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Survivin as a Cell Cycle-Related and Antiapoptotic Protein in Granulosa Cells

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Survivin is a relatively unique member of the inhibitor of apoptosis protein (IAP) family in that it contains a single baculovirus IAP repeat (BIR) domain combined with a COOH-terminal α-helix coiled-coil domain instead of the more common zinc-binding RING finger. Results from a variety of transformed or continuous mammalian cell lines suggest that, due to the combination of these features, Survivin is capable of regulating both cell proliferation and apoptotic cell death. However, to date there is essentially no information regarding Survivin expression, regulation or function within the ovary, or in any nonmammalian vertebrate species. In the present studies, cDNAs for chicken (ch) Survivin-142 (homologous to human Survivin-142) plus three alternatively spliced variants (ch Survivin-short, -γ, and -δ) are described, and of these, transcripts for ch Survivin-142 and -short are expressed in granulosa cells from the hen ovary. Highest levels of Survivin mRNA during follicle development occur in mitotically active granulosa cells from undifferentiated, prehierarchal follicles. Cell cycle analysis determined that Survivin mRNA expression is elevated specifically during the G2/M phase of mitosis. Significantly, transient transfection with ch Survivin-142 in primary cultures of hen granulosa cells attenuates taxol- and *N*-octanoylsphingosine- (C8-ceramide-) induced caspase-3 activity, whereas overexpression of ch Survivin-short (a truncated variant that lacks much of the functional BIR domain plus the entire α-helix coil domain) lacks this antiapoptotic activity. Taken together, these data provide evidence for Survivin in granulosa cells acting as a bifunctional protein associated with regulation of the cell cycle and the inhibition of apoptosis. (*Endocrinology* 143: 3405–3413, 2002)

INHIBITOR OF APOPTOSIS proteins (IAPs) represent a conserved gene family that protects against apoptosis induced by a variety of death-inducing stimuli. Mammalian IAPs currently include cellular IAP [cIAP1 (human IAP2, HIAP2)] cIAP2 (HIAP1), X-linked IAP [(XIAP) (IAP3)], neuronal apoptosis inhibitory protein (NIAP), Apollon, the baculovirus IAP repeat (BIR) repeat containing ubiquitin-conjugating enzyme, and Livin (1–5). Survivin (also identified as mouse TIAP) is one of the newer characterized members of the IAP gene family that in humans and mice shows widespread tissue expression during embryonic and fetal development but is largely undetectable in terminally differentiated adult tissues (6). Significantly, Survivin is found to be expressed at high levels in most common cancers (7, 8), and, accordingly, has been identified as the top fourth transcriptome expressed in human tumors (9). Survivin has several unique characteristics that differentiate it from related IAP family members. Unlike most IAP proteins that contain two to three approximately 70-amino-acid residue BIR motifs, Survivin contains a single BIR motif. The BIR domains of IAP proteins have previously been demonstrated to be essential for interaction with proapoptotic proteins, including one or more members of the caspase family of death-promoting proteases (10–12) and Diablo/second mitochondria-derived activator of caspase (Smac) (13). In addition, a concanical RING finger found in the COOH terminus of cIAP1, cIAP2, and XIAP is replaced with an α-helical coiled-coil domain in Survivin (7, 14). This domain is commonly found in microtubule-associated proteins and is thought to interact with the acidic COOH-terminal domain of tubulin (15). Survivin is expressed in transformed cell lines in a cell cycle-dependent manner, with highest levels found during the G2/M phase of the cycle (16). Cell localization studies have determined that during the G2/M stage of the cell cycle Survivin associates with the mitotic spindle and centrosome, whereas functional studies have demonstrated that suppression of Survivin levels in HeLa cells causes spindle defects and promotes apoptosis (15, 16). In this regard, Survivin is hypothesized to inhibit a default apoptotic cascade initiated during mitosis via the α-helix coiled-coil domain binding to mitotic spindle microtubules (15).

To date the expression and function of the homolog to mammalian cIAP1, chicken IAP1 (ch-IAP1 or inhibitor of T cell apoptosis; Refs. 17–19) has been studied in several ch tissues, while more recently a second IAP family member, the ch ortholog to XIAP, has been identified (GenBank accession no. AF451854). In hen granulosa cells, ch-iap1 mRNA is most highly expressed in preovulatory follicles that are largely resistant to undergoing atresia. Moreover, hen granulosa ch-iap1 mRNA levels are up-regulated, *in vitro*, following activation of protein kinase A, a primary signaling pathway linked to promoting hen granulosa cell survival (20). Together, these published data are consistent with a role for ch-IAP1 in protecting granulosa cells from apoptosis, and by implication, promoting follicle viability.

Abbreviations: BIR, Baculovirus IAP repeat; 8br-cAMP, 8-bromo-cAMP; CDE, cell cycle-dependent element; ch, chicken; CHR, cell cycle gene homology region; cIAP, cellular IAP; EST, expressed sequence tag; F1, F2, F3, first, second, and third largest preovulatory follicles; FBS, fetal bovine serum; h, human; HIAP, human IAP; IAP, inhibitor of apoptosis protein; m, mouse; NIAP, neuronal apoptosis inhibitory protein; nt, nucleotide; RING, a structural domain found in many IAPs that shows E3 ubiquitin ligase activity; Smac, second mitochondria-derived activator of caspase; TIAP, designated so because of its high expression in thymus and testis; XIAP, X-linked IAP.

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As a result of searching a ch expressed sequence tags (EST) database derived from the Bursa of Fabricius (21), a third ch IAP that most closely matched the human (h) and mouse (m) Survivin amino acid sequences was identified. The studies reported herein are among the first to evaluate the role of Survivin in cultures of primary cells. The results document several variants of ch Survivin that are predicted to result from alternative gene splicing, demonstrate a pattern of Survivin mRNA expression associated with granulosa cell proliferation, and provide evidence for the ability of one of these Survivin isoforms, ch Survivin-142, to act as an antiapoptotic protein.

Materials and Methods

Animals and reagents

Single-comb white Leghorn hens (Creighton Brothers, Warsaw, IN), 25–35 wk of age and laying regular sequences of at least 6 eggs, were used in all studies described. Birds were housed individually in laying batteries, provided with free access to feed (Purina Layeena Mash, Purina Mills, St. Louis, MO) and water, and were exposed to a photoperiod of 15 h light, 9 h dark, with lights on at midnight. Individual laying cycles were monitored by the daily timing of oviposition. Hens were killed approximately 16–18 h before a midsequence ovulation by cervical dislocation. All procedures described within were reviewed and approved by the University of Notre Dame Institutional Animal Care and Use Committee, and were performed in accordance with the Guiding Principles for the Care and Use of Laboratory Animals.

Recombinant hTGFα and IGF-I were obtained from PeproTech (Rocky Hill, NJ), whereas 8-bromo-cAMP (8br-cAMP) and Paclitaxol (Taxol) were from Sigma (St. Louis, MO). Aphidicolin, nocodazole and *N*-octanoylsphingosine (C8-ceramide) were obtained from BIOMOL Research Laboratories, Inc. (Plymouth Meeting, PA).

Tissue collection

Tissues collected for analysis of Survivin expression included hen oviduct, brain, spleen, kidney, and bone marrow, rooster testes, and Bursa of Fabricius tissue from 1-d-old posthatch chicks. Ovarian samples consisted of the granulosa and theca layer from the largest (F1), second largest (F2), and third largest (F3) preovulatory follicles, and prehierarchal (3–5 mm and 6–8 mm) follicles, together with ovarian stromal tissue and the most recent postovulatory follicle. Morphologically normal and early atretic follicles (3–5 mm) were processed without separating granulosa and theca layers (20), and the presence of apoptotic cell death was determined by evaluating oligonucleosome formation (22). Testes tissue was collected from reproductively active white Leghorn roosters.

Isolation of ch Survivin cDNA variants

A search of the ch GenBank EST database led to the identification of a nucleotide (nt) sequence (accession no. AJ394393) most similar to h Survivin. PCR was used to amplify the ch sequence from a granulosa cell cDNA template using primers (forward 5′-TTT GAA AAA TGG CGG CCT ATG C-3′ and reverse 5′-CCC AGG AAA CAG TTA TTG AGA CAG-3′) under the conditions of 94 C for 30 sec, 58 C for 1 min, and 72 C for 1 min for 30 rounds. Two PCR products resulted from this amplification, and each was cloned into the TA cloning vector, pCR2.1, for transfection into INVαF′ cells (Invitrogen, Carlsbad, CA). The cloned products were subsequently sequenced using the dideoxychain-termination method with Sequenase version 2.0 (United States Biochemical Corp., Cleveland, OH). One of the two products amplified was most related to the h Survivin-142 cDNA, and was designated ch Survivin-142. The second product was smaller due to a 110-bp deletion, and was designated ch Survivin-short. Subsequently, a second primer pair that included restriction digest sites was designed to amplify the entire coding region using a 19-oligomer forward primer positioned on the initiation codon and an 18-oligomer reverse primer at the stop codon of ch Survivin-142. Amplification from both granulosa cell and bursal tissue cDNA templates once again produced products corresponding to ch Survivin-142 and -short, plus two novel products from the bursal template subsequently designated as ch Survivin-δ and -γ variants. Individual bands from the PCR were cloned and sequenced as described above. Ch Survivin-δ was found to contain a 129-bp insertion within the 3′ end of the sequence, whereas ch Survivin-γ contained both the identical deletion identified in ch Survivin-short plus the 129-bp insertion described for ch Survivin-δ.

Follicle incubatons and granulosa cell cultures

Whole 3–5 mm follicles were freshly collected and immediately frozen at –70 C or incubated at 40 C for 3 h in 12 × 75 mm polypropylene tubes with 1 ml M199-HEPES plus 1 ml DMEM containing 5% fetal bovine serum (FBS). Granulosa cells from preovulatory (F1, F2, plus F3) follicles and prehierarchal (6–8 mm) follicles were collected, combined within their respective group, and dispersed with 0.3% collagenase (type 2; Worthington, Freehold, NJ). An aliquot of cells from each follicle stage was frozen immediately at −70 C (T0 controls), whereas the remaining cells were plated at 40 C in 6-well polystyrene culture plates (Falcon 3046; Fisher Scientific, Hanover Park, IL) at a density of approximately 10^6/well in 1 ml medium (M)199-HEPES supplemented with Hank salts (Life Technologies, Inc., Gaithersburg, MD) plus 1 ml DMEM plus 5% FBS.

Culture experiments were performed to evaluate effects of protein kinase A signaling or growth factor treatment on levels of ch Survivin mRNA expression. Prehierarchal granulosa cells were cultured for 3 h or 20 h in the absence or presence of 8br-cAMP (1 mM), TGFα (50 ng/ml), or IGF-I (50 ng/ml). Treatment doses for culture experiments were based upon recently published studies (22). Media plus cells were collected from each well, and cells were pelleted by centrifugation at 200 × *g* for 5 min. Cells were frozen at −70 C until total RNA was prepared for Northern blot analysis.

Transient transfection of granulosa cells

Constructs for ch Survivin transfection were produced by amplifying the open reading frame (including the Kozak sequence, but deleting the stop codon) of ch Survivin-142 and ch Survivin-short using the primers described above. Each product was ligated into the pcDNA6 vector, which includes a V5 epitope plus six His residues on the 3′ end of the polylinker region (pcDNA6/V5-His; Invitrogen). Both constructs were fully sequenced to verify for in-frame coding. The pcDNA6 vector containing LacZ was obtained from Invitrogen, and served as a transfection control.

Granulosa cells from preovulatory follicles were plated in six-well culture plates in DMEM containing 5% FBS and 1 mM 8br-cAMP (to promote cell proliferation; Johnson, A. L., and J. T. Bridgham, unpublished observations) until cells reached approximately 90% confluence. At this time, medium was replaced with basic DMEM (without serum, antibiotics or antimycotics), and cells were transfected using LipofectAMINE 2000 (Life Technologies, Inc.) complexed with the pcDNA6/V5-His vector alone, or vector containing LacZ, ch Survivin-142 or ch Survivin-short per manufacturer's recommendations. After 6 h, media were replaced with complete DMEM containing 5% FBS and 1 mM 8br-cAMP, and cells were cultured for an additional 18 h. Transfection efficiency for primary cultures of hen granulosa cells routinely exceeded 30%, as estimated from counts of cells stained with 5-bromo-4-chloro-3-indolyl-β-D-galactoside (X-Gal) 18–42 h following transfection with pcDNA6/V5-His containing LacZ.

Transfected cells were subsequently treated with 5 μM Taxol for 24 h or 50 μM ceramide for 8 h (23). Cells plus media were then collected and cells pelleted by centrifugation at 200 × *g* for 5 min. Cell pellets were frozen at –70 C until assayed for ch Survivin-142 and -short protein expression and caspase-3 activity. The cell-free caspase-3 activity assay was conducted as previously described (24).

Western blot analysis of Survivin protein

Two-hundred micrograms of precleared, detergent-solublilized testes or ovarian stromal extracts were immunoprecipitated with a rabbit polyclonal antiserum (20 μg/ml extract) generated against amino acid residues 60–74 of the m Survivin-140 protein (generously provided by

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Dr. E. M. Conway, University of Leuven; Ref. 11). This epitope is located within the BIR domain and is predicted to have 13 of 15 residues in common with the ch Survivin-142, but only 5 of 15 residues in common with ch-IAP1. Moreover, the antiserum is predicted not to recognize the ch Survivin-short isoform due to differences in amino acid sequence beginning at Gly^{40}. Immune complexes were precipitated by the addition of 50 μl of a 50:50 protein A-Sepharose slurry.

Western blot analysis for ch Survivin following immunoprecipitation of proteins from tissues or transfection of cultured cells was conducted essentially as described (22). Incubations with the m Survivin-140 antibody (at 1:400 dilution) were conducted overnight at 4 C, whereas those for the horseradish peroxidase-conjugated antirabbit IgG secondary antibody (Pierce Chemical Co., Rockford, IL) were for 1 h at RT. Alternatively, membranes were incubated with an anti-V5 serum linked to horseradish peroxidase (diluted 1:5000; Invitrogen) to assess expression levels of each fusion protein. Signals were visualized after incubating with enhanced chemiluminescence Western blotting reagent (Amersham Pharmacia Biotech, Arlington Heights, IL) for 1 min and exposing to x-ray film for 1–5 min.

Cell cycle-specific expression of Survivin mRNA

Prehierarchal follicle granulosa were cultured with 50 ng/ml TGFα (to promote cell viability and cell proliferation; Refs. 20 and 22) in the absence or presence of aphidicolin (30 μM; to block at stage G0/G1 of the cell cycle) for 17 h, or in the absence or presence of nocodazole (1.3 μM; to block at G2/M) for 27 h (16, 25). The culture time was optimized for each reagent in preliminary experiments where the maximal block at the expected stage of cycle with each agent occurred within the minimal culture time.

Cell cycle analysis of cultured granulosa was conducted by fixing cells overnight at –20 C in 70% EtOH. Cells were resuspended in 1 ml 50 μg/ml propidium iodide (at room temperature) in HEPES buffered saline solution (pH 7.4) containing 0.1% Triton X-100, 0.1 mM EDTA, and 50 μg/ml ribonuclease, and incubated for 30 min at room temperature. Cell cycle distribution was determined at an excitation wavelength of 488 nm using an Epics XL Flow Cytometer (Coulter Corp., Miami, FL) equipped with an argon laser.

Northern blot analysis of Survivin mRNA

Levels of ch Survivin mRNA were evaluated using Northern blots containing 10–15 μg of total RNA isolated from tissues or cells using Trizol Reagent (Life Technologies, Inc.). Membranes were probed with the full-length coding region of ch Survivin-142 cDNA. Given the nt homology among the various ch Survivin isoforms, this probe would be expected to hybridize to all alternatively spliced variants. In selected experiments, membranes were rehybridized with a ch-iap1 probe (19) to confirm differential expression of these IAPs. ch Survivin or ch-iap1 templates were random-prime labeled using the Megaprime DNA Labeling System (Amersham Pharmacia Biotech) and [^{32}P]dCTP (3000 Ci/mmol; Amersham Pharmacia Biotech). Prehybridizations and hybridizations were carried out as previously described (19). Images were captured using the Storm 840 PhosphorImager system equipped with the ImageQuant data reduction system (Molecular Dynamics, Inc., Sunnyvale, CA). Blots were eventually reprobed with ch 18S ribosomal RNA to enable standardization for RNA loading (22).

Data analysis

All experiments were replicated a minimum of three times, unless otherwise stated. When appropriate, levels of ch Survivin mRNA were standardized to 18S ribosomal RNA and expressed as fold-difference compared with a control tissue (*e.g.* stroma, whole follicles) or freshly collected (T0) cells. Data were analyzed by one-way ANOVA without including data from the control group, and the Fisher's protected least significant difference multiple range test. Data from normal *vs.* atretic, and T0 *vs.* T3 incubated follicles were compared using an unpaired *t* test. Caspase-3 activity and Survivin mRNA levels from nocodazole- and aphidicolin-treated cells were compared with their respective control group by paired *t* test.

Results

Nucleic acid and deduced amino acid sequences of ch Survivin-142 plus variants

The sequenced ch Survivin-142 cDNA (GenBank accession no. AF322051) includes a consensus Kozak sequence (AAAATGG from −3 to +4), a putative cell cycle gene homology region [CHR; TTGAA from nt −9 to −3], and putative cell cycle-dependent element (CDE; GGCGG from +3 to +7) adjacent to the ATG start codon (26, 27). nt sequences corresponding to ch Survivin-short and -γ show a 110-bp deletion beginning after nt 117 (relative to the ch Survivin-142 coding sequence) that results in the acquisition of a new in-frame stop codon and consequently an open reading frame of 59 amino acids. Due to this deletion in both the ch Survivin-short and -γ variants, the predicted amino acid identity to ch Survivin-142 is lost after the first 39 residues (Fig. 1). The sequence for ch Survivin-γ also includes an insertion of 129 bp, which begins after nt 234, but this insertion occurs 3′ to termination of the putative coding region. Similarly, ch Survivin-δ shows a 129-bp insertion identical with ch Survivin-γ beginning after nt 345 (relative to ch Survivin-142), which results in an extended open reading frame of 150 amino acids; however, the final 35 amino acid residues show no homology to ch Survivin-142. Interestingly, the original EST sequence derived from ch bursal tissue (EST accession no. AJ394392), which contains an 83-bp insert at position 227 of ch Survivin-142 and most closely matches h Survivin-β, was not amplified in the present studies from either bursal or ovarian tissues.

Deduced amino acid sequences for each of the ch Survivin isoforms aligned to the h and m orthologs are shown in Fig. 1. The full-length coding regions of h Survivin-142 and m Survivin-140 are 60.6% and 59.8% identical, respectively, to the deduced ch Survivin-142 sequence, while within the predicted BIR domain, the identity increases to 76.7% and 78.1%, respectively. There is complete conservation of several sites within the ch Survivin-142 BIR domain that are reportedly critical to Survivin function in mammals, including a Thr^{36} phosphorylation site (28), three cysteines and a histidine which are proposed to form a zinc-binding fold ($Cys^{59}X_2Cys^{62}X_{16}His^{79}X_6$ Cys^{86}; Ref. 29), and various residues reportedly required for apoptosis inhibition (Trp^{69}, Pro^{75}, Cys^{86}; Refs. 16 and 30). The conserved $Thr^{36}Pro^{37}Glu^{38}Arg^{39}$ motif in all isoforms matches the consensus phosphorylation site for the cyclin-dependent kinase, $p34^{cdc2}$ (31).

Expression of Survivin mRNA and protein

A single 1.1-kb ch Survivin mRNA signal was found to be most highly expressed in granulosa cells from prehierarchal follicles, ovarian stromal tissue, testes of adult chickens, and bursal tissue from 1-d-old chicks (Fig. 2A). It was considered unlikely that the short deletions and insertions characterized within the various cDNAs would alter the overall size of the mRNA transcript detected by Northern blot analysis. Therefore, PCR amplification was performed from cDNA templates derived from 6- to 8-mm follicle granulosa cells and from bursal tissue using primers which included the start and termination codons of ch Survivin-142. Two distinct

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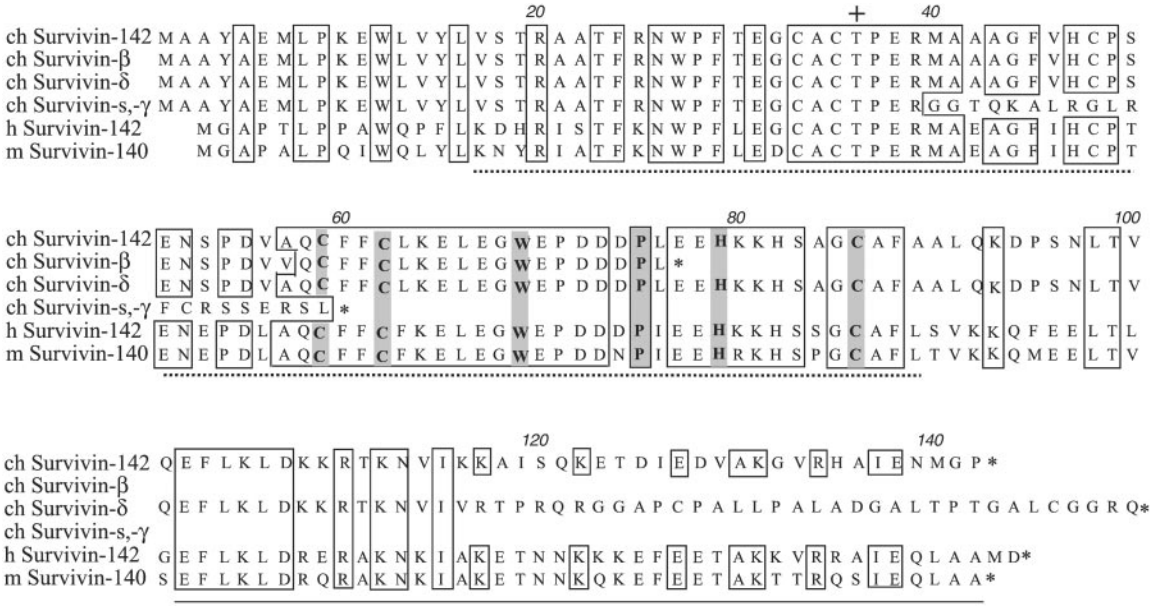

FIG. 1. Deduced amino acid sequence for ch Survivin-142 and its alternatively spliced forms (Survivin-β [accession no. AJ394392], -δ, -γ, and -short [s]) aligned to h Survivin-142 (accession no. U75285) and m Survivin-140 (accession no. AB013819). Numbering corresponds to the ch Survivin, the *dotted underlined* region indicates the predicted BIR domain, whereas the *solid underline* represents the putative α-helical coiled-coil motif. *Boxes* show amino acids that are completely conserved among the ch, h, and m Survivin sequences, whereas *shaded residues* represent the various residues reported to be critical for BIR domain function (16, 29). +, Thr phosphorylation site found to be phosphorylated by $p34^{cdc2}$-cyclin B1 and reported to be essential for maintaining cell viability at cell division (28); *, stop codon.

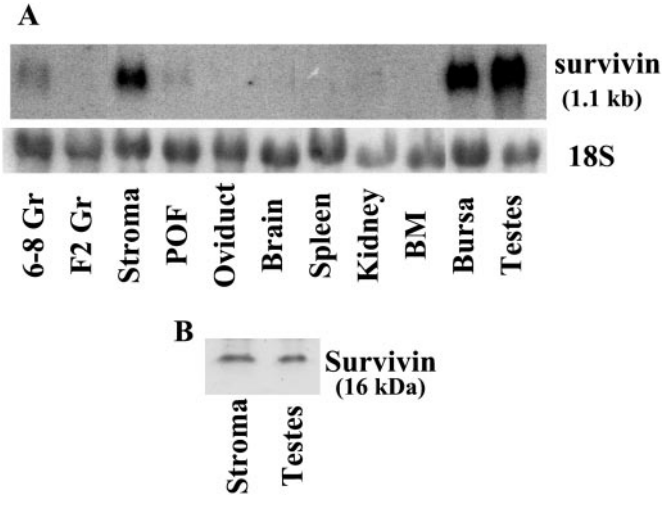

FIG. 2. A, Expression of ch Survivin mRNA in hen tissues. 6–8 Gr and F2 Gr, Granulosa cells collected from 6- to 8-mm follicles and the second largest preovulatory follicle, respectively; stroma, ovarian stromal tissue; POF, postovulatory follicle; BM, bone marrow. Ribosomal RNA (18S) is provided as a loading control. This blot was replicated twice with similar results. B, Western blot of immunoprecipitated Survivin from ovarian stroma and testes tissues.

products (corresponding to the predicted 332-bp ch Survivin-short, and 445-bp ch Survivin-142) were amplified from both tissues, whereas in addition the 571-bp ch Survivin-δ was amplified from bursal tissue (Fig. 3). Although ch Survivin-γ was initially cloned and sequenced from bursal tissue, we were not able to detect the amplification of ch Survivin-γ, as electrophoresis for an extended duration failed to resolve a second, larger product corresponding to 461 bp. Survivin protein was not readily detectable from total cellular protein, however immunoprecipitation from proteins collected from hen ovarian stroma and rooster testes resulted in the identification of a single, approximately 16-kDa Survivin protein (Fig. 2B).

Levels of ch Survivin mRNA are highest in granulosa cells collected from prehierarchal (3–5 mm and 6–8 mm) follicles and are significantly lower in preovulatory follicles (Fig. 4, *left panels*; $P < 0.05$). By contrast, there are low but detectable levels of ch Survivin mRNA in theca tissue but no significant changes are detected during follicle development (Fig. 4, *right panels*; $P > 0.5$). Whole, healthy (nonatretic, indicated by the absence of oligonucleosomes) 3- to 5-mm follicles express greater than 9-fold the levels of ch Survivin mRNA compared with atretic follicles (Fig. 5). In addition, incubation of healthy 3- to 5-mm follicles for 3 h, *in vitro*, results in a rapid appearance of oligonucleosome formation associated with a decrease in ch Survivin mRNA (by 50%) compared with freshly collected follicles.

ch Survivin mRNA levels are not regulated, in vitro, by 8-br-cAMP, TGFα, or IGF-I

Levels of ch Survivin mRNA in control cultured granulosa cells from 6- to 8-mm follicles significantly decrease by 35% after 3 h of culture and by 68% after 20 h ($P < 0.05$) compared with T0 levels. However, levels of ch Survivin mRNA are not significantly altered by treatment with 8br-cAMP, TGFα, or IGF-I (Fig. 6). By comparison, levels of ch-iap1 mRNA were increased 4.3 ± 0.5-fold (compared with T0) by culture alone after 3 h, and were further increased by 1.7 ± 0.2-fold following culture with 8br-cAMP after a 24-h culture compared with control cultured cells (data not shown).

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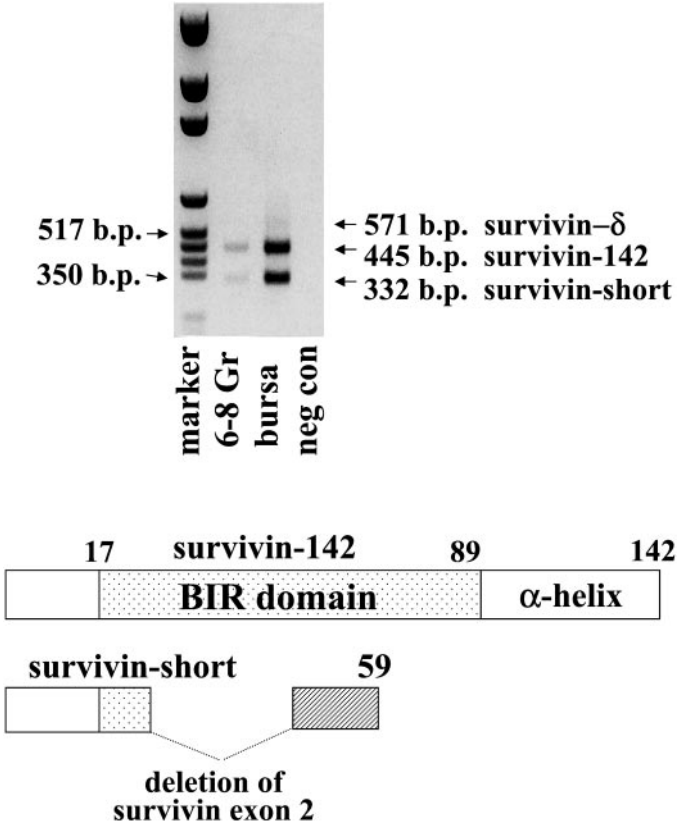

FIG. 3. *Top panel*, PCR amplification of ch Survivin variants from 6- to 8-mm follicle granulosa (6–8 Gr) compared with Bursa of Fabricius (bursa) tissue from 1-d-old chicks. The negative control (neg con) represents amplification in the absence of cDNA template. Sizes of marker DNA are shown at *left*, whereas predicted insert sizes for the various alternatively spliced transcripts are shown at *right* (see text for details). Amplifications were replicated several times using different reverse transcribed cDNA templates with comparable results. *Bottom panel*, Depiction of the two Survivin forms amplified from hen granulosa cells. The full-length coding region for ch Survivin-142 includes the predicted BIR domain (amino acids 17–89; *stippled portion of bar*) and α-helix region. By comparison, the alternatively spliced ch Survivin-short nt sequences lack exon 2 (a 110-bp deletion) that results in a predicted 59-amino-acid protein.

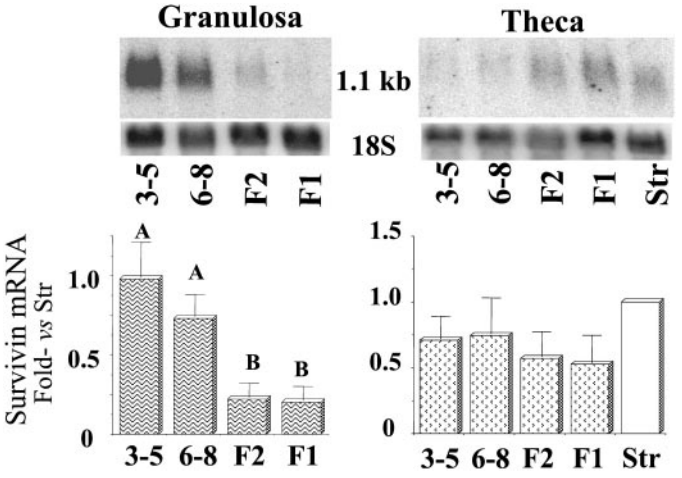

FIG. 4. Expression of ch Survivin mRNA in granulosa and theca tissue from prehierarchal (3–5 mm and 6–8 mm), and the second largest (F2) and largest (F1) preovulatory follicles. Ch Survivin data were standardized to levels of 18S ribosomal RNA and expressed relative to ovarian stromal tissue (Str; arbitrarily set to 1.0). A and B, $P < 0.05$, n = 3 replicate experiments.

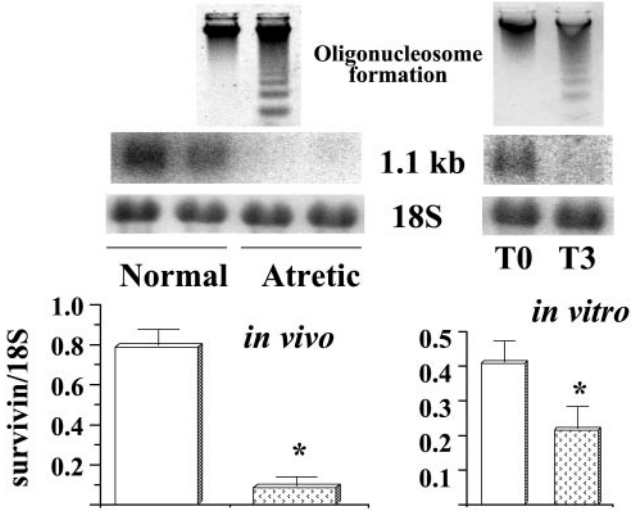

FIG. 5. Representative Northern blots and summarized levels of ch Survivin mRNA in whole normal *vs.* atretic follicles, and in whole 3- to 5-mm healthy follicles incubated, *in vitro*, for 0 h (T0) or 3 h (T3). Ch Survivin data were standardized to 18S ribosomal RNA, and analysis of each comparison was made by unpaired *t* test. Oligonucleosome formation detected in whole follicles incubated for 3 h is indicative of apoptosis. *, $P < 0.05$, n = 3 replicate observations.

Transient transfection of hen granulosa cells with Survivin-142 attenuates caspase-3 activity

Transient transfection with ch Survivin-142 consistently attenuated caspase-3 activity induced by a 24-h treatment with Taxol (to 66 ± 5% of levels in vector-transfected cells; $P < 0.05$) (Fig. 7). In addition, ch Survivin-142 transfection attenuated caspase-3 activity induced by an 8-h treatment with ceramide (to 50 ± 6% *vs.* LacZ-transfected controls; $P < 0.05$), whereas transfection with ch Survivin-short failed to inhibit caspase activity (90 ± 5%; $P > 0.05$). The identity of transfected ch Survivin-short was indicated by the inability of the m Survivin-140 antiserum to recognize the truncated ch Survivin-short protein.

Cell cycle-specific expression of Survivin mRNA in cultured granulosa cells

Culture of granulosa cells from 6- to 8-mm follicles for 17 h with aphidicolin or for 27 h with nocodazole enhanced the percentage of cells blocked in the G0/G1 ($P = 0.09$ by *t* test; n = 3 replicate experiments) and G2/M ($P < 0.05$; n = 3) stage of the cell cycle, respectively (Fig. 8, *left panels*). Under such culture conditions, ch Survivin mRNA levels were increased (by 5.2 ± 0.9-fold) *vs.* the respective control cultured cells only in nocodazole-treated cells (Fig. 8, *right panels*; $P < 0.05$).

Discussion

The present studies are among the first to evaluate Survivin expression and function in primary cultures of epithelial cells from adult tissues, and more specifically provide the first information regarding the relationship of Survivin ex-

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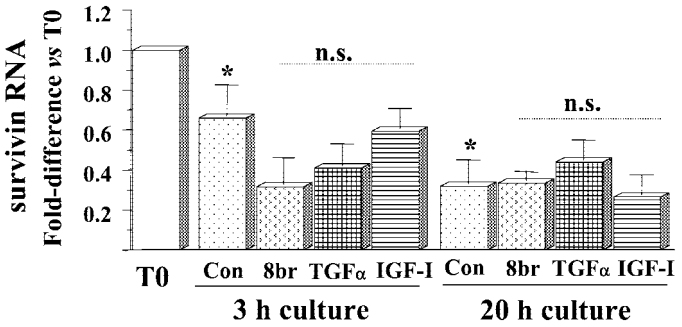

FIG. 6. Levels of Survivin mRNA before (T0) and following a 3-h or 20-h culture of prehierarchal follicle granulosa cells in the absence (Con) or presence of 8br-cAMP (8br, 1 mM), TGFα (50 ng/ml), or IGF-I (50 ng/ml). Although there are no differences among treatments after 3 or 20 h of culture (n.s., $P > 0.05$), levels of Survivin in Con cultured cells decrease following 3 and 20 h of culture (*, $P < 0.05$ for Con *vs.* T0, by paired *t* test).

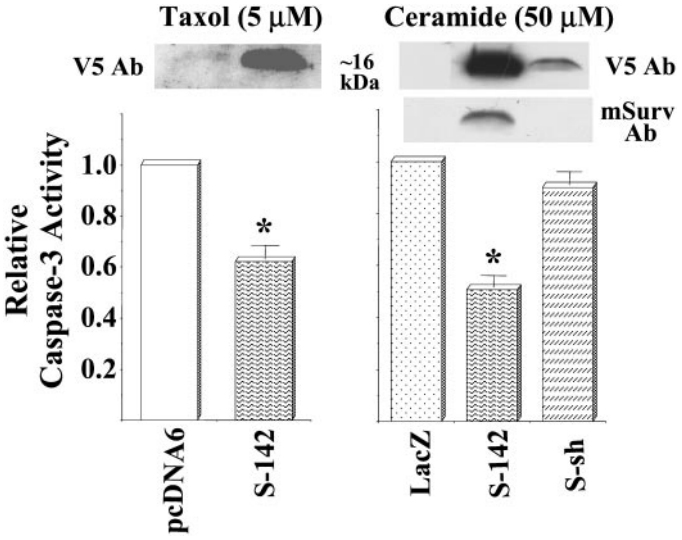

FIG. 7. *Top panels*, Representative Western blots of protein from transfected hen granulosa cells using a V5 epitope antiserum (V5 Ab), which recognizes both the ch Survivin-142 and -short fusion proteins, or an antimouse Survivin-140 serum (mSurv Ab). Note that levels of Survivin protein are not readily detected in control-transfected cells and that, as predicted, the antimouse Survivin serum does not recognize the transfected ch Survivin-short protein. *Bottom panels*, Transient transfection of hen granulosa cells with ch Survivin-142 attenuates caspase-3 activity following treatment with 5 μM Taxol for 20 h (*left panel*) or 50 μM ceramide for 6 h (*right panel*). By comparison, ch Survivin-short (S-sh), which lacks the complete BIR domain, fails to attenuate ceramide-induced caspase-3 activity. *, $P < 0.05$ *vs.* the corresponding control (either the pcDNA6/V5-His vector alone or vector containing LacZ) transfected cells by paired *t* test.

pression to granulosa cell cycle progression and its potential for antiapoptotic activity in the ovary.

The m Survivin-140 gene encodes 4 exons of 111, 110, 118, and 81 bp in length (11). Although the ch gene has yet to be characterized, the 110-bp deletion found in ch Survivin-short and -γ is presumed to represent a deletion of the entire second exon because this is identical in size to the second exon from m Survivin (11, 30). As a result of this splicing, ch Survivin-short and -γ retain the $Thr^{36}Pro^{37}Glu^{38}Arg^{39}$ $p34^{cdc2}$ phosphorylation motif which resides upstream of putative exon two, but lose many of the previously established functional residues found within the BIR domain due to the new in-frame stop codon (Fig. 1; Refs. 16, 29, 30). The absence of such functional residues residing within the BIR domain can explain the inability of transfected ch Survivin-short to attenuate caspase-3 activity in ceramide-treated cells (Fig. 7). Importantly, the presence of transfected ch Survivin-short also does not increase caspase-3 activity in control (untreated) cultures (data not shown), indicating that expression of this variant by itself is not actively proapoptotic.

It is also predicted that the altered amino acid sequence following residue 116 in ch Survivin-δ represents an alternative splicing event at the beginning of exon 4. The 129-bp insertion within ch Survivin-δ results in a deviation of the terminal 35 residues compared with ch Survivin-142, thus eliminating much of the α-helix coiled-coil region. The fact that Survivin-δ was amplified and cloned on several independent occasions from bursal, but not ovarian, tissue suggests that Survivin spliced-variants are expressed in a tissue-specific manner.

It is important to note that at this time relative levels of ch Survivin-142 protein, compared with its putative isoforms have not been evaluated due to the present lack of appropriate isoform-specific antibodies. Nevertheless, it is of interest to speculate about the potential physiological role(s) for the various ch Survivin cDNA splice variants identified from hen bursal and ovarian tissues. From the present studies, it is predicted that the truncated ch Survivin-short (and potentially ch Survivin-γ), which lacks much of the functional BIR domain and fails to block caspase-3 activity, could, by protein-protein interactions (11), competitively inhibit the antiapoptotic activity of ch Survivin-142. On the other hand, ch Survivin-δ, which shows no homology throughout the COOH-terminal 27 amino acids compared with the putative α-helical coiled-coil domain of ch Survivin-142, would be predicted to possess altered properties such as the inability to interact with the mitotic spindle and/or exhibit changes in subcellular localization (11, 16). There are currently several reports from h and m tissues regarding splice-variants originating from the same gene, several of which exhibit markedly reduced antiapoptotic activity and/or an absence of cell cycle-specific expression (11, 32, 33). More recently, a novel gene homologous to m Survivin-140 has been reported (tiap-2; Ref. 34), and encodes only the last 14 amino acids of the BIR domain through the COOH terminus. This suggests that additional ch Survivin homologs, including the variant represented by the originally identified EST from bursal tissue (EST accession no. AJ394393), remain to be characterized. At this point, it will be important to evaluate under physiologically relevant conditions whether a balance among the different isoforms of ch Survivin, and particularly between ch Survivin-142 and -short in granulosa cells, can ultimately determine the cell's response to proapoptotic and mitotic signals.

The pattern of ch Survivin mRNA expression in various tissues from the adult chicken (Fig. 2A) is consistent with findings from mammals in that Survivin expression is largely limited to undifferentiated, mitotically active tissues such as the thymus, germ cells from the testes, and intestine (6, 7, 11). Hen tissues showing the highest levels of ch Survivin mRNA (posthatch Bursa of Fabricius; adult ovarian stroma and tes-

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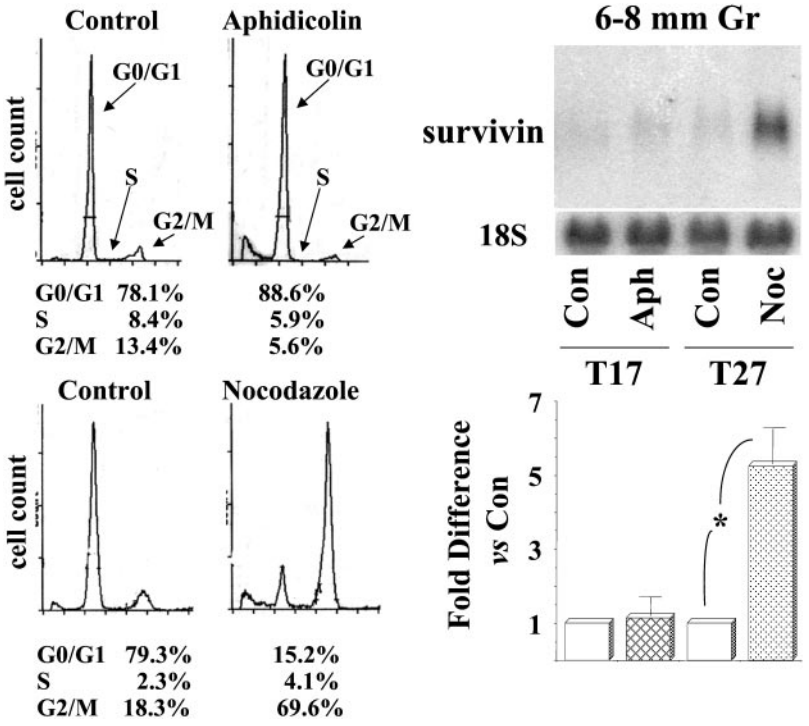

FIG. 8. Differential expression of Survivin mRNA relative to stage of cell cycle. Granulosa cells from 6- to 8-mm follicles were cultured with TGFα for 17 h (T17) in the absence or presence of aphidicolin (Aph, 30 μM; to block at G0/G1), or for 27 h (T27) in the absence or presence of nocodazole (Noc, 1.3 μM; to block at G2/M). Cells were subsequently collected for cell cycle analysis by flow cytometry, and the percentage of cells at each stage found in a representative experiment are indicated (*left panels*). In addition, cells were processed for quantitation of Survivin mRNA (*right panels*). Survivin data were standardized to 18S ribosomal RNA and comparisons of the standardized data from each treatment were made to the appropriate cultured control by paired *t* test. *, $P < 0.05$, n = 3 replicate experiments.

tes) include those in which ongoing cell proliferation would be predicted. With regards to developing ovarian follicles, it is also significant to note that higher levels of ch Survivin expression were found in granulosa cells from undifferentiated, prehierarchal follicles compared with differentiated, preovulatory follicles (Fig. 4). The pattern of elevated Survivin expression specifically during early follicle development is similar to that previously reported for ^{3}H-thymidine uptake (indicative of active DNA synthesis; Ref. 35). This association is consistent with previously reported findings that granulosa cells from prehierarchal follicles are largely undifferentiated and actively proliferating until the stage of follicle selection into the preovulatory hierarchy. Subsequent to selection, granulosa cells in preovulatory follicles rapidly differentiate and become nonproliferative except within the germinal disc region (36–38).

The loss of Survivin expression in atretic follicles can be replicated by incubation of whole prehierarchal follicles for 3 h (Fig. 5). Follicle incubation for as little as 3 h routinely promotes follicle death (indicated by oligonucleosome formation), and both induced follicle death and follicle atresia, *in vivo*, are largely the consequence of apoptotic cell death initiated within the granulosa layer (37). Similarly, prehierarchal follicle granulosa show a decline in Survivin mRNA levels following 3 h of culture (Fig. 6), and subpopulations of cells from such follicles are known to be inherently susceptible to undergoing apoptosis (20). This rapid decline in Survivin transcript levels may be attributed either to a short half-life and/or the targeted degradation of mRNA during the initial stages of apoptosis. At this time, it has not been established whether the decrease in Survivin expression during the early stages of atresia or during the transition to the differentiated state (*e.g.* in preovulatory follicle granulosa cells) is a direct cause or alternatively an effect of the altered cell physiology.

ch Survivin mRNA levels are not significantly altered by short- (3 h) or long- (20 h) term culture with factors previously demonstrated to activate granulosa cell survival signaling pathways or induce cell survival proteins (Fig. 6). For instance, we have previously reported that gonadotropin signaling via adenylyl cyclase/cAMP increases levels of ch Bcl-xLong mRNA and protein (20, 39) and ch-iap1 (19) mRNA, whereas TGFα and IGF-I promote cell survival, in part, by their ability to activate PI3 kinase-Akt signaling (22). By comparison, in human vascular tissues growth factors such as basic fibroblast growth factor, vascular endothelial growth factor and stem cell factor, each of which is known to display both mitogenic and antiapoptotic actions, transiently up-regulate Survivin expression over a period of 1.5–24 h (40–42).

Although the extent to which the regulation of Survivin expression in granulosa occurs via endocrine/paracrine/autocrine factors has yet to be established, the presence of several conserved regulatory regions and motifs within the ch Survivin cDNA and protein provide evidence for a role of ch Survivin in granulosa cell cycle regulation. Studies of the m Survivin promoter region identified three CDE and one CHR G1 repressor element which together are responsible for cell cycle-dependent expression in G2/M-regulated genes (27, 43). The use of Survivin-luciferase promoter constructs demonstrated that all three CDE plus the CHR were required for maximal promoter activity in G2/M-synchronized HeLa cells (16). Significantly, both consensus CHR and CDE elements are present within the regulatory region of the ch Survivin cDNA. Additional regulatory elements likely

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reside further 5′ within the sequence; however, this region of the ch Survivin gene has not yet been characterized.

Moreover, complete conservation of the $Thr^{36}Pro^{37}Glu^{38}Arg^{39}$ ch Survivin motif provides a mechanism for regulation by the cyclin-dependent kinase $p34^{cdc2}$-cyclin B1. Recent *in vitro* studies have determined that $p34^{cdc2}$-cyclinB1 phosphorylates wild-type h Survivin-142 at Thr^{34} (28). During the cell cycle, active phosphorylation of Survivin occurs exclusively when cells enter into mitosis, and this coincides with the stage at which $p34^{cdc2}$ is found to physically associate with h Survivin-142. Consequently phosphorylation of this conserved $Thr^{36}Pro^{37}Glu^{38}Arg^{39}$ motif is proposed to stabilize a Survivin-142-antiapoptotic complex that counteracts a default induction of apoptosis in the G2/M phase of the cell cycle.

Some published reports suggest that Survivin directly suppresses activation and/or activity of the effector caspase, caspase-3 (6, 12), whereas others propose that Survivin primarily acts at the level of an initiator caspase by directly or indirectly binding to caspase-9 and/or by neutralizing the proapoptotic protein, Diablo/Smac (5, 12, 13). Although the present studies do not address ch Survivin's site of action, the results demonstrate that transfection with ch Survivin-142 attenuates downstream caspase-3 activity induced by agents (Taxol and ceramide) that act via distinctly different mechanisms (Fig. 7). Taxol, a microtubule stabilizing agent, has previously been shown to promote apoptosis by disrupting the cell cycle-dependent localization of Survivin (44), whereas ceramide constitutes a second messenger molecule that promotes mitochondrial perturbations in response to proapoptotic stimuli such as Fas ligand, ionizing radiation, or chemotherapeutic agents (23, 45). Irrespective of the specific initiating event, apoptotic pathways eventually converge to activate the downstream effector caspase, caspase-3. However, while these findings are the first to provide support for the ability of transfected ch Survivin-142 to act as an antiapoptotic protein in granulosa cells, it will be important to corroborate these observations under alternative conditions (*e.g.* following targeted depletion).

In summary, the observation that ch Survivin mRNA is expressed in primary cultures of granulosa cells in a cell cycle-dependent manner is consistent with its previously proposed role as a protein that interacts with components of the G2/M checkpoint and preserves the integrity of the mitotic apparatus. In addition, results from *in vitro* studies provide evidence that ch Survivin-142, but not the truncated ch Survivin-short, may act as an antiapoptotic protein by attenuating activation/activity of the effector caspase, caspase-3. Finally, evidence for alternative splicing of ch Survivin adds not only to the complexity regarding Survivin's role in cell proliferation and viability, but more generally to the intricacy of systems controlling granulosa cell apoptosis.

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GenBank accession no. for ch Survivin-142 is AF322051; Survivin-short, AF377322; Survivin-δ, AF377323; and Survivin-γ, AF377324.

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