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Bovine Granulocyte Chemotactic Protein-2 Is Secreted by the Endometrium in Response to Interferon-tau (IFN-τ)

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Interferon-tau (IFN-τ) is secreted by the bovine conceptus and may regulate synthesis of uterine endometrial cytokines to provide an environment that is conducive to embryo development and implantation. Interferon-τ stimulates secretion of an 8-kDa uterine protein (P8) in the cow. P8 was purified, digested to yield internal peptides, and partially sequenced to determine identity. Two internal peptides had 100% (13-mer) and 92% (12-mer) amino acid sequence identity with bovine granulocyte chemotactic protein-2 (bGCP-2). Bovine GCP-2 is an α-chemokine that acts primarily as a potent chemoattractant for granulocyte cells of the immune system. A peptide was synthesized based on a region of bGCP-2 that overlapped with a P8 peptide amino acid sequence, coupled to keyhole limpet hemocyanin, and used to generate high titer polyclonal antiserum in sheep. Western blots revealed that bGCP-2 was not released by endometrium from day 14 nonpregnant cows, but was released in response to 25 n*M* IFN-τ ($p < 0.05$). Uterine GCP-2 exhibited high affinity to heparin agarose, a characteristic shared by all α chemokines. This is the first report describing presence of GCP-2 in the uterine endometrium and regulation by IFN-τ. The regulation of bGCP-2 by IFN-τ may have important implications for cytokine networking in the uterus during pregnancy. Also, the regulation of inflammation and angiogenesis by bGCP-2 working together with other cytokines may be integral to establishing early pregnancy and implantation in the cow.

Key Words: Chemokine; granulocyte chemotactic protein; interferon; pregnancy; uterus.

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Introduction

In mammals, the incidence of early embryonic mortality has been estimated to be 25–60% *(1)*. This mortality may be caused by dysfunctional communication between the conceptus and uterus during early pregnancy. There is now evidence to suggest that the uterine environment has a significant influence on the outcome of pregnancy *(2)*. A receptive uterus allows attachment and invasion of the embryo, while escaping immunological rejection *(3–5)*. This "receptive" state reflects reorganization of uterine tissue and potentially lymphoid cells that are regulated by steroids (estradiol and progesterone) as well as cytokines during early pregnancy.

In cattle, interferon-tau (IFN-τ) has been implicated in protecting the conceptus from early abortion by reducing the release of prostaglandin $F_2\alpha$ (PGF), a substance that dictates functional and structural regression of the ovarian corpus luteum *(6,7)*. Interferon-τ, like other type I IFNs, induces an antiviral state in target cells, but also has many effects that are not related to antiviral activity. These effects, may be integral to pregnancy, and could include regulating immunocyte populations and the products of lymphocytes in the endometrium. For example, granulocyte-macrophage colony stimulating factor-I (GM-CSF) is released by the endometrium in response to IFN-τ *(8)*. GM-CSF promotes growth of granulocytes and macrophages, which could be involved in remodeling of the uterine endometrium in preparation for implantation.

Granulocytes are classified as neutrophils, eosinophils, or basophils depending on cellular morphology and cytoplasmic staining *(9)*. Neutrophils are the predominant circulating granulocyte, phagocytic, and generally the first cells to arrive at a site of inflammation. The phagocytic activity of neutrophils is of critical importance in the first line of defense against pathogenic agents. Neutrophils comprise most of the cellular infiltrate into the early pregnant uterus in mice *(5)*. Thus, the uterine response to implantation has been compared with a classic inflammatory response.

The authors have been characterizing uterine proteins that are regulated by IFN-τ during early pregnancy *(10–12)*. One of these proteins (17-kDa) was recently placed in the ubiquitin family and called ubiquitin crossreactive protein, or UCRP *(12)*. Another IFN-stimulated gene product of ~8-kDa (P8) also was described *(11)*. Here, it is reported that P8 is structurally related to granulocyte chemotactic protein-2 (GCP-2) and belongs to the α chemokine family.

The term "chemokine" (chemotactic cytokine) designates a large family of related polypeptides that directs the migration and activation of leukocytes *(13)*. It is now clearly evident that these chemotactic cytokines are involved in many different processes. Granulocyte CP-2 was first identified in human osteosarcoma cells (MG-63) *(14)*. Purified GCP-2 had a strong chemotactic index in stimulating the migration of granulocytes through polycarbonate micropore membranes. Subsequent studies using GCP-2 purified from bovine kidney cells (MDBK) confirmed that it was chemotactic for granulocytes and stimulated these cells to secrete protease *(15)*. Other than these reports, little progress has been published on the characterization of this chemokine. The present paper is the first to describe GCP-2 in the endometrium. Also, it is the first to identify a specific cytokine (IFN-τ) as a regulator of GCP-2 secretion.

Results

Purification of P8

Because P8 was estimated to have a pI > 7.5 *(11)*, cation exchange fast phase liquid chromatography (FPLC) was used as a first step in enrichment (Fig. 1). Radiolabeled (^{3}H-Leu) P8 was identified in fractions using ID-PAGE and fluorography (not shown). P8 did not elute from this cation exchanger until the salt gradient reached 1*M*. Fractions 65–68 were pooled and separated from higher Mr contaminants using ID-PAGE. A gel slice containing P8 was digested with Lys-C and trypsin to yield peptides. These peptides were purified using reversed-phase microbore high pressure liquid chromatography (HPLC; not shown).

Amino Acid Sequence of P8 Peptides

Two peptides were sequenced. The first peptide, called P8-9 (i.e., peak 9 following HPLC purification) had the following sequence: TVSDLQVIAAGPQ and 100% identity with bGCP-2 (Fig. 2). The second peptide, called P8-13 had the following sequence: EVCLxPEAPLIK and 92% sequence identity with bGCP-2. Because the fifth residue for P8-13 could not be determined, it is possible that this sequence also was 100% identical to bGCP-2.

Generation of GCP-2 Peptide Antiserum in Sheep

The synthetic GCP-2 peptide (bGCP-pep) amino acid sequence is shown in Fig. 2. This peptide sequence (TPGIHPKTVSDLQV IAAGPQCSK) overlapped the P8-9 sequence and was predicted to have a relatively high antigenic index. Also, this peptide was selected to be unique to bGCP-2 sharing only 40–85% sequence identity with other α chemokines (Fig. 2). The bovine GCP-2 peptide was coupled to keyhole limpet hemocyanin (KLH) and used to generate antiserum in sheep. Crude serum from immunized sheep provided antibody that could be detected out to a 1/10,000 dilution as determined by ELISA using unconjugated bGCP-2 peptide as the antigen (Fig. 3). Serum collected prior to immunization (serum control) had no significant titer when tested against the bGCP-2 peptide antigen.

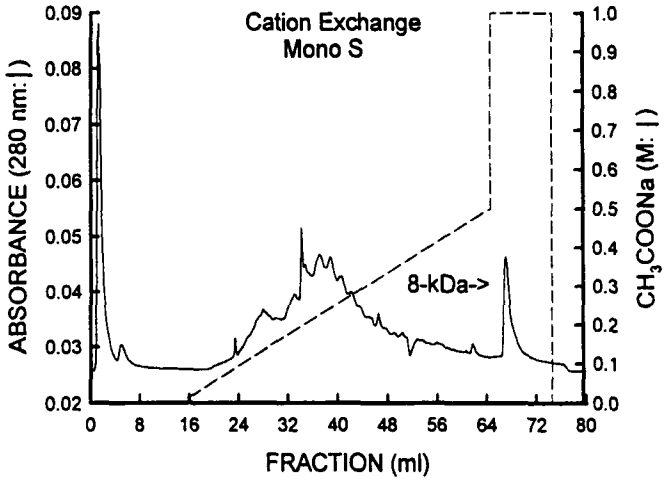

Fig. 1. Enrichment of P8 using cation exchange FPLC. Analysis of proteins eluting from the Mono S exchanger using ID-PAGE and fluorography revealed that radiolabeled P8 was found in fractions 65–68 (~1*M* CH_3COONa).

Western Blot of Endometrial Proteins Using GCP-2 Peptide Antiserum

Bovine GCP-2 peptide antiserum was used in Western blots to determine if immunoreacting proteins were present in the endometrium and regulated by IFN-τ. Figure 4 illustrates a representative Western blot that demonstrates the absence of bGCP-2 in cultured endometrium representing day 14 of the estrous cycle (lane 1) and the presence of bGCP-2 in this same endometrium when treated in vitro with 25 n*M* rbIFN-τ for 24 h (lane 2). An expanded analysis (Fig. 4B) revealed that bGCP-2 was released into medium in response to the 25 n*M* dose of rbIFN-τ ($p < 0.05$).

Affinity of P8/bGCP-2 to Heparin Agarose

α Chemokines share the ability to bind to heparin, a highly sulfated dextro-rotatory mucopolysaccharide that is present in the liver, lungs, and mast cells. Crude endometrial proteins known to contain P8 were fractionated over heparin-agarose. Radiolabeled P8 was retained on this matrix until step elution reached 0.5–1*M* NaCl (Fig. 5). Western blot with bGCP-2 peptide antiserum confirmed that radiolabeled P8/bGCP-2 eluted at 0.5–1*M* NaCl and had strong affinity with heparin agarose (Fig. 5).

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                10        20        30        40        50        60        70    75
        VAAVVRELR CVCLTTTPG IHPKTVSDLQ VIAAGPQCSK VEVIATLKNG REVCLDPEAP LIKKIVQKIL DSGKN

8K-9      (100)                 TVSDLQ VIAAGPQ
8K-13     ( 92)                                                EVCLxPEAP LIK
bGCP-Pep  (100)         TPG IHPKTVSDLQ VIAAGPQCSK

pAMC2     ( 85)  [88]   TPG IHPKMISDLQ VIPAGPQCSK
hENA-78   ( 65)  [73]   TQG VHPKMISNLQ VFAIGPQCSK
hGCP-2    ( 55)  [68]   TLR VNPKTIGKLQ VFPAGPQCSK
hNAP-2    ( 45)  [66]   SG- IHPKNIQSLE VIGKGTHCNQ
bPF-4     ( 45)  [49]   TSG INPRHISSLE VIGAGTHCPS
mGRO/KC   ( 40)  [62]   MAG IHLKNIQSLK VLPSGPHCTQ
mMIP-2    ( 40)  [57]   LPR VDFKNIQSLS VTPPGPHCAQ
hIL-8     ( 40)  [40]   SKP FHPKFIKELR VIESGPHCAN
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Fig. 2. Amino acid sequence of bGCP-2, P8 peptides, the synthetic GCP-2 peptide (bGCP-Pep) and identities with several α chemokines. P8 peptides were identified based on their order of elution following microbore reversed phase HPLC: P8-9, P8-13. The entire amino acid sequence is shown across the top for bGCP-2 *(15)*. Percentages in parentheses reflect amino acid sequence identity when compared with the synthetic bGCP-2 peptide and the same region in native bGCP-2 (i.e., residues 18–40). Percentages in brackets reflect full length amino acid sequence identities compared with bGCP-2. Chemokines and references: porcine alveolar macrophage chemotactic factor-2 (pAMC2; *28*), human neutrophil activating protein (hENA-78; *29*), human granulocyte chemotactic protein-2 (hGCP-2; *14*), human neutrophil activating peptide-2 (hNAP-2; *30*), bovine platelet factor-4 (bPF-4; *31*), murine growth regulated protein (mGRO, also called KC; *32*), murine macrophage inflammatory protein-2 (mMIP-2; *33*), and human interleukin-8 (hIL-8; *34*).

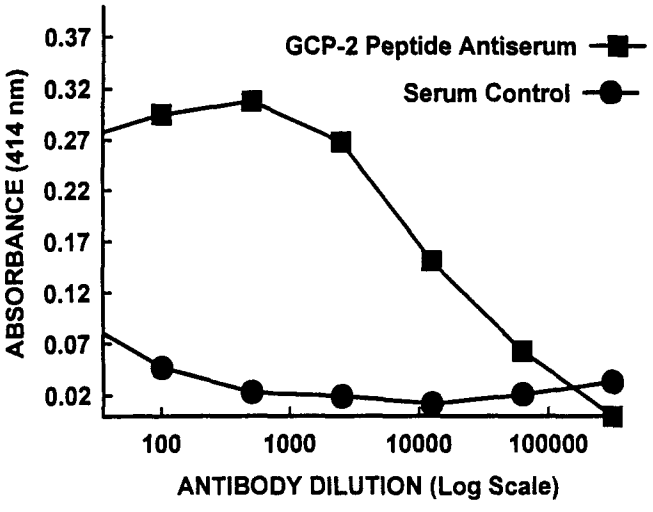

Fig. 3. Evaluation of GCP-2 peptide antiserum using ELISA. Granulocyte CP-2 peptide was used as the antigen. Serum collected prior to immunization (serum control) was used as an index of background. Crude GCP-2 peptide antiserum was diluted as shown.

Discussion

The bovine endometrium undergoes significant changes in secretory activity and cell turnover during the estrous cycle and pregnancy. In addition to estradiol and progesterone, other hormones/cytokines affect secretion of endometrial proteins. Because IFN-τ has been shown to be secreted by the conceptus during early pregnancy (reviewed in ref. *6*), it is suspected that it might stimulate secretion of endometrial proteins that were critical to the maintenance of pregnancy. The authors identified an 8-kDa protein (P8) that was produced by bovine uterine endometrial explants in response to IFN-τ *(11)*. It was expected that amino acid sequence identity with other proteins would provide insight into function (i.e., structure/function). Purification and amino acid sequence of internal peptides revealed that P8 was structurally related to GCP-2 (92–100% identity), which is a member of the α chemokine family.

Chemokines are chemotactic and proinflammatory polypeptides that contain four conserved cysteine residues *(13)*. They have been subdivided depending on the presence or absence of an intervening amino acid between the first two cysteine residues yielding C-X-C (α chemokine) or C-C (β chemokine) families *(13,16)*. Chemokines were originally described based on their ability to dictate the migration and activation of leukocytes. It is now recognized that these chemotactic cytokines are involved in many different processes. Some of these processes include, but are not restricted to inflammatory responses *(13)*, angiogenesis *(17)*, lymphocyte trafficking *(18)*, coagulation *(19)*, hematopoiesis *(20)*, wound healing *(21)*, atherogenesis *(22)*, anaphylaxis *(23)*, and malignancy *(24)*.

Granulocyte CP-2 was first described in human osteosarcoma cells (MG-63) *(14)*. This chemokine was purified and found to stimulate the migration of granulocytes through polycarbonate micropore membranes. Granulocyte CP-2 was structurally identified as a novel member of the α (C-X-C) family of chemoattractants, some of which include: IL-8, GRO/KC, MIP-2, and ENA-78. Functionally, IL-8 had 10 times the granulocyte chemotactic activity and was more efficacious in activating granulocytes to release gelatinase than GCP-2 *(14)*.

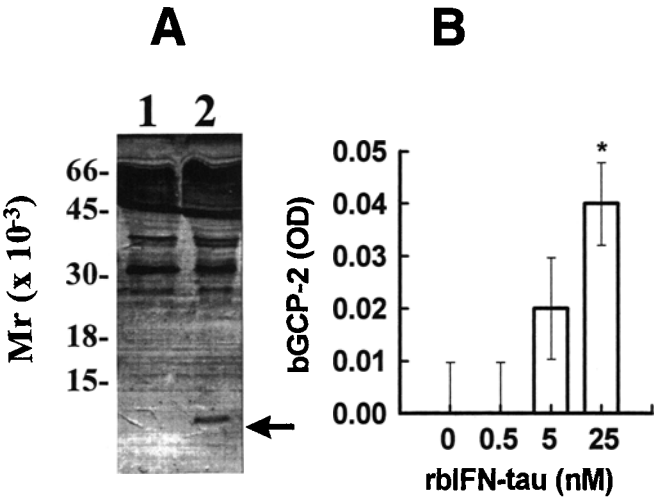

Fig. 4. Representative Western blot and expanded densitometric analysis of secretion of bGCP-2 in response to rbIFN-τ. In **(A)**, endometrium was collected on day 14 of the estrous cycle and cultured for 24 h in the absence (lane 1) or presence (lane 2) of 25 nM IFN-τ. Immunoreactive P8 (GCP-2) is shown with the arrow. **(B)** represents quantitation and analysis of secretion of bGCP-2 by endometrium cultured with 0, 0.5, 5, or 25 n*M* rbIFN-τ for 24 h. Optical density was derived from densitometric scanning of Western blots in the 8-kDa area. Values represent mean ± SEM. Release of bGCP-2 was higher in response to 25 n*M* IFN-τ compared with controls (*; $p < 0.05$).

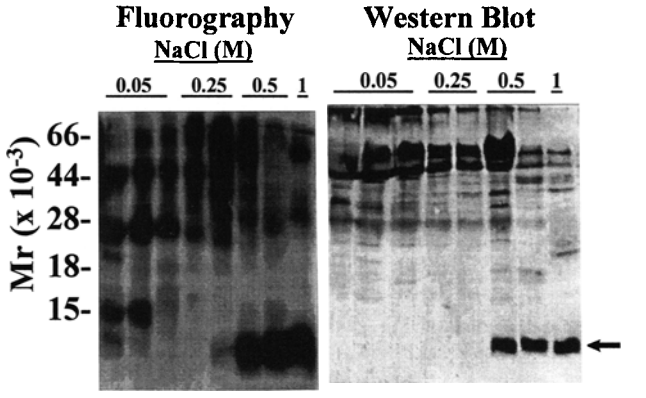

Fig. 5. Fluorography and Western blot of endometrial proteins eluting from heparin-agarose during a step salt gradient. Radiolabeled P8 eluted from the matrix when the gradient reached 0.5 to 1*M*. Likewise, immunoreactive P8 (GCP-2) eluted during the same step gradient. The 8-kDa protein showing affinity to heparin–agarose is shown with the arrow.

GCP-2 also has been identified in MDBK cells, but only when these cells were pretreated with phorbol ester *(15)*. The amino acid sequence was determined for bGCP-2 by examining overlapping peptides. Bovine GCP-2 had only 68% sequence identity with hGCP-2, but both were able to recruit human granulocytes and to induce secretion of gelatinase, a characteristic of activated granulocytes. Bovine GCP-2 had high affinity for heparin and existed in multiple forms (7.9–7.1 kDa; 9.4–9.1 pI) because of N-terminal deletion of 2–8 amino acids. It has been suggested that the 67-residue form of GCP-2 (i.e., lacking eight N-terminal amino acids) is the biologically active molecule *(15)*. Eight N-terminal "leading" amino residues are removed from pre-IL-8 to yield IL-8. N-terminal amino acids remaining (i.e., ELR) have been implicated to be needed for chemotactic activity and receptor binding *(16)*.

A partially purified cytokine preparation from leukocytes was used to stimulate production of hGCP-2 by osteosarcoma cells *(14)*. Because this preparation contained several cytokines such as IL-1, TNF-α, IFN-γ, and IL-2, it was not clear which cytokine was directly involved in eliciting release of GCP-2. Other than these reports by Proost and coworkers *(14,15)*, little is known regarding the secretion and regulation of GCP-2 in "normal" cells. The current research is the first to report secretion of GCP-2 by the uterine endometrium in response to conceptus-derived IFN-τ.

Interferon-τ is secreted by the conceptus and interacts with uterine endometrial receptors to elicit release of GCP-2. IFN-τ receptors have not been localized to a specific cell type (i.e., epithelial, stromal, cells of the immune system) in the endometrium. Likewise, because this is the first report describing presence of GCP-2 in the endometrium, localization to specific cells within this tissue is unknown. Thus, the cell of origin for GCP-2 and the IFN-τ receptor remains to be determined.

Granulocyte CP-2 could be secreted into the uterine lumen to affect growth, differentiation, and invasive properties of the conceptus. Likewise, it may be involved in regulating the uterine immune system, inflammation, remodeling, and angiogenesis associated with early conceptus/uterus communication and implantation. Because GCP-2 is chemotactic for granulocytes, we suspect that recruitment/activation of granulocytes and other immunocyte cells in the uterus is required for pregnancy to be established and maintained. GCP-2 also could induce an immunosuppressive phenotype and recruit scavenger immunocytes (granulocyte/macrophage) to control the inflammation associated with implantation. The regulation of inflammation and angiogenesis by GCP-2 and/or other α chemokines may be integral to survival of the early developing conceptus. These hypotheses will be examined in future experiments, as will clarification of exactly when GCP-2 is secreted by the endometrium during early pregnancy in response to IFN-τ.

Methods

Animal Care

All procedures described in which animals were utilized were approved by the University of Wyoming Animal Care and Use Committee.

Purification of P8

Based upon preliminary 2D-PAGE results *(11)*, it was suspected that P8 had a basic p*I* (p*I* > 7.5). Thus, the first step in purification was cation exchange FPLC *(25)*.

Endometrium was collected from day 18 (d 0 = estrus) pregnant cows. This day of pregnancy was selected because it represents the time during which production of IFN-τ by the conceptus is maximal *(6)*. Endometrium was cultured in the presence of 25 n*M* IFN-τ to potentiate release of regulated proteins even after removal from the pregnant cow. Endometrial explants were cultured as previously described in leucine-deficient (1/10 normal concentration) Eagle's Minimal Essential Medium in the presence of L-[3,4,5 ^{3}H-leucine (100 μCi; 155 Ci/mmol) for 24 h under 50% O_2, 45% N_2 and 5% CO_2 *(10,11)*. After culture, medium was treated with the protease inhibitor phenyl methyl-sulfonyl fluoride (0.1 m*M*), and centrifuged at 2000*g* for 20 min to remove debris. Crude medium containing ^{3}H-Leu-labeled secreted proteins was concentrated using a Centriprep-3 concentrator (M_r cutoff: 3000; Amicon), dialyzed (3500 M_r cutoff) against 50 m*M* sodium acetate buffer (pH 4.8), loaded on a cation exchanger (Mono S HR, 5/5 column, Pharmacia) equilibrated in 50 m*M* sodium acetate (pH 4.8), and fractionated using the following gradient of 1*M* sodium acetate (pH 4.8): 0%, 15 min; 0–50%, 50 min; 100%, 10 min (Fig. 1). The fractions containing the 8-kDa protein were identified by using lD-PAGE and fluorography *(11)*.

Polyacrylamide Gel Electrophoresis

Secreted and radiolabeled proteins were lyophilized, reconstituted in Laemmli buffer *(26)*, and separated using lD-PAGE (15%; reducing conditions, sodium dodecyl sulfate [SDS]) *(11,12)*. Gels were stained with Coomassie brilliant blue R-250, impregnated with a fluorographic enhancer, dried, and placed on Kodak XAR film for 11 d at –80°C.

Amino Acid Sequence of the 8-kDa Protein

A PAGE gel slice containing the 8-kDa protein was sent to a protein sequencing laboratory (Dr. Y. M. Lee, UC Davis, CA) where it was digested in-gel with Lys-C (Lysylendopeptidase; Achromobacter protease I from Wako Bioproducts, Richmond, VA) and in solution with trypsin (sequencing grade from Promega, Madison, WI) to yield peptides based on methods described by Kawasaki et al. *(27)*. Briefly, the gel slice was washed extensively with water to remove acetic acid and SDS. The gel was diced to 1–2 mm squares, transferred to a micro centrifuge tube, dehydrated, and then rehydrated in 30 μL of the following rehydration buffer: 0.1*M* Tris-HCI, pH 9.0, 0.05% SDS with 0.5 μg Lys-C. Rehydration buffer was added until the gel was completely rehydrated. The gel was incubated with Lys-C at 30°C overnight. The supernatant was removed and saved. The gel was rinsed twice with water over a 4 h period, and incubated 1 h with enough 0.1% TFA and 80% acetonitrile to cover the gel. All supernatants were collected in the same tube, dried, and resuspended in 25 μL 6*M* guanidine-HCl, 0.4*M* Tris, pH 8.2. The sample was reduced with DTT, and alkylated with iodoacetamide. SDS was precipitated using 0.1% TFA. The second digestion with trypsin was done in solution in a manner similar to that described for in-gel digestion.

Peptides were purified using reversed phase HPLC *(27)*. The peptide digest was injected onto a microbore C18 reversed phase HPLC column (Brownlee C18, 1 mm × 10 cm, Aquapore RP-300; Applied Biosystems, Foster City, CA). After sample injection, the column was washed with 92% solvent A (0.1% TFA)–8% solvent B (70% acetonitrile, 0.075% TFA) for 5 min (0.1 mL per min). Peptides were eluted with a gradient of 8 to 18% solvent B over 10 min, followed by a second gradient of 18 to 60% B over 65 min. Peptides were detected with a UV monitor at 210 nm and submitted for automated amino acid sequencing.

Generation of Polyclonal bGCP-2 Peptide Antiserum

A bGCP-2 peptide amino acid sequence (TPGIHPK-TVSDLQ) was selected based on a relatively high antigenic index and uniqueness in identity compared with other α chemokines. Amino acid sequence identity was determined through computer analysis using the BLAST network server at the National Center for Biotechnology Information. This amino acid sequence was selected to represent a region of GCP-2 that was downstream from the 8-amino acid N-terminal sequence and the ELR sequence that has been implicated in the chemotactic activity and the binding of α chemokines to receptors *(16)*.

The bGCP-2 peptide was synthesized at Multiple Peptide Systems (San Diego, CA) and N-linked through a Cys to KLH. Four wethers were immunized with the conjugated peptide to generate the polyclonal antiserum. Primary and secondary immunizations were done 2 wk apart and consisted of 300 g of conjugate emulsified in 3 mL of complete or incomplete Freund's adjuvant, respectively. Subcutaneous injections (0.25 mL/site) were made over 12 sites on the insides of the rear legs. Sera were collected 14 d following the secondary boost and analyzed for peptide antibodies using an indirect solid-phase enzyme-linked immunosorbent assay (ELISA).

Antipeptide antibodies were detected using unconjugated peptide (antigen) in a criss-cross serial dilution analysis. Antigen (1 μg) in 50 μL of coating buffer (35 n*M* $NaHCO_3$, 27 m*M* Na_2C0_3; pH 9.6) was adsorbed onto Immulon II 96-well, flat bottom microtiter plates. Plates were incubated (overnight, 4°C), washed with phosphate-buffered saline containing 10% Tween-20, rinsed in deionized water, incubated (overnight, 4°C) in 100 μL coating buffer containing 1% bovine serum albumin and washed. Crude GCP-2 peptide antiserum was diluted in 47 mM NaCl/0.5 m*M* EDTA/19 m*M* Tris/0.05% pH 7.4 Tween-20, and added (50 μL) to coated plates. Microtiter plates were incubated at 37°C for 1 h and washed. Rabbit antisheep IgG conjugated to horseradish peroxidase (1/2000 dilution) (Cappel-Organon-Ternika, West Chester, PA) was added to wells (50 μL) for 1 h at 37°C. Wells were washed, sub-

strate was added (50 μL citrate buffer containing 0.0004% hydrogen peroxide and 0.02% 2,2'-azinobis [3-ethylbenzothiozoline-6-sulfonic acid] diammonium salt), and plates were developed for 30 min (room temperature). Fifty microliters of 0.1*N* NaF were dispensed into each well and absorbance was detected using an ELISA plate reader (414-nm filter). Two wethers provided high titer antisera that could be detected out to a 1/10,000 dilution. Crude antiserum was diluted (1/500) and used in lD-PAGE/Western blots to determine if endometrial P8 immunoreacted.

Western Blot of Endometrial Proteins Using bGCP-2 Peptide Antiserum

Endometrial explants from 3 d 14 nonpregnant cows (0 = estrus) were cultured with 0, 0.5, 5, or 25 n*M* rbIFN-τ (3×10^6 IU/mg) as described previously *(12)*. Endometrium from each cow received all IFN-τ doses in vitro. Day 14 of the estrous cycle was selected because it represents a time during which progesterone release from the corpus luteum is not confounded by release of $PGF_2\alpha$, which causes luteolysis and declining progesterone concentrations on days 16–21 *(7)*. Day 14 also is the period during which IFN-τ is first secreted by the expanding blastocyst in the pregnant cow *(6)*. Finally, endometrium on this day of the estrous cycle would not be exposed to conceptus-derived IFN-τ. Thus, proteins released by endometrial explants would reflect those stimulated by addition of IFN-τ in vitro. Proteins released into medium were loaded (100 μg protein) and separated based on size using SDS-PAGE. Proteins were transferred to nitrocellulose membranes (0.22 μ; Micron Separations, Inc., Westborough, MA) in Towbin's buffer (12.5 m*M* Tris, 96 m*M* glycine) at 4°C for 1 h at 100 v. Membranes were rinsed, blocked, and incubated in bGCP-2 peptide antiserum (1/500 dilution). Membranes were washed and then incubated in donkey antisheep IgG conjugated to alkaline phosphatase (1/30,000 dilution, Sigma, St. Louis, MO). After the last wash, AP buffer (100 m*M* Tris-HCl, pH 9.5; 100 m*M* NaCl; 5 m*M* MgCl) containing nitroblue tetrazolium and 5-bromo-4 chloro-3-indolyl phosphate was added for color development (stopped after 15 min with distilled water). Western blots were scanned in the linear range of detection using densitometry as described previously *(12)*. Optical density was analyzed using protected *t*-test.

Affinity of the P8 to a Heparin Matrix

Crude radiolabeled bovine endometrial proteins known to contain P8 were applied to a column of Heparin Agarose (type I, Sigma, 5 mL bed vol) equilibrated with 50 m*M* Tris-HCl, pH 7.4, 50 m*M* NaCl. Proteins were eluted with steps of increasing concentrations of NaCl (up to 1*M*) in 50 m*M* Tris-HCl, pH 7.4. Aliquots (5 mL) of fractions during the step-gradient were dialyzed against 10 m*M* Tris-HCl, pH 7.5, concentrated, loaded (100 μg protein) and separated using lD-PAGE. Gels were fixed, soaked in fluorographic reagent (Amplify, Amersham), dried, and exposed to X-ray film at -70°C for 12 d for fluorography. Gels were transferred to nitrocellulose membranes and detected using bGCP-2 peptide antiserum as described previously for Western blot.

Acknowledgments

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