Purification and Characterization of a Novel, Distinct Isoform of Prostaglandin Endoperoxide Synthase Induced by Human Chorionic Gonadotropin in Granulosa Cells of Rat Preovulatory Follicles*

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To purify and characterize the isoform of prostaglandin endoperoxide synthase (PGS) induced by human chorionic gonadotropin in granulosa cells of rat preovulatory follicles, solubilized cell extracts were subjected to anionic exchange chromatography, column fractions were resolved by onedimensional sodium dodecyl sulfate-polyacrylamide gel electrophoresis, and rPGS was visualized by immunoblotting and silver staining techniques. Immunoreactive rPGS, and peroxidase activity co-eluted at pH 6.5 and 6.0. Amino-terminal amino acid sequence of three immunoreactive rPGS bands (M, = 72,000, 70,000, and 59,000) were identical, M, = 59,000 being a proteolytic fragment. Alignment of the amino-terminal sequences of rPGS, with those of ovine PGS (oPGS) indicated that only 15 of 26 residues were identical (58% identity). In contrast, rPGS, was closely related to the deduced amino acid sequence of PGS-related cDNA clones isolated from chicken and mouse cell lines, with 77% (20/26 residues) and 96% (25/26 residues) identity, respectively. Whereas tryptic digests of oPGS generated fragments of M, = 38,000 and M, = 33,000, only a small peptide appeared cleaved from the carboxyl terminal rPGS. Peroxidase activity of partially purified rPGS, exhibited lower apparent K, and maximal velocity than that of oPGS. Collectively, these results document the existence of a novel rat PGS isofrom (based on purification, enzymatic activity, and amino-terminal amino acid sequence) which is hormonally induced and obligatory for a known biological process, ovulation.

Prostaglandin endoperoxide synthase (PGS) is the first rate limiting step in the synthesis of prostaglandins (PG), prostacyclins, and thromboxanes from arachidonic acid (1–3). The synthase, originally purified from ovine (4, 5) and bovine (6) seminal vesicles, is a homodimer composed of two subunits of about 70,000 daltons and one associated heme group that confers enzymatic activity (7–9). Prostaglandin endoperoxide synthase has two catalytic activities, a cyclooxygenase activity mediating PGG2 formation from arachidonic acid and a peroxidase activity involved in the reduction of PGG2 to PGH2. Because of its key role in the generation of potent inflammatory signals, PGS is the primary target of a large group of nonsteroidal anti-inflammatory drugs, including aspirin, indomethacin, and ibuprofen (10). The cDNA for ovine PGS (oPGS) was the first one to be cloned and used to deduce its full length amino acid sequence (11–13). The protein is made up of 600 amino acids, including a signal sequence of 24 amino acids, and is encoded by a 2.8-kilobase mRNA transcript. The ovine cDNA clones have been used to isolate corresponding cDNAs for mouse (14) and human (15) PGS. Comparisons among deduced amino acid sequences revealed that sheep, mouse, and human enzymes were highly homologous, with approximately 90% of the residues identical (16).

Ovulation, the culminating point of every mammalian reproductive cycle, has been compared to a controlled inflammatory process (17). Several lines of evidence have documented the obligatory role of prostaglandins during the preovulatory cascade leading to follicular rupture. Intrafollicular levels of prostaglandins are markedly increased in several species just prior to ovulation (18–21), and the preovulatory surge of luteinizing hormone (LH) induces the expression of PGS enzyme in granulosa cells of rat preovulatory follicles (22–24). Administration of aspirin or indomethacin, specific inhibitors of PGS, have been shown to block ovulation in mice (25), rats (26–28), rabbits (29,30), pigs (31), and humans (32). Although the precise role of prostaglandin synthesis during the ovulation process is still unclear, its induction appears required for the preovulatory stimulation of ovarian collagenase (33).

Until recently, it has generally been assumed that there was only one gene encoding the PGS enzyme (16). However, increasing evidence has now challenged this latter idea. Using oPGS cDNA probes, Rosen et al. (34) showed that the induction of a 4.0-kilobase message (and not the 2.8-kilobase PGS transcript) was correlated with increased prostaglandin synthase activity in mitogen-stimulated tracheal mucosal cells. Xie et al. (35) recently reported the regulation of a PGS-related gene in chicken embryo fibroblasts (called CEF-147) transformed by Rous sarcoma virus, characterized by a 4.1-kilobase mRNA encoding a 603-amino acid protein. The deduced sequence of this cDNA showed only a 57% identity to the amino acid sequence of oPGS. Similarly, Kujubu et al. (36) recently described a novel PGS mRNA (called TIS-10) induced in mouse Swiss 3T3 cells by forskolin and phosphor esters, which is 3.9 kilobases in size and is highly homologous to the chicken PGS-related transcript reported by Xie et al.
(38). Finally, using different polyclonal antibodies raised in rabbits against oPGS, we have recently provided evidence for the presence of two immunodistinct molecular weight variants of PGS in the rat ovary (37). Each PGS variant is localized to specific cell types and is regulated by different transcriptional/translational controls. For example, one immunodistinct form of PGS (M, 72,000/70,000) was rapidly, but transiently, induced in granulosa cells of preovulatory follicles by LH in vivo and in vitro, whereas the other form (M, 69,000) was constitutively present in other tissues (37). Furthermore, using the mouse cDNA available at that time, the 40-fold induction of PGS enzyme by LH/human chorionic gonadotropin (hCG) in granulosa cells was not associated with an increase in the 2.8-kilobase PGS mRNA transcript (24). Therefore, the general objective of this study was to further characterize the PGS variant induced by LH/hCG in granulosa cells of rat preovulatory follicles (rPGS). The specific objective was to purify rPGS, from rat granulosa cells and determine its amino-terminal amino acid sequence in order to ascertain if this isoform corresponds to a novel PGS enzyme.

**EXPERIMENTAL PROCEDURES**

**Materials**—Hypophysectomized immature female rats were obtained from Johnson Laboratories (Chicago, IL) 1 day after surgery (day 26 of age), and New Zealand white female rabbits were purchased from JoJo Rabbitry (Bandera, TX). Hematin, trypsin type Ix, trypsin inhibitor type III-0, hydrogen peroxide, N, N,N',N'-tetraethyl-p-phenylenediamine (TMPD), diethyldithiocarbamic acid (DEDTC), n-octyl 3-glucopyranoside (octyl glucoside), and 17-estradiol were obtained from Sigma; electrophoretic reagents from Bio-Rad; cyanogen bromide-activated Sepharose 4B from Pharmacia LKB Biotechnology, Inc.; '*25I]Protein A from ICN Biochemicals, Inc.; ovine FSH (NIH-oFSH-16) from the National Hormone and Pituitary Program (Bethesda, MD); hCG from Organon Special Chemicals (West Orange, NJ); purified ovine PGS from Oxford Biomedical Research, Inc. (Oxford, MD); Freund's complete and incomplete adjuvants from Difco Laboratories (Detroit, MI); nitrocellulose filters from Schleicher & Schuell; Problot polyvinylidene difluoride (PVDF) membranes from Applied Biosystems (Foster City, CA); DEAE-Toyopearl 650M resin from Tosoh Bioscience (Philadelphia, PA); Centronic microconcentrators (30,000 and 100,000 M, cut off) from Amicon (Danvers, MA); and Rainbow molecular weight markers from Amersham (Arlington Heights, IL).

**Preparation of Solubilized Cell Extracts**—Ovaries from hypophysectomized (H) and 17-estradiol (E; 1.5 mg/day for 3 days-) and FSH (F; NIH-oFSH-16; 1.0 mg, twice daily for 2 days)-treated rats were collected before (HEF rats) or after an ovulatory dose of hCG (10 IU intraperitoneally, HEF + hCG rats). Granulosa cells and residual ovarian tissue (also containing some remaining granulosa cells) were obtained as previously described (23). Kidneys and uterus were also collected from HEF and HEF + hCG rats. To prepare solubilized cell extracts, tissues were homogenized on ice in TED buffer (50 mM Tris, pH 8.0, 10 mM EDTA, 1 mM DEDTC) containing 2 mM octyl glucoside, and centrifuged at 30,000 x g for 1 h at 4 °C. The crude pellets (membranes, nuclei, mitochondria) were solubilized (8 x cycle, 3 cycles) in TED sonication buffer (20 mM Tris, pH 8.0, 50 mM EDTA, 0.1 mM DEDTC) containing 45 mM octyl glucoside. The sonicates were centrifuged at 100,000 x g for 60 min at 4 °C. The recovered supernatant (solubilized cell extract) was stored at -80 °C until electrophoretic analyses. Protein concentration was determined by the method of Bradford (51) (Bio-Rad Protein Assay, Bio-Rad Laboratories).

**Immunoblot Analyses**—Proteins (50-100 μg) present in solubilized cell extracts or in collected column fractions (see below) were resolved by one-dimensional sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and electrochemically transferred to nitrocellulose filters as previously described (23, 24). Filters were incubated with PGS antibodies diluted 1:10 in TBS (10 mM Tris-buffered saline, pH 7.5) containing 2% nonfat dry milk. Polyclonal antibodies raised in rabbits against purified oPGS were affinity-purified by cyanogen bromide-activated Sepharose beads (Pharmacia) and used to probe PGS as the ligand, as previously described (23). '*25I]Protein A (1 x 10**5** cp/mg/mTBS-2% milk) was used to visualize immunopositive proteins. Filters were washed in TBS-0.05% Tween and exposed to film at -80 °C (Kodak XAR-5, XRP, Eastman Kodak). For quantification purposes, bands from the nitrocellulose filters were excised and counted in a γ counter.

**DEAE-Chromatography and Size Fractionation**—Rat PGS was purified from solubilized cell extracts by anionic exchange chromatography as described by Mvkvh et al. (38), with the following modifications. Extracts from granulosa cells, residual ovarian tissue, or uterus (1-5 mg of protein per column, pH adjusted to 8.5) were loaded onto a 1-ml DEAE-Toyopearl 650M column. Proteins were eluted using a pH step gradient from 8.5 to 3.0, including 12 pH steps: pH 8.5, 8.2, 7.8, and every 0.5 pH interval from 7.5 to 3.0. The elution buffer consisted of 20 mM Tris-HCl, 0.05 mM EDTA, 0.1 mM DEDTC, and 27 mM octyl glucoside. Collected fractions (6 ml/fraction) were concentrated to a final volume of 200-400 μl on 30,000 M, cut off concentrator. All procedures were done at 4 °C. PGS-containing fractions were identified by SDS-PAGE and immunoblot analyses. These modifications of the original protocol were the replacement of Tween 20 by octyl glucoside as the nonionic detergent used in homogenization, sonication, and elution buffers. Tween 20 was initially employed but, because of its nondialyzable nature and the use of microconcentrators to purify the PGS isoforms, the nonionic detergent that interfered with the resolution of proteins on one-dimensional SDS-PAGE. This problem could be completely circumvented when the dialyzable detergent octyl glucoside was selected.

As a second purification step, fractions enriched for the induced form of PGS (rPGS) were pooled and applied to a 100,000 M, cut off microconcentrator prior to electrophoresis. It was hypothesized that rPGS, like oPGS, would exist as a dimer (M, 140,000) under nonreducing conditions and therefore be retained by the concentrator. PGS monomers (M, 70,000) would filter through the 100,000 M, concentrator and could subsequently be recovered on a 30,000 M, cut off concentrator (Fig. 1). Purification was monitored by one-dimensional SDS-PAGE and monochromatic silver staining (40).

**Peroxidase Assay and Tryptic Digestion**—Spectrophotometric analysis of rPGS, peroxidase activity was performed using H2O2 (700 μM) as substrate and TMPD (200 μM) as co-substrate, essentially as described by Raz and Needleman (39). The initial rate of the peroxidase reaction was calculated using an extinction coefficient of 13.5 (μM TMPD) oxidized cm-1 and a stoichiometry of 2 mol of TMPD oxidized/mole of H2O2 reduced (41). The content of holoenzyme and total enzyme (holoenzyme plus apoenzyme) was determined by immunoblotting and silver staining techniques.

**Fig. 1**. Use of microconcentrators to purify the PGS isoform induced by hCG in granulosa cells of rat preovulatory follicles (rPGS). Rat PGS was first purified from solubilized cell extracts by anionic exchange chromatography as described under "Experimental Procedures." DEAE-fractions containing immunoreactive rPGS, were then pooled and applied on a 100,000 M, cut off concentrator. It was hypothesized that rPGS, would exist as a dimer (M, 140,000) and therefore be retained by the concentrator (100,000 M, retentate). The 100,000 M, filtrate was subsequently applied on a 30,000 M, cut off concentrator, to determine the relative amount of PGS monomers (M, 70,000). Purification of rPGS, was monitored by immunoblotting and silver staining techniques.
cubating the sample in the absence or presence of 1 mM hematin, respectively. Apparent $K_a$ and maximal velocity ($V_{max}$) of oPGS and partially purified rPGS were determined by incubating a constant amount of each enzyme (150 ng of rPGS, and 100 ng of oPGS) with increasing concentrations of substrate (0, 44, 88, 175, 350, 700, 1400, and 2170 µM H$_2$O$_2$). The concentration of rPGS, in partially purified preparations was determined by monochromatic silver staining of proteins resolved on one-dimensional SDS gels; the color intensity of rPGS, in the tested sample was compared to that of increasing concentrations of pure oPGS standards. Each sample was assayed in triplicate.

To determine and compare sensitivities of rPGS, and oPGS to trypsin, PGS enzyme preparations (100 ng) were incubated with trypsin (10 ng) for 15 min at 22 °C. Digestion was terminated by the addition of 100 ng of trypsin inhibitor and cooling the reaction on ice. Tryptic fragments were resolved by one-dimensional SDS-PAGE and visualized by immunoblot analyses.

Amino-terminal Amino Acid Analyses—To characterize the amino-terminal amino acid sequence of the PGS enzyme induced in rat granulosa cells by hCG, partially purified rPGS, (from 100,000 M, microconcentrator retentate) was resolved by electrophoresis on a one-dimensional SDS polyacrylamide gel and transferred on a PVDF membrane (ProBlot, Applied Biosystems). The membrane was stained with Coomassie Blue, bands corresponding to immunoreactive rPGS were cut with a razor blade, and amino-terminal microsequencing analyses were performed by Dr. Richard Cook (Dept. of Immunology, Baylor College of Medicine, Houston, TX) using an Applied Biosystems 473A gas-phase Sequencer and following the methodology provided by the manufacturer.

**RESULTS**

Expression of PGS Enzymes in Rat Tissues—The differential pattern of expression of immunoreactive PGS in various rat tissues, as detected by two affinity-purified polyclonal antibodies (9181 and 8223) raised in rabbits against purified oPGS, is shown in Fig. 2. Immunoblots in panels A and B indicate that, although both antibodies had comparable affinities for the primary oPGS antigen, they had different abilities to recognize immunoreactive PGS in various tissues. Antibody 9181 had a strong affinity for the PGS variant induced by hCG in granulosa cells (rPGS). Immunoreactive rPGS appears as a large $M_w$ = 70,000 band and a smaller $M_w$ = 59,000 band believed to correspond to a breakdown product. A qualitatively similar pattern of PGS induction by hCG was also observed in residual ovarian tissue, as a result of granulosa cells remaining in that preparation. However, antibody 9181 had a very limited ability to recognize another isoform of PGS constitutively expressed in kidney and uterus (panel A). Thus, antibody 9181 is similar to antibodies 1 and 2 described previously (37). In contrast to antibody 9181, polyclonal antibody 8223 (previously designated as antibody 3 (37)) had a weaker affinity for the PGS variant induced in granulosa cells by hCG, but exhibited a strong affinity for the immunoreactive PGS detected in residual ovarian tissue, kidney, and uterus (Fig. 2, panel B).

**Purification of rPGS, by DEAE-Chromatography and Size Fractionation—Anionic exchange chromatography was initially used to purify rPGS, present in granulosa cell extracts (Fig. 3, panel A). Most rPGS, eluted in fractions 5 and 6, corresponding to pH 6.5 and 6.0, respectively. A shorter exposure of the same immunoblot (panel A, Fig. 3) revealed that the $M_w$ = 70,000 band (fractions 5 and 6) was composed of two distinct bands (autoradiogram not shown). When the amount of immunoreactive rPGS, present in lane 5 was expressed as counts/min/µg of protein loaded in that lane, and then compared to the amount of rPGS, present in the crude granulosa cell extract (GC lane), it was estimated that a 127-fold purification was achieved by this one-step purification. Similar patterns of elution (fractions 5 and 6) and purification (122-fold) were observed when solubilized cell extracts from ovarian residual tissues were used (Fig. 3, panel B), with rPGS, originating from granulosa cells left in that preparation. The immunoreactive PGS signals detected in fractions 5 and 6 of residual tissue (panel B) appear intense because 4 times as much protein was loaded in those lanes (4 µg/lane in panel B) as compared to granulosa cells (1 µg/lane in panel A).

When extracts from uteri were analyzed using antibody 8223, a different elution pattern was observed with immunoreactive PGS eluting in fraction 13 (pH 4.0; panel C, Fig. 3). No immunoreactive PGS was detected in uterine fractions 5 and 6 when a duplicate blot of panel C was probed with

![Fig. 2. Expression of PGS enzymes in rat tissues.](image-url)

**FIG. 2.** Expression of PGS enzymes in rat tissues. Solubilized extracts were prepared from granulosa cells, residual ovarian tissues, kidney, and uterus of hypophysectomized rats primed with estradiol and FSH, as described under "Experimental Procedures." Tissues were collected 6 h after the rats had received (+), or not received (−), an ovulatory dose of human chorionic gonadotropin (hCG). Proteins were analyzed by one-dimensional SDS-PAGE and immunoblotting techniques using affinity-purified antibodies raised in rabbits against ovine PGS (nPGS). Duplicate blots were probed with antibody 9181 (panel A; 50 µg of protein/lane) or antibody 8223 (panel B; 100 µg of protein/lane). In both panels, oPGS (25 ng) was run as a standard. Arrows on the right indicate migration of molecular weight standards.

![Fig. 3. Partial purification of rat PGS by anionic exchange chromatography.](image-url)

**FIG. 3.** Partial purification of rat PGS by anionic exchange chromatography. Rat PGS was purified from solubilized cell extracts using a DEAE- column and a pH step gradient for elution. Proteins present in the collected fractions were resolved by one-dimensional SDS-PAGE and analyzed by immunoblotting techniques, as described under "Experimental Procedures." A, granulosa cell (GC) extracts were prepared from hypophysectomized rats treated with estradiol, FSH, and hCG (HEF + hCG rats); GC lane (crude extract) = 50 µg of protein loaded; lanes 5, 6, and 7 = 1 µg of protein/ lane. B, solubilized extracts were prepared from residual ovarian tissues (RES) of HEF + hCG rats; RES lane (crude extract) = 100 µg of protein loaded; lanes 5, 6, and 7 = 4 µg of protein/lane. C, solubilized extracts were prepared from uteri (UT) of HEF rats; UT lane (crude extract) = 125 µg of protein loaded; lane 13 = 9 µg of protein loaded. Ovine PGS (oPGS; 25 ng in panels A and B; 50 ng in panel C) was run as a standard. Arrows on the right indicate migration of molecular weight standards. The numbers in parentheses on the left of each immunoblot indicates the antibody used.
antibody 9181 (data not shown). This differential pattern of elution of immunoreactive PGS in granulosa cells compared to uterus further substantiates our previous data (37) and working hypothesis that there is more than one PGS isoform in various rat tissues.

Proteins present in fractions 1 to 8 of panel A (Fig. 3) were silver-stained following one-dimensional SDS-PAGE and are shown in Fig. 4. A protein doublet corresponding to the two closely associated bands that form the \( M_r = 70,000 \) immunoreactive PGS signal (Fig. 3) is shown in lane 5 of Fig. 4 (bracket). The suspected \( M_r = 59,000 \) PGS breakdown product is indicated by an arrowhead (lane 5). These observations are comparable to those of Huslig et al. (22) who have previously reported the purification of PGS from whole ovaries of rats primed with pregnant mare serum gonadotropin and hCG.

A rapid second purification step was performed when rPGS-enriched fractions were pooled and concentrated on a 100,000 \( M_r \) cut off microconcentrator (as described under “Experimental Procedures,” Fig. 1). The upper panel of Fig. 5 indicates that all immunoreactive rPGS, present in the enriched fraction (prior to one-dimensional SDS-PAGE) was retained by the 100,000 \( M_r \) cut off concentrator, indicating that the column-purified rPGS, does not exist in a monomeric form but more likely as a \( M_r = 140,000 \) homodimer (1, 2). This retention pattern of rPGS, was also visualized when a duplicate gel was silver-stained (Fig. 5, lower panel). This rapid step allowed us to purify rPGS, by removing proteins that, under nonreducing conditions, have a molecular weight smaller than 100,000 (proteins that filtrated through the 100,000 \( M_r \) cut off concentrator and were subsequently recovered by the 30,000 \( M_r \) cut off concentrator are shown in lane 4, Fig. 5). Consistently less than 5 to 8 non-PGS proteins (silver-stained bands) were observed in the 100,000 \( M_r \) retentate. Likewise, retention of the \( M_r = 59,000 \) breakdown product by the \( M_r = 100,000 \) concentrator also indicates that the fragment is not a monomer under nonreducing conditions, but is associated with either another \( M_r = 59,000 \) fragment or an intact rPGS, monomer. Therefore, all subsequent experiments with the exception of one (peroxidase activity in collected fractions) were performed using rPGS preparations from a 100,000 \( M_r \), retentate.

**Peroxidase Activity of rPGS, and oPGS**—The amount of peroxidase activity present in eluted fractions from a column loaded with granulosa cell extract is shown in Fig. 6. Peroxidase activity was observed only in fractions containing immunoreactive rPGS, therefore suggesting that rPGS, was the source of the enzymatic activity. Accordingly, when rPGS-enriched fractions were further purified using 100,000 and 30,000 \( M_r \) cut off concentrators (as shown in Fig. 5), peroxidase activity was localized only where immunoreactive and silver-stained rPGS, was detected (i.e. in the 100,000 \( M_r \), retentate, data not shown).

Fully active holoenzyme could be reconstituted by incubating rPGS, or oPGS with hematin. Comparisons of peroxidase activity after incubation in the absence or presence of hematin indicated that 30% of rPGS, was holoenzyme, as compared to 60% for oPGS (Table I). The effect of \( H_2O_2 \) concentration on the initial velocity of the rPGS, peroxidase reaction is shown is Fig. 7 (inset; Michaelis-Menten plot). A Lineweaver-Burk transformation of the Michaelis-Menten kinetic was used to calculate the apparent \( K_m \) and \( V_{max} \) of rPGS, (Fig. 7) and oPGS (plot not shown). Both parameters were lower for rPGS, than for oPGS (Table I).

**Amino-terminal Amino Acid Sequence of rPGS**—To char-
characterize the amino-terminal amino acid sequence of rPGS, proteins from the 100,000 M₀, retentate were resolved by one-dimensional SDS-PAGE, transferred to a PVDF membrane, and stained with Coomassie Blue. Three stained bands were determined for oPGS and rPGS as described in Fig. 7. Pro- teins present in collected fractions were resolved by one-dimensional SDS-PAGE and analyzed by immunoblotting techniques. The up- per panel indicates the amount of immunoreactive PGS (Immu- no PG S) present in the first eight fractions as determined by cutting each Mₒ = 70,000 band and counting them in a γ counter (data are presented as counts/min/μg of protein loaded per lane). The amount of peroxi- dase activity (initial reaction rate; expressed as nanomoles of H₂O₂ reduced/min/μg of protein) present in the same fractions was quan- tified following the procedure of Raz and Needleman (39; lower panel). Each sample was assayed in triplicate (mean ± S.E.).

TABLE I
Characteristics of the peroxidase activity of ovine PGS (oPGS) and of the partially purified hCG-induced rat PGS (rPGS)

<table>
<thead>
<tr>
<th>Initial rate of peroxidase</th>
<th>Kₘ</th>
<th>Vₘₐₓ</th>
</tr>
</thead>
<tbody>
<tr>
<td>nmol H₂O₂ reduced/min/150 ng PGS</td>
<td>μM</td>
<td>nmol/min/150 ng PGS</td>
</tr>
<tr>
<td>rPGS</td>
<td>0.123 (39%)</td>
<td>0.407</td>
</tr>
<tr>
<td>oPGS</td>
<td>0.410 (61%)</td>
<td>0.658</td>
</tr>
</tbody>
</table>

greater than 80% homology observed when comparisons are made between oPGS and the deduced amino acid sequence of mouse and human PGS (Fig. 8). Lastly, the amino terminus of rPGS, had much greater homology to the putative PGS enzymes encoded by the recently cloned cDNAs of chicken and mouse, with 20 (77%) and 25 (90%) of the first 26 residues identical with the deduced amino acid sequence of the chicken and the mouse protein, respectively (Fig. 8).

Tryptic Digests of rPGS, and oPGS—To further document structural differences between rPGS and oPGS, both proteins were treated with trypsin. Incubation of oPGS with trypsin for 15 min at 22 °C generated the previously documented Mᵢ = 38,000 and Mᵢ = 33,000 fragments (40, 42, 43; Fig. 9). In contrast, incubation of rPGS, with trypsin under identical conditions produced only a small downward shift in the major rPGS band (Mᵢ = 70,000), with no effect on the Mᵢ = 59,000 breakdown product (Fig. 9). When the concentration of trypsin used was increased 10-fold, the temperature shifted from 22 °C to 42 °C, and the incubation period prolonged from 15 to 30 and 60 min, a marked increase in digestion of oPGS was observed, but no changes were noted for rPGS, (data not shown).

DISCUSSION
Collectively, the structural, immunological, and enzymatic activity data presented in this study provide conclusive evidence that the PGS induced by LH/hCG in granulosa cells of rat preovulatory follicles corresponds to a novel, distinct variant of the enzyme. This report is also the first to document the isolation of a different PGS isoform, based on its purification from differentiated cells stimulated in vivo and its amino-terminal amino acid sequence analysis. The PGS variant induced by hCG, rPGS, in rat granulosa cells will likely be encoded by the rat homologue of the PGS cDNAs recently cloned as early response gene products in mitogen-stimulated chicken embryo fibroblasts (35) and mouse Swiss 3T3 cells (36).
A Novel Isoform of PGS in Rat Granulosa Cells

Fig. 8. Amino-terminal amino acid sequence analysis of the rat PGS isoform induced by hCG in granulosa cells of preovulatory follicles (rPGSi). Partially purified rPGSi, (100K retentate; see Fig. 5) was resolved by one-dimensional SDS-PAGE, transferred to a PVDF membrane, and stained with Coomassie Blue (blot shown on the left, lane 3). Molecular weight markers (lane 1) and ovine PGS (oPGS, lane 2) were run as standards. Three bands (right arrows) corresponding to immunoreactive rPGS, (including M_r = 70,000 protein doublet and M_r = 59,000 breakdown product) were cut and subjected to amino-terminal amino acid microsequencing. The amino-terminal sequence of bands 1, 2 (first 26 residues), and 3 (first 14 residues) are shown on the right and are compared to the aligned amino acid sequence of oPGS (11-13) and to the deduced amino acid sequence of human (hPGS, 15) and mouse (mPGS, 14). Also included are the aligned deduced amino acid sequence of two novel PGS-related cDNAs recently cloned in chicken (CEF-147, Ref. 35) and mouse (TIS-10, Ref. 36). In all cases, a dash indicates that the amino acid is identical with the corresponding residues in the rat (rPGSi) sequence.

Fig. 9. Proteolytic cleavage of ovine PGS (oPGS) and of partially purified hCG-induced rat PGS (rPGSi). PGS enzyme preparations (100 ng) were incubated in the absence (−) or presence (+) of trypsin (10 ng) for 15 min at 22°C. Digestion was terminated by the addition of trypsin inhibitor (100 ng) and cooling the reaction on ice. Tryptic fragments were resolved by one-dimensional SDS-PAGE and visualized by immunoblot analyses, as described under “Experimental Procedures.” Arrows on the right indicate migration of molecular weight standards.

knowing that this fragment corresponds to the intact amino terminus of rPGSi, we deduced that the small shift observed with the major bands resulted from removal of a small peptide at the carboxyl terminus. It is unlikely that this action of trypsin on rPGSi is caused by the absence of theoretical cleavage sites (Arg-X or Lys-X, where X is any amino acid; Ref. 44) within the protein primary structure, since deduced amino acid sequences of the mouse (TIS-10) and chicken (CEF-147) homologues of rPGSi and oPGS reveal the presence of more than 10 potential trypsin-sensitive sites (35, 36). Protein folding and steric hindrance are more likely involved, as hypothesized for oPGS where only 1 of 57 potential sites is preferentially targeted by trypsin (45).

Measurements of the two catalytic functions of oPGS have shown that the kinetics of the peroxidase reaction (spectrophotometric assay (46)) roughly parallel those of the cyclooxygenase reaction (oxygen electrode assay (46)) although only the latter can be inhibited by nonsteroidal anti-inflammatory drugs (47-49). Thus, the peroxidase activity displayed by partially purified rPGSi, provides preliminary evidence of its functional enzymatic activity. Differences in apparent K_m and V_max, between rPGSi and oPGS may indicate that rPGSi has a higher affinity for the substrate (H_2O_2). However, it cannot be excluded that the presence of a few contaminating proteins in the final rPGSi preparations may affect the kinetics of the peroxidase reaction. Final interpretations should await further analyses in which both cyclooxygenase and peroxidase activities are measured on purified or recombinant rPGSi.

The structural differences between rPGSi and oPGSi now provide a biochemical basis for the differential pattern of expression of immunoreactive PGS in various rat tissues, as detected by different polyclonals (23, 24, 37). Three polyclonal antibodies generated against pure oPGS as the antigen (918 here; 1 and 2 in Refs. 23 and 37) preferentially recognize the PGS variant (rPGSi) which is rapidly, but transiently, induced in granulosa cells of preovulatory follicles by LH/hCG, FSH, or forskolin. Another antibody (8223 herein; 3 in Ref. 37) preferentially detects a PGS isoform which is present, but not induced by LH/hCG or forskolin, in preovulatory follicles, exhibits a more ubiquitous tissue distribution with high levels in kidney and uterus (37) and elutes from anionic exchange chromatography at a lower pH. The latter enzyme is likely the one encoded by the cDNAs (11-15) which recog-
nize the low abundance 2.8-kilobase mRNA transcript in the proestrus rat ovary (24).

Several distinct features of rPGS, expression in the ovary are to be noted. This PGS isoform is only induced in granulosa cells of preovulatory follicles; induction requires elevated concentrations of gonadotropins; expression is rapid but transient (23, 24, 37); and the effects of gonadotropins can be mimicked by gonadotropin-releasing hormone and epidermal growth factor (acting via tyrosine kinase) (52). This pattern of strict cell-specific expression combined with induction by several peptides/agonists is similar to that observed for the chicken egg yolk not only allows one to study the hormonal regulation of the rPGS isoform is only induced in granulosa cells of preovulatory follicles and its transient expression restricted localization of rPGS within the ovary to granulosa cells of the preovulatory follicles and its transient expression by phorbol myristate 1,3-acetate, serum, and epidermal growth factor or by transformation with Rous sarcoma virus (35, 50). Furthermore, in the fibroblasts, there is strong evidence for a role of the cellular tyrosine kinase pp60src (35). Thus, several intracellular pathways appear to converge to regulate the gene encoding this novel PGS enzyme. Understanding the precise regulatory molecules and cis-acting DNA elements involved in the expression of these genes, as well as the role of rPGS, in cell proliferation and differentiation, will be important goals for future studies.

The purification and identification of rPGS, as a novel, distinct isoform of prostaglandin endoperoxide synthase represents an important step toward understanding the role of eicosanoids during the biological process of ovulation. The restricted localization of rPGS within the ovary to granulosa cells of preovulatory follicles and its transient expression following LH/hCG surge, indicate that regulation of the rPGS gene and the half-life of rPGS mRNA are under very stringent transcriptional and post-transcriptional controls. The ovary not only allows one to study the hormonal regulation of this novel prostaglandin endoperoxide synthase in a physiological context (ovulation), but may also serve as a unique model to characterize the role of this new PGS gene during basic inflammatory processes.

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