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Interferon Stimulated Gene 15 Conjugates to Endometrial Cytosolic Proteins and Is Expressed at the Uterine-Placental Interface throughout Pregnancy in Sheep

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Interferon-stimulated gene 15 (ISG15) is a ubiquitin homolog expressed in uteri of ruminants in response to interferon (IFN)-τ and is also induced during pregnancy in the uteri of mice, pigs, humans, and baboons. This study examined expression of ISG15 and its conjugation to target proteins in the ovine uterus beyond the period of IFNτ secretion by the conceptus. Although steady-state levels of ISG15 mRNA decreased after d 25 of pregnancy, ISG15 persisted in endometrium through d 120. *In situ* hybridization and immunocytochemistry localized ISG15 across the entire uterine wall through d 25, after which expression was restricted to endometrial stroma along the maternal-placental interface. Western blots revealed ISG15 and ISG15-conjugated proteins in endometrium. Treatment of ovariectomized sheep with progesterone and IFNτ increased both free and conjugated ISG15. These results are the first to show *in vivo* regulation of ISG15 function (*i.e.* conjugation to target proteins) by a type I IFN in the uterus of any species and that ISG15 is expressed at contacts between the placenta and uterus when trophectoderm no longer produces IFNτ. Interestingly, mRNA for the type II IFNγ was present in the endometrial stromal compartment on d 15–50, which may stimulate the synthesis of ISG15 through later pregnancy. We hypothesize that ISG15 is not merely a consequence of an antiviral state induced by trophoblast IFNτ but represents a critical component of the microenvironment at the uterine-placental interface during the progressive events of conceptus development, implantation, and placentation in sheep and perhaps other mammalian species. (*Endocrinology* 146: 675–684, 2005)

DURING EARLY PREGNANCY in sheep, the conceptus (*i.e.* embryo/fetus and associated extraembryonic membranes) synthesizes and secretes interferon (IFN)τ, the signal for maternal recognition of pregnancy, from d 10 to d 21 (1). IFNτ acts on the luminal epithelium (LE) and superficial glandular epithelium (GE) to inhibit transcription of the estrogen receptor (ER)-α gene. This precludes ERα from stimulating an increase in oxytocin receptors, thereby preventing oxytocin from inducing release of luteolytic pulses of prostaglandin $F_{2\alpha}$ (2, 3). The result is maintenance of the corpus luteum, the source of progesterone required for successful pregnancy (1). In addition to its antiluteolytic effects, IFNτ increases expression of several IFN-stimulated genes (ISGs) in the progestinized ovine uterus, such as signal transducer and activator of transcription (Stat) 1 and Stat2 (4), major histocompatibility complex class I and β_2-microglobulin (5), IFN regulatory factor (IRF) 1 and IRF-9 (4), Mx (6), 2′,5′-oligoadenylate synthetase (7), and ISG15 (8, 9). Induction of ISGs in response to IFNτ is mediated by an intracellular signal transduction system involving type I IFN receptors, Stat1, Stat2, and IRFs (10–12). Most ISGs are not increased in the endometrial LE or superficial GE of early pregnant ewes. Indeed, the majority of ISGs increase in the stroma and deep GE (4, 5, 7, 8). Recently Choi *et al.* (4) provided evidence that IRF-2, a transcriptional repressor of ISGs, in the LE and superficial GE of pregnant ewes restricts expression of ISGs to the stroma and deep GE.

ISG15 was first identified in mouse Ehrlich ascites tumor cells in which expression was regulated by type I IFNs (13). Knight and co-workers later purified and characterized the 15-kDa protein from IFN-treated human Daudi cells (14, 15). In examining ubiquitin expression in virally infected human A549 cells, Haas *et al.* (16) noted an IFN-induced 15-kDa protein that cross-reacted with ubiquitin-specific rabbit polyclonal antibody (ubiquitin cross-reactive protein). The polypeptide was named ISG15 and subsequently found to be identical with the protein discovered by Knight's laboratory. Austin *et al.* (17) linked ISG15 to early pregnancy when they identified a 16-kDa protein secreted by bovine endometrium in response to conceptus-derived IFNτ. ISG15 is induced by the type I IFNα (14), cross-reacts with ubiquitin antisera (16), and contains the C terminus Leu-Arg-Gly-Gly amino acid

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Abbreviations: E1, Ubiquitin-activating enzyme; E2, carrier protein; ER, estrogen receptor; GE, glandular epithelium; IFN, interferon; IRF, IFN regulatory factor; ISG, IFN-stimulated gene; LE, luminal epithelium; Stat, signal transducer and activator of transcription; TBST, Tris-buffered saline and 0.1% Tween 20.

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sequence common to ubiquitin that is essential for its conjugation to intracellular proteins (18). Conjugation of proteins to ubiquitin either targets proteins for rapid degradation in the proteasome or stabilizes proteins for long-term modification (19). ISG15 is up-regulated in the uterine endometrium of pregnant sheep and cows in response to trophoblast IFNτ (8, 9, 17, 20). Johnson *et al.* (21) found that bovine ISG15 conjugates to intracellular proteins during pregnancy, suggesting that ISG15 may be involved in the regulation of proteins critical to the establishment and maintenance of pregnancy.

ISG15 is also induced or increased in the pregnant endometrium of species that do not use IFNs for maternal recognition of pregnancy. ISG15 is localized to decidual cells in the endometria of pregnant humans and baboons (22). ISG15 is also present in the decidualized stroma and surrounding GE of pregnant mice but not in pseudopregnant mice (23), indicating that the conceptus may be involved in the expression of this protein. Similar to rodents, ISG15 is induced in the endometrial stroma of pigs during the periimplantation period, but ISGs do not increase in the endometrium during porcine pseudopregnancy (24). Collectively, these studies support the hypothesis that endometrial ISG15 is not merely a consequence of an antiviral state induced by high levels of IFNτ in the lumen of ruminants during pregnancy recognition but rather is a universally critical uterine response to the progressive processes of conceptus development, implantation, and placentation. The objective of this study was to examine ISG15 and its conjugation to target proteins in the ovine uterus during pregnancy after the period of IFNτ secretion by the conceptus. Results suggest that IFNτ induction of ISG15 also leads to the formation of ISG15 conjugates and that other factor(s), possibly the type II IFNγ, regulate endometrial ISG15 expression at the uterine-placental interface after the period of conceptus IFNτ secretion.

Materials and Methods

Animals

All experimental and surgical procedures complied with the Guide for Care and Use of Agriculture Animals and were approved by the Institutional Agricultural Animal Care and Use Committee of Texas A&M University. Mature Suffolk cross-bred ewes were observed daily for estrous behavior using vasectomized rams, with all ewes exhibiting at least two estrous cycles of normal duration (~16–18 d) before use.

To evaluate uterine gene expression throughout gestation, ewes were randomly assigned to be ovariohysterectomized (n = 4 ewes/d) on d 15 of the estrous cycle or d 11, 13, 15, 17, 19, 25, 30, 35, 40, 45, 50, 55, 60, 80, 100, or 120 of pregnancy (d 0 = estrus/mating). Pregnancy was confirmed by the presence of an apparently normal conceptus in uterine flushings (d 11–17) or visualization of an apparently normal conceptus at hysterectomy (d 19–120).

To evaluate *in vivo* regulation of uterine ISG15 expression by IFNτ, cyclic ewes were ovariectomized and fitted with intrauterine catheters on d 5 postestrus as described previously (5). Ewes (n = 5 ewes/treatment) received daily im injections of 50 mg progesterone from d 5 to 16 and intrauterine infusions of either 200 μg of control serum proteins (ovine serum proteins) or recombinant ovine IFNτ (2×10^7 antiviral units) from d 11 to 16. Proteins were prepared for intrauterine injections as previously described (5). All ewes were hysterectomized on d 17.

For both *in vivo* studies, several sections (~0.5 cm) from the middle of all uterine horns, including both interplacentomal and placentomal uterine wall regions of tissues from d 45 to d 120 of pregnancy, were fixed at hysterectomy in 4% paraformaldehyde in PBS (pH 7.2) and embedded in Paraplast-Plus (Oxford Laboratory, St. Louis, MO). Placentomes, if present, were then removed, and the remaining endometrium was physically dissected from the myometrium, frozen in liquid nitrogen, and stored at −80 C.

To evaluate IFNτ regulation of ISG15 in primary cell cultures, a ewe was hysterectomized on d 5 of the estrous cycle, and endometrial stromal cells were isolated as described previously (25).

Slot blot hybridization analysis

Total cellular RNA was isolated from frozen endometrium using Trizol reagent (Invitrogen, Carlsbad, CA). For each ewe, denatured total cellular RNA (20 μg) was analyzed by slot blot hybridization using a radiolabeled antisense bovine ISG15 (26) cRNA probe using methods previously described (3). A duplicate RNA slot blot membrane was hybridized with a radiolabeled antisense 18S rRNA (pT718S; Ambion, Austin, TX) cRNA probe. Radiolabeled riboprobes were generated by *in vitro* transcription with [α-^{32}P]uridine 5-triphosphate (Perkin-Elmer Life Sciences, Inc., Boston, MA) and a MAXIscript kit (Ambion). After washing, nonspecific hybridization was removed by RNase A digestion. Hybridization signals were detected by exposing the slot blots to a PhosphoImager screen and visualized using a Typhoon 8600 variable mode imager (Molecular Dynamics, Sunnyvale, CA).

RT-PCR

Partial cDNAs for ovine IFNα, IFNτ, and IFNγ were amplified by RT-PCR of RNA from d 17 pregnant ovine endometrium. Total cellular RNA (200 ng) was incubated at 65 C for 5 min with 1 μl of oligo(dT)$_{20}$ (50 μM), 1 μl of deoxynucleotide triphosphate mix (10 mM), and 7 μl diethylpyrocarbonate-treated water and then cooled on ice for 1 min. The following reagents were then added to each reaction to generate cDNA: 2 μl of 10 × reverse transcription buffer, 4 μl $MgCl_2$ (25 mM), 2 μl dithiothreitol (0.1 M), 1 μl RNaseOUT (40 U/μl), and 1 μl Superscript III reverse transcriptase (200 U/μl; Invitrogen). The reactions were incubated for 50 min at 50 C followed by 85 C for 5 min, and then 1 μl RNase H was added to each reaction and incubated for 20 min at 37 C. The cDNA (2 μl) was used for PCR analysis using primers specific for ovine IFNα (GenBank accession no. X59067.1; forward: 5′-AGC TTC AAC CTC TTC CAC ACA G-3′; reverse: 5′-AGC TCA GGT CTC CAT CCA TCA TTC-3′), ovine IFNγ (27) (forward: 5′-GAA CGG CAG CTC TGA GAA AC-3′; reverse: 5′-GCA GGC AGG AGA ACC ATT AC-3′), and ovine IFNτ (GenBank accession no. AF158823; forward: 5′-GGA AAC TCA TGC TGG ATG C-3′; reverse: 5′-AAG GTG GTT GAT GAA GTG AGG-3′). A master mix for each reaction was created that contained the following reagents (Invitrogen): 5 μl of 10 × PCR buffer, 1 μl deoxynucleotide triphosphate mixture (10 μM), 1.5 μl $MgCl_2$, 1 μl of forward and reverse primers (10 μM each), 0.2 μl DNA polymerase (platinum *Taq* DNA polymerase), and 38.3 μl nuclease-free water (Ambion). The reaction conditions were one cycle of 94 C for 2 min followed by 35 cycles of 94 C for 30 sec, 55 C for 30 sec, and 72 C for 1 min. The amplified 363-bp IFNα, IFNτ (460 bp), and IFNγ (200 bp) PCR products were subcloned into a pCR-II cloning vector using the TA cloning kit (Invitrogen) and confirmed by sequence analysis. A BLAST search for each was conducted to ensure that only target genes were evaluated.

In situ hybridization

ISG15 and IFN mRNAs were localized in paraffin-embedded ovine uterine tissue by *in situ* hybridization using methods previously described (9). Briefly, deparaffinized, rehydrated, and deproteinated uterine cross-sections (5 μm) were hybridized with radiolabeled antisense or sense bovine ISG15 or ovine IFN cRNA probes synthesized by *in vitro* transcription with [α-^{35}S]uridine 5-triphosphate (PerkinElmer Life Sciences). After hybridization, washes, and RNase A digestion, autoradiography was performed using NTB-2 liquid photographic emulsion (Eastman Kodak, Rochester, NY). Slides were exposed at 4 C for 6 d, developed in Kodak D-19 developer, counterstained with Harris' modified hematoxylin (Fisher Scientific, Fairlawn, NJ), dehydrated, and protected with coverslips.

Protein isolation and Western blot analyses

Endometrial samples were thawed and immediately homogenized in lysis buffer (1% Triton X-100, 0.5% Nonidet P-40, 150 mM NaCl, 10 mM

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Tris, 1 mM EDTA, 0.1 mM EGTA, 0.2 mM Na_3VO_4, 0.2 mM phenylmethylsulfonyl fluoride, 50 mM NaF, 30 mM $Na_4P_2O_7$, 1 μg/ml leupeptin, 1 μg/ml pepstatin) at a ratio of 1 g tissue per 5 ml buffer. Cellular debris was cleared by centrifugation (12,000 × *g*, 15 min, 4 C). The protein concentration of the supernatant was determined using a Bradford protein assay (Bio-Rad Laboratories, Hercules, CA) with BSA as the standard.

Ovine endometrial stromal cell monolayer cultures were grown to 90% confluence on 100-mm tissue culture plates (Nunc, Naperville, IL) and then left untreated as a control or treated with recombinant ovine IFNτ (10^4 antiviral units per milliliter) for 48 h. Cells were rinsed with ice-cold Hanks' balanced salt solution and then incubated in lysis buffer for 30 min at 4 C. Cellular debris was pelleted by centrifugation (12,000 × *g* for 10 min) and protein concentrations determined using a Bradford protein assay.

Proteins (20 μg for gestational and intrauterine infusion tissues, 15 μg for primary cells) were denatured in Laemmli buffer, separated on 15% SDS-PAGE gels, and transferred to nitrocellulose. Blots were blocked in 5% nonfat milk/TBST (Tris-buffered saline, 0.1% Tween 20) at room temperature for 1 h, incubated with either a rabbit polyclonal antibody against recombinant bovine ISG15 (2.5 μg/ml) (28, 29) or normal rabbit serum (2.5 μg/ml; Sigma, St. Louis, MO) in 2% nonfat milk/TBST overnight at 4 C, rinsed three times for 10 min each with TBST at room temperature, incubated with goat antirabbit IgG horseradish peroxidase conjugate (1:20000 dilution of 1 mg/ml stock; Kirkegaard & Perry Laboratories, Bethesda, MD), and then rinsed three times for 10 min each with TBST. Immunoreactive proteins were detected using enhanced chemiluminescence (SuperSignal West Pico Luminol System, Pierce Chemical Co., Rockford, IL) according to the manufacturer's recommendations using a FluorChem IS-8800 (Alpha Innotech, San Leandro, CA), and blots were quantified using AlphaEase FC software (Alpha Innotech). Immunoreactive proteins greater than 38.5 kDa were deemed conjugates and quantified together.

Immunohistochemistry

ISG15 protein was localized by immunohistochemistry in paraffin-embedded ovine uterine tissues using the Rabbit Super ABC kit (Biomeda, Foster City, CA) according to methods previously described (30) and a rabbit polyclonal antibody against recombinant bovine ISG15 (1 μg/ml; Refs. 28 and 29). Normal rabbit serum (Sigma) was used as the negative control and diaminobenzidine tetrachloride (Sigma) as the chromagen. Sections were counterstained with Harris' modified hematoxylin, dehydrated, and protected with coverslips.

Microscopy and digital imaging

Representative photomicrographs of bright-field (immunohistochemistry) or bright-field and dark-field (*in situ*) images were obtained using either an Eclipse E1000 photomicroscope (Nikon Instruments Inc., Melville, NY) with a DXM 1200 digital camera (Nikon) and ACT-1 software (Nikon) or an Axioplan 2 microscope (Carl Zeiss, Thornwood, NY) interfaced with an Axioplan HR digital camera and Axiovision 3.0 software. Photographic plates were assembled using Adobe Photoshop (version 6.0, Adobe Systems Inc., San Jose, CA).

Statistical analyses

Data were subjected to least-squares ANOVA using the general linear models procedures of the Statistical Analysis System (SAS, Cary, NC). Analyses of steady-state levels of endometrial ISG15 mRNA, as determined by slot blot hybridization analysis, used 18S rRNA as a covariate to correct for differences in RNA loading. Protein levels of ISG15, as determined by Western blotting, were evaluated within each blot to control for any differences in exposure times. All tests of significance were performed using the appropriate error terms according to the expectation of the mean squares for error. Data are presented as least-squares means with overall SEs.

Results

Slot blot hybridization for endometrial ISG15 mRNA

Steady-state levels of ISG15 mRNA were low on d 11 of pregnancy, increased markedly by d 15, concurrent with maximal secretion of IFNτ from the conceptus, and then declined throughout the remainder of gestation, with an overall effect of day ($P < 0.01$, quadratic) (Fig. 1). ISG15 mRNA was detectable through d 120 of pregnancy (Fig. 1).

In situ hybridization for endometrial and conceptus ISG15 and IFN mRNAs

ISG15 mRNA was not detected during the estrous cycle (Fig. 2A). However, a hybridization signal for ISG15 was present in the LE on d 11 of pregnancy and increased in the subluminal stroma and GE across the entire uterine wall by d 15 (Fig. 2A). Expression was maintained throughout the stroma and GE through d 25 and then declined by d 30 of pregnancy, with expression limited to patches of the stratum compactum stroma along the maternal-conceptus interface in the interplacentome, in which it remained throughout pregnancy (Fig. 2A). In the placentomal endometrium, ISG15 mRNA was localized to patches of the maternal caruncular stroma surrounding the interdigitating placental chorionic

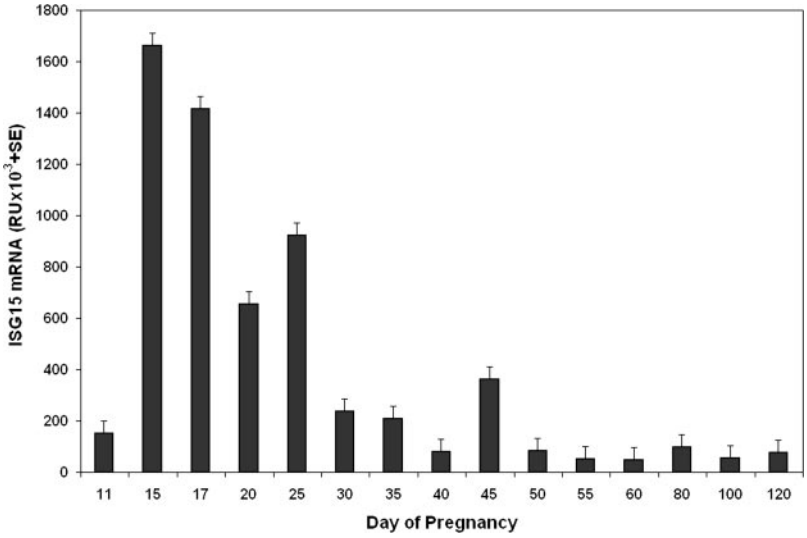

FIG. 1. Steady-state levels of ISG15 mRNA in ovine endometrium (20 μg/sample) from d 11 through d 120 of pregnancy. ISG15 mRNA levels, expressed as least square means of relative units (RU) with SE, are normalized for differences in sample loading using the 18S rRNA. An effect of day ($P < 0.001$) was detected.

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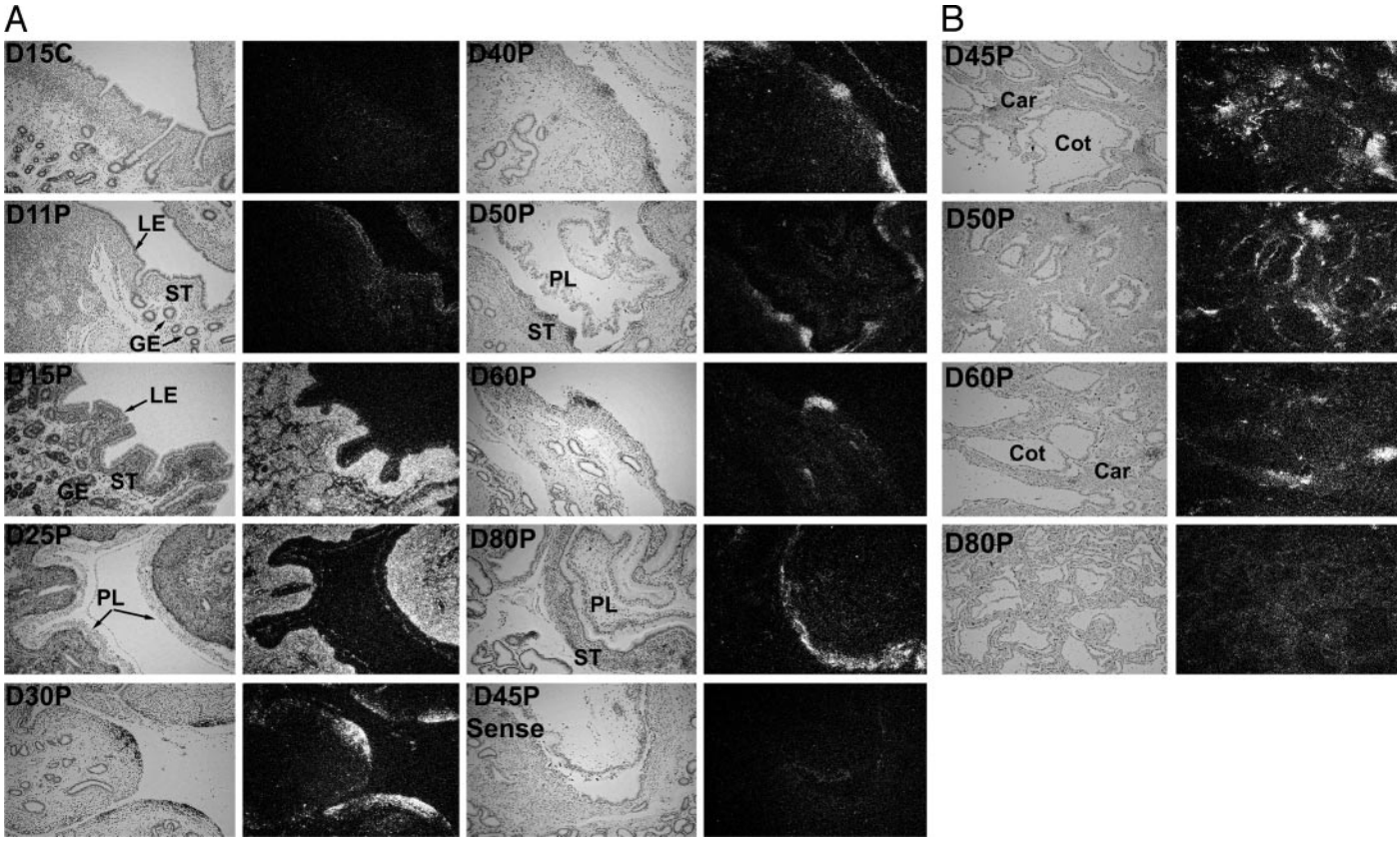

FIG. 2. *In situ* hybridization analysis of ISG15 mRNA in interplacentomal (A) and placentomal (B) cross-sections of ovine uterus and placenta. Corresponding bright- and dark-field images from cyclic (C) and pregnant (P) ewes are shown. A representative section from d (D) 45 hybridized with radiolabeled sense cRNA probe (sense) serves as a negative control. ST, Maternal stroma; PL, placenta; Car, maternal caruncle; Cot, fetal cotyledon. Width of each field, 940 μm.

villi beginning on d 45 of pregnancy and then declined through d 80 (Fig. 2B).

IFNτ mRNA was abundant in the trophectoderm of d 15 and 17 ovine conceptuses (Fig. 3). IFNτ mRNA abundance decreased noticeably on d 20 and 25 and was undetectable in the placenta on d 30–120 of pregnancy (Fig. 3). The pattern of hybridization, using the cRNA probe for IFNα, was identical with that for IFNτ, indicating cross-reactivity with IFNτ, but the absence of another type I IFN *in utero*-placental tissues. In contrast, IFNγ mRNA was localized to cells scattered within the stromal compartment of intercaruncular endometrium of pregnant, but not cyclic, ewes (Fig. 4). IFNγ mRNA, first detected on d 15, was expressed at maximal levels on d 45 and 50 and then decreased to undetectable levels by d 60 of pregnancy (Fig. 4). IFNγ mRNA was not detected in conceptus tissues (data not shown).

Immunohistochemistry for ISG15 protein

Consistent with *in situ* hybridization results, immunoreactive ISG15 protein was observed throughout the endometrium on d 25 of pregnancy (Fig. 5). Protein levels decreased by d 35 of pregnancy, with ISG15 protein localized to patches of the maternal stroma along the maternal-conceptus interface (Fig. 5). In the placentomes, ISG15 protein was localized to maternal stroma surrounding the interdigitating placental villi (Fig. 5).

Western blot analysis of ISG15 protein

Proteins of both 17 kDa and more than 30-kDa were detected in Western blots. Similar patterns of high-molecular-weight proteins that cross-react with ISG15-specific antibodies have previously been reported, biochemically characterized, and defined as ISG15-conjugated target proteins (18, 20, 21, 23). Therefore immunoreactive proteins greater than 30 kDa on Western blots are called conjugated ISG15 protein. Neither free nor conjugated ISG15 protein was detected during the estrous cycle or through d 10 of pregnancy (Fig. 6). Both free and conjugated ISG15 were present by d 16 of gestation (Fig. 6), and these maximal levels did not change through d 30 ($P > 0.05$). Levels of free ISG15 protein tended to decline between d 30 and 40 of pregnancy ($P = 0.07$), concomitant with a significant decrease in conjugated ISG15 ($P < 0.05$). It should be noted that one of the three d 40 pregnant uteri expressed levels of free ISG15 comparable with those observed in uteri from d 30, whereas free ISG15 levels were barely detectable in the other two d 40 samples analyzed (Fig. 6). Incubation of primary ovine uterine stromal cells with IFNτ for 48 h induced cytosolic expression of

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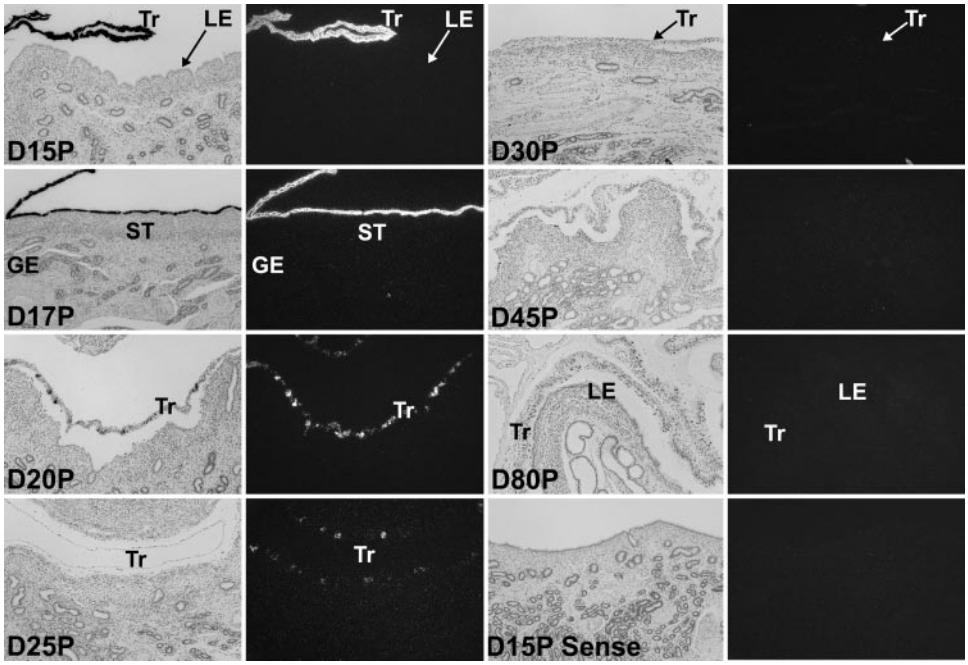

FIG. 3. *In situ* hybridization analysis of IFNτ mRNA in interplacentomal cross-sections of ovine uterus and conceptus. Corresponding bright- and dark-field images from pregnant (P) ewes are shown. A representative section from d (D) 15 hybridized with radiolabeled sense cRNA probe (sense) serves as a negative control. ST, Maternal stroma; Tr, conceptus trophectoderm. Width of each field, 940 μm.

both free and conjugated ISG15 (Fig. 7). Treatment of ovariectomized ewes with progesterone and IFNτ increased free and conjugated ISG15, compared with treatment with progesterone and control proteins ($P < 0.05$) (Fig. 8).

Discussion

A correlation between ISG15 and early pregnancy in eutherian mammals is firmly established. Austin and coworkers (17) first defined ISG15 as an IFNτ-induced endometrial protein of early pregnancy when they used ubiquitin antiserum to identify an approximately 16-kDa protein that was present in endometrium and uterine flushings from pregnant cows and released into culture medium when endometrial explants from cyclic animals were stimulated with recombinant IFNτ. ISG15 has subsequently been shown to be present in significant amounts in endometria of pregnant humans and baboons (22, 31), sheep (8), mice (23), and pigs (24). Up-regulation or induction of ISG15 in the endometrial stroma is consistent across these species (8, 20, 23, 24, 29, 31, 32), but ISG15 expression also increases in the middle to deep GE and myometrium of cows (20, 32) and sheep (8, 29). *In vivo*, IFNτ induction of ISG15 in the endometrium has been observed in sheep (9), and both IFNτ and IFNα induce *in vitro* synthesis of ISG15 by bovine endometrial explants (17). IFNτ,

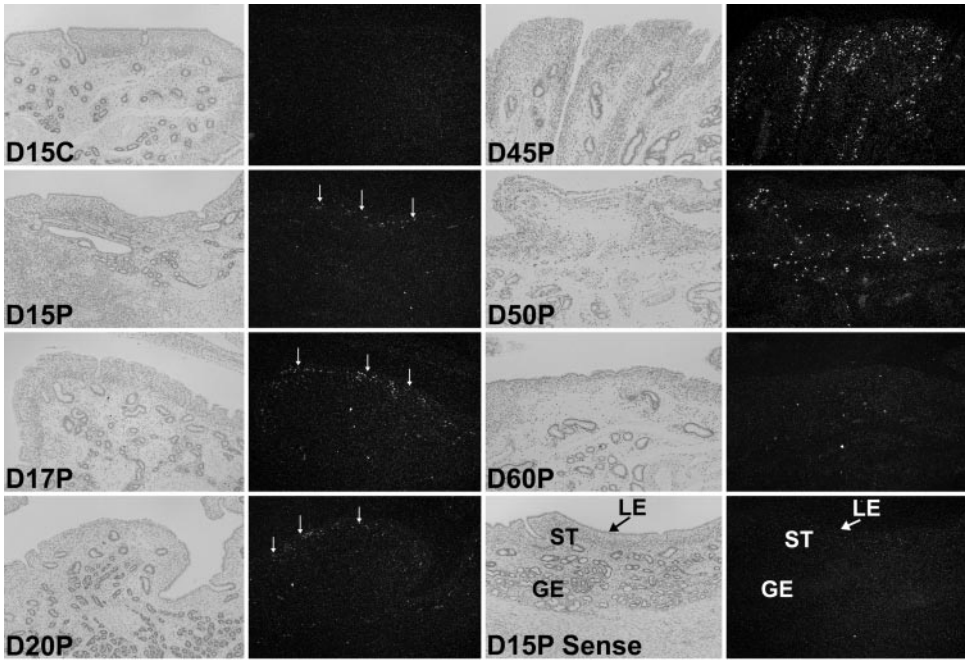

FIG. 4. *In situ* hybridization analysis of IFNγ mRNA in interplacentomal cross-sections of ovine uterus. Corresponding bright- and dark-field images from cyclic (C) and pregnant (P) ewes are shown. A representative section from d (D) 15P hybridized with radiolabeled sense cRNA probe (sense) serves as a negative control. *Arrows* indicate expression of IFNγ mRNA in the uterine stroma on d 15, 17, and 20 of pregnancy. ST, Maternal stroma. Width of each field, 940 μm.

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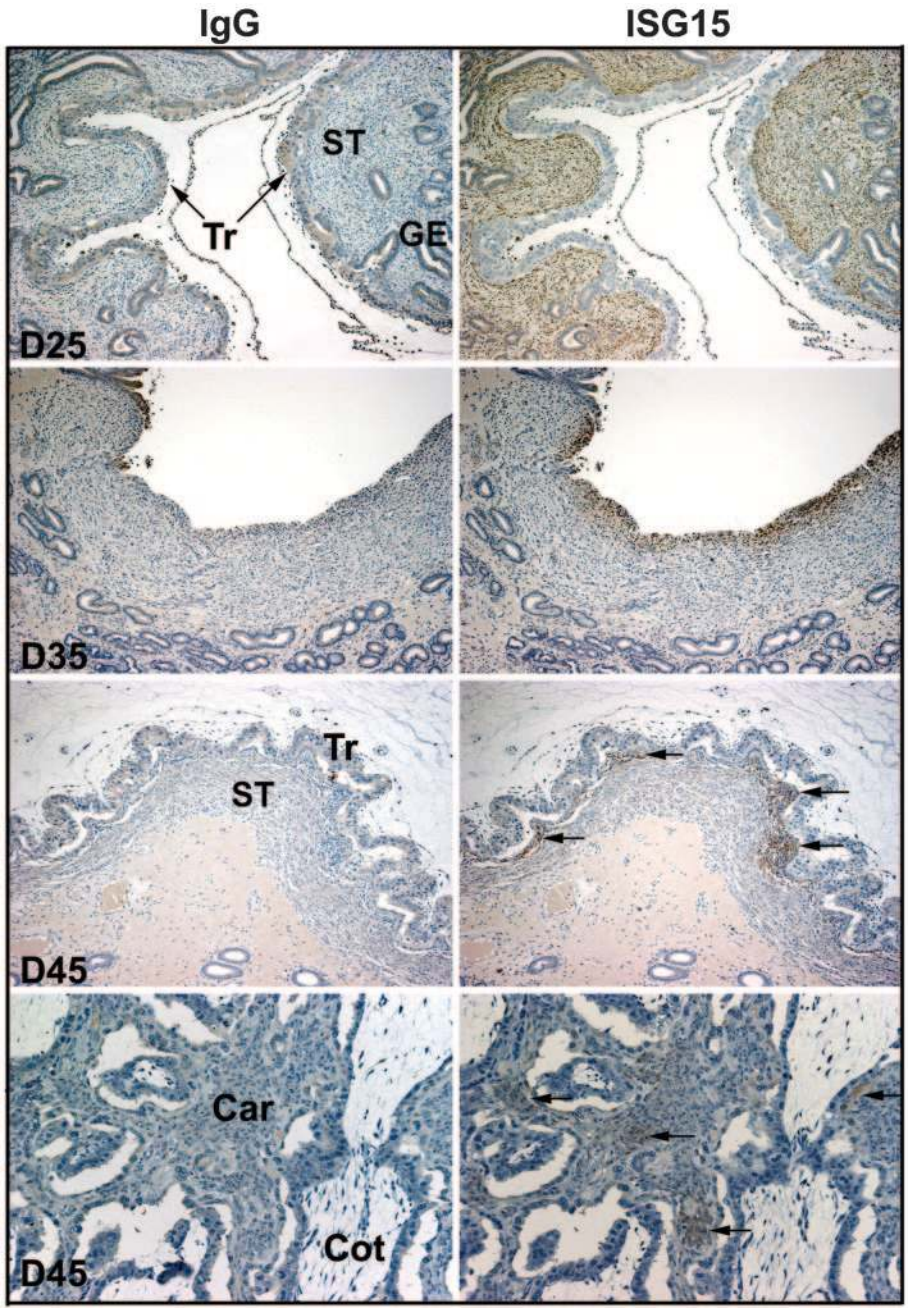

FIG. 5. Immunohistochemical localization of ISG15 protein in paraffin-embedded uterine cross-sections from pregnant ewes. The *brown color* indicates positive immunostaining for ISG15. All sections were counterstained with Harris' hematoxylin. For d (D) 45, sections were from either the interplacentomal region of the uterus or a placentome, with *arrows* denoting locations of positive immunostaining. Sections stained with nonimmune rabbit IgG serve as negative controls. ST, Stroma; Tr, trophectoderm; Car, maternal caruncle; Cot, fetal cotyledon. Width of D45 PL fields, 0.3 mm; width of D25, D35, and D45 IP fields, 1.3 mm.

IFNα, and IFNγ each increase ISG15 and/or ISG15 conjugate formation in murine decidual cells (23). Functional roles for ISG15 in the uterus during early pregnancy are suggested by results from Western blotting, indicating conjugation of endometrial proteins to ISG15 in mice (23), humans (22), and cows (20, 21).

Results reported here extend our understanding of ISG15 expression in sheep, and mammals in general, by: 1) confirming that endometrial proteins become conjugated to ISG15 in correlation with *in vivo* induction of ISG15 by IFNτ during early pregnancy; 2) localizing ISG15 gene expression to contacts between the placenta and uterine caruncular and intercaruncular endometrium between d 30 and 120 of pregnancy when production of IFNτ by trophectoderm is no longer detected; and 3) localizing IFNγ mRNA to cells within the endometrial stromal compartment of pregnant sheep. These data are the first to show *in vivo* regulation of ISG15 function *(i.e.* conjugation to target proteins) by a type I IFN in any species and are the first to describe expression of any of the known IFNτ-stimulated genes throughout gestation. Although periimplantation induction of endometrial ISG15 in ruminants is the result of conceptus-derived IFNτ, ISG15 expression in ovine stroma during later pregnancy may be the result of IFNγ and is reminiscent of its expression in the stroma of pregnant mice and primates whose placenta do not produce large amounts of a type I IFN for pregnancy recognition. Therefore, stromal expression of ISG15 may represent a universal uterine response to conceptus implantation and placentation.

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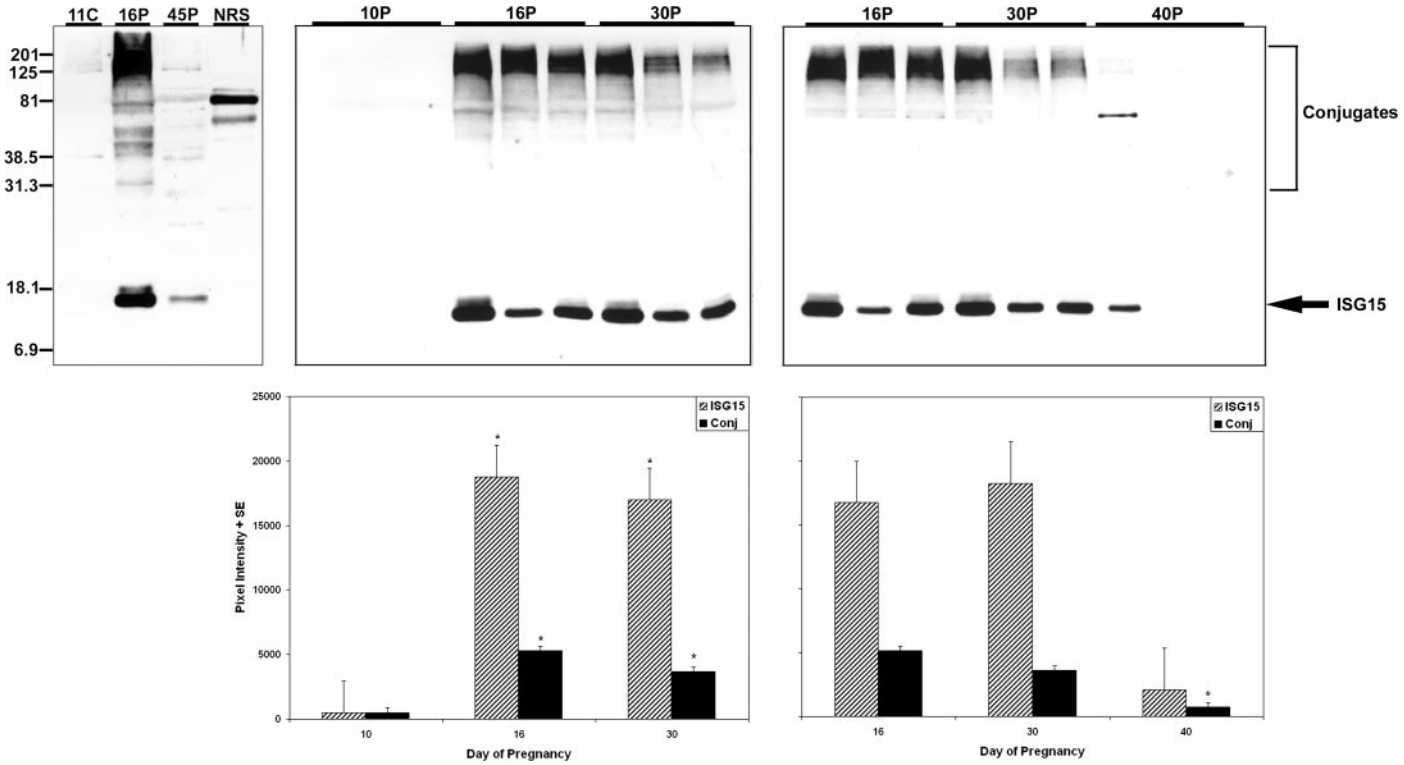

FIG. 6. Detection of ISG15 protein in ovine endometrial extracts from cyclic (C) and pregnant (P) ewes using Western blotting (15% SDS-PAGE). The blot on the *left* illustrates representative protein profiles in endometria from the estrous cycle (11C, 40 μg/lane) as well as periimplantation (16P, 20 μg/lane) and post implantation (45P, 40 μg/lane). Each lane in the remaining blots corresponds to endometrial extracts (20 μg/lane) from a different ewe, except that the same d 16 extracts are represented in both blots. Blots were statistically analyzed separately. Immunoreactive proteins were detected using either polyclonal rabbit antirecombinant bovine ISG15 antibody or normal rabbit serum (NRS) as a control. Positions of prestained molecular-weight standards ($\times 10^{-3}$) are indicated. As denoted by the *asterisk* in the bar graphs, free (ISG15) and conjugated (conj) ISG15 increased on d 16 and 30 of pregnancy, compared with d 10 of pregnancy ($P < 0.05$). By d 40 of pregnancy, levels of conjugated ISG15 were decreased, compared with levels on d 16 and 30 ($P < 0.05$).

ISG15 cross-reacts with ubiquitin antisera and is considered to be a ubiquitin-related molecule (16, 33). Ubiquitin conjugates through its carboxyl terminus to proteins in a multistep enzymatic process (34). In an ATP-dependent manner, ubiquitin forms a thiol ester linkage with the ubiquitin-activating enzyme (E1) (34). This activated ubiquitin is transferred to a carrier protein (E2) (34) and then ubiquitin ligase assists in the formation of an isopeptide bond between ubiquitin and Lys residues of target proteins (34). Proteins often become monoubiquinated (35), but in some cases, chain elongation factor is subsequently involved in the formation of isopeptide bonds between Lys residues of ubiquitin and additional ubiquitin molecules, resulting in multiubiquitin chains (36), with the size and location of these chains influencing the cellular response to ubiquitination (37). These responses include bulk protein degradation of the target protein, cell-cycle control, stress response, DNA repair, signal transduction, transcriptional regulation, vesicular trafficking, and antigen presentation (38, 39).

Whereas ubiquitin has many diverse functions, the biological role(s) of ISG15 is not well understood. Studies of ISG15 indicate that it has an extracellular role as an immunomodulatory cytokine and an intracellular role as a ubiquitin-like protein. Immunomodulatory functions of ISG15 include: 1) induction of IFNγ production by T lymphocytes (40); 2) differentiation and proliferation of natural killer cells and activation of natural killer cell-derived lymphokine-activated killer cells (41); and 3) induction of E-cadherin on dendritic cells as a potential mechanism of tumor immune escape (42). In addition, ISG15 is a chemotactic factor for neutrophils (43). Intracellularly, ISG15 can conjugate to proteins in a similar, yet distinct, manner as ubiquitin. In IFN-treated cells, ISG15 and conjugates have been localized to the cytoplasm (44) as well as the intermediate filament network (45), in which they may function as part of the antiviral response.

Ubiquitin and ISG15 are also potentially involved in events important for successful pregnancy. In support of this idea, inhibition of the ubiquitin-proteasome pathway reduces embryo implantation in mice, an effect partially mediated through a decrease in matrix metalloproteinase-2 and -9 gene expression (46). Despite major differences in implantation strategies, ISG15 and its conjugates are induced or increased during early pregnancy in multiple species, suggesting that this conceptus-induced response may be part of a universal sequence of events essential for early embryonic development and survival (9, 20–23). Indeed, enzymes involved in the conjugation process, including ubiquitin-conjugating enzymes (E2) and bovine ubiquitin activating E1-like enzyme, an ISG15-activating enzyme (E1), are increased by IFNs (47–49). Significantly, bovine ubiquitin activating E1-like as well as the E2-like molecules 1–8U and Leu-13

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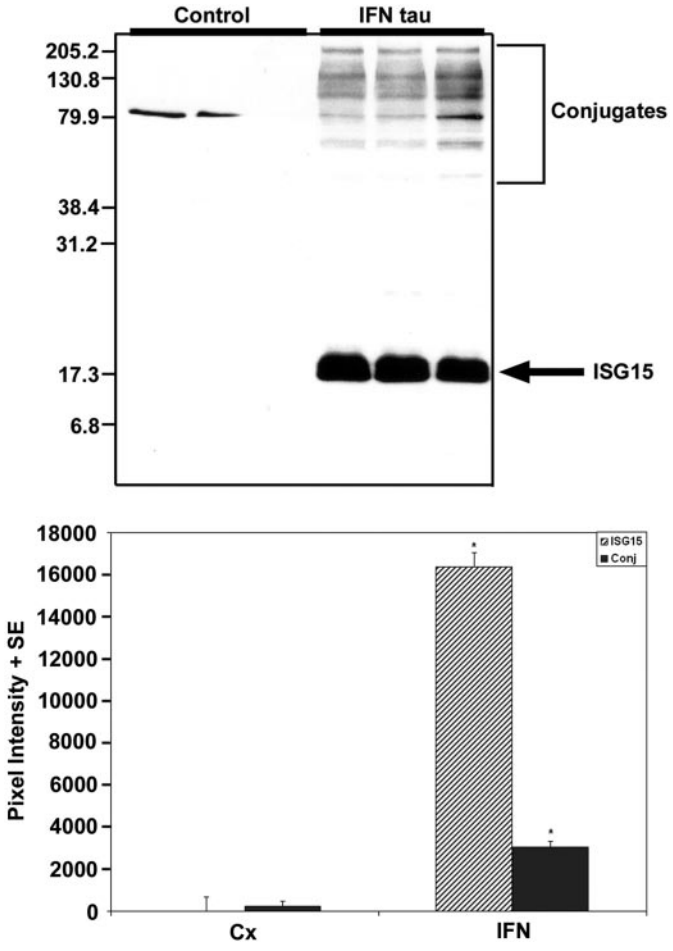

FIG. 7. Detection of ISG15 (15% SDS-PAGE) in primary ovine uterine stromal cells. Each lane (15 μg/lane) corresponds to a separate 100-mm culture dish of stromal cells. Positions of prestained molecular-weight standards ($\times 10^{-3}$) are indicated. Immunoreactive proteins were detected using polyclonal rabbit antirecombinant ISG15 antibody. Treatment of uterine stromal cells with IFNτ-induced expression of free (ISG15) and conjugated (conj) ISG15, compared with cells that did not receive IFNτ ($P < 0.05$). CX, Control serum proteins.

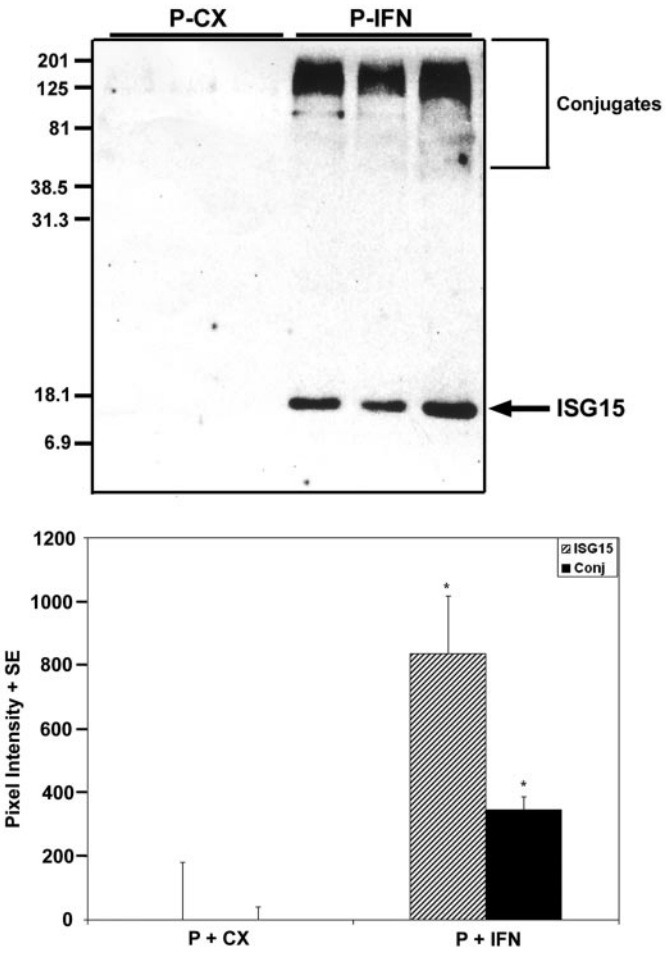

FIG. 8. Detection of ISG15 protein in ovine endometrial extracts using Western blotting (15% SDS-PAGE). Each lane (20 μg/lane) corresponds to endometrial extracts from a different ewe. Immunoreactive proteins were detected using either a polyclonal rabbit antirecombinant bovine ISG15 antibody or normal rabbit serum (NRS) as a control. Positions of prestained molecular-weight standards ($\times 10^{-3}$) are indicated. Treatment of ovariectomized ewes with progesterone and recombinant ovine IFNτ (P+IFN)-induced expression of free (ISG15) and conjugated (conj) ISG15, compared with ewes treated with progesterone and control serum proteins (P+CX), as indicated by the *asterisk* in the bar graph ($P < 0.05$).

increase in the bovine uterus, and 1–8U family members increase in the ovine uterus in response to conceptus production of IFNτ (48–50).

At this time, the biological implications of ISG15 conjugation are unclear. It is possible that, like polyubiquitination, ISG15 conjugation targets proteins for proteasomal degradation. Whereas Liu and coworkers (51) reported increased levels of ISG15 conjugation in IFNα-induced immortalized human fibroblast stromal cells (2fTGH) after treatment with various proteasome inhibitors, Malakhov *et al.* (52) did not detect an increase in ISG15 conjugates in IFNβ-treated murine embryonic fibroblast cells when the proteasomal inhibitor lactacystin was used. Alterations in the ISG15 conjugation system are correlated with various disease states or physiological abnormalities including carcinogenesis (53), influenza virus infection (54), and aberrant brain cell function (55). To date, only a few protein targets for ISG15 conjugation have been identified. The signal transduction factors phospholipase Cγ1, Janus kinase 1, ERK1, and Stat1 have been identified as ISG15 conjugates (52), indicating that ISG15 may regulate multiple signal transduction pathways. Serpin 2a, a member of the family of serine protease inhibitors, is also a target of ISG15 conjugation (56). Intriguingly, additional members of the serpin family, ovine uterine serpins (57), are among the most highly expressed secretory products of the pregnant sheep uterus (58, 59) and perhaps represent potential targets for ISG15 conjugation that may be important for pregnancy.

The temporal and spatial patterns of ISG15 characterized in this study are of particular interest because this so-called IFNτ-induced gene is detectable beyond the period of IFNτ secretion by the conceptus and is localized to discrete patches of the maternal stroma along the maternal-fetal interface. In this study, cRNA probes designed to detect type I IFNs (IFNτ and IFNα) and the type II IFNγ mRNAs were used with *in situ* hybridization to gain insight into factors that mediate ISG15 expression in uteri between d 30 and 120 of pregnancy.

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Although a second period of immunoreactive IFNτ secretion has been reported in sheep (60), we found no evidence for type I IFN expression in the ovine uterus or placenta during middle to late stages of gestation. In contrast, IFNγ mRNA was detected in cells of the stromal compartment of intercaruncular and interplacentomal endometria from d 15 through d 50 of pregnancy. It is notable that endometrial IFNγ is evident on d 15 of pregnancy but not d 15 of the estrous cycle, suggesting pregnancy-specific regulation, and that IFNγ levels are highest on d 45, which corresponds to a second peak of total ISG15 mRNA expression in sheep endometrium.

IFNγ pretreatment of monocyte U937 cells increases and hastens ISG expression in response to type I IFNs (61). IFNγ can also increase ISG15 expression but is less effective than type I IFNs (14) and does not affect secretion of the protein (62). Furthermore, Austin *et al.* (23) observed that IFNγ induces ISG15 conjugate formation in cultured mouse decidual cells and speculated that IFNγ may have a supplementary role in ISG15 expression during implantation. It is reasonable to hypothesize that in sheep IFNγ is responsible for maintaining ISG15 gene expression in the endometrium at the uterine-placental interface after the period of conceptus IFNτ production. Because ISG15 is known to induce IFNγ production by T lymphocytes (40), it is possible that conceptus IFNτ up-regulates ISG15 in endometrium and that ISG15 induces T cells within the stratum compactum stroma to express IFNγ, which maintains expression of ISG15 on the maternal side of the maternal-placental interface throughout pregnancy. This hypothesis will be the subject of future investigations. Results from this study do not explain expression of ISG15 in ovine placentomes because type I IFNs were not present in placentomes, and IFNγ was almost undetectable with no apparent colocalization with ISG15 (data not shown). In addition, it will be important to identify the cell-specific expression of IFNγ in maternal endometrium.

In summary, there is a significant increase in ISG15 gene expression in the ovine uterus by d 15 of pregnancy, and results of the present study are the first to identify expression of this gene in patches of stroma along the uterine-placental interface throughout pregnancy, perhaps in response to IFNγ within the endometrial stromal compartment. Furthermore, results of this study demonstrate that levels of ISG15-conjugated proteins increase and then decrease during pregnancy, indicative of a biologically active molecule that is responsive to the IFNτ signal from the conceptus and that it temporally targets proteins for pregnancy-associated regulation and/or modification. At present, we can only speculate on the identity of the uterine proteins being modified for pregnancy by ISG15; however, the continual presence of ISG15 in uterine stroma adjacent to conceptus tissue is highly suggestive of an influence of this ubiquitin homolog over interactions between maternal and fetal tissues for implantation and placentation. A hallmark of pregnancy in invasive implanting species is the decidualization response that uterine stroma undergoes in response to the invading conceptus. ISG15 is expressed in decidua of mice (23), baboons (22), and women (31) as well as uterine stroma undergoing a decidualization-like transformation in sheep (63). It is reasonable to speculate that ISG15 is involved in conjugation events affecting decidual roles of hormone secretion, embryo nutrition, fetal allograft protection, uterine remodeling, and limiting of conceptus trophoblast invasion through generation of a local cytokine environment that promotes conceptus attachment over invasion (64). Given that ISG15 activity increases in uterine endometrium during pregnancy in several different mammalian species, ISG15 may represent one facet of a complex temporal and spatial response of the stroma as its functions change during embryo implantation and placentation.

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