BIOLOGY OF REPRODUCTION **70,** 972–979 (2004)
Published online before print 26 November 2003.
DOI 10.1095/biolreprod.103.023614

Alternatively Spliced Variants of *Gallus gallus* TNFRSF23 Are Expressed in the Ovary and Differentially Regulated by Cell Signaling Pathways[1]

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ABSTRACT

As a result of searching recently available chicken (ch) expressed sequence tag databases, a new Tumor Necrosis Factor Receptor Super Family (TNFRSF) member with similarity to the murine (m) TNFRSF23 decoy receptor (DcR) has been identified. However, by comparison with the mTNFRSF23, there exist at least two splice variants of chTNFRSF23, one of which includes an intracellular death domain (TNFRSF23.v1) characteristic of death receptors, and the other with a truncated cytoplasmic domain of a DcR (named TNFRSF23.v2). These two splice variants of chTNFRSF23 display differential patterns of mRNA expression across various hen tissues, with the highest levels observed within reproductive tissues. More specifically, TNFRSF23.v1 is most highly expressed in preovulatory follicle granulosa cells in the ovary, whereas TNFRSF23.v2 mRNA is found at highest levels in ovarian stromal tissue. Primary culture experiments with granulosa cells determined that expression of TNFRSF23.v1 mRNA was decreased by protein kinase A signaling and enhanced by transforming growth factor (TGF) α treatment. Interestingly, TGFβ1 and signaling via protein kinase C also enhanced levels of TNFRSF23.v1 expression but only in undifferentiated granulosa cells from prehierarchal follicles. Based on patterns of mRNA expression and its endocrine/paracrine regulation, we predict that ovarian chTNFRSF23 represents a modulator of granulosa cell survival and/or differentiation. Finally, the characterization of these receptor variants is of considerable interest from an evolutionary perspective in that they provide additional evidence to support a continuing divergence of TNFRSF members throughout vertebrate evolution.

apoptosis, follicle, follicular development, granulosa cells, ovary

INTRODUCTION

Prior to or around the time of birth/hatch, germ cells within the vertebrate ovary become organized into primordial follicles surrounded by a nurturing granulosa cell layer. Subsequently, these primordial follicles will succumb to one of two fates during the reproductive life span of the female. Follicles will either die by atresia, a process often initiated within the granulosa cell layer [1, 2], or alternatively will continue to grow, undergo differentiation, and eventually ovulate. The distinct mechanisms and signaling pathways involved in determining the fate of ovarian follicles at each stage of development have been the subject of recent studies (e.g., [3, 4]).

In the hen ovary, survival of granulosa cells from growing follicles is dependent on the support of a variety of factors, including gonadotropins and vasoactive intestinal peptide, which predominantly signal via adenylyl cyclase/cyclic-AMP [2, 5]. In addition, various growth factors, including insulin-like growth factor-I (IGF-I), acting primarily through phosphoinositide 3 kinase/Akt, and epidermal growth factor, and transforming growth factor (TGF) α signaling through the mitogen-activated protein kinase/extracellularly regulated kinase (MAPK/Erk) pathway, are known to be important regulators of hen granulosa cell survival and differentiation [6, 7]. Such endocrine/paracrine factors and associated cell signaling pathways promote expression of cellular antiapoptotic proteins that are correlated with decreased susceptibility to granulosa cell apoptosis and increased resistance to follicle atresia [8, 9].

Death receptors (DRs) belonging to the tumor necrosis factor (TNF) receptor superfamily (TNFRSF) have previously been implicated in initiating ovarian follicle atresia in a wide range of vertebrate species (mouse, [10]; bovine, [11]; rat, [12]; chicken, [13]). Such receptors contain intracellular death domains (DD) capable of mediating interactions with internal signaling mechanisms that promote apoptotic cell death.

However, in vitro studies designed to study granulosa cell death through binding of death receptors with their cognate ligands have often proven effective only following cotreatment of cells with cycloheximide, interferon-γ, or combinations of ligands such as Fas ligand (FasL) and TNFα (e.g., [10, 14, 15]). One interpretation for resistance to DR-mediated apoptosis is that granulosa cells produce inhibitory proteins such as FLIP [16], Bcl-X_{long} [2], and X-linked inhibitor of apoptosis protein [17], which can interfere with initiator caspase activation/activity. A second possibility is that DR-initiated apoptosis may be prevented at an extracellular site by the presence of decoy receptors (DcRs), which might prevent ligand binding to DRs. DcRs may also preclude DR signaling by interfering with DR homotrimerization, which prevents interactions with intracellular adaptor proteins and ultimately blocks initiation of the death-inducing cascade of signaling events [18].

Several TNFRSF DcRs that may prevent DR signaling have previously been identified in various vertebrate species. Specifically, soluble forms of the Fas receptor, which exist in both mammals [19] and birds [13], can act in a fashion similar to a DcR, while human DcR3 prevents signaling by FasL, LIGHT, and TL1A [20–22]. In addition, osteoprotegerin (OPG) prevents signaling by receptor acti-

[1]This work was supported by the National Science Foundation (IBN-0131185) and the Department of Defense (DAMD17-03-1-0206) to A.L.J.
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Received: 26 September 2003.
First decision: 15 October 2003.
Accepted: 24 November 2003.
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ISSN: 0006-3363. http://www.biolreprod.org

vator of nuclear factor kappa B ligand and TNF-related apoptosis-inducing ligand (TRAIL) [23, 24]. Two human decoy receptors for TRAIL (DcR1 and DcR2) were independently isolated by several labs using database search profiles to identify members of the TNFR family [25–29]. Mice apparently do not have orthologous genes for DcR1 and DcR2 decoy receptors, yet recent reports have described a cluster of up to three similar TNFR decoy-like genes (mTNFRH1 [also known as TNFRSF23], -H2, and -H3) encoded on mouse chromosome 7, two of which bind to TRAIL [30, 31]. None of these genes appears in the predicted location on chromosome 11 of the human genome.

Significantly, investigations directed toward identifying the physiological role of DRs in any cell system are further complicated by the recognition that DR activation may actually play a role in regulation of cell survival, development, and/or differentiation by signaling through alternative pathways. Moreover, in multiple tissues, including granulosa cells, TNFα is known to have pleiotropic effects that can be mediated by TNF receptor-associated protein (TRAF) family members [32], Jun kinase signaling [33], or nuclear factor-κB activation [16], rather than the death-inducing signaling complex formed by adaptor proteins and caspases [34].

In the present studies, a new chicken receptor was identified with similarity to murine (m) TNFRSF23 as a result of searching recently available chicken expressed sequence tag (EST) databases. Interestingly, during the characterization of chicken (ch) TNFRSF23, splice variants were identified that are predicted to encode either a cytoplasmic DD or a truncated domain characteristic of a DcR. Furthermore, the two splice variants of chTNFRSF23 display differential patterns of mRNA expression across various hen tissues, with highest levels of expression occurring within the ovary. Finally, expression of chTNFRSF23 mRNA is regulated by several signaling pathways previously determined to regulate hen granulosa cell survival and differentiation.

MATERIALS AND METHODS

Animals and Tissue Collection

Single-comb white Leghorn hens (Creighton Bros., Warsaw, IN), 25–35 wk of age and laying regular sequences of at least 5–6 eggs, were used in all studies described. Birds were housed individually in laying batteries, were provided with free access to feed (Purina Layeena 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 by cervical dislocation approximately 16–18 h prior to a midsequence ovulation. 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.

Tissue samples were collected from the chicken ovarian stroma, postovulatory follicle, brain, spleen, bone marrow, oviduct, kidney, and heart for isolation of total RNA. Ovarian follicle tissues included granulosa and theca from 3- to 5-mm-, 6- to 8-mm-, and 9- to 12-mm-diameter prehierarchal follicles, and the largest (F1), second largest (F2), and third largest (F3) preovulatory follicles. Morphologically normal and atretic follicles (3- to 8-mm diameter) were processed without separating the granulosa and theca layers, as previously described [35]. Samples were collected and processed immediately or frozen at $-70°C$.

Cloning of TNFRSF23 Splice Variants

The cysteine-rich domain of a putative novel chicken receptor was isolated based on sequence information available from the UMIST database (http://www.chick.umist.ac.uk). This sequence was found specifically within an ovarian library in two separate clones (#603777144F1 and #603154403F1) that were tentatively identified as most similar to two different *Danio* death receptors. Primers were designed to amplify a 448-base pair (bp) product (forward primer 5′-GCA CTG AAG GAA GAG ATT ACA CCG-3′, and reverse primer 5′-AGA CAA CCT GAC GAA ACC TAT TGG-3′). Gene-specific primers (GSP) were also designed to generate 3′ Rapid Amplification of cDNA Ends (RACE) PCR products. These nested primers (GSP1 5′-AAG GGG AAA ATG TGC CTC TTG C-3′, and GSP2 5′-TGA GTG CCA GTG CCA CCA GGG G-3′) were used to amplify products from a poly A^+ mRNA template made from a combination of ovarian stroma, F1, and 6- to 8-mm follicle granulosa tissues. The RACE reactions were performed in duplicate using either the Marathon cDNA amplification kit (Clontech, Palo Alto, CA) or the GeneRacer kit (Invitrogen Life Technologies, Carlsbad, CA). These RACE reactions produced two different 3′ PCR products resulting in two predicted coding regions. A primer located on the putative start codon, 5′-TGG GTC GGG CGC CGC GAT G-3′, was paired with reverse primers located near the end of two separate coding regions, either 5′-GAG CCA CCT ATA GAC CGG GC-3′, or 5′-CCC TGG TAT CTA TAT CCC ATA GC-3′. These final PCR reactions were designed to specifically amplify two alternatively spliced coding regions and were performed according to manufacturer's recommendations using Platinum *Taq* DNA Polymerase High Fidelity (Invitrogen).

All PCR products described above were cloned into TA cloning vector (Invitrogen), transformed into INVαF′ cells (Invitrogen), and subsequently sequenced as previously described [36]. Sequences for the novel death receptor and alternatively spliced decoy receptor were submitted to a TNF nomenclature group (http://www.gene.ucl.ac.uk/nomenclature/genefamily/tnfinfo.html), and were assigned the names TNFRSF23 variant (v) 1 and v2, respectively.

Granulosa Cell Cultures

Granulosa cells collected from preovulatory (F1, F2, plus F3) follicles and undifferentiated prehierarchal (6- to 8-mm) follicles were collected, combined within their respective group, and dispersed in 0.3% collagenase (type 2; Worthington, Freehold, NJ) prior to treatment. An aliquot of each group was frozen immediately at $-70°C$ (T0 controls), while the remaining cells were plated at 40°C at a density of approximately 10^6/well in 2 ml Dulbecco modified Eagle medium (DMEM) containing 2.5% fetal bovine serum (Gibco-BRL, Gaithersburg, MD).

Experiments were designed to evaluate effects of previously identified granulosa cell signaling pathways on levels of chTNFRSF23 mRNA expression using the cysteine-rich domain (C-R) probe. Results were confirmed using the v1-DD probe. The cellular pathways investigated included protein kinase A (using 1 mM 8br-AMP; Sigma Chemical Co, St. Louis, MO [37]), MAPK/Erk (50 ng/ml TGFα; PeproTech, Rocky Hill, NJ [6]), Smad signaling (50 ng/ml TGFβ1; PeproTech; unpublished results), protein kinase B/AKT (50 ng/ml insulin-like growth factor (IGF)-I; PeproTech [6]), and protein kinase C (100 ng/ml PMA; Sigma Chemical Co. [38]). Preovulatory granulosa cells were cultured for 20 h in the absence or presence of each agonist. Media plus cells were collected from each well, and cells were pelleted by centrifugation at $200 \times g$ for 5 min. Cells were frozen at $-70°C$ until RNA was prepared for Northern blot analysis. A similar set of experiments was conducted with prehierarchal follicle granulosa cells to compare differences in response relative to stage of follicle differentiation.

Northern Blot Analysis

The 448-bp C-R domain of chTNFRSF23 was used as a probe template that would hybridize with all potential splice variants. Additionally, a PCR product was made corresponding to the unique C-terminal region of the alternatively spliced receptor (chTNFRSF23.v1) using primers forward 5′-GGC ACT ATC CCA CAG AAG AAC TTG-3′ and reverse 5′-TCC CCC ATT TTG TTT TTC CAC-3′ to generate a 380-bp product to be used as a probe template, designated v1-DD because this region encodes a putative death domain. Finally, a probe template was designed to hybridize specifically with the 3′ untranslated region of the decoy-like v2 transcript. Primers for this 631-bp v2-DcR probe were, forward, 5′-GAA TCA TCC AAT AGG TTT TGT CAG G-3′ and, reverse, 5′-CAA GGT CAG TAG CCA CAT AAC ACC-3′.

Templates were labeled with random primers using the Megaprime DNA Labeling System (Amersham, Piscataway, NJ) and [^{32}P]-dCTP (3000 Ci/mmol; Amersham). Prehybridization and hybridization of Northern blots were performed as previously described [36]. Blots were subsequently exposed to phosphorimaging plates and images were captured using the Storm 840 PhosphorImager system equipped with the Image-

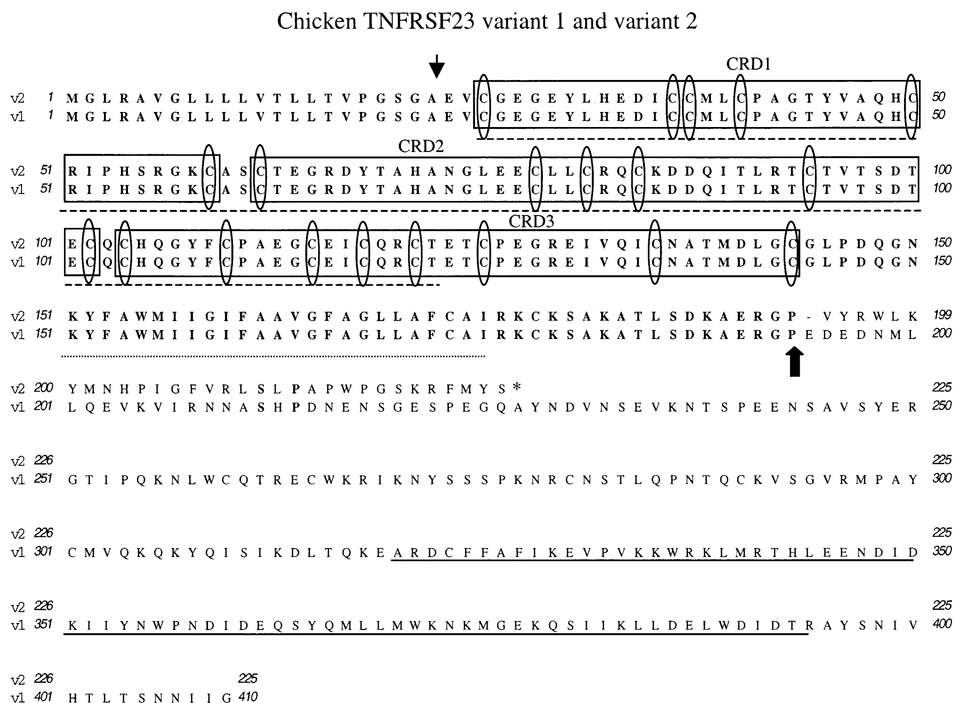

FIG. 1. Deduced amino acid sequences of chicken TNFRSF23 variant 1 (v1; accession #AY251408), and variant 2 (v2; accession #AY251409). Heavy arrow indicates position at which the two splice variants diverge. Thinner arrow indicates the putative N-terminal signal peptide cleavage site. Dashed underline is the conserved TNFR domain, according to GenBank conserved domain database from RPS-BLAST. Dotted underline represents the predicted transmembrane domain. Solid underline represents the conserved (GenBank) death domain region of v1. Boxes represent three conserved domains (CRD). Conserved cysteine residues associated with the ligand-binding domain [31] are circled.

Quant data reduction system (Molecular Dynamics, Sunnyvale, CA). Blots were subsequently reprobed with chicken 18S ribosomal RNA to standardize for loading of RNA [13].

Data Analysis

For purposes of quantitation, replicate data within an experiment were normalized to 18S ribosomal RNA and expressed as fold difference relative to a common reference sample (usually T0 control, standardized to 1.0). The data were subsequently compared by one-way ANOVA (without including the reference sample) (Statview; Abacus Concepts, Inc., Berkeley, CA). Further analysis of T0 versus T20 h samples was conducted by paired *t*-test using original, normalized data. Data from normal versus atretic follicles were compared using an unpaired *t*-test.

RESULTS

Chicken TNFRSF23 Characterization and Sequence Homology

A cDNA fragment was cloned that corresponded to sequences initially obtained through the UMIST database (#603777144F1 and #603154403F1) and that confirmed a C-R domain with homology to the TRAIL receptor subgroup of the TNFRSF. However, 3′-RACE PCR reactions, utilized to amplify the full coding region, produced two alternatively spliced variants. One contained a stop codon that truncates the predicted coding region at amino acid (aa) 225, producing a decoy-like receptor sequence that does not encode a DD. The predicted amino acid sequence of this DcR is 40% identical and 60% similar to murine TNFRSF23. The other RACE product began with the identical 5′ sequence, but varied beginning at nucleic acid 640. This mRNA splice variant results in a deduced, novel coding region that includes a conserved death domain.

Of interest, repeatable allelic differences were found in both variants. The two alleles were either G, C, C, A, C, A, T or A, T, T, G, T, T, C at nucleic acid positions 122, 154, 156, 159, 165, 641, 1090, respectively. The last two allelic differences are not present in chTNFRSF23.v2 as a result of alternative splicing. These nucleic acid differences lead to three amino acid substitutions at positions 21 (glysine to apartic acid), 32 (histidine to tyrosine), and 194 (glutamic acid to valine). Position 21 is within the predicted signal peptide region and does not influence the cleavage site, as determined using the signal peptide search program available at www.cbs.dtu.dk/services/SignalP/. Position 32 falls within the conserved C-R domain; the histidine from one allele is identical to *Danio* OTR, whereas the allele containing tyrosine at this position is identical to the sequence found in the conserved consensus TNFR domain (GenBank, RPS-BLAST). Allelic differences at position 194 only occur in chTNFRSF23.v1, and the amino acid substitution does not fall within a predicted functional domain.

In order to confirm these novel splice variants of chTNFRSF23, primers were designed to anneal at the putative start codon and end with either a decoy-specific or death domain-specific 3′-end primer. PCR amplification resulted in the predicted full-length sequences. The new decoy receptor contained a conserved TNFR domain (aa 25–122), as predicted using a reverse-position-specific BLAST (RPS-BLAST) of the conserved domain database, and was followed by a transmembrane (TM) domain region (aa 151–175, based on hydrophobicity plots), and was designated TNFRSF23.v2 (GenBank accession #AY251409). The longer alternatively spliced variant contained the TNFR and TM domains and also had a conserved DD represented by aa 320–388 as predicted by RPS-BLAST (Fig. 1). The domain architecture places this protein within the death receptor family, and it has been assigned the name TNFRSF23.v1 (accession #AY251409).

TNFRSF23 mRNA Expression

Northern blots hybridized with the C-R domain chTNFRSF23 probe demonstrated an ovarian tissue-enhanced expression pattern with highest levels of mRNA expression in ovarian stroma and granulosa cells (Fig. 2, upper panel), and transcript sizes of approximately 3.3 kb, 2.4 kb, 2.1 kb, and 1.2 kb. Based on common 5′ nucleic acid sequences, the C-R probe was predicted to hybridize with both

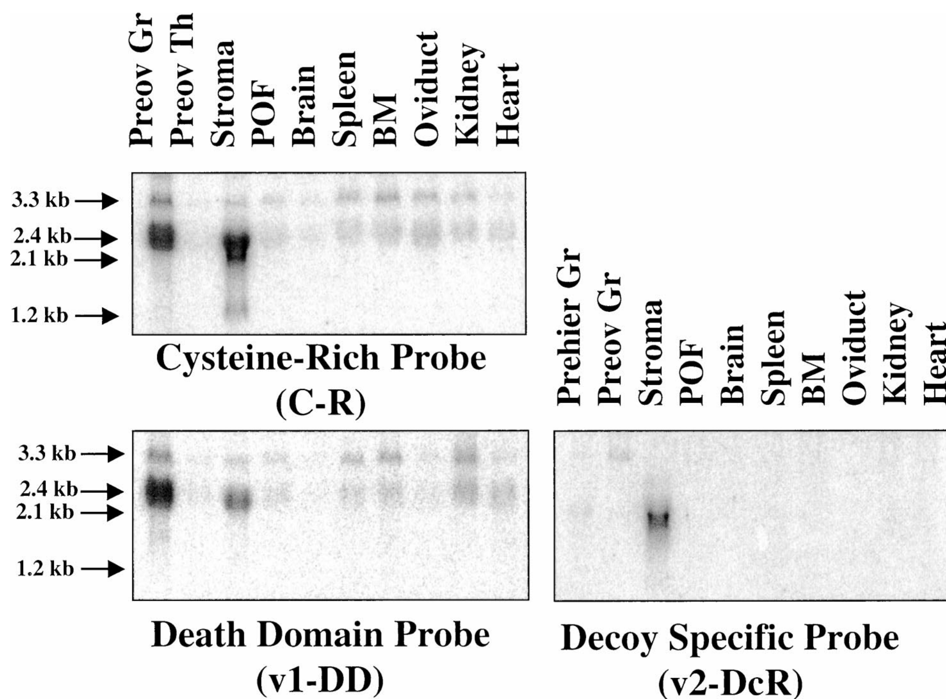

FIG. 2. Northern blot analysis of tissue distribution patterns for chTNFRSF23 mRNA revealed using the cysteine-rich (C-R) domain probe (upper panel) that is predicted to hybridize with both variants. This pattern can be compared with those generated using the death domain-specific (v1-DD) (lower left panel) and decoy-specific (v2-DcR) probes (lower right panel) that recognize the chTNFRSF23 splice variant 1, and variant 2, respectively. Note the absence of 2.1- and 1.2-kb transcripts following hybridization using the v1-DD probe and the absence of the 2.4-kb transcript following hybridization using the v2-DcR probe. Preov Gr, Granulosa from preovulatory follicles; Preov Th, theca tissue from preovulatory follicles; Prehier Gr, prehierarchal follicle granulosa; POF, postovulatory follicle; BM, bone marrow.

TNFRSF23.v1 and -.v2. The primary transcript of 2.4 kb, as well as the larger 3.3-kb transcript, were also recognized by the TNFRSF23.v1-death domain (v1-DD)-specific probe (Fig. 2, lower left panel), indicating that at least a portion of the mRNA found in these bands included the DD-containing splice variant. Hybridization with the probe specific for the 3′-untranslated region of the DcR-like sequence (v2-DcR) revealed a low level 3.3-kb transcript in preovulatory granulosa, and a 2.1-kb transcript in stromal tissue, which was also barely detectable in prehierarchal granulosa (Fig. 2, lower right panel). While, various transcripts of ch-TNFRSF23 mRNA were found to be expressed in the granulosa cell layer, they were virtually undetectable in theca layers at any stage of development as revealed by all three probes (data not shown; and Fig. 2, upper panel).

Both the C-R and v1-DD probes demonstrated differential expression of the 2.4-kb transcript during development such that lowest levels were detected in 6- to 8- and 9- to 12-mm follicle granulosa, and highest levels were expressed in granulosa from the three largest preovulatory (F1, F2, and F3) follicles (Fig. 3). Interestingly, the 2.1-kb transcript was apparent in prehierarchal (3- to 5- and 6- to 8-mm) follicle granulosa cells using the decoy-specific probe (v2-DcR), whereas the 3.3-kb transcript appeared at highest levels in the granulosa of preovulatory follicles. The 9- to 12-mm follicles, representing those follicles that have been most recently selected into the preovulatory hierarchy, expressed relatively low levels of the v1-DD transcript and expressed almost nondetectable levels of the 3.3- and 2.1-kb transcripts when hybridized with the v2-DcR specific probe (Fig. 3).

Northern blots of total RNA collected from whole, healthy prehierarchal follicles (normal) did not show a difference in expression levels of the predominant 2.4-kb transcript compared with spontaneously atretic follicles using either the C-R or v1-DD (data not shown).

Regulation of TNFRSF23 Expression In Vitro

Granulosa cells from preovulatory follicles were cultured in the presence of various agonists known to promote activation of several previously characterized granulosa cell signaling pathways. Expression of the 2.4-kb transcript was measured using the C-R probe. High levels of transcript in freshly collected (T0) granulosa cells from preovulatory follicles were decreased by 63% in control cells cultured for 20 h. Treatment with 8br-cAMP further reduced levels of the 2.4-kb transcript, while TGFα significantly induced expression by 1.9-fold above control cultured cells. Treatments with TGFβ1, IGF-I, and PMA had no significant effect on expression of this transcript compared with control cultured cells (Fig. 4). Similar patterns were observed when the same RNA samples were analyzed using the v1-DD probe (Fig. 4; stippled bars).

Comparable culture experiments were accomplished with granulosa cells collected from prehierarchal (6- to 8-mm) follicles. By comparison with granulosa cells from preovulatory follicles, the initially low levels of transcript found in prehierarchal follicle granulosa cells using the C-R probe increased 1.9-fold relative to freshly collected (T0) cells after 20 h of culture ($PP < 0.05$). This increase was attenuated by treatment with 8br-cAMP ($P = 0.016$ by post hoc paired *t*-test). Treatments with TGFα, TGFβ1, and PMA all significantly increased levels of the 2.4-kb transcript relative to control cultured cells, while IGF-I treatment had no effect (Fig. 5).

DISCUSSION

In the present studies, a novel chicken DD-containing receptor (chTNFRSF23.v1), together with a splice variant proposed to represent a decoy receptor (chTNFRSF23v.2), were identified. There is also evidence of additional transcriptional variants that were not pursued in the present study. Combined with the previously characterized chFas (TNFRSF6) and chTNFR1 (TNFRSF1A) [13], TvB (a TNFRSF10B-like DR [39, 40]), chDR6 (TNFRSF21 [41]), and ch p75^{NTR} (TNFRSF16 [42]; unpublished data), ch-TNFRSF23.v1 now represents the sixth DR thus far known to be expressed by tissues from the hen ovary. Moreover, along with the recently reported ch osteoprotegerin

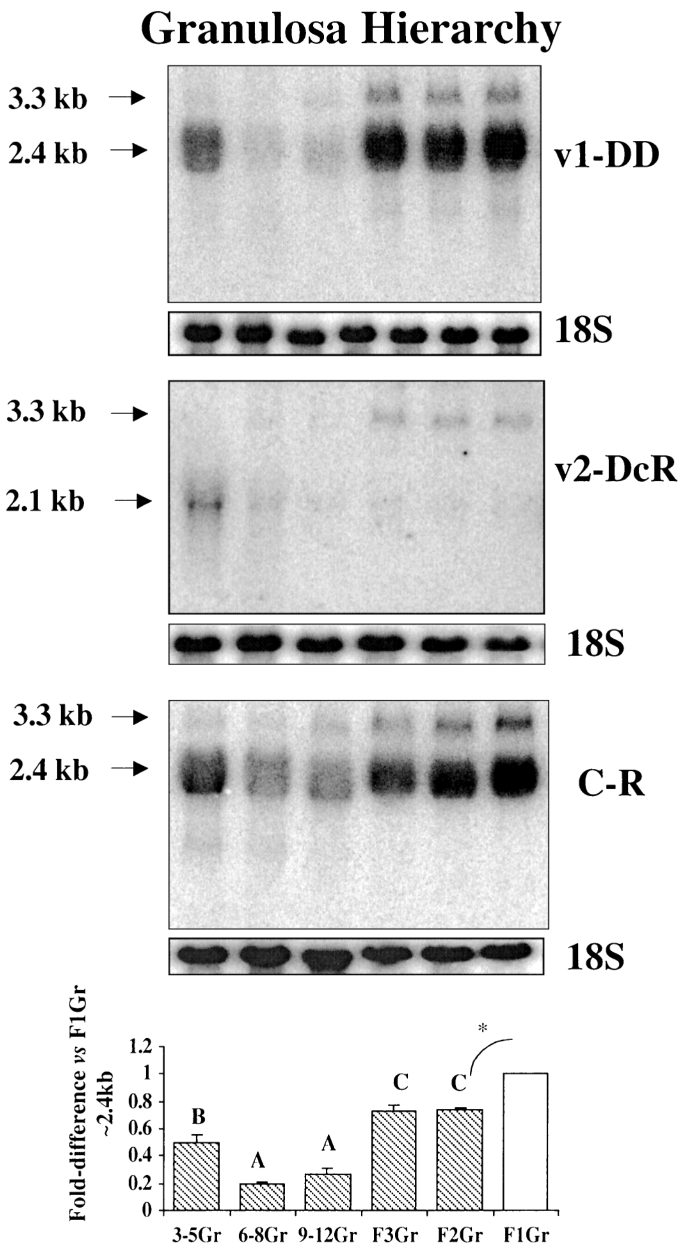

FIG. 3. Northern blot analysis of chTNFRSF23 cysteine-rich (C-R), death domain (v1-DD), and decoy (v2-DcR) mRNA expression in granulosa cells during follicle development. F1, F2, and F3 represent the largest, second largest, and third largest preovulatory follicle, respectively; 9–12, 6–8, and 3–5, refer to the diameter of the prehierarchal follicles (mm). Data from C-R probe 2.4-kb transcript mRNA expressed in granulosa cells is depicted on the graph. **A**, **B**, **C**) $P < 0.05$ relative to F1 granulosa by ANOVA; *$P < 0.05$ F1 versus F2 granulosa by paired *t*-test. Blots for each probe were replicated a minimum of four times using a pool of granulosa cells isolated from follicles collected from an individual hen for each experiment.

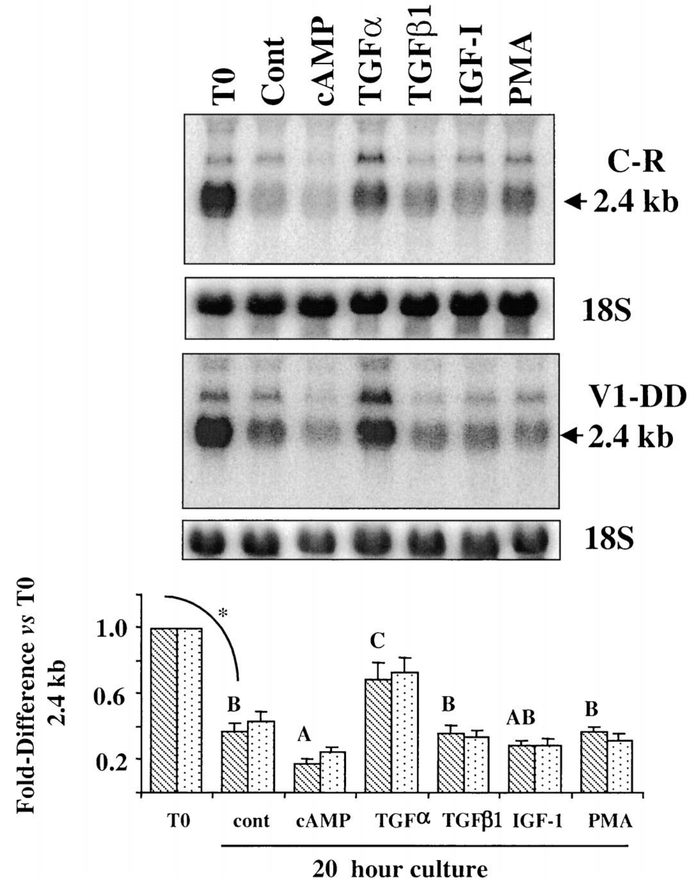

FIG. 4. Levels of chTNFRSF23 mRNA expression, measured following hybridization with the cysteine-rich (C-R; hatched bars) domain and death domain specific (v1-DD; stippled bars) probes, in granulosa cells from preovulatory follicles after 20 h of culture in the absence or presence of 8br-cAMP (cAMP; 1 mM), TGFα (50 ng/ml), TGFβ1 (50 ng/ml), IGF-I (50 ng/ml), and PMA (100 ng/ml). Only the 2.4-kb transcript was quantitated and standardized to 18S rRNA. Values are expressed as the fold-difference versus freshly collected (T0) control cells. Data represent the mean ± SEM from four replicate experiments. **A**, **B**, **C**) $P < 0.05$ relative to T20 control by ANOVA; *$P < 0.05$ versus T0 by paired *t*-test for C-R probe only, v1-DD data are shown for comparison.

(TNFRSF11B), chDcR3 (TNFRSF6B) [36], and soluble chFas [13], chTNFRSF23v.2 represents the fourth putative chicken DcR.

Implications of chTNFRSF23.v1 and -.v2 to Understanding TNFRSF Phylogeny

The chTNFRSF23.v1 DD-containing receptor and the related DcR-like chTNFRSF23.v2 appear to be most closely related to TRAIL receptors in other vertebrate species. Four known human TRAIL-specific receptors, two DRs and two DcRs, are all encoded at chromosomal position 8p21–22. The three murine decoy TNFRs also occur in a cluster on mouse chromosome 7, providing evidence for recent duplication events that support the gene duplication theory [31]. Although the murine and human TRAIL decoy receptors likely have evolved independently, our identification of chicken sequences that are similar to the murine genes is of considerable interest and may indicate that the murine decoys stem from an ancestral form rather than a more recent evolutionary event, as suggested by Schneider et al. [31]. These murine sequences have not been reported to include death domain-containing splice variants. Significantly, the putative death receptor-like splice variant identified here in an avian species, chTNFRSF23.v1, is most similar to three different receptors identified in fish (*Danio rerio* ovarian TNF receptor [OTR, GenBank accession #AAG24365]; *Danio rerio* death receptor [ZH-DR, #AAG21396]; and *Paralichthys olivaceus* tumor necrosis factor receptor-1 [#BAC65225]), all of which contain a death domain.

The existence of a death domain-containing splice var-

iant in the chicken TNFRSF23 with similarity to piscine receptors supports the scenario proposed by Collette et al. [43], that some vertebrate TNFRSF members diversified from DD-containing receptors, yielding receptors with distinct cytoplasmic tails. Furthermore, the data herein provide evidence to support a continuing divergence of TNFRSF members throughout vertebrate evolution. A phylogeny based on the death domain sequences of TNFRSF death receptors [44] suggests that the TRAIL receptor subgroup has continued to diverge within species. For instance, zebrafish have two receptors, OTR and ZH-DR, that are closely related, and humans have both DR4 and DR5, whereas mice express only DR5. The chTNFRSF23.v1 described herein together with the previously identified TVB receptor indicate that the chicken has at least two DRs within this proposed subgroup. This high level of divergence may be related to species-specific selective pressures on the adaptive immune system. The recent report of both chDcR3 and chOPG expressed in the hen [36] is relatively novel as well because humans are the only other species known thus far to express both of these genes. As suggested by Schneider et al. [31] and Liu et al. [45], decoy receptors will likely provide important additional evidence concerning the early evolution and divergence of the TNF receptor family.

Physiological Implications

The physiological function of TRAIL is not yet well understood, but recent evidence suggests that it plays a role in tumor surveillance by the immune system [46]. On the other hand, very little is known about the function of TRAIL and its receptors in nontransformed ovarian cells. Immunocytochemical analysis of TRAIL and its receptors has recently been described in the porcine ovary using antibodies directed against human antigens [47]. From these studies, it was concluded that TRAIL and its associated receptors, DR4 and DR5, are not regulated during follicle atresia, but that DcR1 is markedly reduced with atresia. However, these data require further confirmation because the genes for DR4 and DcR1 have yet to be identified in the pig and are believed to be absent in the mouse genome. In this study, neither of the transcripts for chTNFRSF23 was found to be regulated during atresia. By comparison, it is interesting to note that expression levels of four previously characterized chicken DRs (Fas, TNFR1, TVB, and DR6) were significantly increased in atretic, compared with normal, follicles [13, 40, 41].

The regulation of chTNFRSF23 mRNA expression during development, and in particular the high levels of chTNFRSF.v1 found in preovulatory follicles, would suggest that this receptor is more likely to be involved in developmental processes within the ovary rather than inducing atresia. Evidence for this proposed role is suggested by experiments with cultured granulosa cells. Such results provide evidence that chTNFRSF23.v1 transcription is downregulated by the protein kinase A signaling pathway in both prehierarchal and preovulatory granulosa. Protein kinase A is known to be a potent cell survival signal in hen granulosa cells [37] and a decrease in expression of a functional DR could represent one mechanism by which this pathway protects cells from death. On the other hand, transcript levels are increased in prehierarchal follicle granulosa by growth factors TGFα and TGFβ1 as well as the pharmacologic protein kinase C activator, PMA. It is noted that, under the conditions utilized, PMA does not induce Erk1/2 phosphorylation in hen granulosa cells from either preovulatory

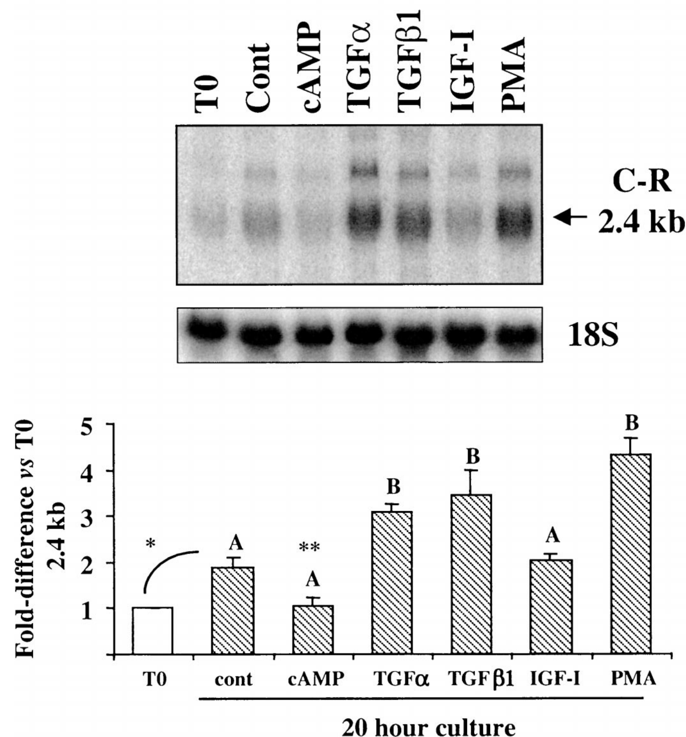

FIG. 5. Levels of chTNFRSF23 mRNA expression, measured following hybridization with the cysteine-rich (C-R) domain probe, in granulosa cells from prehierarchal follicles after 20 h of culture in the absence or presence of 8br-cAMP (cAMP; 1 mM), TGFα (50 ng/ml), TGFβ1 (50 ng/ml), IGF-I (50 ng/ml), and PMA (100 ng/ml). Only the 2.4-kb transcript was quantitated and standardized to 18S rRNA. Values are expressed as the fold-difference versus freshly collected (T0) control cells. Data represent the mean ± SEM from four replicate experiments. **A**, **B**) $P < 0.05$ relative to T20 control by ANOVA; $*P < 0.05$ versus T0 by paired *t*-test. $**P = 0.016$ versus T20 by post hoc paired *t*-test.

or prehierarchal follicles (data not shown). Interestingly, TGFα and protein kinase C signaling are linked to the inhibition of granulosa cell differentiation and steroidogenesis [48–50], while TGFβ1 has recently been determined to promote hen granulosa cell differentiation and steroid production (unpublished data). Nevertheless, regulation of chTNFRSF23.v1 in vivo has yet to be evaluated. Ultimately, the source and extent of cell signaling cross-talk may be a critical determinant of chTNFRSF23 expression.

In summary, there has been identification of this new DR, chTNFRSF23.v1, and the DcR-like splice variant, chTNFRSF.v2, each with homology to known TRAIL receptors. Given that we have recently identified chTRAIL (Genbank #AY057941) and found TRAIL mRNA expressed within the follicle theca layer (unpublished data), the combined results suggest a yet unknown role for TRAIL signaling in the normal hen ovary. In light of the enhanced expression within the ovary relative to other hen tissues and developmental stage-specific expression and regulation in granulosa cells, we predict that chTNFRSF23.v1 and -.v2 will be determined to have pleiotropic effects involved in the regulation of granulosa cell survival as well as development and differentiation. Clearly, additional studies regarding protein levels expressed as well as functions elicited following overexpression will contribute to a more complete understanding of this family member. Significantly, related DRs from the TNFR family, including the

p75^{NTR} [42] and ectodysplasin receptor [51], are also known to preferentially participate in the regulation of differentiation and development in a variety of cell types.

ACKNOWLEDGMENTS

We thank Ms. D. Woods for collaborative efforts and Mr. J. Martin for excellent technical assistance during the course of these studies.

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