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Caspase-3 and -6 Expression and Enzyme Activity in Hen Granulosa Cells[1]

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ABSTRACT

We have cloned and sequenced cDNAs corresponding to the complete coding regions of the chicken homologues to mammalian caspase-3 and caspase-6. Both caspases are included among members of the cysteine protease (caspase) family that are most closely identified with mediating apoptosis. The deduced amino acid sequences for chicken caspase-3 and -6 show 65% and 68% identity with the respective human sequences, with complete conservation found within the QACRG active peptide region. Both caspase-3 and -6 are widely expressed within various tissues from the hen. Within the ovary, levels of caspase-3 and caspase-6 mRNA and protein do not change significantly in theca tissue during follicle development. On the other hand, procaspase-3 and -6 protein levels are elevated by 2- to 5-fold in preovulatory, compared to prehierarchal (6- to 8-mm diameter), follicle granulosa cells. Nevertheless, the function of this family of cell death-inducing proteins requires activation of the proenzyme caspase, which occurs after cleavage at predictable sites within the N-terminal domain. Accordingly, it was determined that okadaic acid, a pharmacologic inducer of apoptotic cell death in cultured apoptosis-resistant, preovulatory follicle granulosa cells, induced both caspase-3- and caspase-6-like activity within 8–16 h of treatment. By comparison, spontaneous apoptotic cell death that occurs in apoptosis-sensitive, prehierarchal follicle granulosa cells after short-term suspension culture is accompanied by a more rapid increase (within 2 h) in both caspase-3- and -6-like activity. Treatment with 8-bromo-cAMP, which has previously been shown to attenuate, or at least slow, the onset of apoptosis in prehierarchal follicle granulosa cells, mitigates this suspension culture-induced increase in caspase activity. While the present results provide further support for the relationship between caspase activation and apoptotic cell death in hen granulosa cells, the molecular ordering of enzymatic events and the caspase-specific substrates remain to be elucidated.

INTRODUCTION

Apoptosis is a ubiquitous form of cell death mediated by, among other intracellular factors, the activation of one or more cysteine proteases (caspases). Caspases share homology with the *Caenorhabditis elegans* gene, *ced*-3, the product of which is required for mediating apoptosis during development [1]. Caspase-3 (formerly called CPP32 or Yama) represents the vertebrate caspase most similar to Ced-3 on the basis of both amino acid sequence homology and substrate specificity. The active mammalian form, consisting of p17 and p12 subunits, is processed from an inactive 32-kDa proenzyme after auto- or heterocatalysis, and it preferentially cleaves substrates containing a DEVD motif [2]. By comparison, the active form of mammalian caspase-6 (Mch2α) consists of p18 and p11 subunits (processed from a 34-kDa proenzyme), and is the only caspase to date determined to cleave lamins at a VEID recognition sequence [3, 4].

Studies have demonstrated the existence of at least 12 additional homologous mammalian proteases, caspase-1 through -14 [5–10], that together constitute the caspase family of cysteine proteases. All mammalian caspases identified to date are synthesized as inactive proenzymes that are activated after cleavage at specific N-terminal domain aspartate cleavage sites. Each caspase also contains an active site QAC(X)G pentapeptide, in which X represents an Arg (R), Gln (Q), or Gly (G) residue. Within this family of enzymes, caspase-7 (Mch3) and caspase-9 (Mch6) are considered to be most homologous in sequence to caspase-3 and -6, while the amino acid sequences of caspase-8 (MACH) and caspase-10 (Mch4) are the next most related [6, 7]. Although the mechanism of caspase action is highly conserved, differences among primary sequences account for different substrate specificity. As a group, caspase members are generally proposed to form a cascade of enzymatic activity that results in both the initiation and amplification of early apoptotic events (e.g., caspase-8, -9) as well as mediating the later executioner steps of apoptosis (e.g., caspase-3) [2]. At present it is unclear whether caspase-6 activation occurs before, simultaneously with, or after caspase-3 activation [11].

It is now well established that ovarian follicle atresia in mammals and in the domestic hen is mediated via apoptosis, and that such apoptotic cell death occurs at least initially within the granulosa cell layer (e.g., [12–14]). At present the signal for apoptotic cell death, whether originating from within granulosa cells or alternatively initiated by the germ cell or extracellular signals (e.g., cytokines, hormones) has not been established [12, 15]. A notable aspect of the hen ovarian follicle hierarchy is that inherent susceptibility to apoptosis has been reported to vary with the stage of follicle differentiation [13]. Granulosa cells from prehierarchal (6- to 8-mm diameter) follicles are highly susceptible to apoptosis when plated in culture or incubated as a suspension culture for 3–6 h. By comparison, granulosa cells from the three most mature preovulatory follicles are highly resistant to apoptosis when cultured in vitro, but they can be induced to undergo apoptosis when exposed to various agents such as ultraviolet irradiation or the synthetic ceramide, *N*-octanoylsphingosine (C8 ceramide) [16].

To date, identification and characterization of only two avian caspases, chicken caspase-1 [17] and caspase-2 [18], have been reported. This is despite biochemical evidence for at least five avian caspase-like proteases, each with distinctive substrate recognition properties [19]. We have recently provided evidence that caspase-1 itself appears not to have a critical, nonredundant role in mediating apoptosis in hen granulosa cells [17], while the precise role of caspase-2 in granulosa cell death has yet to be determined.

[1]Supported by NIH grant HD36095 and USDA grant 95-37203-1998. Genbank accession number for chicken caspase-3 is AF083029 and for chicken caspase-6 is AF082329.
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The objectives of the present study were to isolate and characterize the chicken caspase-3 and -6 cDNAs, evaluate caspase-3 and -6 mRNA and protein expression in hen tissues, and monitor caspase activity under in vitro conditions known to promote apoptosis in hen granulosa cells.

MATERIALS AND METHODS

Animals

Single-comb white Leghorn hens (H&H Poultry, Portland, IN), 25–35 wk of age and laying regular sequences of 5 or more eggs, were used in all studies described. Birds were housed individually in laying batteries, provided with free access to feed (Purina Layena Mash; Purina Mills, St. Louis, MO) and water, and were exposed to a photoperiod of 15L:9D, 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 animal procedures described herein 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.

Tissue Collection

Tissues collected for analysis of caspase mRNA and protein expression included cerebrum, oviduct, spleen, kidney, and bone marrow. In addition, granulosa and theca tissue from the largest (F1), second largest (F2), and third largest (F3) preovulatory follicles; follicles recently selected into the follicle hierarchy (9- to 12-mm diameter follicles); prehierarchal (6–8 mm and 3–5 mm) follicles; and ovarian stromal tissue were collected and prepared as previously described [13, 20]. Specifically, thecal tissue consists of both interna (including interstitial cells) plus externa layers, while ovarian stroma represents ovarian tissue stripped of all visible follicles greater than 0.5 mm in diameter.

Isolation and Characterization of Chicken Caspase-3 and -6 cDNAs, and Deduced Amino Acid Sequence

Nested polymerase chain reactions (PCRs) were performed with an adaptor-ligated cDNA template (derived from granulosa cell poly[A]$^+$ RNA) generated by using a Marathon cDNA amplification kit (Clontech, Palo Alto, CA). The initial amplification was conducted for 30 cycles of 1 min at 94°C, 1 min at 58°C, 1 min at 72°C using the degenerate forward primer 5′-AAGCCSAAGITSDTSATCRTSCAGG (where D = A, T, or G; I = deoxyinosine; R = A or G; S = G or C) [21], together with an AP1 adapter primer. An aliquot of this PCR reaction was used as template to reamplify according to the above conditions using the forward degenerate primer 5′-CAGGCNTGYCGNGG (where N = A, T, G, or C; Y = T or C) [21], and the reverse degenerate primer 5′-GATRWACCAGGAKCC (where W = A or T; K = T or G) [17]. PCR products from the second amplification were subcloned into the pCR2.1 TA cloning vector (Invitrogen, San Diego, CA) and subsequently sequenced using the dideoxy chain-termination method with the Sequenase v.2.0 kit (Amersham, Arlington Heights, IL). The original product determined to be homologous to mammalian caspase-3 was 174 base pairs (bp) in length; that for caspase-6 was 197 bp.

The marathon cDNA template was subsequently used to obtain complete sequences in both the 5′ and 3′ directions by rapid amplification of cDNA ends (RACE). The conditions for RACE amplification were as previously described [17], and the reaction used nested, gene-specific primers in combination with adaptor primers. The sequences reported (caspase-3, Genbank accession #AF083029; caspase-6, Genbank accession #AF082329) represent data from at least two clones sequenced from both the forward and reverse directions for each cDNA.

Northern Blot Analysis

Total cellular RNA from each tissue and stage of follicle development was isolated using Trizol Reagent (Gibco-BRL, Gaithersburg, MD) as previously described [22]. Briefly, 10–15 μg of total cellular RNA was electrophoresed on 1% agarose gels in the presence of formaldehyde, then transferred to nitrocellulose Nitro ME membranes (MSI, Westboro, MA) by capillary action overnight. Analysis of caspase-3 and -6 mRNA was conducted with cDNA probes labeled by the random-prime labeling method using the Megaprime DNA labeling System (Amersham) and [α-^{32}P]dCTP (3000 Ci/mmol; Amersham). The caspase-3 probe consisted of a 460-bp cloned product derived from bp 283–743 of the reported GenBank sequence, while the caspase-6 probe was a 442-bp cloned product corresponding to bp 189–631. Blots were prehybridized for 30 min at 60°C and subsequently hybridized overnight at 60°C. Membranes were exposed to autoradiographic film at −70°C for 1–3 days and subsequently analyzed by densitometric scanning (UltraScan XL laser densitometer; Pharmacia LKB, Piscataway, NJ), or exposed to phosphorimaging plates for 1–2 days, then analyzed using a Storm 840 PhosphorImager system equipped with the ImageQuant data reduction system (Molecular Dynamics, Sunnyvale, CA). Blots were routinely rehybridized to a random-primed chicken 18S ribosomal RNA cDNA probe to standardize for equal loading of RNA samples [22].

Western Blot Analysis

Granulosa and all other tissues were homogenized in lysis buffer and then centrifuged at 12 000 × *g* at room temperature for 10 min as previously described [22]. The supernatant was collected and protein was quantified using the Bio-Rad DC Protein Assay kit (Hercules, CA). Protein samples were subsequently diluted with sample buffer, heated at 70°C for 10 min, and centrifuged at 12 000 × *g* for 2 min. Proteins were separated on a 12% SDS-PAGE gel before being transferred to a nitrocellulose membrane (Micron Separations, Inc., Westborough, MA).

Membranes were blocked for 1 h at room temperature in Western blocking solution (5% nonfat dry milk and TBST: 10 mM Tris [pH 8.0], 100 mM sodium chloride, 0.1% Tween 20). The caspase-3 goat polyclonal antiserum, which recognizes the amino terminus of the p11 subunit as well as proenzyme (cat.# sc-1224; Santa Cruz Biotechnology, Santa Cruz, CA), and the caspase-6 rabbit polyclonal antiserum, which recognizes only the proenzyme form (cat.# AAP-106; StressGen Biotechnologies Corp., Victoria, BC, Canada) were diluted 1:500 or 1:2000, respectively, in blocking solution. Each diluted antiserum was incubated with membranes at 4°C overnight; then membranes were washed (3 × 10 min) in blocking solution. Rabbit anti-goat or goat anti-rabbit serum coupled to horseradish peroxidase (Pierce Chemical Co., Rockford, IL) was diluted 1:10 000 in blocking solution and incubated with membranes for 1 h at room temperature. Membranes were

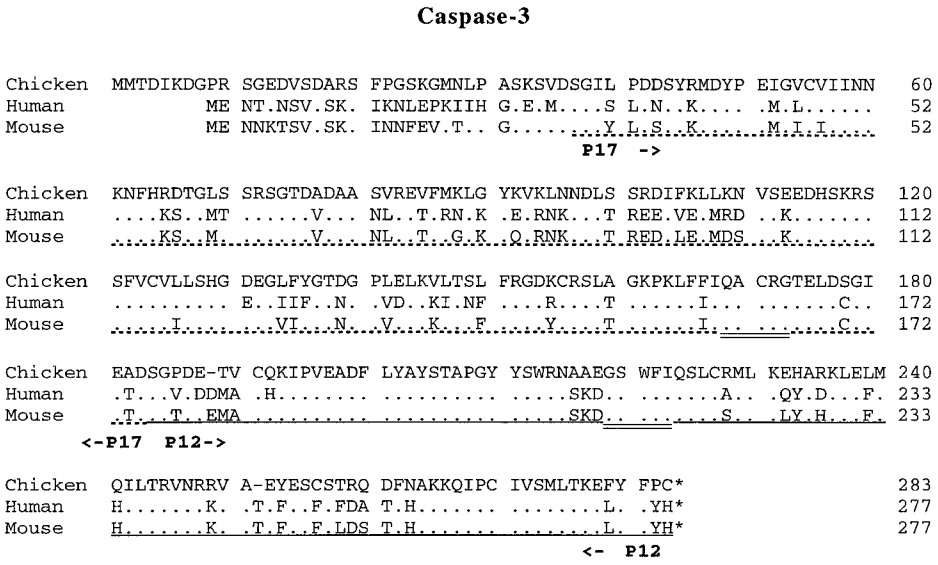

FIG. 1. Deduced amino acid coding sequence of chicken caspase-3 aligned to the human (Genbank accession #U26943) and mouse (Genbank accession #U49929) homologues. The P17 subunit is represented by the broken underline; the P12 subunit is indicated by a solid underline. The double solid underline indicates the conserved active site pentapeptide region (QACRG) and the conserved protein binding domain (GSWFI). Conserved amino acids are represented by periods (.), while deleted amino acids are denoted by -; * signifies the predicted stop codon.

washed three times in TBST (10 min each wash). Finally, blots were incubated with ECL Western blotting detection reagent (Amersham) for 1 min, then wrapped and exposed to autoradiographic film for 1–5 min. The extent of antibody binding was standardized to β-tubulin [22], and both were quantitated by densitometry (UltraScan XL laser densitometer; Pharmacia LKB). Standardized caspase protein levels were expressed as fold difference (mean ± SEM) versus a designated reference tissue or treatment (arbitrarily set at 1).

Caspase Activity

Experiments in which caspase-3- and -6-like activity was measured were conducted with incubated 6- to 8-mm follicle granulosa cells or cultured preovulatory (F1-F3) follicle granulosa cells. Prehierarchal (6–8 mm) follicle granulosa layers were collected and immediately frozen (T0) or rapidly dispersed with collagenase (0.3%, Type II; Worthington Biochemical Corp., Lakewood, NJ). Cells were then cultured in suspension (approximately 10^6 per 12 × 75-mm polypropylene tube) in 2 ml M199/HEPES containing 2.5% fetal bovine serum (FBS) for 2, 4, 6, or 8 h in the absence (Control, Con) or presence of a maximally effective dose of 8-bromo-cAMP (8-br-cAMP; 1 mM; Sigma Chemical Co., St. Louis, MO). The preovulatory follicle granulosa layer was collected, and a small portion was immediately frozen (T0). The remainder was dispersed with collagenase and plated (approximately 10^6 per well) overnight in 6-well plates (Falcon 3046; Becton Dickinson, Franklin Lakes, NJ) with 20 μg fibronectin (bovine; Sigma) and 2 ml M199/HEPES plus 2.5% FBS. Medium was changed, and preovulatory follicle cells were cultured an additional 8, 16, or 28 h in the absence (Con) or presence of okadaic acid (50 nM; Sigma).

Cells in both experiments were subsequently collected, pelleted, and frozen at −70°C. Frozen pellets were subsequently resuspended in caspase resuspension buffer (10 mM $MgCl_2$, 0.25% NP-40), then centrifuged at 90 000 × *g* for 30 min at 4°C. Supernatants were collected and combined with an equal volume of double-strength caspase storage buffer (40 mM HEPES, pH 7.4, 20 mM NaCl, 2 mM EDTA, 20% glycerol). For caspase-3 activity, 30 μg of protein was transferred to a 96-well plate (Costar 3632; Corning Inc., Corning, NY) and first preincubated at 37°C for 10 min in the absence or presence of the caspase-3 inhibitor, Ac-DEVD-CHO (0.1 μM final concentration; Biomol, Plymouth Meeting, PA), then incubated for an additional 4 h at 37°C with the caspase-3 fluorogenic substrate (Ac-DEVD-AFC, 200 μM final concentration; Biomol). Total volume of reagents per well was adjusted to 100 μl. Enzyme activity was measured with a Victor^2 multilabel counter (model 1420; EG&G Wallac, Turku, Finland) at an excitation wavelength of 390 nm and emission wavelength of 510 nm.

Caspase-6 activity was measured using the assay protocol described above, except that the caspase-6 inhibitor was Ac-VEID-CHO (10 μM final concentration; Alexis Corp., San Diego, CA) while the fluorogenic substrate was Ac-VEID-AMC (200 μM final concentration; Alexis). Caspase-6 activity was measured with excitation and emission wavelengths of 390 nm and 460 nm, respectively. During the validation of these assays, it was established that the freezing of supernatants used for enzyme assays did not noticeably affect enzyme activity.

Various members of the mammalian caspase family have been grouped according to substrate specificity. For instance, caspase-3, -7, and -2 cleave substrates with a preferred DEXD motif (where X represents any amino acid), whereas the preferred motifs for caspase-6, -8, and -9 include (IVL)EXD [2]. Because of the absence of complete substrate specificity among the various caspases, the ability of protein preparations from the present experiments to cleave the fluorogenic substrates Ac-DEVD-AFC and Ac-VEID-AMC is conservatively referred to herein as caspase-3-like activity or caspase-6-like activity, respectively.

Finally, in an effort to verify the occurrence of apoptosis in the above experiments, additional cells were suspension-cultured (prehierarchal follicle granulosa) or plated and cultured with treatments (preovulatory follicle granulosa) as described above. Cells were subsequently collected, and DNA was analyzed for oligonucleosome formation as previously described [20, 23].

Data Analysis

Northern and Western blot analyses were repeated a minimum total of three times. Such data were analyzed by one-way ANOVA (analysis of fold difference data did not include the reference value) and the Fisher's protected least-

Caspase-6

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Chicken  MSGAERRPAA GRVQLDSKPT PTTTADGNQN ITEVDAFDK-R RTFDPAEQYK MNHQRRGVAL  60
Human    ..S.SGLRRG HP-------- ----.G.EE. M..T...Y.-. EM.....K.. .D.R...I..  48
Mouse                                     M..T.G.Y.S. EV..........D.K.....  31

Chicken  IFNHEHFFWH LRLPDRRGTL ADRNNLKRSL TDLGFEVRIF DDLKAEDVLK KVFEASRDDY  120
Human    .....R.... .T..E..R.C ...D..T.RF S......KC. N.....EL.L .IH.V.TVSH  108
Mouse    .....R.....T..E...N....D..T.RF S......KC. N..R..EL.L..IH.V.TSSH   91

Chicken  SNADCFVCVF LSHGENDHVY AYDAQIKIET ITNMFRGDKC QSLVGKPKIF IIQACRGDKH 180
Human    AD........ .....GN.I. ....K.E.Q. L.GL.K.... H......... .......NQ. 168
Mouse    ID.....I.........GN.........K.E.Q. L.GL.K.....................SQ.  151

Chicken  DDPVLVQDSV DSKDETTV-N QTEVDAAGVY TLPAGADFIM C---AQGYFS HRETVNGSWY  236
Human    .V..IPL.V. .NQT.KLDT. I......S.. ........L. .YSV.E..Y. ..........  228
Mouse    .V..VPL.M...HQTDKLD-. V.Q...S.. .........L. .YSV.E..Y. ..........  210
             <- P18                  P11 ->

Chicken  IQDLCEALGK HGSSLEFTEL LTVVNRKVSH RKVDICRDIN AIGKKQIPCF ASMLTKKLYF  296
Human    ......M... Y......... ..L......Q .R..F.K.PS ......V... ........H.  288
Mouse    ......M.AR Y......... ..L......Q .R..F.K.PD ......V... ........H.  270

Chicken  HPK-SK*                                                            301
Human    F..-.N*                                                            293
Mouse    C..P..*                                                            276
         <-  P11
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FIG. 2. Deduced amino acid coding sequence of chicken caspase-6 protein compared to the full-length human (Genbank accession #U20536) and mouse (Genbank accession #Y13087) homologues. Met (M)-51 (bold type) represents a potential alternative start site for the chicken caspase-6 (see text for specifics). The P18 subunit is represented by the broken underline; the P11 subunit is indicated by a solid underline. The double solid underline indicates the conserved active site pentapeptide region (QACRG) and the conserved protein binding domain (GSWYI). Other abbreviations are as described in the legend to Figure 1.

significant difference multiple-range test. Caspase-3- and -6-like activity data were calculated from the respective emission value (arbitrary units). Activity in 6- to 8-mm follicle granulosa cells was expressed as fold difference compared to the background emission detected in T0 samples treated with the respective caspase inhibitor, while preovulatory follicle granulosa cell activity was compared to the appropriate control. Data were analyzed by paired *t*-tests unless otherwise indicated.

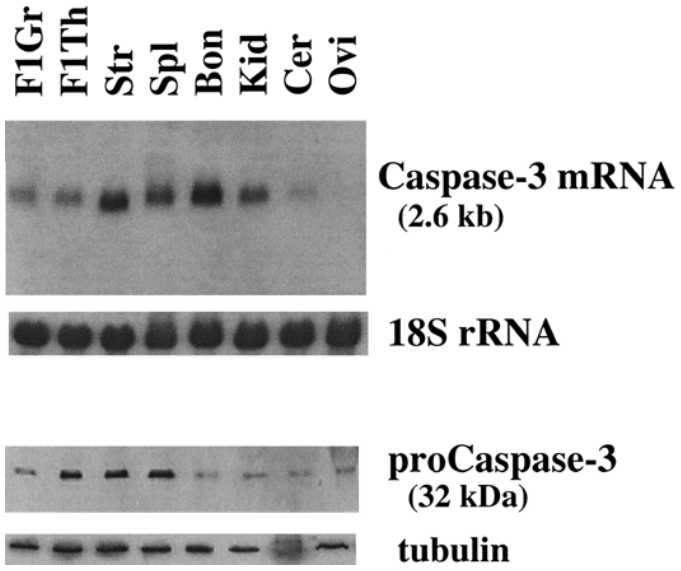


FIG. 3. Caspase-3 mRNA and protein expression in several hen tissues. Ribosomal (r) RNA (18S) and tubulin data are provided for the evaluation of equal loading. F1Gr, Granulosa from largest preovulatory follicle; F1Th, theca tissue from largest preovulatory follicle; Str, ovarian stromal tissue; Spl, spleen; Bon, bone marrow; Kid, kidney; Cer, cerebrum; Ovi, oviduct. This experiment was repeated once, with similar results.

RESULTS

Overall nucleic acid homology within the predicted coding region of chicken caspase-3 cDNA and the corresponding human and mouse sequences is approximately 72% and 70%, respectively. Amino acid identity between the deduced chicken caspase-3 proenzyme and the corresponding human and mouse sequences is 65% and 69%, respectively; absolute conservation within the more highly conserved P12 subunit is somewhat higher (75% and 77%, respectively). On the basis of the mammalian caspase-3 [24], processing of the chicken caspase-3 proenzyme to active enzyme is predicted to occur by cleavage at Asp-36 Ser-37 (removal of the propeptide) and Asp-183 Ser-184 (processing of the active enzyme; Fig. 1). The QACRG active site region is completely conserved. The N-terminal peptide prodomain of the putative chicken caspase-3 amino acid sequence contains 7 or 8 additional residues (depending upon which Met residue serves as the initiation site) compared to the mammalian homologues.

On the basis of the nucleic acid sequence generated from PCR and RACE reactions, it is concluded that the caspase-6 cDNA isolated herein represents the α form of Mch-2 previously described by Fernandes-Alnemri et al. [25]. A second, β form (Mch-2β) [25] was not identified from the 3 clones sequenced within the present studies; thus the chicken enzyme is referred to hereafter as caspase-6.

Nucleic acid homology within the predicted coding region of chicken caspase-6 and the corresponding human and mouse sequences is 72% and 70%, respectively. Amino acid identity between the deduced chicken caspase-6 sequence and the human and mouse caspase-6 sequences is 68% and 70%, respectively, and conservation within the P11 subunit is somewhat higher (80% and 81%, respectively). On the basis of the mammalian caspase-6 [25], processing of this proenzyme form to active enzyme is predicted to occur at Asp-35 Ala-36 or Asp-44 Pro-45 (removal of the propeptide), Asp-191 Ser-192, and Asp-

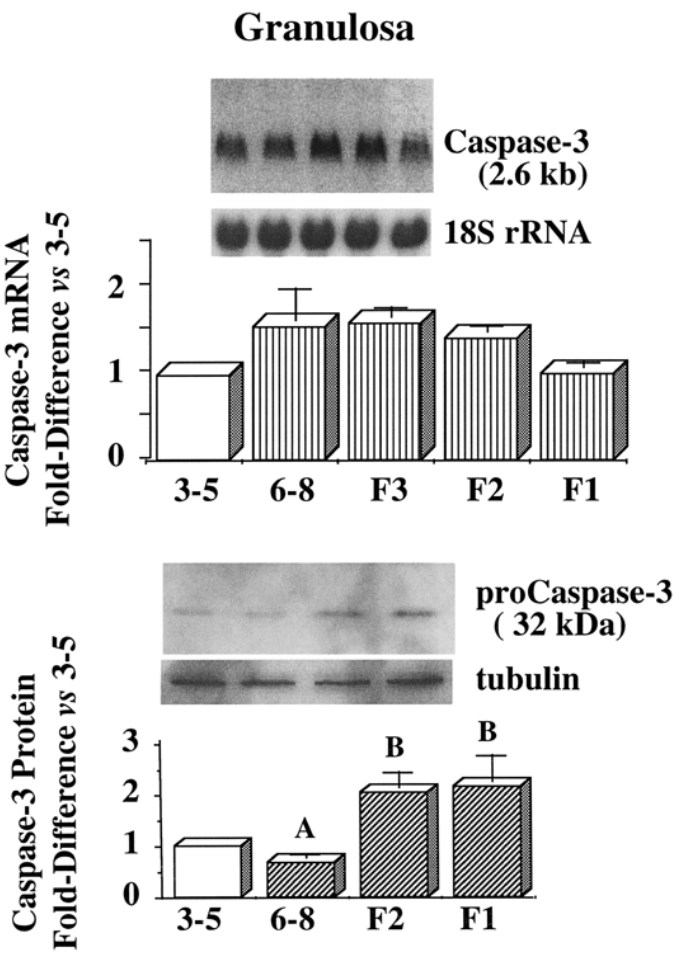


FIG. 4. Caspase-3 mRNA (**top**) and protein (**bottom**) in granulosa tissue during follicle development. Summarized data represent the mean ± SEM from 3 replicate blots standardized to levels of 18S rRNA or tubulin and expressed as a fold difference versus 3- to 5-mm follicles. F3, F2, F1 represent the third largest, second largest, and largest preovulatory follicle; 3–5, 6–8 denote diameter size of prehierarchal follicles. A,B: $P < 0.05$ by ANOVA; There were no significant differences ($P > 0.10$) in levels of caspase-3 mRNA across follicle development.

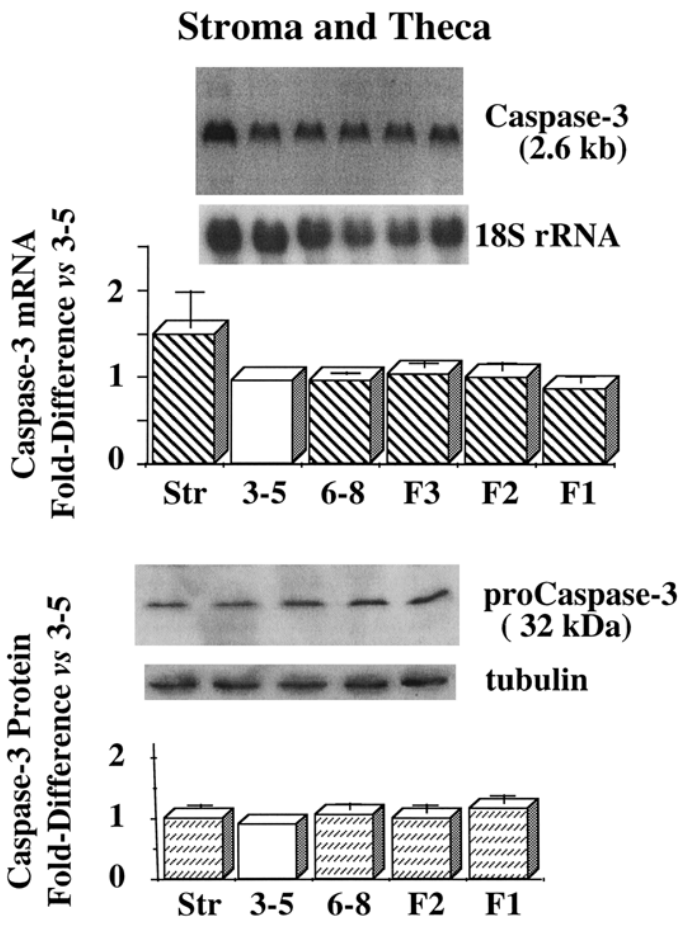


FIG. 5. Caspase-3 mRNA (**top**) and protein (**bottom**) in stromal tissue and the theca during follicle development. Summarized data represent the mean ± SEM from 3 replicate blots standardized to levels of 18S rRNA or tubulin and expressed as a fold difference versus 3- to 5-mm follicles. There were no significant differences ($P > 0.10$) in levels of caspase-3 mRNA or protein across follicle development or compared to stromal tissue (Str). Other abbreviations are as described in Figure 4 legend.

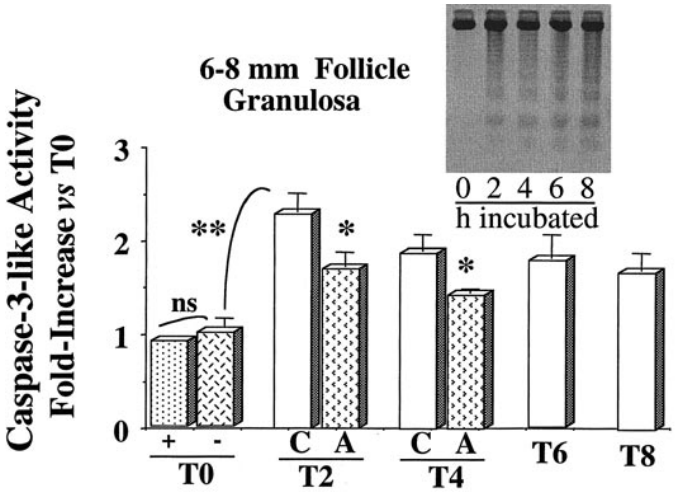
204 Ala-205 (processing of the active enzyme; Fig. 2). An alternative start site may also exist at Met-51, although neither this nor the Met-1 start codon is preceded by a conventional mammalian Kozak sequence [26]. The latter putative start site may be considered less likely, as this would preclude the presence of any N-terminal prodomain and would result in a truncated P18 domain. On the other hand, based upon Western blot analysis (see below), the predicted size of chicken caspase-6 (29 kDa) is more consistent with a protein initiated at Met-51. Additional analysis of this protein is required to differentiate between these two possibilities.

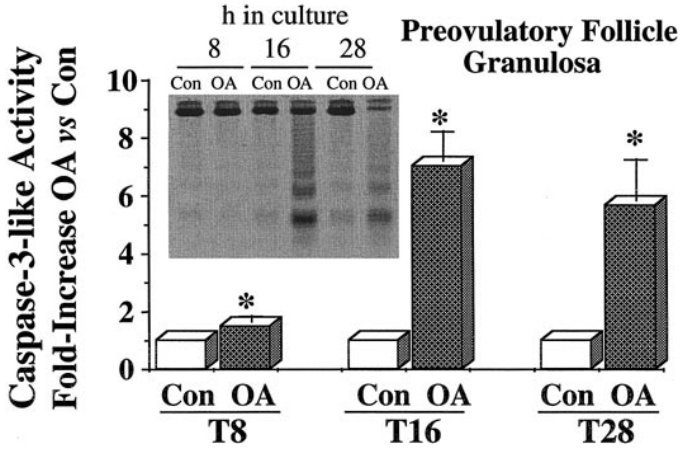
As found for caspase-3, the QACRG active site for caspase-6 is completely conserved. The predicted length of the full coding region (301 amino acids) is more similar to the human (293 amino acids) than to the mouse (276 amino acids) sequence.

Northern blot analysis of caspase-3 showed a single mRNA transcript corresponding to a size of approximately 2.6 kilobases (kb), whereas Western analysis detected a 32-kDa caspase-3 proenzyme. Caspase-3 mRNA was expressed to varying levels within different tissues from the hen, yet levels of mRNA transcript were not always directly related to those for the translated protein when compared across the various tissues (Fig. 3). For instance, caspase-3 mRNA levels in bone marrow and kidney were relatively high, whereas corresponding levels of translated protein were comparatively low. By comparison, F1 theca caspase-3 mRNA expression was relatively low, while protein levels were comparatively high.

Caspase-3 mRNA levels remained essentially unchanged within the granulosa layer during follicle development, while levels of procaspase-3 protein in preovulatory (F1 and F2) follicle granulosa cells were approximately 2-fold those measured in 6- to 8-mm follicles ($P < 0.05$; Fig. 4). By comparison, neither caspase-3 mRNA nor proenzyme (Fig. 5) levels changed significantly within theca tissue during follicle development ($P > 0.10$).

The absence of detectable caspase-3-like activity in freshly collected and frozen (T0) granulosa tissue collected from 6- to 8-mm follicles was indicated by the lack of difference between samples incubated in the presence (+) or absence (−) of the caspase-3 inhibitor, Ac-DEVD-CHO ($P > 0.2$ by paired *t*-test; Fig. 6, top panel). Compared to that in T0 tissue, caspase-3-like activity was increased by 2.3 ± 0.2-fold within 2 h of the initiation of apoptosis induced by suspension culture of prehierarchal (6–8 mm) follicle granulosa cells, and activity did not significantly





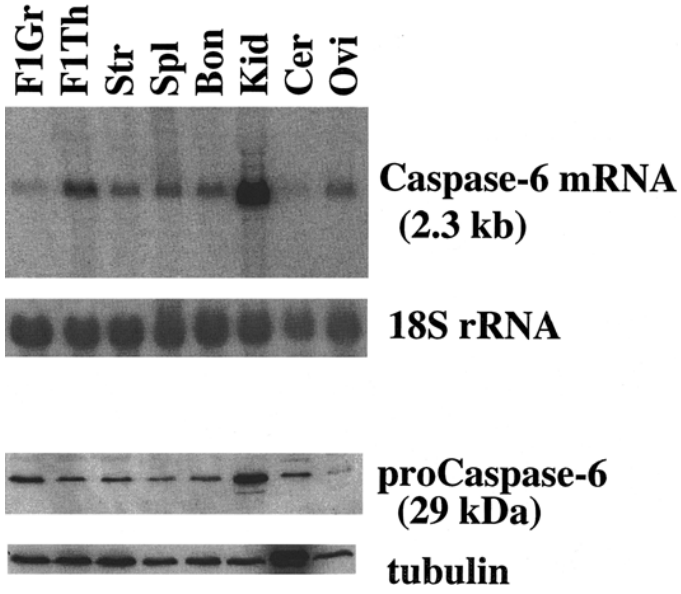


FIG. 7. Caspase-6 mRNA and protein levels in several tissues from the hen. Ribosomal RNA (18S) and tubulin data are provided for the evaluation of equal loading. This experiment was repeated twice with similar results. Abbreviations are explained in the legend to Figure 3.

FIG. 6. **Top**) Caspase-3-like enzyme activity in prehierarchal (6–8 mm) follicle granulosa cells incubated for 0 (T0) to 8 h in the absence (C) or presence (A) of 1 mM 8-br-cAMP. There was no difference between T0 samples incubated in the presence (+) or absence (−) of the caspase-3 inhibitor, Ac-DEVD-CHO, indicating the lack of detectable caspase activity in freshly collected and frozen samples. Inset represents oligonucleosome formation in cells incubated in medium alone after 0–8 h of incubation. **Bottom**) Caspase-like activity in cultured preovulatory follicle granulosa cells following okadaic acid (OA; 50 nM)-induced apoptosis compared to the appropriate control. Inset represents oligonucleosome formation in the absence (Con) and presence of okadaic acid after 8, 16, or 28 h of culture. $*P < 0.01$ compared to the appropriate control, by paired *t*-test; $**P < 0.05$ compared to T0 minus inhibitor by paired *t*-test; ns, $P > 0.10$ for the comparison of T0 samples plus and minus inhibitor.

change thereafter through T8 h ($P > 0.2$). Coincident with caspase-3-like activity was a decrease in levels of the caspase-3 proenzyme as measured by Western analysis (data not shown) and the ability to detect increased oligonucleosome formation (Fig. 6, insert). Significantly, coincubation with 1 mM 8-br-cAMP was found to attenuate ($P < 0.05$, by paired *t*-test compared to the appropriate control) the

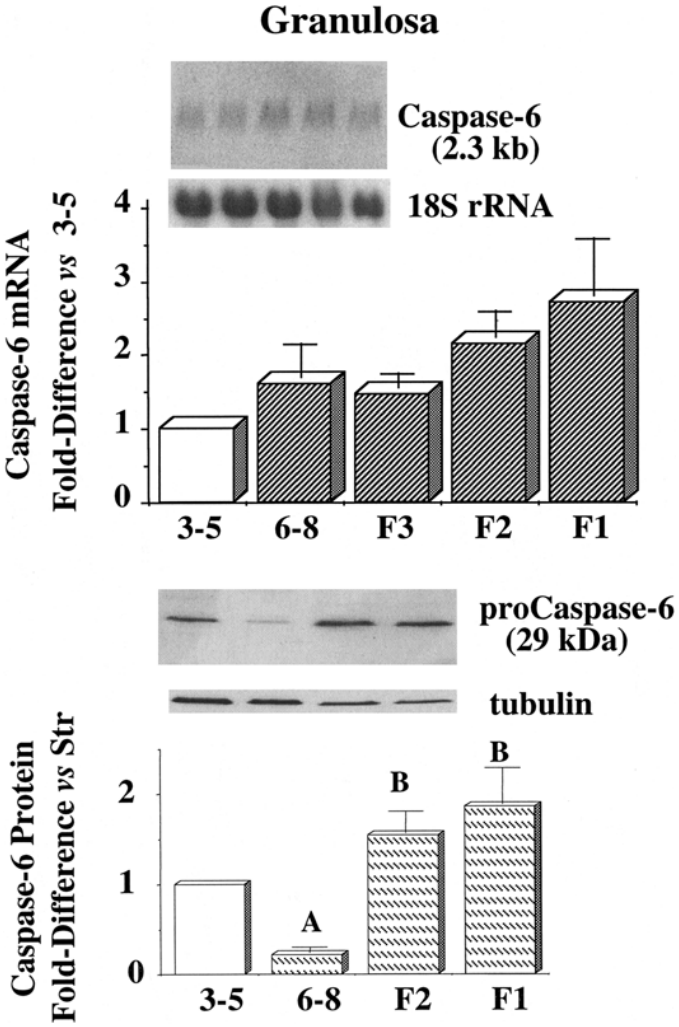


FIG. 8. Caspase-6 mRNA (**top**) and protein (**bottom**) in granulosa tissue during follicle development. Summarized data represent the mean ± SEM from 3 replicate blots standardized to levels of 18S rRNA or tubulin and expressed as a fold difference versus 3–5 mm follicles. A,B: $P < 0.05$ by ANOVA; there were no significant differences ($P > 0.05$) in levels of caspase-6 mRNA across follicle development. Abbreviations are defined in legend to Figure 4.

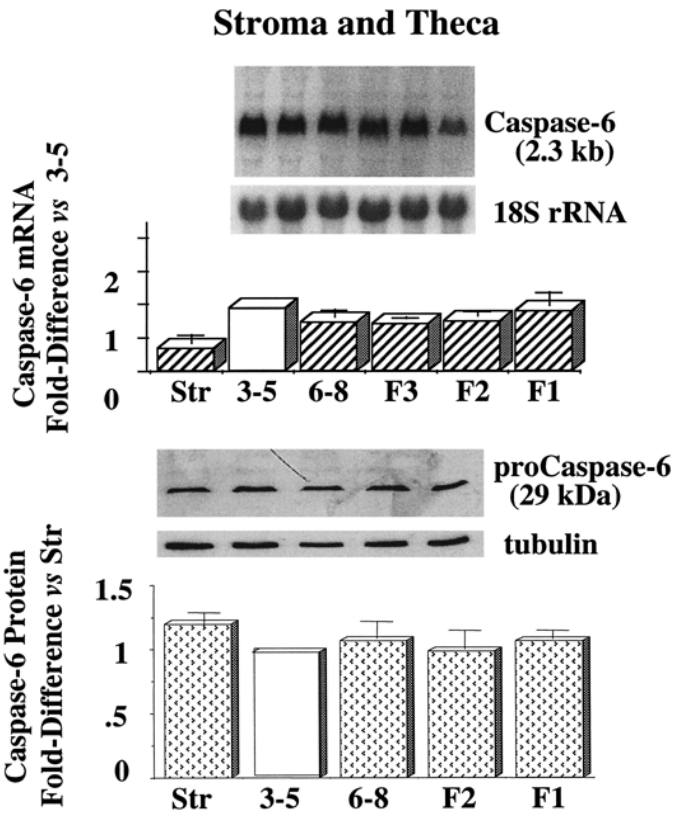


FIG. 9. Caspase-6 mRNA (**top**) and protein (**bottom**) in stromal tissue and theca layers during follicle development. Summarized data represent the mean ± SEM from 3 replicate blots standardized to levels of 18S rRNA or tubulin and expressed as a fold difference versus 3- to 5-mm follicles. There were no significant differences ($P > 0.10$) in levels of caspase-6 mRNA or protein across follicle development or compared to stromal tissue (Str). Other abbreviations are as described in Figure 4 legend.

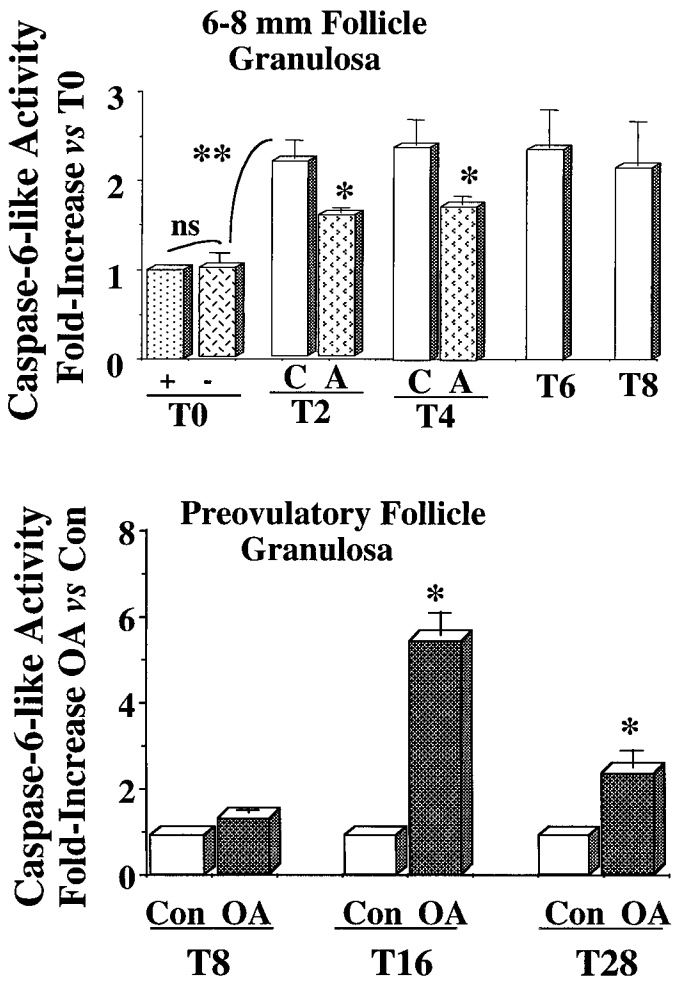


FIG. 10. **Top**) Caspase-6-like enzyme activity in prehierarchal (6–8 mm) follicle granulosa cells incubated for 0 (T0) to 8 h in the absence (C) or presence (A) of 1 mM 8-br-cAMP. There was no difference between T0 samples incubated in the presence (+) or absence (−) of the caspase-6 inhibitor, Ac-VEID-CHO, indicating the lack of detectable caspase-like activity in freshly collected and frozen tissue. **Bottom**) Caspase-6-like activity in cultured preovulatory follicle granulosa cells following culture for 8, 16, or 28 h in the absence (Con) or presence of okadaic acid (OA; 50 nM)-induced apoptosis. *$P < 0.05$ compared to the appropriate control, by paired *t*-test; **$P < 0.05$ compared to T0 minus inhibitor; ns, $P > 0.10$ for the comparison of T0 samples plus and minus inhibitor.

level of caspase-3 activity at 2 and 4 h of incubation. By comparison, caspase-3-like activity did not increase in preovulatory (F1 and F2) follicle granulosa cells cultured in complete medium for 2 days compared to freshly collected (T0) cells ($P > 0.1$ by ANOVA; data not shown), and oligonucleosome formation remained consistently low throughout this period (Fig. 6, bottom panel). On the other hand, treatment of cultured cells with okadaic acid (50 nM) induced caspase-3-like activity within 8 h (maximal increase of 7.6 ± 1.3-fold by 16 h), and the initial significant increase in enzyme activity appeared to precede detection of oligonucleosome formation.

Northern analysis of caspase-6 showed a single, 2.3-kb mRNA transcript from each of the hen tissues investigated. Western blot analysis detected a 29-kDa caspase-6 protein that is proposed to encode the previously described mammalian Mch-2α form [25] (Fig. 7). Like caspase-3, caspase-6 mRNA was expressed to varying degrees within different tissues from the hen, yet levels of transcript were not always directly related to levels of the protein when compared across tissues. For instance, although some of the highest levels of both caspase-6 mRNA and protein appeared to occur within the kidney, the protein was easily detected in F1 follicle granulosa cells and cerebrum tissues despite comparatively low levels of mRNA transcript.

As described for caspase-3, caspase-6 mRNA levels remained unchanged within the granulosa layer during follicle development, yet levels of translated procaspase-6 protein in preovulatory follicle granulosa cells were consistently 7- to 8-fold those measured in 6- to 8-mm follicles ($P < 0.05$; Fig. 8). Neither caspase-6 mRNA nor protein levels changed significantly within theca tissue during follicle development (Fig. 9). The absence of detectable caspase-6-like activity in freshly collected (T0) granulosa tissue collected from 6- to 8-mm follicles was indicated by the lack of difference between samples incubated in the presence (+) or absence (−) of the caspase-6 inhibitor, Ac-VEID-CHO ($P > 0.2$ by paired *t*-test; Fig. 10, top panel). Caspase-6-like activity in 6- to 8-mm follicle granulosa cells was increased by 2.2 ± 0.2-fold within 2 h of incubation, and the level of activity did not change appreciably through T8 h. This increase in activity was attenuated by coincubation with 1 mM 8-br-cAMP. Finally, caspase-6-like activity did not increase in preovulatory follicle granulosa cells during the two-day culture period ($P > 0.1$ versus freshly collected cells by ANOVA; data not shown) but was increased by 5.4 ± 0.5-fold after a 16-h treatment with 50 nM okadaic acid ($P < 0.01$; Fig. 10, bottom panel).

DISCUSSION

The significance of the findings reported herein includes the first identification and characterization of two additional chicken homologues related to the mammalian caspase family of proteases, together with the detection of their coordinated activity associated with the initiation of apoptosis, in vitro, in hen granulosa cells. Caspase-3 activation has been associated with the execution phase of apoptosis and has previously been reported to mediate proteolysis of numerous cellular substrates, including the nuclease DFF40/CAD, which cleaves DNA into oligonucleosomes, poly(ADP ribose)polymerase, protein kinase Cδ, and U1–70 kDa [27–30]. Although caspase-6, together with caspase-3, have been suggested to represent two of the major active caspases detected in apoptotic cells [31], physiological substrates cleaved by caspase-6 (other than lamins) [3] are largely unknown. Moreover, the causative relationship between (or absolute requirement for) caspase-6 activation and apoptosis has yet to be fully established in any cell system.

Among the mammalian caspases thus far described, the N-terminal peptide prodomain is the most variable in primary sequence and length across species [11]. In addition, the chicken caspase-3 protein shows relatively poor homology at the amino acid level to both the human and mouse caspase-3 sequences within the N-terminal prodomain (21% and 35% homology, respectively; Fig. 1). Similarly, the predicted chicken caspase-6 N-terminal prodomain is 35 amino acids in length, and it shares even less amino acid homology with either the mouse or human sequence (Fig. 2). Cleavage of the human N-terminal prodomain is predicted to occur at Asp-23 Ala-24, Asp-32 Pro-33, or Asp-40 His-41 [24], and the first two of these sites are conserved within the chicken sequence. Whereas a relatively large prodomain, such as found in mammalian caspase-2, -8, -9, and -10, has been proposed to be important in upstream receptor-mediated events, caspases with a comparatively short prodomain, such as found for caspase-3 and -6, are typically involved in the execution phase of cell death [11].

Procaspase-3 and -6 are widely expressed within tissues of the hen, with readily detectable levels of the protein found in the spleen and bone marrow, suggesting a prominent role within the immune system [24]. The apparent absence of a direct relationship between caspase-3 and -6 mRNA and protein levels in each tissue may reflect tissue-specific differences in the turnover rate of the mRNA transcript and/or protein.

Recent studies have also documented the expression of caspase-3 within the mammalian ovary [32–36]. While immunoreactive levels are comparatively high in the healthy as well as regressing corpus luteum, considerably lower levels of expression occur within granulosa cells from nonatretic preovulatory follicles. By comparison, both the caspase-3 and -6 proenzymes are readily detectable within hen ovarian follicle tissues during all stages of development. Although there are no differences in levels of caspase-3 or -6 mRNA transcript within granulosa cells during follicle development, levels of both procaspase-3 and -6 protein are consistently lower in 6- to 8-mm follicles (Figs. 4 and 8). Given that prehierarchal follicle granulosa cells are inherently more susceptible to apoptosis compared to preovulatory follicle granulosa cells, this result would appear paradoxical. In this regard, it is possible that levels of procaspase do not necessarily reflect the cell's potential for facilitating apoptosis, as only a fraction of the active enzyme need be functional to effect cell death.

Alternatively, higher levels of caspase protein may be related to increased expression of the inhibitor of apoptosis protein gene, *ita,* in preovulatory follicles as recently reported [37]. Given that inhibitor of apoptosis proteins have been proposed to function, at least in part, by preventing activation of caspases and/or directly inhibiting caspase activity [38], proportionately more caspase may be required to enable effective execution of the cell when required. Caspase activation and granulosa apoptosis would be predicted to occur, in vivo, in hen preovulatory follicle granulosa cells during induced follicle atresia (e.g., with food or water withdrawal, or reduced photoperiod) and in association with the process of ovulation [20, 39].

Caspase-3 and -6, as well as the previously reported caspase-1 [17] and caspase-2 [18], are also found expressed within the hen theca layer. Although it has been proposed that follicle atresia in both mammals and the hen results from the initiation of apoptosis within the granulosa layer and/or oocyte [12, 40], several investigators have documented caspase-1 and -3 expression and/or enzyme activity within thecal tissue (e.g., [33, 34, 41]); presumably such activity serves as a mechanism to facilitate follicle resorption after the death of the follicle. It is currently not known what cellular signals or mechanisms may be responsible for activating any of the caspase enzymes expressed within hen theca tissue.

Initiation of caspase-3-like activity occurs in cultured mouse granulosa cells coincident with cell death induced by serum withdrawal, and this enzyme activity has been linked to activation of the apoptotic protease-activating factor-1 [35]. Moreover, the coordinate expression of caspase-3- and -6-like activity following the initiation of granulosa cell apoptosis reported herein is consistent with a previous study in cultured mammalian cells [31]. The initiation and maintenance of enzyme activity for both caspases following the dispersion and 8-h suspension culture of granulosa cells from prehierarchal follicles (Figs. 6 and 10, top panels) are associated with oligonucleosome formation (Fig. 6, inset). This observation is consistent with a rapid onset of apoptosis at this stage of follicle development as previously reported [16], presumably in response to the removal of cell survival factors. Of significance is the finding that coincubation with 8-br-cAMP attenuates this increase in activity at 2 and 4 h of incubation, which further supports the proposal that agonists which activate the adenylyl cyclase/cAMP pathway (e.g., LH, vasoactive intestinal peptide) [16, 42, 43] serve as survival signals.

Finally, okadaic acid, a known inducer of apoptosis (reviewed in [44]), promotes both oligonucleosome formation and caspase-3 and -6 activity in cultured preovulatory follicle granulosa cells (Figs. 6 and 10, lower panels). However, the timing of maximal caspase activity appears to be somewhat delayed compared to that in prehierarchal follicle granulosa cells, and this is consistent with a previous study that examined the onset of oligonucleosome formation following induced cell death [16]. Caspase-3-like activity is significantly increased after 8 h of treatment and precedes detection of significant oligonucleosome formation; nevertheless, a direct cause and effect relationship between these two endpoints remains to be established. The ability of cells to maintain caspase activity appears to be transient: after 28 h of okadaic acid treatment, the majority of cultured cells have detached from the culture plate (indicative of cell

death; data not shown), and caspase activity begins to decline.

In summary, the present studies are the first to characterize the caspase-3 and -6 cDNAs and to monitor proenzyme expression as well as enzyme activity in an avian species. Furthermore, results indicate that the coordinate activation of the two caspases following initiation of apoptosis in granulosa cells results in an efficient progression of cell death which, in vivo, would culminate in follicle atresia. Whether activation of both caspases is prerequisite for, or alternatively represents parallel redundant pathways leading to, cell death remains to be established. Finally, the difference in time to initiation of caspase activity after induced cell death in prehierarchal versus preovulatory follicle granulosa cells further supports the proposal for inherent differences in susceptibility to cell death related to stage of development. Further studies will be required to identify additional caspase family members expressed within the ovary in this species and to elucidate the molecular ordering of the putative active caspase cascade, as well as to determine the physiological substrates for each caspase within this cell type.

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