEM medium (Gibco-BRL) containing 10% FBS (Gibco) and 5% Pen-Strep (Gibco). For cell cycle synchronization experiments, HeLa cells were G1-arrested by a 16 hour mimosine block at 37°C (Li et al., 1999; Li et al., 1998), and harvested at 0, 3, 6, 9 and 12 hours after release. Cells were analyzed for DNA content by propidium iodide staining and flow cytometry, as described (Li et al., 1999). A wild type survivin cDNA was cloned downstream of a FLAG epitope in pcDNA3 (Invitrogen Corp., San Diego, CA) and used for mammalian cell expression. Transient transfections were carried out by lipofectAmine (Li et al., 1999) using pFLAG-survivin. Briefly, HeLa cells were plated onto 6-well plates at 5×10^5/well and transfected with 2.5 μg of plasmid DNA and 9 μl of lipofectAmine (Life Technologies) in 1 ml of serum-free OptiMEM medium (Life Technologies) for 6 hours at 37°C. Transfection efficiency was monitored by western blotting.

Antibodies

Rabbit polyclonal antibodies (pAb) NOVUS (NOVUS Biologicals, Littleton, CO) and BTD raised against full-length recombinant survivin were characterized in previous studies (Grossman et al., 2001; Grossman et al., 1999). An affinity-purified rabbit antibody recognizing survivin phosphorylated on Thr^{34} by $p34^{cdc2}$-cyclin B1 (α-survivinT34*) was generated and characterized in previous studies (O'Connor et al., 2000). A mouse monoclonal antibody (mAb) 8E2 (IgG1, Neomarkers Inc. Fremont, CA) raised against full-length recombinant survivin was described previously (Li et al., 1998). A new mAb panel to survivin was generated in mice by standard hybridoma technology using bacterially expressed, full-length recombinant survivin as an immunogen. Antibody producing cells were fused to NS1 myeloma cells, and hybridomas were cloned twice by limiting dilution. Three new mAbs to survivin, 32.1, 60 and 58 (all of IgG1 subtype), were established and confirmed for reactivity with recombinant survivin by ELISA, and for recognition of ~16.5 kDa survivin by western blotting of HeLa cell extracts. A mouse mAb to β-actin was from Sigma (clone AC-15, A5441). A rabbit polyclonal antibody to FLAG was from Sigma (F7425). A mouse mAb 2E1 reacting with the p80 subunit of the Ku nuclear antigen was characterized previously (Rothermel and Altieri, 1998). A mouse mAb 1G12 to intercellular adhesion molecule-1 (ICAM-1) was characterized previously (Duperray et al., 1997).

Proteins, peptides and epitope mapping

Full-length human survivin in pGEX2T vector (Pharmacia) was expressed as a GST fusion protein as described previously (Li et al., 1998). The GST frame was removed by overnight incubation with 10 U/ml thrombin (Sigma), followed by incubation with 100 μl of benzamidine-coated beads (Sigma, A8332) for 4 hours at 4°C. Two truncated survivin mutants, Met^1-Gly^{99} and Glu^{100}-Asp^{142}, were also expressed as GST fusion proteins, released from the GST frame and purified as described above. A series of partially overlapping synthetic peptides was generated to duplicate the entire survivin sequence between Met^1 and Phe^{101}, including Met^1-Pro^{12}, Ala^3-Ile^{19}, Pro^{12}-Gly^{30}, Leu^{28}-Ala^{39}, Met^{38}-Thr^{48}, Pro^{47}-Phe^{58}, Cys^{57}-Trp^{67}, Lys^{79}-Lys^{90} and Val^{89}-Phe^{101}. For epitope mapping, aliquots of the various recombinant survivin proteins (1 μg/ml), or survivin synthetic peptides (5 μg/ml) dissolved in 1% DMSO, were immobilized on plastic microtiter wells (Immulon-2, Dynatech Laboratories, Chantilly, VA) in bicarbonate buffer, pH 9.5 (100 μl/well) for 18 hours at 4°C. Wells were blocked with 3% gelatin for 1 hour at 37°C, rinsed and incubated with 1:3 serial dilutions of culture supernatants of mAbs 32.1, 58, 60 or mAb 1G12. In other experiments, serial dilutions of pAbs NOVUS or BTD, mAb 8E2 or control rabbit serum were used as primary antibodies. After washes in TBS, pH 7.4, containing 0.1% bovine serum albumin and 0.5% Tween-20, binding of the primary antibodies was revealed by addition of biotin-conjugated, rabbit anti-mouse or goat anti-rabbit IgG for 1 hour at 37°C, followed by streptavidine-alkaline phosphatase and determination of absorbance at A_{405} using *p*-nitrophenyl phosphate (Sigma).

Subcellular fractionation

HeLa cells at $0.5\text{-}1\times10^7$ were lysed in two volumes of Hepes buffer (25 mM Hepes, pH 7.5, 100 mM KCl, 2 mM EGTA, 1% Triton X-100, 1 mM Na_3VO_4, 1 mM PMSF) for 20 minutes at 4°C and centrifuged at 900 ***g*** for 10 minutes at 4°C. The pellet-1 was collected as nuclear fraction and the supernatant-1 (post nuclear sedimentation (PNS) fraction) was centrifuged at 2000 ***g*** for 10 minutes at 4°C. The supernatant-2 was centrifuged at 100,000 ***g*** in a Beckman TLA 100.4 rotor for 30 minutes at 4°C, and the resulting supernatant (supernatant-3, cystosol) and the pellet-3 (cytoskeleton associated proteins) were collected. The nuclear and cytoskeletal fractions were suspended in equal volumes of Hepes buffer and analyzed for survivin expression by western blotting.

For sub-nuclear fractionation, HeLa cells at 2×10^7 were washed once in PBS, pH 7.4, and lysed in 1 ml of solution 1 (100 mM Pipes, pH 7.5, 1 mM EGTA, 1 mM $MgCl_2$, 1% Triton X-100, 1 mM Na_3VO_4, 1 mM PMSF). After centrifugation at 900 ***g*** for 5 minutes at 4°C, the supernatant was collected (PNS fraction) and nuclei (pellet-1) were further washed once with solution 1 and suspended in 1 ml of solution 2 (10 mM Pipes pH 7.5, 300 mM sucrose, 100 mM KCl, 3 mM $MgCl_2$, 1 mM EGTA, 1 mM Na_3VO_4, 1 mM PMSF) containing 200 μg/ml of DNAse. The resulting cell extract was incubated for 45 minutes at 33°C and then centrifuged at 1500 ***g*** for 10 minutes at 4°C. The supernatant-2 (DNAse released proteins, DRp fraction) was collected and the pellet-2 was resuspended in 1 ml of solution 2. 0.25 M ammonium sulfate (final concentration) was added dropwise and the extract was incubated for 5 minutes at room temperature. After centrifugation at 4000 ***g*** for 5 minutes at 4°C, the fraction containing

nucleoplasmic proteins was collected (supernatant-3, NPp fraction) and the pellet-3 was suspended in 1 ml of solution 2. NaCl was added dropwise to a final concentration of 2 M, and the extract was further incubated for 10 minutes at 0°C and re-centrifuged at 8000 ***g*** for 5 minutes at 4°C. The supernatant-4 (remnant nucleoplasmatic and outer nuclear matrix proteins, ONMp fraction) and the pellet-4 suspended in 1 ml solution 2 (nuclear matrix, NM fraction) were collected and analyzed for survivin expression by western blotting.

Enriched centrosome fractions were prepared from HeLa cells as previously described (Li et al., 1999). Briefly, cells were solubilized in 50 ml of 1 mM Hepes, pH 7.2, 0.5% NP-40, 0.5 mM $MgCl_2$, 0.1% β-mercaptoethanol plus protease inhibitors. The cell lysate was passed five times through a 10 ml serological pipette, centrifuged at 2500 ***g*** for 10 minutes and incubated in 10 mM Hepes and 1 μg/ml DNAse I for 30 minutes at 0°C. The mixture was overlaid on a 5 ml 60% sucrose cushion containing 10 mM Hepes, pH 7.2, 0.1% Triton X-100, and 0.1% β-mercaptoethanol and centrifuged at 10,000 ***g*** for 30 minutes. The interface of the sucrose cushion containing enriched centrosomal fractions was collected, separated on a 5-15% SDS polyacrylamide gel and analyzed for survivin expression by western blotting.

For microtubule co-sedimentation experiments, HeLa cell extracts (10 mg/ml) in MES buffer (0.1 M 2-[morpholino]ethane sulphonic acid, 1 mM EGTA, 1 mM $MgSO_4$), pH 6.6, were subjected to three sequential rounds of temperature-dependent microtubule polymerization/depolymerization at 37°C or 0°C for 30 minutes each, respectively. After the last round of polymerization, microtubules were centrifuged through a sucrose cushion at 39,000 ***g*** for 30 minutes at 30°C and aliquots of pellet and supernatant were separated on a 5-15% SDS polyacrylamide gel and analyzed for survivin expression by western blotting. Protein extracts were quantified by Bradford protein assay.

Immunoprecipitation, immunoblotting and immunofluorescence

Asynchronously growing HeLa cells or HeLa cells arrested at the metaphase-anaphase transition by treatment with 0.2 μM taxol (Sigma) for 16 hours at 37°C were solubilized in lysis buffer containing 0.03% CHAPS. 200 μg of precleared HeLa cell extracts were immunoprecipitated with mAbs 32.1 (10 μg/ml), or pAb NOVUS to survivin (4 μg/ml) for 16 hours at 4°C, with precipitation of immune complexes by addition of 50 μl of a 50:50 protein A slurry, as described (O'Connor et al., 2000). For immunoblotting, aliquots of the various subcellular fractions or survivin immunoprecipitates were separated by electrophoresis on a 5-15% SDS-PAGE, transferred to nylon membranes and incubated with various primary antibodies (1-5 μg/ml), followed by HRP-conjugated secondary antibodies (Amersham) and chemiluminescence (Amersham).

Immunofluorescence and confocal microscopy were carried out as described (Li et al., 1998). HeLa cells grown on optical grade coverslips (12 mm diameter) were washed for 30 seconds at 37°C in microtubule stabilizing buffer (MSB) containing 0.1 M Pipes, pH 6.9, 1 mM EGTA, 2.5 mM GTP, 4% polyethyl glycole 6000. Cells were incubated for 3-8 minutes at 37°C in MSB containing 0.5% Triton X-100 or 0.1% NP-40, washed twice in MSB for 30 seconds at 37°C, and fixed in MSB containing 3.7% EM grade formaldehyde for 20 minutes. Coverslips were incubated with mAbs 32.1 or 8E2 to survivin (10 μg/ml), 20C6 to tubulin, pAbs NOVUS or BTD to survivin (5-10 μg/ml), or a human autoimmune CREST antibody (1:5000, generously provided by Joseph Craft, Yale University School of Medicine). DNA was stained with Hoechst 33342. Binding of the primary antibodies was revealed by addition of fluorescein (FITC)- or Texas red (TR)-conjugated antibodies of the appropriate specificity (Molecular Probes, Inc. Junction City, OR). After washes, coverslips were mounted in Mowiol 4-88 (Hoechst, Frankfurt/Main, Germany), and analyzed on a Zeiss Axiophot microscope or by confocal laser scanning microscopy (CLSM Bio-Rad 1024). Files obtained from confocal microscopy were assembled with Adobe PhotoShop 5.0.

In vitro kinase assay

$p34^{cdc2}$ kinase activity was assessed in immunoprecipitates from untreated or taxol-treated HeLa cells, as described previously (O'Connor et al., 2000). Briefly, cells were harvested under nondenaturing conditions and lysed using sonication in 1× ice-cold lysis buffer (50 mM Tris, pH 7.5; 1% NP-40; 0.25% DOC; 150 mM NaCl; 0.2 mM EGTA; 1 mM EDTA, pH 8.0) containing 20 mM NaF, 1 mM Na_3VO_4, plus PMSF. After centrifugation, the supernatant was precleared by incubation with 10 μl MEP HyperCel (Gibco/BRL) beads for 1 hour at 4°C, and incubated with a mAb to $p34^{cdc2}$ (Zymed) for 1 hour at 4°C followed by addition of 10 μl of MEP HyperCel beads overnight at 4°C. The immune complexes were recovered and washed twice with lysis buffer and twice with reaction buffer (20 mM Hepes, pH 7.4; 10 mM $MgCl_2$; 0.5 mM DTT). The $p34^{cdc2}$ immunoprecipitates were incubated with 1 μg of histone H1 (Boehringer Mannheim) and 5 μCi [γ-^{32}P]ATP (Amersham Pharmacia) in 20 μl of reaction buffer, for 30 minutes at 30°C. The reaction was terminated by addition of 2× SDS sample buffer, and samples were separated on a 15% SDS-polyacrylamide gel followed by autoradiography or western blotting with mAb 32.1 (1 μg/ml) or pAb NOVUS (1 μg/ml).

Lipid-based antibody loading of synchronized HeLa cells

HeLa cells were seeded at $0.5\text{-}1\times10^5$ in a 24-well plate and synchronized at the G1/S boundary by a 16 hour culture in the presence of 2 mM thymidine. 4 hours after thymidine release, cells were loaded with 2 μg of mAb 8E2 or pAb NOVUS to survivin, or 2 μg control mouse or rabbit IgG in 250 μl of serum-free medium, in the presence of BioPORTER™ Protein Transfection Reagent (Gene Therapy System, BP509604). After 4 hours of incubation, the antibody uptake reaction was terminated by addition of 250 μl of complete growth medium containing 20% FCS. The efficiency of intracellular antibody loading was determined using 1 μg of FITC-conjugated goat IgG, followed by fluorescence microscopy analysis of transduced cultures. Typically, this protocol resulted in expression of green fluorescence in ~80% of treated cells. Cells were fixed 11 hours after thymidine release, labeled with an antibody to tubulin and analyzed by immunofluorescence, as described above. Images were collected using an IX70 Olympus inverted microscope equipped with 40× (0.85 NA) and 60× (1.4 NA) objectives and Inovision (Raleigh, NC) image analysis software.

Results

Characterization of novel mAbs to survivin

A novel mAb panel to full-length recombinant survivin was generated by hybridoma technology, and cloned by limiting dilution. The three new mAbs designated 32.1, 58 and 60, and the previously characterized mAb 8E2 (Li et al., 1998) reacted in a concentration-dependent manner with full-length recombinant survivin, and with a Met^1-Gly^{99} truncated survivin mutant, lacking the C-terminus α-helix, by ELISA (Fig. 1A). A control mAb to ICAM-1, 1G12 (Duperray et al., 1997), did not react with survivin proteins, and no reactivity of the various mAbs with the survivin C-terminus Glu^{100}-Asp^{142} was detected by ELISA (Fig. 1A). mAbs 32.1, 58 and 60 all recognized full-length recombinant survivin by western blotting in a concentration-dependent manner, indistinguishably from a rabbit pAb BTD to full-length survivin (Fig. 1B) (Grossman et al., 1999). Using partially overlapping synthetic peptides, the

mAb 32.1 epitope was localized to the peptide Ala^{3}-Ile^{19}, and further narrowed to the sequence Ala^{3}-Pro^{12} (Fig. 1C). By contrast, mAbs 58 and 60 reacted with the survivin sequence Cys^{57}-Trp^{67}, by peptide mapping (Fig. 1C).

Identification of immunochemically distinct subcellular pools of survivin

In subcellular fractionation experiments, pAb NOVUS to full-length survivin (O'Connor et al., 2000) detected a ~16.5 kDa survivin band both in cytosolic (Cy), cytoskeletal (Csk) and nuclear (N) HeLa cell fractions, by western blotting (Fig. 2A). In control experiments, cytosolic and cytoskeletal fractions reacted with an antibody to β-actin, whereas nuclear fractions contained the p80 subunit of Ku nuclear antigen, by immunoblotting (not shown). Surprisingly, the new mAb panel exhibited a differential and mutually exclusive recognition of survivin in isolated subcellular fractions. mAb 32.1 recognized a 16.5 kDa survivin band in the nuclear, but not in the cytosolic fraction (PNS, post-nuclear sedimentation comprising cytosolic and cytoskeletal extracts) (Fig. 2B). Conversely, mAbs 58 or 60 bound to survivin exclusively in the cytosol, whereas no reactivity was observed with nuclear extracts, by western blotting (Fig. 2B). Similarly, in extracts of HeLa cells over-expressing FLAG-tagged survivin, a nuclear pool of survivin recognized by the antibody to FLAG or pAb NOVUS was entirely unreactive with mAb 60 (Fig. 2C). By contrast, all three antibodies indistinguishably recognized a more prominent (see below) cytosolic pool of FLAG-tagged, over-expressed survivin by western blotting (Fig. 2C).

The potential association of the various subcellular pools of survivin with the mitotic apparatus was next investigated. As shown in Fig. 3A, mAb 32.1 did not recognize survivin bands in centrosome-enriched HeLa cell fractions, consistent with its lack of reactivity with cytosolic survivin (Fig. 2B). By contrast, mAbs 58 and 60 immunoblotted a 16.5 kDa centrosome-associated survivin band (Fig. 3A), in agreement with previous observations (Li et al., 1999). In other experiments, none of the three mAbs 32.1, 58 or 60 recognized microtubule-associated survivin, co-sedimented after three rounds of temperature-dependent microtubule polymerization/depolymerization (Fig. 3B). However, a strong survivin band was immunoblotted in association with polymerized microtubule by pAb BTD to full-length survivin (Fig. 3B).

Distinct subcellular localization of endogenous survivin pools

In interphase HeLa cells, mAb 8E2 recognized survivin with a filamentous pattern consistent with localization to cytoplasmic

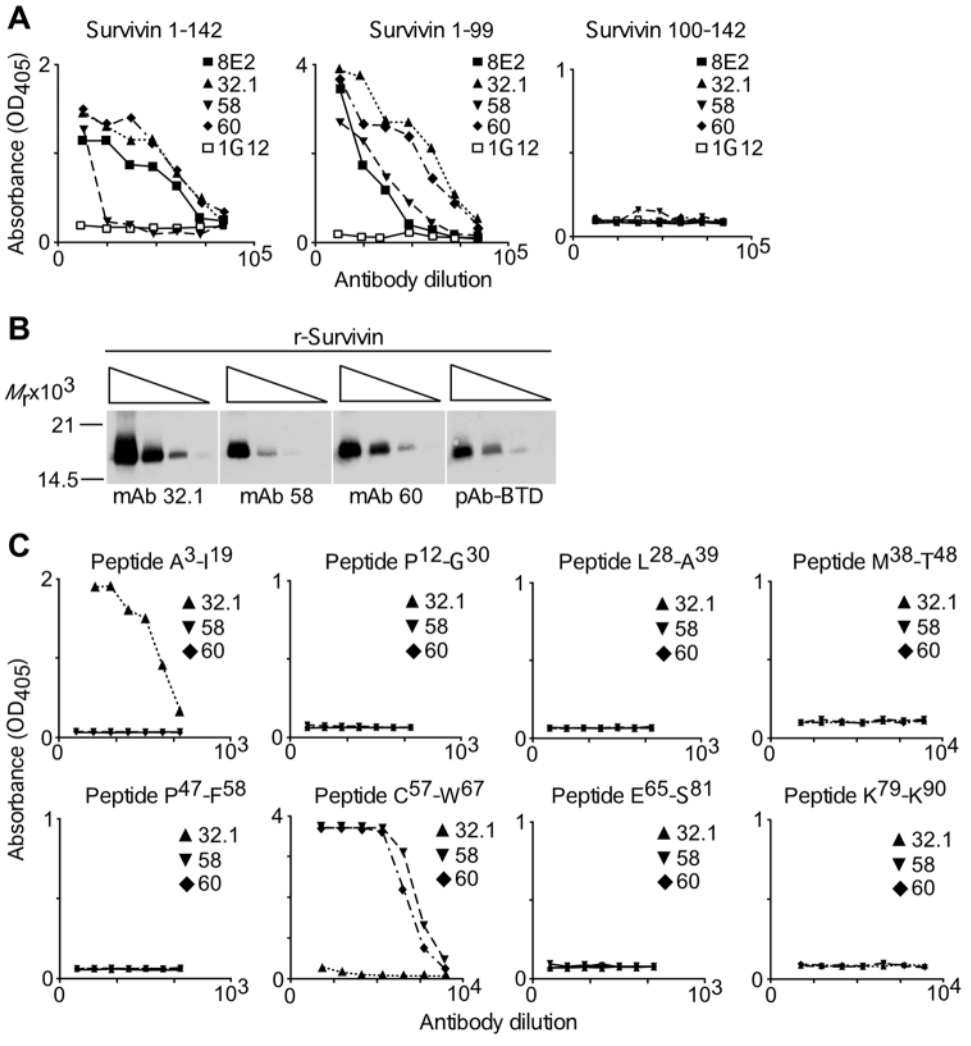

Fig. 1. Characterization of new mAbs to survivin. (A) Reactivity with survivin domains. Full-length (Met^{1}-Asp^{142}) or truncated (Met^{1}-Gly^{99} or Glu^{100}-Asp^{142}) survivin were immobilized on plastic microtiter plates (1 μg/ml) and incubated with the indicated dilutions of mAbs 8E2, 32.1, 58 or 60 to survivin, or mAb 1G12 to ICAM-1. (B) Western blotting. Increasing dilutions of recombinant survivin (r-Survivin) at 1, 0.2, 0.04, 0.008 μg were immunoblotted with mAbs 32.1, 58 or 60 or rabbit polyclonal antibody BTD, followed by chemiluminescence. Molecular weight (M_r) markers in kDa are shown on the left. (C) Epitope mapping. Partially overlapping synthetic peptides duplicating the indicated survivin sequences were immobilized on plastic microtiter plates (5 μg/ml) and incubated with increasing dilutions of mAbs 32.1, 58 or 60. For panels A and C, binding of the various antibodies was determined by ELISA at A_{405}. Data are representative of a single experiment out of at least three independent determinations.

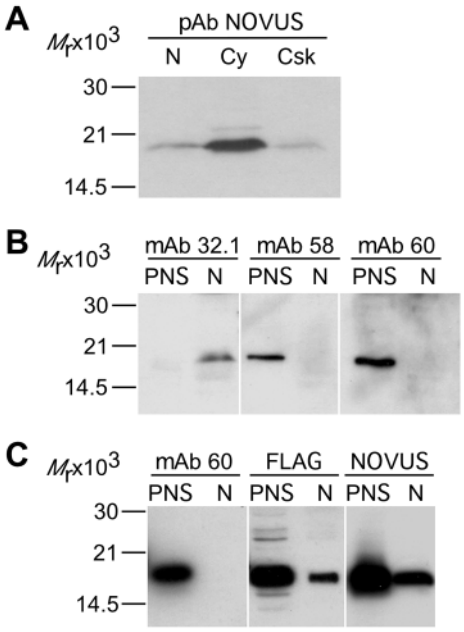

Fig. 2. Sub-cellular distribution of immunochemically distinct survivin pools. (A) Subcellular distribution of endogenous survivin. Equal volumes of nuclear (N), cytosolic (Cy) and cytoskeleton (Csk) fractions were immunoblotted with pAb NOVUS followed by chemiluminescence. (B) Differential antibody reactivity with endogenous survivin. Aliquots (100 μg) of PNS (post-nuclear sedimentation, comprising cytosolic and cytoskeletal extracts) and N (nuclear) fractions were immunoblotted with mAbs 32.1, 58 or 60, or pAb NOVUS followed by chemiluminescence. (C) Subcellular distribution of over-expressed survivin. Aliquots (100 μg) of PNS or N extracts of HeLa cells transfected with N-terminal FLAG-tagged survivin were immunoblotted with the indicated mAb 60, pAb NOVUS or an antibody to FLAG.

microtubules, by immunofluorescence and confocal microscopy (Fig. 4Aa). During mitosis, mAb 8E2 strongly labeled spindle poles and the entire length of mitotic spindle microtubules at metaphase (Fig. 4Ab), and anaphase (Fig. 4Ac), and intensely stained midbodies at telophase (Fig. 4Ad), in agreement with previous observations (Li et al., 1998; Wheatley et al., 2001). By dual immunofluorescence labeling, mAb 8E2 reactivity remained restricted to mitotic spindle microtubules, and did not co-localize with the punctate staining of a human anti-centromere CREST antibody with kinetochores of metaphase chromosomes (Fig. 4Ae). Microtubule destruction by colchicine treatment did not affect the reactivity of the CREST antibody with metaphase chromosomes, whereas it abolished the labeling of mitotic spindle microtubules by mAb 8E2 (Fig. 4Af). In striking contrast to the mAb 8E2 pattern, the novel mAb 32.1 did not react with mitotic spindle microtubules, but exhibited a punctate labeling of metaphase chromosomes that co-localized with the reactivity of the antiserum to CREST with kinetochores (Fig. 4Ba). At anaphase, mAb 32.1 labeling transferred to the microtubules of the central spindle midzone, and concentrated in midbodies at telophase, whereas labeling by the CREST antibody remained associated with separating sister chromatids (Fig. 4Bb-d). Analysis of a chromosomal spread revealed that mAb 32.1 reacted with kinetochores of individual chromosomes (Fig. 4Cc, inset), and co-localized with the CREST labeling (Fig. 4Ca-c). By contrast, mAb 8E2 did not react with individual chromosomes, but recognized a colchicine-resistant structure that contains γ-tubulin (Fig. 4Cd,e).

Consistent with the observation that pAbs to full-length survivin recognized both nuclear and cytosolic survivin by western blotting (Fig. 2; Fig. 3), pAbs NOVUS (Fig. 5Aa-c) and BTD (Fig. 5Ad-f) simultaneously identified both pools of survivin within the same mitotic cell, with intense labeling of spindle poles and mitotic spindle microtubules (mAb 8E2 pattern), and a more limited reactivity with the central spindle midzone (mAb 32.1 pattern) (Fig. 5A). At telophase, both pAbs recognized survivin in midbodies (Fig. 5Ac,f), consistent with the individual reactivity of mAbs 8E2 and 32.1 (Fig. 4A,B). In peptide mapping experiments, pAbs NOVUS and BTD reacted with survivin epitopes Ala^{3}-Ile^{19}, Met^{38}-Thr^{48}, Pro^{47}-Phe^{58} and Cys^{57}-Trp^{67} (Fig. 5B). Although the mAb 8E2 epitope was localized to the peptide Cys^{57}-Trp^{67} (Fig. 5B) and the inefficient protein recognition by western blotting (not shown), suggest that mAb 8E2 may react with a conformational epitope within Cys^{57}-Trp^{67}. In agreement with their lack of reactivity with nuclear survivin and microtubule-associated survivin, mAbs 58 and 60 did not provide clear patterns of survivin localization in mitotic or interphase HeLa cells, by immunofluorescence (not shown).

Differential cell cycle regulation of cytosolic and nuclear survivin pools

In subcellular fractionation experiments, pAb NOVUS reacted with a 16.5 kDa survivin band in cytosolic extracts and in a

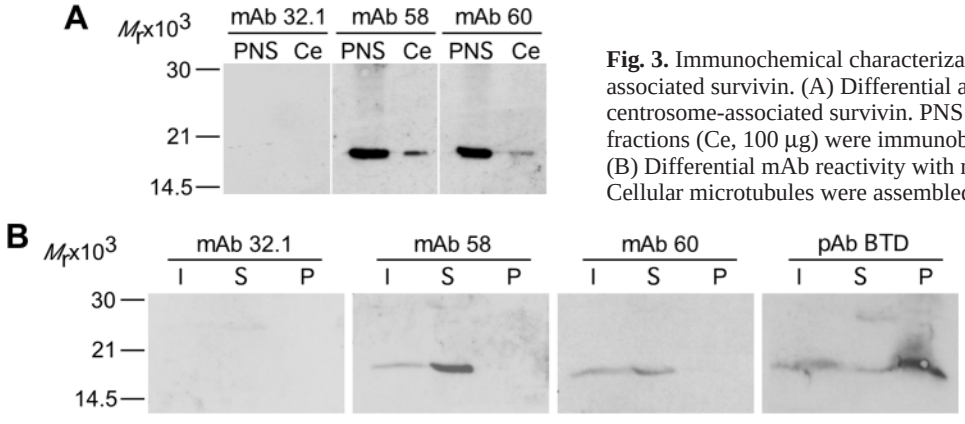

Fig. 3. Immunochemical characterization of centrosome- and microtubule-associated survivin. (A) Differential antibody reactivity with cytosolic or centrosome-associated survivin. PNS extracts (100 μg) or centrosome-enriched fractions (Ce, 100 μg) were immunoblotted with mAbs 32.1, 58 or 60. (B) Differential mAb reactivity with microtubule-associated survivin. Cellular microtubules were assembled through three successive rounds of temperature-dependent polymerization/depolymerization, centrifuged through a sucrose gradient and aliquots of input (I), supernatant (S) and pellet (P) were immunoblotted with mAbs 32.1, 58, 60 or pAb BTD. Molecular weight markers in kDa are shown on the left of each panel.

subnuclear fraction predominantly containing nucleoplasmic proteins (Fig. 6A). By contrast, no survivin bands were detected in subnuclear fractions containing DNAse-released proteins (DRp), outer nuclear matrix proteins (ONMp), or nuclear matrix proteins (NMp) (Fig. 6A). A ratio of ~1:6 was derived for the survivin pool associated with nucleoplasmic proteins versus cytosolic survivin, by quantitative western blotting with pAb NOVUS (Fig. 6B). In synchronized cytosolic extracts, a background level of survivin in interphase cells detected by pAb NOVUS abruptly increased at mitosis (Fig. 6C), 9 hours after release from a mimosine block, as determined by DNA content analysis and flow cytometry (Fig. 6E) (Li et al., 1998). This coincided with mitotic phosphorylation of survivin on Thr^{34} by $p34^{cdc2}$-cyclin B1, as determined by western blotting with a Thr^{34}-phospho-specific antibody (α-survivinT34*) (Fig. 6C), and in agreement with previous observations (O'Connor et al., 2000). However, in synchronized nuclear extracts, survivin detected by pAb NOVUS began to accumulate in S phase 6 hours after mimosine release, thus potentially suggesting active nuclear import at this cell cycle phase, and continued to increase throughout mitosis (Fig. 6D) 9 and 12 hours after G1 release (Fig. 6E). In addition, no survivin bands phosphorylated on Thr^{34} were detected in synchronized nuclear extracts at the various cell cycle phases, by western blotting with α-survivinT34* (Fig. 6D). In control experiments, synchronized nuclear extracts, but not cytosolic extracts, strongly reacted with mAb 2E1 to the p80 subunit of Ku nuclear antigen (Rothermel and Altieri, 1998), thus corroborating the specificity of the subcellular fractionation protocol (Fig. 6C,D).

Differential association of nuclear versus cytosolic survivin with $p34^{cdc2}$-cyclin B1

Survivin immunoprecipitated with pAb NOVUS from asynchronous or taxol-arrested HeLa cells was found associated with $p34^{cdc2}$, by western blotting of the immune complexes with an antibody to $p34^{cdc2}$ (Fig. 7A,B), and in agreement with previous observations (O'Connor et al., 2000). Consistent with its lack of reactivity for cytosolic survivin (Fig.

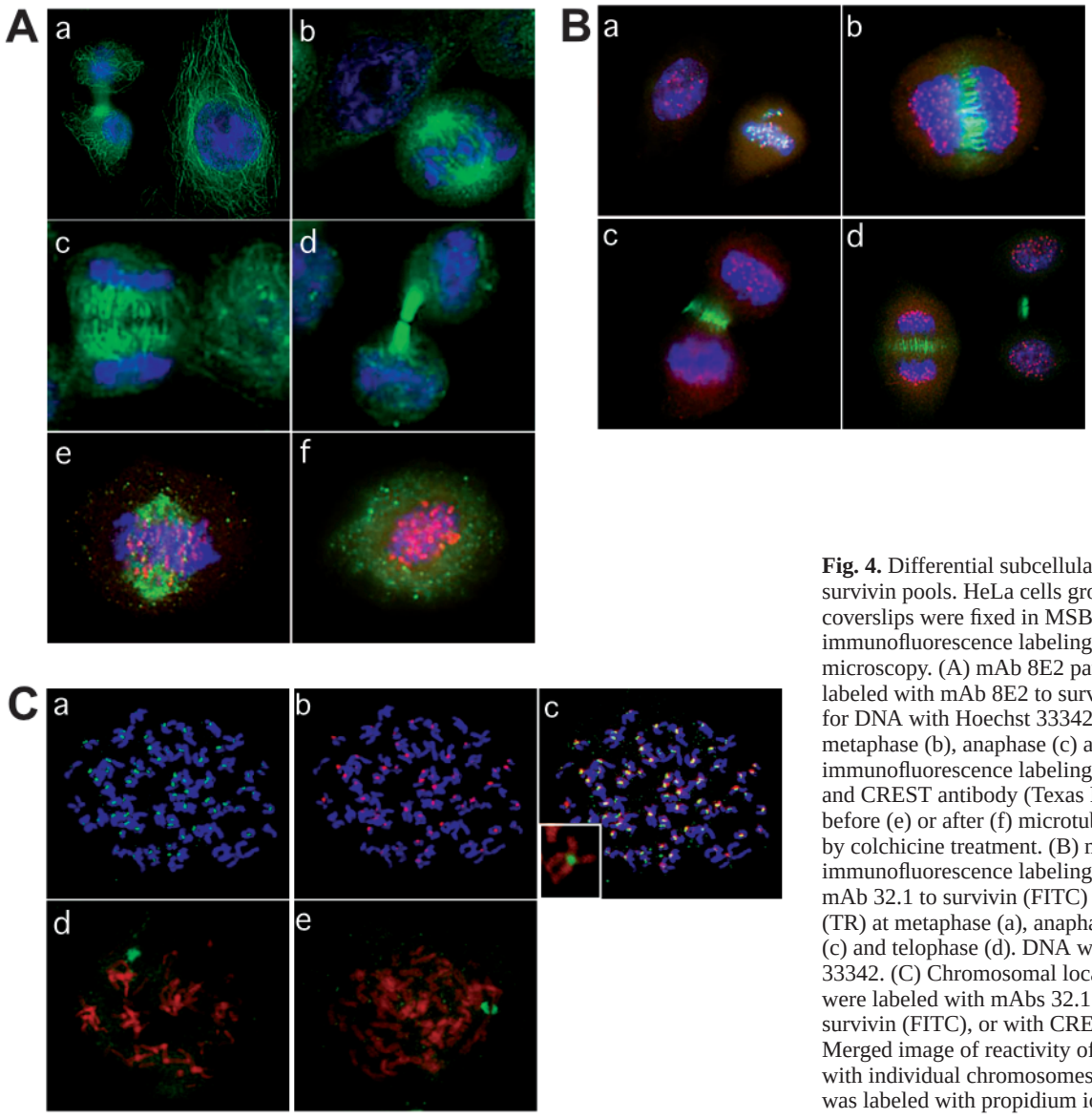

Fig. 4. Differential subcellular localization of survivin pools. HeLa cells grown on optical grade coverslips were fixed in MSB, and processed for dual immunofluorescence labeling and confocal microscopy. (A) mAb 8E2 pattern. HeLa cells were labeled with mAb 8E2 to survivin (FITC) and stained for DNA with Hoechst 33342 in interphase (a), metaphase (b), anaphase (c) and telophase (d). Dual immunofluorescence labeling with mAb 8E2 (FITC) and CREST antibody (Texas Red, TR) is shown before (e) or after (f) microtubule depolymerization by colchicine treatment. (B) mAb 32.1 pattern. Dual immunofluorescence labeling of HeLa cells with mAb 32.1 to survivin (FITC) and CREST antibody (TR) at metaphase (a), anaphase A (b), anaphase B (c) and telophase (d). DNA was stained with Hoechst 33342. (C) Chromosomal localization. Chromosomes were labeled with mAbs 32.1 (a) or 8E2 (d,e) to survivin (FITC), or with CREST antibody (TR, b). (c) Merged image of reactivity of mAb 32.1 and CREST with individual chromosomes. For all panels, DNA was labeled with propidium iodide.

2), mAb 32.1 did not immunoprecipitate survivin from asynchronously growing HeLa cells (Fig. 7A). By contrast, mAb 32.1 immunoprecipitated survivin from taxol-arrested HeLa cells after nuclear envelope breakdown (Fig. 7B). However, no association of $p34^{cdc2}$ with survivin immunoprecipitated by mAb 32.1 was demonstrated by Western blotting of the immune complexes (Fig. 7B). Consistent with the data presented above, α-survivinT34* strongly recognized a Thr^{34}-phosphorylated survivin band in pAb NOVUS immunoprecipitates from taxol-synchronized cells, but not in mAb 32.1 immunoprecipitates under the same experimental conditions (Fig. 7C). Analysis of kinase activity of $p34^{cdc2}$ immunoprecipitates at various time intervals after taxol treatment revealed the presence of a ~32 kDa phosphorylated histone H1 band used as a substrate, and a faster migrating band of 16.5 kDa consistent with the size of survivin (Fig. 7D). Immunoblotting with pAb NOVUS confirmed the identity of the phosphorylated 16.5 kDa band co-immunoprecipitated with $p34^{cdc2}$ as survivin (Fig. 7D). However, mAb 32.1 did not react with any bands in $p34^{cdc2}$ immunoprecipitates under the same experimental conditions (Fig. 7D). DNA content analysis by propidium iodide staining and flow cytometry confirmed the sustained mitotic arrest induced by taxol treatment (Fig. 7E).

Global antibody targeting of endogenous survivin causes defects of spindle microtubules

Lipid-based intracellular loading of pAb NOVUS in synchronized HeLa cells resulted in gross abnormalities of mitotic progression and a sixfold increase in the number of cells with aberrant multipolar mitotic spindles, compared with control cultures loaded with non-immune rabbit IgG (Fig. 8A,B). A threefold increase in aberrant multipolar mitoses was also observed after intracellular loading of mAb 8E2, compared with non-immune mouse IgG (not shown). By contrast, treatment with pAb NOVUS did not result in gross abnormalities of cytokinesis, and formation of midbodies was unaffected (Fig. 8A). Similar results were obtained after microinjection in the nucleus of mAb 32.1 (data not shown). In parallel experiments, loading of synchronized HeLa cells with pAb NOVUS resulted in a reproducible increase in mitotic index, as compared with control cultures (Fig. 8C).

Discussion

In this study, we have shown that endogenous survivin exists in strikingly different subcellular pools, comprising a predominant (~80%) cytosolic fraction and a smaller nuclear pool that localizes to kinetochores of metaphase chromosomes. The two survivin pools are immunochemically distinct, independently modulated during cell cycle progression, and only cytosolic survivin associates with $p34^{cdc2}$ and is phosphorylated on Thr^{34} by $p34^{cdc2}$. In addition, intracellular antibody targeting of survivin did not affect cytokinesis, but resulted in microtubule defects and formation of multipolar mitotic spindles.

The immunochemical differences between nuclear and cytosolic survivin may explain, in part, the conflicting data of survivin localization reported in the literature (Li et al., 1998; Skoufias et al., 2000; Uren et al., 2000; Wheatley et al., 2001). Two regions in survivin that exhibited strikingly differential antibody reactivity were identified here as Cys^{57}-Trp^{67}, which is exposed in cytosolic and centrosome-associated survivin, but masked in nuclear and microtubule-bound survivin, and Ala^{3}-Ile^{19}, which is accessible in kinetochore-associated survivin, but not in cytosolic survivin.

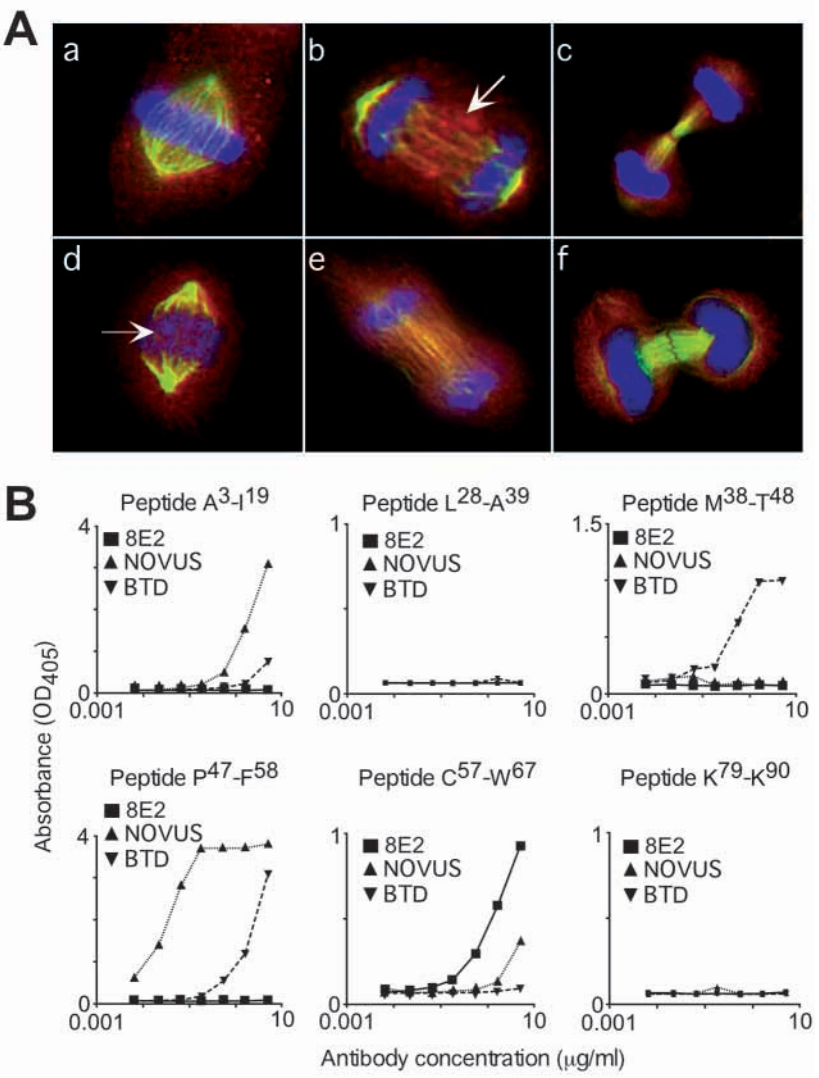

Fig. 5. Simultaneous identification of both survivin pools within mitotic cells. (A) Immunofluorescence. The experimental conditions are the same as in Fig. 4A,B. Image merging of the reactivity of pAb NOVUS (a-c) or BTD (d-f) (TR) and mAb 20C6 to tubulin, (FITC) at metaphase (a,d), anaphase (b,e) and telophase (c,f). DNA was labeled with DAPI. Arrows indicate labeling for survivin on the central spindle midzone (mAb 32.1 pattern). (B) Epitope mapping. Increasing concentrations of pAb NOVUS or BTD, or mAb 8E2 were analyzed for reactivity with the indicated immobilized survivin peptides at A_{405} by ELISA.

A plausible interpretation of these data is that separate post-translational modifications may differentially affect epitope accessibility of nuclear versus cytosolic/microtubule-bound survivin in vivo. Consistent with this view, all the new mAbs described here that exhibited differential reactivity with survivin pools in vivo, indistinguishably reacted with bacterially expressed recombinant survivin, in vitro. Together, these data may explain the incomplete results of survivin localization to kinetochores reported by Uren et al., which were obtained using only one antibody directed to the same survivin sequence Ala^{3}-Ile^{19} (Uren et al., 2000). In addition, simple over-expression experiments in the absence of corroborating biochemical evidence are generally viewed as inadequate to determine protein subcellular localization(s) (Skoufias et al., 2000; Wheatley et al., 2001), and the use of tagged molecules (Skoufias et al., 2000; Wheatley et al., 2001) can notoriously generate incomplete or biased patterns of subcellular topography (Ramanathan et al., 2001). This may be especially true for survivin, where addition of a sequence tag may affect the N-terminus dimerization interface or the C-terminus microtubule-binding domain in the extended α-helices (Verdecia et al., 2000), which

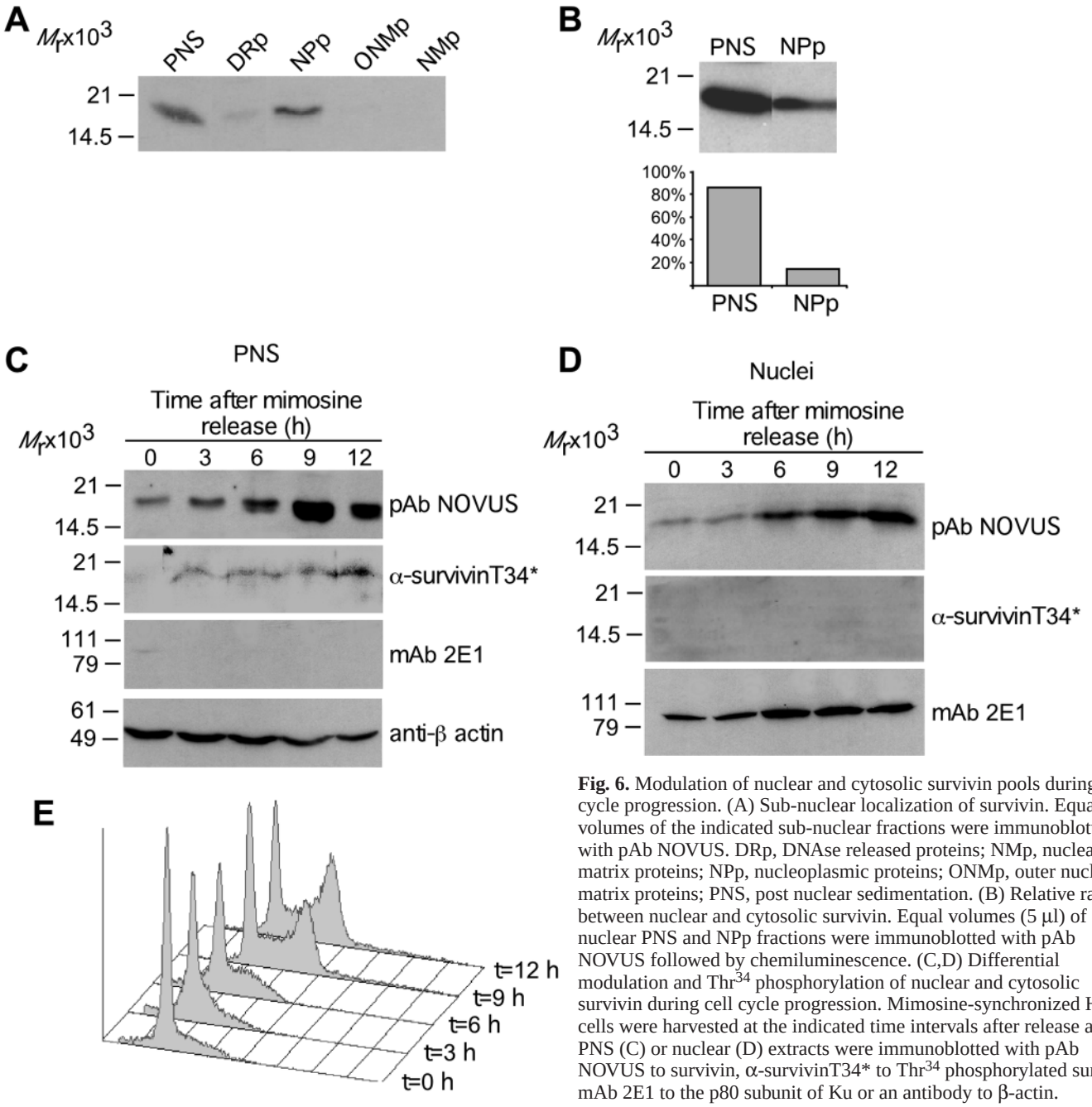

Fig. 6. Modulation of nuclear and cytosolic survivin pools during cell cycle progression. (A) Sub-nuclear localization of survivin. Equal volumes of the indicated sub-nuclear fractions were immunoblotted with pAb NOVUS. DRp, DNAse released proteins; NMp, nuclear matrix proteins; NPp, nucleoplasmic proteins; ONMp, outer nuclear matrix proteins; PNS, post nuclear sedimentation. (B) Relative ratio between nuclear and cytosolic survivin. Equal volumes (5 μl) of sub-nuclear PNS and NPp fractions were immunoblotted with pAb NOVUS followed by chemiluminescence. (C,D) Differential modulation and Thr^{34} phosphorylation of nuclear and cytosolic survivin during cell cycle progression. Mimosine-synchronized HeLa cells were harvested at the indicated time intervals after release and PNS (C) or nuclear (D) extracts were immunoblotted with pAb NOVUS to survivin, α-survivinT34* to Thr^{34} phosphorylated survivin, mAb 2E1 to the p80 subunit of Ku or an antibody to β-actin. (E) Mimosine-synchronized HeLa cells were harvested at the indicated increasing time intervals after release and analyzed for DNA content by propidium iodide staining and flow cytometry.

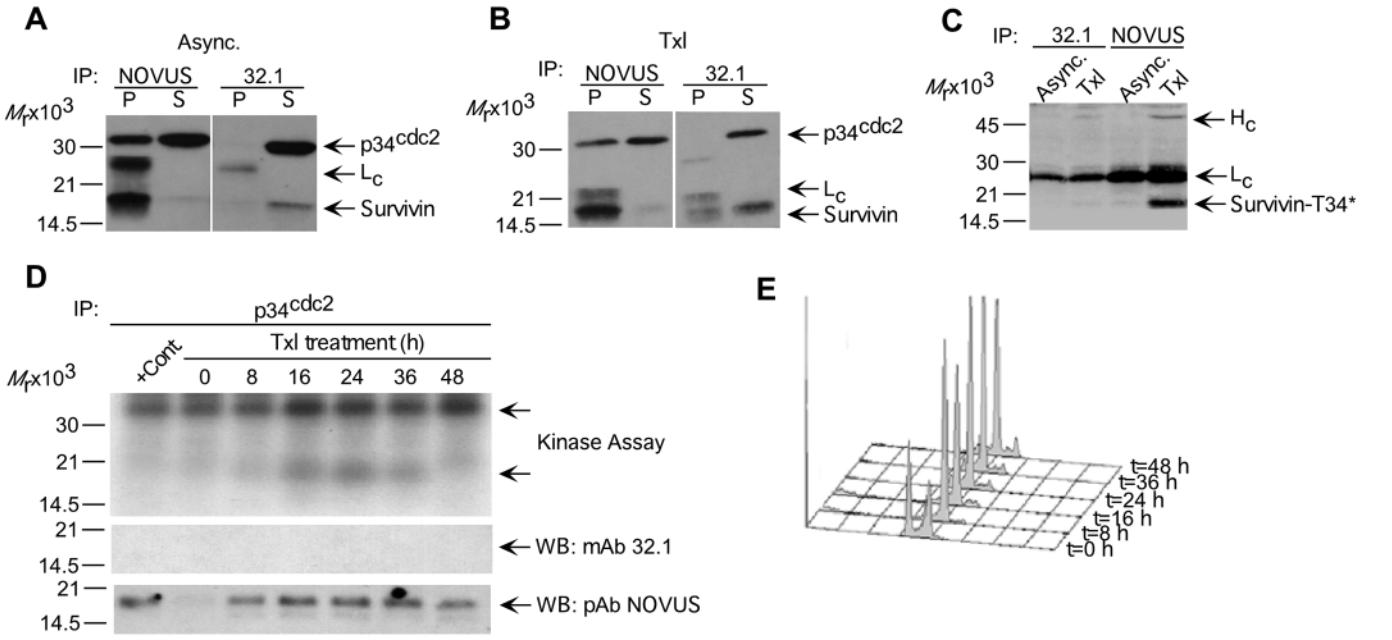

Fig. 7. Differential association of cytosolic and nuclear survivin with $p34^{cdc2}$. Asynchronous (A, Async.) or taxol-arrested (B, Txl) HeLa cells were immunoprecipitated with pAb NOVUS or mAb 32.1. Aliquots of pellet (P) or supernatant (S) were sequentially immunoblotted with pAb NOVUS or a mAb to $p34^{cdc2}$ (arrows). (C) Differential phosphorylation on Thr^{34} by $p34^{cdc2}$-cyclin B1. The experimental conditions are the same as in A and B, except that survivin immunoprecipitated from asynchronously (Async.) or taxol (Txl)-arrested HeLa cells was analyzed by western blotting with the Thr^{34}-phospho-specific antibody, α-survivinT34*. H_c, Ig heavy chain; L_c, Ig light chain. (D) Kinase assay. $p34^{cdc2}$ was immunoprecipitated from control or taxol-arrested HeLa cell extracts and assayed for kinase activity using histone H1 as a substrate. Arrows indicate the position of phosphorylated bands of ~32 and 16.5 kDa. Western blotting of the $p34^{cdc2}$ immune complexes was carried out with mAb 32.1 or pAb NOVUS. (E) Cell cycle analysis. Synchronized HeLa cells were harvested at the indicated time intervals after taxol treatment and analyzed by propidium iodide staining and flow cytometry. IP, immunoprecipitate; WB, western blotting. Molecular weight markers in kDa are indicated on the left of each panel.

may explain the failure of Wheatley et al. to localize NH_2-tagged survivin to the anaphase central spindle (Wheatley et al., 2001). Finally, although Wheatley et al. independently confirmed the spindle microtubule localization of survivin using mAb 8E2 (Wheatley et al., 2001), their inability to observe metaphase spindle labeling with pAb NOVUS is inconsistent with our findings, and may reflect inadequacies in experimental protocol, as we have previously described a taxol-free microtubule-stabilizing procedure for optimal detection of survivin on metaphase microtubules (see Materials and Methods) (Li et al., 1998).

In addition to immunochemical differences, cytosolic and nuclear survivin exhibited distinct kinetics of accumulation at mitosis, and only microtubule-bound survivin physically associated with $p34^{cdc2}$, and was phosphorylated on Thr^{34} by $p34^{cdc2}$-cyclin B1. Previously, phosphorylation of survivin by $p34^{cdc2}$-cyclin B1 was identified as a requisite for apoptosis inhibition (O'Connor et al., 2000), and expression of a phosphorylation-defective survivin $Thr^{34}\rightarrow$Ala dominant negative mutant caused apoptosis of cells traversing mitosis (O'Connor et al., 2000). Intriguingly, the putative survivin-like IAPs in yeast and *C. elegans* that have been solely implicated in cytokinesis (Speliotes et al., 2000; Uren et al., 1999), lack a Thr^{34} consensus phosphorylation site, which is instead perfectly conserved in genuine survivin homologs in *Xenopus* or avian genomes (data not shown). This suggests that a more recent function of survivin in cytoprotection may have evolved from a primordial role in cell division, and coincided with the acquisition of a $p34^{cdc2}$-cyclin B1 phosphorylation site on Thr^{34}, a modification that remained segregated with microtubule-associated survivin, but not with kinetochore-bound survivin.

Whether the small subset of kinetochore-associated survivin participates in an evolutionary conserved pathway of cleavage furrow formation (Speliotes et al., 2000; Uren et al., 1999) is currently unknown. Clearly, the proposed definition of survivin solely as a chromosomal passenger protein (Skoufias et al., 2000; Uren et al., 2000; Wheatley et al., 2001) is inconsistent with the complexity of multiple survivin localization, predominant association with microtubules and defects of mitotic spindle assembly resulting from antibody (this study) or antisense (Li et al., 1999) targeting. Rather, we propose a different model for the role of survivin at mitosis, which involves regulation of microtubule function and formation of a normal bipolar apparatus, a proposition fully consistent with the catastrophic defect of microtubule assembly observed in survivin-knockout mice (Uren et al., 2000). Because of the complexity and multiplicity of functions of the kinetochore (Rieder and Salmon, 1998), it is also possible that kinetochore-associated survivin may not participate in cytokinesis at all. Alternative functions of kinetochore-associated survivin may involve regulation of sister chromatid separation at metaphase (Dobles et al., 2000) or, as previously suggested (Li et al.,

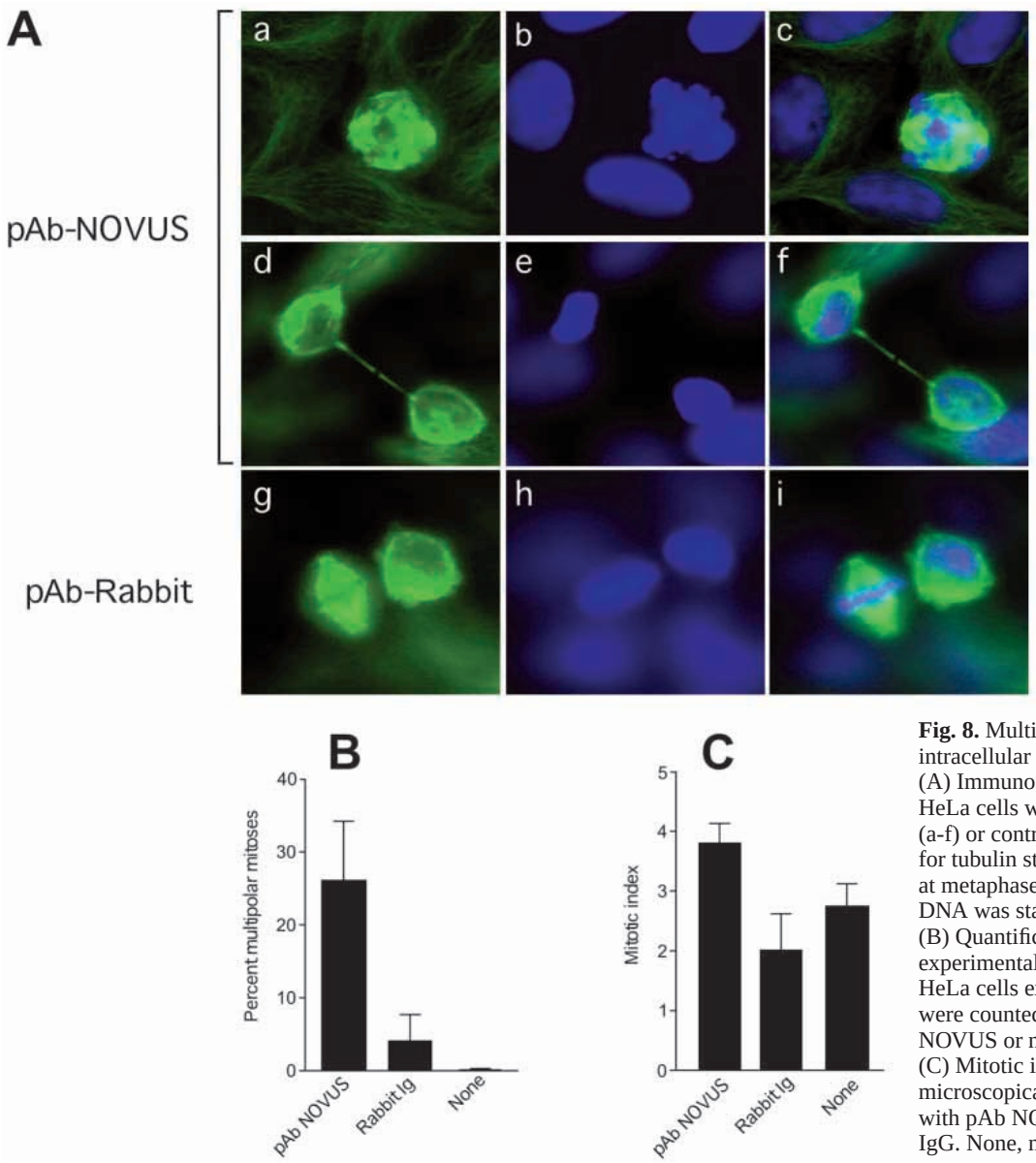

Fig. 8. Multipolar mitoses induced by global intracellular antibody targeting of survivin. (A) Immunofluorescence. Synchronized HeLa cells were loaded with pAb NOVUS (a-f) or control rabbit IgG (g-i), and analyzed for tubulin staining by confocal microscopy at metaphase (a-c,g-i) or telophase (d-f). DNA was stained with DAPI. (B) Quantification of aberrant mitoses. The experimental conditions are the same as in A. HeLa cells exhibiting multipolar mitoses were counted after loading with pAb NOVUS or non-immune rabbit IgG. (C) Mitotic index. HeLa cells were scored microscopically after intracellular loading with pAb NOVUS or non-immune rabbit IgG. None, no antibody loading.

1998), the integrity of the mitotic spindle checkpoint (Cahill et al., 1998).

In summary, the data presented here identify a far more complex pattern of survivin modifications and subcellular topography than recently claimed (Skoufias et al., 2000; Uren et al., 2000; Wheatley et al., 2001) and anticipate a requirement of the survivin pathway in controlling the assembly of a normal bipolar mitotic apparatus. Future experiments will investigate the dynamic relationship between nuclear and microtubule-bound survivin and dissect their potential involvement in cytoprotection and mitotic control.

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