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Characterization of the avian Ich-1 cDNA and expression of Ich-1_L mRNA in the hen ovary

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Abstract

We have sequenced the chicken interleukin-1**b**-converting enzyme (ICE) and ced-3 homolog (Ich-1) cDNA, and evaluated Ich-1 mRNA expression in the hen ovary during follicle development. While two alternatively spliced forms of Ich-1, Ich-1_L and Ich-1_S, were amplified by PCR from an embryonic chicken cDNA library, only the Ich-1_L form was found to be expressed in adult ovarian granulosa and theca tissues. The deduced amino acid (aa) sequence of ICH-1_L is 70.8% identical to human ICH-1_L and contains the conserved QACRG peptide active catalytic sequence characteristic of many ICE-related family of cysteine proteases. © 1997 Elsevier Science B.V.

Keywords: Caspases; Interleukin-1**b**-converting enzyme; ced-3; Apoptosis; Ovary; Granulosa; Theca; Follicle atresia

1. Introduction

Interleukin-1**b**-converting enzyme (ICE) represents a cysteine protease that cleaves pro-interleukin-1**b** following an aspartic acid residue to generate active cytokine (Wilson et al., 1994). The active protease consists of two subunits (p20 and p10) which are derived from the precursor protein following auto- or heterocatalysis (Kumar, 1995). Subsequent studies in the human have demonstrated the existence of a family of homologous proteases, and it has recently been proposed that ICE/CED-3 proteases now be referred to as caspases (Alnemri et al., 1996) to signify their distinctive catalytic features. In addition to ICE itself (caspase-1), this family of related proteases currently includes Ich-1 (caspase-2; Wang et al., 1994), CPP32 (caspase-3; Fernandes-Alnemri et al., 1994), Ich-2 (caspase-4; Kamens et al., 1995), Mch2 (caspase-6; Fernandes-Alnemri et al., 1995a), Mch3 (caspase-7; Fernandes-Alnemri et al., 1995b), Mch4, Mch5 (caspase-10 and caspase-8, respectively; Fernandes-Alnemri et al., 1996) and Mch6 (caspase-9; Srinivasula et al., 1996).

Several lines of evidence indicate that caspases are directly involved in mediating apoptosis via the cleavage of one or more intracellular proteins (Kumar, 1995). For instance, the nt sequences within this family of proteases are homologous to ced-3, an invertebrate gene whose product is required for apoptosis during development in *Caenorhabditis elegans* (Yuan et al., 1993). Moreover, over-expression of ICE protease in a fibroblast cell line induces apoptosis, and this activity can be blocked by co-expression of Bcl-2, an inhibitor of apoptotic cell death, and CrmA, a cowpox virus protein that is a selective inhibitor of ICE protease activity (Miura et al., 1993).

Two forms of the human Ich-1 have been characterized: Ich-1_L, whose over-expression induces apoptotic cell death, and an alternatively-spliced Ich-1_S which suppresses cell death (Wang et al., 1994). A major difference between these two transcripts occurs in the form of a 61-bp insert which results in the termination of translation 21 aa downstream from the insertion site. As is the case for several other members of this family, the physiological substrate(s) for the ICH-1_L protease has yet to be identified.

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Abbreviations: aa, amino acid(s); bp, base pair(s); cDNA, complementary DNA; cRNA, complementary RNA; ICE, interleukin-1**b**-converting enzyme; Ich-1, interleukin-1**b**-converting enzyme and ced-3 homolog; Ich-1_L, Ich-1 long; Ich-1_S, Ich-1 short; kb, kilobase(s) or 1000 bp; nt, nucleotide(s); ORF, open reading frame; PCR, polymerase chain reaction; RACE, rapid amplification of cDNA end(s); RNase analysis, ribonuclease protection analysis.

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                ATG CTG GGA GCA TGC GGC ATG CAG CGA TAC CAC CAG GAA GCT CTG AAG   48
                 M   L   G   A   C   G   M   Q   R   Y   H   Q   E   A   L   K    16

AAG AAC CGG GTG ATG CTG GCG AGA GAG CTG GTT TTA AAA GAG TTG ATG GAA CAC ATG ATA  108
 K   N   R   V   M   L   A   R   E   L   V   L   K   E   L   M   E   H   M   I    36

GAG AAG GAT ATC ATC ACC ATT GAG ATG GTG GAA ATG ATA CAG GCA AAG TCT GGA AGC TTC  168
 E   K   D   I   I   T   I   E   M   V   E   M   I   Q   A   K   S   G   S   F    56

AGC CAA AAT GTG GAA TTC CTT AAT TTG TTG CCC AAG AGA GGC CCT AAT GCC TTT TCA GCC  228
 S   Q   N   V   E   F   L   N   L   L   P   K   R   G   P   N   A   F   S   A    76

TTC TGT GAA GCT CTA CAA GAA ACC AAA CAG CAG CAT CTG GCG GAA ATG ATC TTG AAG ACA  288
 F   C   E   A   L   Q   E   T   K   Q   Q   H   L   A   E   M   I   L   K   T    96

GAA TCC AGC TTG AGA CAT GGG ATT GCA ACG CTT GAA CAG CGT TAT GGC TCA AAT CTT CCA  348
 E   S   S   L   R   H   G   I   A   T   L   E   Q   R   Y   G   S   N   L   P   116

CTT CCT CTG AGT GAA TCA TGT AAT TCA AAG AGA CCA CGC TTG ATT GTG GAA CAT TCT TTG  408
 L   P   L   S   E   S   C   N   S   K   R   P   R   L   I   V   E   H   S   L   136

GAC AGT GGA GAT GGT CCT CCG ATT CCT CCA GTG AAG CAC TGC ACT CCA GAA TTC TAT CGT  468
 D   S   G   D   G   P   P   I   P   P   V   K   H   C   T   P   E   F   Y   R   156

GAT CAT CAG CAC TTA GCA TAC AAA CTG ATA TCA GAG CCC CGA GGC TTA GCA CTT ATT CTC  528
 D   H   Q   H   L   A   Y   K   L   I   S   E   P   R   G   L   A   L   I   L   176

AGC AAT ATC CAT TTC AGC AGT GAA AAG GAC TTG GAA TAT CGT TCA GGT GGA GAT GTG GAC  588
 S   N   I   H   F   S   S   E   K   D   L   E   Y   R   S   G   G   D   V   D   196
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Fig. 1. Nucleotide and deduced aa sequences of avian Ich-1$_L$. Putative ATG start codon and TAA stop codon begin at bp 1 and 1273, respectively. The QACRG active catalytic site, conserved within many caspases described to date, is single underlined. *** indicates an in-frame stop codon. The putative Ich-1$_S$ 62 bp insert was found immediately after the G residue (double underline). GenBank accession number for this nt sequence is U64963. **Methods**: Chicken Ich-1$_L$ and Ich-1$_S$ cDNA PCR products were amplified from an embryonic chicken cDNA library (Clontech) as previously described (Wang et al., 1994). RACE was conducted in the 5′ and 3′ directions as described by the manufacturer (Clontech Laboratories, Inc., Palo Alto, CA). Poly A$^+$-enriched RNA from granulosa tissue of the second largest (F2) preovulatory plus prehierarchal (6- to 8-mm) follicles was isolated using the Micro-Fast Track Kit (Invitrogen, San Diego, CA). Double stranded cDNA was synthesized, and Marathon cDNA Amplification adaptors (Clontech) were ligated. Nested gene-specific primers for the 5′ direction (GSP1, 5′-AACCCCACCCTCTACACCGTGG-3′ [bp 762–741]; and GSP2, 5′-GAAGCACAGTCCACATCTCCACCTG-3′ [bp 596–572]) and 3′ direction (GSP3, 5′-TGTGAGGAGAGTGATGCAAACAAGG-3′ [bp 940–964]; and GSP4, 5′-CCACTGTGTTTGCAGAGGACTCCC-3′ [bp 1085–1108]) were designed from sequence obtained from the chicken Ich-1$_L$ PCR product. An initial amplification of the 5′-region by PCR was performed using GSP1 and Adaptor Primer 1 (Clontech) under the following conditions: 1 min, 94°C; 0.5 min, 94°C and 4 min, 72°C for five cycles; 0.5 min, 94°C and 4 min, 70°C for five cycles; 0.5 min, 94°C and 4 min 68°C for 25 cycles. A second amplification was conducted with the GSP2 and Adaptor Primer 2 as internal primers and amplification conditions as described above. PCR products were gel-purified and ligated into the PCRII vector (TA Cloning Kit, Invitrogen), then further amplified and purified from plasmid preparations. Amplified and cloned chicken Ich-1 sequence from three to four different PCR products was determined by nucleic acid sequencing using the dideoxy chain termination method (Sanger et al., 1977) using the Sequenase version 2.0 system (United States Biochemical, Cleveland, OH). Initial and secondary amplifications of the 3′-region were conducted using GSP3 and Adaptor Primer 1 and GSP4 and Adaptor Primer 2, respectively using similar amplification conditions described above, with the exception that during the final 25 cycles annealing was at 65°C for 0.5 min. PCR products (each approximately 1.5 kb in length) were gel-purified and ligated into the PCRII vector, amplified and purified from plasmid preparations. Sequence data for Ich-1$_L$ were assembled from replicate products over the entire coding region and analyzed using the MacVector and AssemblyLIGN programs (version 4.5.3; IBI, New Haven, CT).

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TGT GCT TCC CTA GAG CTG CTT TTC AAG CAT CTT GGA TAT CAA GTG ACT GTC TTT CAT GAT  648
 C   A   S   L   E   L   L   F   K   H   L   G   Y   Q   V   T   V   F   H   D   216

CAA AGT GCA GAG GAA ATG GAG AGT GCA TTG GAG AGA TTC TCT AAA TTG CCA GAT CAT CAG  708
 Q   S   A   E   E   M   E   S   A   L   E   R   F   S   K   L   P   D   H   Q   236

GAT GTG GAT TCC TGT ATT GTA GCT TTA CTT TCC CAC GGT GTA GAG GGT GGG GTT TAT GGC  768
 D   V   D   S   C   I   V   A   L   L   S   H   G   V   E   G   G   V   Y   G   256

ACT GAT GGC AAA CTC CTA CAG TTG CAG GAG GCT TTC AGG CTC TTT GAT AAT GCA AAC TGC  828
 T   D   G   K   L   L   Q   L   Q   E   A   F   R   L   F   D   N   A   N   C   276

CCC AAT CTC CAG AAT AAG CCC AAA ATG TTC TTT ATT CAG GCT TGC CGG GGA GAT GAG ACA  888
 P   N   L   Q   N   K   P   K   M   F   F   I   Q   A   C   R   G   D   E   T   296

GAC CGG GGA GTG GAT CAG AGA GAT GGC AAA GAA CGG TCA GAT TCC CCA GGC TGT GAG GAG  948
 D   R   G   V   D   Q   R   D   G   K   E   R   S   D   S   P   G   C   E   E   316

AGT GAT GCA AAC AAG GAA GAA AAT CTC AAG CTG CGT TTG CCT ACA CGC TCC GAT ATG ATC 1008
 S   D   A   N   K   E   E   N   L   K   L   R   L   P   T   R   S   D   M   I   336

TGT GGA TAT GCA TGT TTG AAA GGC ACT GCA GCC ATG CGC AAC ACC AAG CGT GGA TCC TGG 1068
 C   G   Y   A   C   L   K   G   T   A   A   M   R   N   T   K   R   G   S   W   356

TAT ATC GAG GCA CTG ACC ACT GTG TTT GCA GAG GAC TCC CGG GAC ACT CAT GTG GCT GAC 1128
 Y   I   E   A   L   T   T   V   F   A   E   D   S   R   D   T   H   V   A   D   376

ATG TTG GTG AAG GTG AAT AGA CAA ATC AAG CAA CGA GAA GGT TAT GCC CCA GGC ACA GAA 1188
 M   L   V   K   V   N   R   Q   I   K   Q   R   E   G   Y   A   P   G   T   E   396

TTC CAT CGC TGC AAG GAA ATG TCA GAA TAC TGT AGT ACG CTC TGT CGG GAC CTT TAC CTA 1248
 F   H   R   C   K   E   M   S   E   Y   C   S   T   L   C   R   D   L   Y   L   416

TTT CCC GGC TAT GTG CCA GGA AAA TAA CCC TAC TGG CTC CAC TGG AAG AGA GGT ACT GCC 1308
 F   P   G   Y   V   P   G   K  ***                                              424

AAA GCT GAA CTC AGT ATG GAT GTG TAC ATT TCT GTT CTC ACA GGC GTG ACA TCA GAT GCC 1368
AAG CAA GGA ATG GTC TTT CAC GTT GAG C                                           1396
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Fig. 1. (*continued*)

It has previously been determined that ovarian follicle atresia in mammals and the chicken is mediated via apoptosis, and that such apoptotic cell death occurs primarily, if not exclusively, within the granulosa cell layer (e.g., Tilly et al., 1991; Johnson et al., 1996). Given the potential for Ich-1 to play a regulatory role in the

process of ovarian follicle atresia (Flaws et al., 1995), the objectives of the present study were to characterize the Ich-1 cDNA from RNA expressed in the hen ovary, and to evaluate the expression of Ich-1 mRNA in ovarian tissues.

2. Experimental and discussion

2.1. Chicken Ich-1 long cDNA and deduced aa sequence

Procedures for the initial cloning of chicken Ich-1_L and Ich-1_S partial cDNAs from an embryonic chicken cDNA library have previously been described (Wang et al., 1994). The nt sequence derived from these fragments was utilized for the design of primers to amplify the 5′ and 3′ ends of chicken Ich-1 by RACE using reverse transcribed RNA obtained from ovarian granulosa cells. This resulted in the identification of a single form of the gene corresponding to Ich-1_L. The composite cDNA sequence consists of 1396 nt, with an ORF of 424 aa (Fig. 1).

Relationship of the Ich-1_S insert to the complete Ich-1_L cDNA is illustrated in Fig. 2A. The Ich-1_S alternatively-spliced sequence is 62 bp long, and was found inserted following bp 879 of the coding region (Fig. 2A). This insertion point is out of frame with respect to the Ich-1_L nt sequence, and similar to the human Ich-1 sequence (Wang et al., 1994), is proposed to cause premature termination of translation. In addition, it is suggested that similar to human ICH-1_S, translation of the chicken ICH-1_S protein is initiated at a start codon found beginning at bp 19 from the Ich-1_L nt sequence.

The deduced aa sequence of ICH-1_L contains the highly conserved active catalytic domain, QACRG, found in many known caspases (Kumar, 1995), two putative domains corresponding to the Asp pocket in ICE (Wilson et al., 1994), and 164 and 106 aa peptides homologous to the p20 and p10 subunits of ICE, respectively (Thornberry et al., 1992) (Fig. 3). These latter regions were determined to be 67.1% homologous to the p20 and 83.0% homologous to the p10 subunits from the human ICH-1_L sequence (Wang et al., 1994).

2.2. Expression of Ich-1 long in the ovary

RNase protection analysis was conducted to evaluate expression of Ich-1 in the ovary at various stages of follicle development, following ovulation, and during follicle atresia. Design of the probe utilized to differentiate between Ich-1_L and Ich-1_S transcripts is shown in Fig. 2B. Irrespective of the physiological status of ovarian tissues evaluated, only the Ich-1_L transcript was

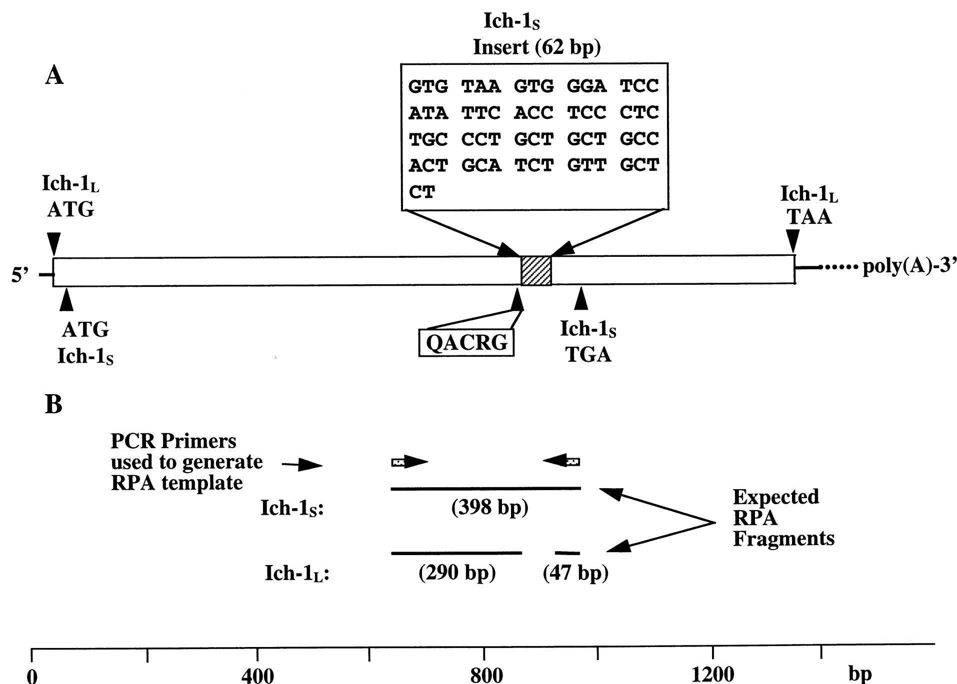

Fig. 2. (**A**) Diagramatic representation of the chicken Ich-1_L cDNA, characterization of the Ich-1_S insert, and putative sites for the Ich-1_S start (ATG) and stop (TGA) codons. · · · · · represents approximately 1.8 kb of unsequenced cDNA ending in a poly(A^+) tail at the 3′ end. (**B**) Design of the Ich-1 probe used in the RNase protection assay to differentiate between expression of Ich-1_L and Ich-1_S transcripts. **Methods**: The probe, which was amplified from a clone containing the Ich-1_S cDNA, spans the region of the 62-bp insert and was generated from forward (GTGCTTCCCTAGAGCTGCTTTTC; bp 590–612) and reverse (TGACCGTTCTTTGCCATCTCTC; bp 926–905) primers by PCR.

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c Ich-1 long          MLGACGMQRYHQEALKKNRVMLARELVLKELMEHMIEKDIITIEMVEMIQ   50
h Ich-1 long    maadrgrri..v...hph...t......v..kq.l.s..l..ll......l..r.l..   58

c Ich-1 long  AKSGSFSQNVEFLNLLPKRGPNAFSAFCEALQETKQQHLAEMILKTESSLRHGIATLEQR  110
h Ich-1 long  ..v.......l........q..d......r....g..ed.l.t.l.g.q.vlpp.scd  118

                                     --> p20
c Ich-1 long  YGSNLPLPLSESCN-SKRPRL---IVEHSLDSGDGPPIPPVKHCTPEFYRDHQHLAYKLI  166
h Ich-1 long  .dls..f.vc...ply.kl..stdt......nk...vclq..p......qt.fq...r.q  178

c Ich-1 long  SEPRGLALILSNIHFSSEKDLEYRSGGDVDCASLELLFKHLGYQVTVFHDQSAEEMESAL  226
h Ich-1 long  .r......v...v..tg..e..f......hst.vt...l...d.h.lc..t.q..qek.  238

                                 +
c Ich-1 long  ERFSKLPDHQDVDSCIVALLSHGVEGGVYGTDGKLLQLQEAFRLFDNANCPNLQNKPKMF  286
h Ich-1 long  qn.aq..a.rvt.............ai..v.........v.q.......s........  298

                  p20  <--                  --> p10
c Ich-1 long  FIQACRGDETDRGVDQRDGKERSDSPGCEESDANKEENLKLRLPTRSDMICGYACLKGTA  346
h Ich-1 long  ..............q...nhag.........g..klp.m..................  358

c Ich-1 long  AMRNTKRGSWYIEALTTVFAEDSRDTHVADMLVKVNRQIKQREGYAPGTEFHRCKEMSEY  406
h Ich-1 long  ..............aq..s.rac.m.........al..d..................  418

c Ich-1 long  CSTLCRDLYLFPGYVPGK*                                          424
h Ich-1 long  ......h......hp.*                                            434
```

Fig. 3. Alignment of deduced aa sequences from the chicken ICH-1_L and human ICH-1_L (Wang et al., 1994) proteins. Identical residues are in upper case letters, while variations are in lower case letters. – represents aa absent in the chicken ICH-1_L sequence. The QACRG active catalytic site is designated by bold type, while the His residue marked by + has also been implicated in catalysis. Double underlined regions represent regions which form the Asp pocket in ICE (Wilson et al., 1994), and are highly conserved within the family of caspases. * indicates the stop codon.

detected under the conditions described (Fig. 4). Levels of Ich-1_L mRNA were determined not to change significantly during follicle development, and this finding was confirmed by Northern blot analysis (Fig. 5). There was, however, a significant decrease (by 36%) in Ich-1_L mRNA levels in atretic, versus normal, prehierarchal follicles ($p=0.017$) which is consistent with the overall decline in the level of several other unrelated transcripts during the progression of follicle atresia (You et al., 1996).

3. Conclusions

(1) The deduced amino acid sequence of Ich-1_L shows 70.8% identity with the human Ich-1_L sequence, and includes conserved catalytic and Asp binding regions identified in other caspases.

(2) Only the Ich-1_L transcript (the putative 'apoptosis-inducing' form) was detected in hen ovarian tissues suggesting the relative importance of this alternatively-spliced form. However, levels of Ich-1_L mRNA are not altered within either granulosa or theca tissues during follicle development, and therefore increased expression of Ich-1_L mRNA is not directly associated with granulosa cell susceptibility to apoptotic cell death within prehierarchal follicle granulosa cells.

(3) It is proposed that similar to other caspases, the regulation of ICH-1 protease activity occurs following post-translational modification(s) by, as yet, unknown factors.

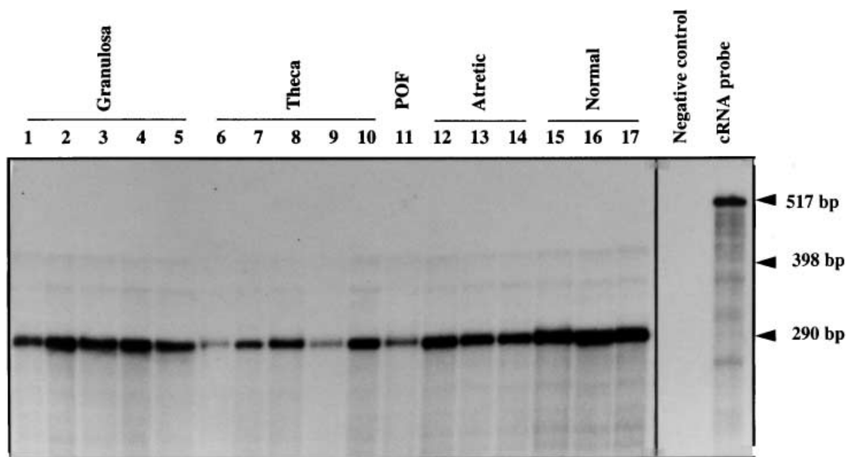

Fig. 4. RNase analysis for Ich-1. **Methods**: The assay was conducted using a ^{32}P-labeled 517-bp cRNA probe (containing 119 nt of vector sequence) that spans the Ich-1_S nt sequence (see Fig. 2B). Accordingly, signal corresponding to 290-bp represents the Ich-1_L transcript, while that corresponding to 398-bp would represent Ich-1_S. Total cellular RNA (15 μg) was hybridized with 6×10^5 cpm of RNA probe at 60°C overnight. The reaction mixture was incubated with 5 μg RNase A/ml and 100 units RNase T1/ml for 30 min at 37°C. Protected fragments were precipitated, resuspended in gel loading buffer, and electrophoresed on a 8 M urea-6% polyacrylamide gel and visualized by autoradiography (Johnson et al., 1996). Lanes: 1 and 6, tissues from the largest (F1) preovulatory follicle; 2 and 7, second largest (F2) follicle; 3 and 8, 9 to 12-mm diameter follicles; 4 and 9, 6 to 8-mm diameter follicles; 5 and 10, 3 to 5-mm diameter follicles; 11, tissue from postovulatory follicle; 12–14, tissue from atretic follicles; 15–17, tissue from whole, morphologically normal 3 to 5-mm diameter follicles; 18, negative control (yeast transfer RNA); 19, chicken Ich-1 probe.

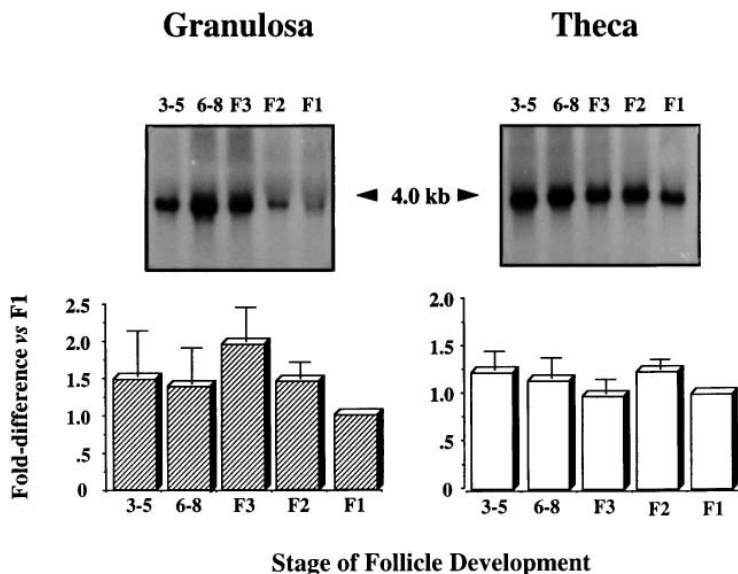

Fig. 5. Northern blot analysis of Ich-1_L mRNA in hen granulosa and theca tissue during ovarian follicle development. Representative blots from granulosa cells (top left panel) and theca tissue (top right panel), together with a summary of densitometric scanning data (bottom panels). **Methods**: Fifteen μg of total cellular RNA was hybridized with a chicken Ich-1 ^{32}P-labeled cRNA probe at 65°C overnight and subsequently washed under highly stringent conditions at 65°C. Data are expressed as fold-difference compared to the largest preovulatory (F1) follicle ±SEM, and represent four to five replicate experiments. The relative extent of hybridization was evaluated by densitometry (UltraScan XL laser densitometer; Pharmacia LKB, Piscataway, NJ), and equal loading of RNA onto gels was verified following the evaluation of ethidium bromide-stained 28S and 18S bands of ribosomal RNA.

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References

Alnemri, E.S., Livingston, D.J., Nicholson, D.W., Salvesen, G., Thornberry, N.A., Wong, W.W., Yuan, J., 1996. Human ICE-CED-3 protease nomenclature. Cell 87, 171.

Fernandes-Alnemri, T., Litwack, G., Alnemri, E.S., 1994. CPP32, a novel human apoptotic protein with homology to *Caenorhabditis elegans* cell death protein Ced-3 and mammalian interleukin-1**b**-converting enzyme. J. Biol. Chem. 269, 30761–30764.

Fernandes-Alnemri, T., Litwack, G., Alnemri, E.S., 1995a. Mch2, a new member of the apoptotic Ced-3/Ice cysteine protease gene family. Cancer Res. 55, 2737–2742.

Fernandes-Alnemri, T., Takahashi, A., Armstrong, R., Krebs, J., Fritz, L., Tomaselli, K.J., Wang, L., Yu, Z., Croce, C.M., Salveson, G., Earnshaw, W.C., Litwack, G., Alnemri, E.S., 1995b. Mch3, a novel human apoptotic cysteine protease highly related to CPP32. Cancer Res. 55, 6045–6052.

Fernandes-Alnemri, T.F., Armstrong, R., Krebs, J., Fritz, L., Tomaselli, K.J., Wang, L., Yu, Z., Croce, C.M., Salvesen, G., Earnshaw, W.C., Litwack, G., Alnemri, E.S., 1996. In vitro activation of CPP32 and Mch3 by Mch4, a novel human apoptotic cysteine protease containing two FADD-like domains. Proc. Natl. Acad. Sci. USA 93, 7464–7469.

Flaws, J.A., Kugu, K., Trbovich, A.M., DeSanti, A., Tilly, K.I., Hirshfield, A.N., Tilly, J.L., 1995. Interleukin-1**b**-converting enzyme-related proteases (IRPs) and mammalian cell death: dissociation of IRP-induced oligonucleosomal endonuclease activity from morphological apoptosis in granulosa cells of the ovarian follicle. Endocrinology 136, 5042–5053.

Johnson, A.L., Bridgham, J.T., Witty, J.P., Tilly, J.L., 1996. Susceptibility of avian ovarian granulosa cells to apoptosis is dependent upon stage of follicle development and is related to endogenous levels of *bcl*-x_{long} gene expression. Endocrinology 137, 2059–2066.

Kamens, J., Paskind, P., Hugunin, M., Talanian, R.V., Allen, H., Banach, D., Bump, N., Hackett, M., Johnston, C.G., Li, P., Mankovich, J.A., Yerranova, M., Ghayur, T. (1995) Identification and characterization of ICH-2, a novel member of the interleukin-1**b**-converting enzyme family of cysteine proteases. J. Biol. Chem. 15250–15256.

Kumar, S., 1995. ICE-like proteases in apoptosis. Trends Biochem. Sci. 20, 198–202.

Miura, M., Zhu, H. Fotello, R., Hartwieg, E.A., Yuan, J., 1993. Induction of apoptosis in fibroblasts by IL-1**b**-converting enzyme, a mammalian homolog of the C.elegans cell death gene ced–3. Cell 75, 653–660.

Sanger, F., Nicklen, S., Coulson, A.R., 1977. DNA sequencing with chain-terminating inhibitors. Proc. Natl. Acad. Sci. USA 74, 5463–5467.

Srinivasula, S.M., Fernandes-Alnemri, T., Zangrilli, J., Robertson, N., Armstrong, R.C., Wang, L., Trapani, J.A., Tomselli, K.J., Litwack, G., Alnemri, E.S., 1996. The Ced-3/interleukin 1**b** converting enzyme homolog Mch6 and the lamin-cleaving enzyme Mch2a are substrates for the apoptotic mediator CPP32. J. Biol. Chem. 271, 27099–27106.

Thornberry, N.A., Bull, H.G., Calaycay, J.R., Chapman, K.T., Howard, A.D., Kostura, M.J., Miller, D.K., Molineaux, S.M., Weidner, J.R., Aunins, J. et al., 1992. A novel heterodimeric cysteine protease is required for interleukin-1**b** processing in monocytes. Nature 356, 768–774.

Tilly, J.L., Kowalski, K.I., Johnson, A.L., Hsueh, A.J.W., 1991. Involvement of apoptosis in ovarian follicular atresia and post-ovulatory regression. Endocrinology 129, 2799–2801.

Wang, L., Miura, M., Bergeron, L., Zhu, H., Yuan, J., 1994. *Ich-1*, an *Ice*/*ced*-3-related gene, encodes both positive and negative regulators of programmed cell death. Cell 78, 739–750.

Wilson, K.P., Black, J.F., Thomson, J.A., Kim, E.E., Griffith, J.P., Navia, M.A., Murcko, M.A., Chambers, S.P., Aldape, R.A., Raybuck, S.A., Livingston, D.J., 1994. Structure and mechanism of interleukin-1**b** converting enzyme. Nature 370, 270–275.

You, S., Bridgham, J.T., Foster, D.N., Johnson, A.L., 1996. Characterization of the chicken follicle-stimulating hormone receptor (cFSH-R) complementary deoxyribonucleic acid, and expression of cFSH-R messenger ribonucleic acid in the ovary. Biol. Reprod. 55, 1055–1062.

Yuan, J., Shaham, S., Ledoux, S., Ellis, H.M., Horvitz, H.R., 1993. The C. elegans cell death gene ced-3 encodes a protein similar to mammalian interleukin-1**b**-converting enzyme. Cell 75, 641–652.