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Apoptosis-Associated Gene Expression in the Corpus Luteum of the Rat[1]

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ABSTRACT

The involution of the corpus luteum (CL) at parturition is an example of physiological apoptosis, a complex process involving massive vascular regression while luteal cells undergo apoptosis.

In the present study, changes in gene expression associated with physiological apoptosis were examined. Three genes isolated in our laboratory because of their association with apoptotic processes in the ovary, mammary gland, and prostate served as the focus of our investigation: *Y81*, *Gas-1*, and the gene *IAP* encoding integrin-associated protein. *Y81* is a novel gene for which three transcripts are apparent. A *Y81* cDNA clone representing the longest transcript has been isolated; it shows an open reading frame exhibiting a region of very high homology with members of the *frizzled* family, the prototypes of which are cell autonomous polarity genes encoding seven-pass transmembrane receptor proteins, for example the receptor for *Wingless*. *Gas-1* is known as a growth-arrest gene that inhibits DNA synthesis when microinjected into cells. Integin-associated protein is a β_3-integrin-binding protein for which, recently, a thrombospondin-binding activity has been recognized. These three genes, all sharply up-regulated in the course of physiological involution processes in the ovarian CL, in mammary gland, and in prostate, seem promising candidates—by virtue of their specific expression in distinct tissues undergoing programmed cell death—as mediators of stimuli leading to apoptosis and subsequent phagocytosis. In this study, sulfated glycoprotein-2, previously observed in many instances of physiological apoptosis, was further employed as an indicator for incipient apoptosis, and stromelysin was followed as a marker for the tissue remodeling activity that is intimately associated with apoptosis during involution.

INTRODUCTION

The role of the corpus luteum (CL) in providing progesterone during the luteal phase is a transient requirement ending within a few days in the rat, unless pregnancy ensues [1]. The events of this brief time are complex and closely spaced. After follicle rupture, two main developmental lines emerge: 1) extensive vascularization extending inward from the theca interna and 2) proliferation then hypertrophy of the luteal cells [2]. Both pathways are completed in about 4 days during the rat ovarian cycle in forming the CL [3]. The process must be reversed at luteolysis, beginning with deconstruction of the vascular system in the CL while significant hemorrhage is avoided [4]. The luteal cells, serving no further purpose, enter programmed cell death and die by apoptosis [5–7]. In the pregnant rat, the CL proliferates longer (until about 8 days of pregnancy) and then hypertrophies, reaching maximum size and peak progesterone production at about 15 days of pregnancy [2, 3]. Luteolysis (commitment to involution) begins between 16 and 18 days, with parturition at 22 days. The CL achieves much greater size in pregnancy, and the growth kinetics can be more easily defined, than in the ovarian cycle. Thus, for our study of the events and gene expression during CL involution and apoptosis, we have chosen to study in detail the period from about 1 wk prior to parturition until 3 days afterward.

In previous studies of mammary gland involution following forced weaning and ventral prostate involution as a consequence of castration, we have employed differential screening [8] and differential display [9] methods to isolate genes up-regulated specifically in association with apoptosis. A coincidence analysis for apoptosis-associated genes, as opposed to tissue remodeling genes, was evolved based on the low level of tissue remodeling seen with prostatic involution. The principle was that general patterns of apoptosis-specific gene expression could be identified against a background of remodeling and other tissue responses in involution if gene expression in several different organs undergoing the same biological regression process could be compared. Based on the mammary gland and prostate analyses, a set of new genes, previously undetected, or previously described but unknown within the context of apoptosis, was isolated [9].

The purpose of the present investigation was to document lines of similarity with involution processes in different reproductive organs and to solidify our knowledge about the constellation of genes expressed. Since the CL involution process combines vascular remodeling and degradation of basement membrane with the apoptotic cell death of luteal and endothelial cells [4], we monitored genes reflecting both tissue remodeling and apoptosis-associated functions in Northern blots. While stromelysin, as a tissue remodeling marker [10–12], exhibited only very modest up-regulation in CL involution, peaking early in luteolysis, the apoptosis marker sulfated glycoprotein-2 (SGP-2) [11, 13] exhibited little expression in early luteolysis, but expression increased more than 10-fold in CL from postpartum mothers. Finally, in situ hybridization was used to examine the localization of *Y81* and *Gas-1* expression.

MATERIALS AND METHODS

Animals

The ovarian CL and inguinal mammary glands were isolated from female Sprague-Dawley rats at each stage of the

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estrous cycle (as determined by following animals for 5–8 days with daily vaginal smears) and during pregnancy, lactation, and various stages after forced weaning.

Terminal Deoxynucleotidyl Transferase-Mediated dUPT Nick End-Labeling (TUNEL) Reaction for Demonstrating DNA Fragmentation

Tissue samples were fixed in freshly prepared 4% paraformaldehyde in PBS, pH 7.4. Paraffin-embedded tissue (4–5-μm sections) was prepared and treated with 10 μg/ml proteinase K (Sigma Chemical Co., St. Louis, MO) in 20 mM Tris, 1 mM EDTA, pH 8.0, for 15 min at 25°C. Digoxigenin-labeled dUTP (Boehringer Mannheim, Penzburg, Germany) was employed in the terminal transferase reaction according to Gavrieli et al. [14] and modified as described previously [9]. Detection was with alkaline phosphatase-conjugated anti-digoxigenin antibody (Boehringer Mannheim) developed for standard times (10 min or 60 min).

Library Screening and DNA Sequencing

A rat CL cDNA library was obtained from Stratagene (La Jolla, CA). Phage plaque lifts were probed using the differential display Y81 clone [9]. Several independently derived, related cDNA inserts were rescued to pBluescript SK (Stratagene) for sequencing. To obtain the full-length sequences, successive deletions were prepared with the Exonuclease III Erase-a-Base system (Promega, Madison, WI). Cloned fragments were sequenced on both strands using the dideoxynucleotide chain termination method performed with the Sequenase 2.0 kit (United States Biochemicals, Cleveland, OH). Sequence analysis was performed by comparison with the EMBL-Genebank database using the FastA program of the Genetics Core Group (Madison, WI).

In Situ Hybridization

Bluescript (Stratagene) constructs were used with T3 and T7 polymerases to prepare riboprobes in sense and antisense orientations for *Y81*-cl 40 and *Gas-1*. In vitro transcription reactions were performed with fluorescein-labeled UTP using the RNA Color Kit (Amersham International, Little Chalfont, England) according to manufacturer's instructions. After transcription, the probe was fragmented by alkaline hydrolysis to a length of approximately 200 bases. Tissue sections were pretreated as follows: proteinase K (10 μg/ml as above) for 15 min at 25°C, followed by 5-min postfixation with 4% freshly prepared formaldehyde in PBS at 0°C, PBS and water washes, 0.02 M HCl for 5 min, PBS and water washes, and finally acetylation for 10 min at pH 8. Sections were then prehybridized for at least 2 h and hybridized with probes overnight in buffer (Amersham International) containing 50% formamide at 45°C, followed first by washing in 50% formamide/double-strength SSC (single-strength SSC is 0.15 M sodium chloride and 0.015 M sodium citrate) at 45°C and then up to 55°C with 0.1-strength SSC. Alkaline phosphatase-conjugated anti-fluorescein isothiocyanate (FITC) *Fab* Fragments (RNA Color Kit; Amersham International) were finally used for detection.

RNA Extraction and Northern Blot Analysis

Total RNA was prepared using the guanidinium-thiocyanate extraction method described by Chomczynski and Sacchi [15] and selected for poly(A) RNA using oligo(dT)-cellulose (Boehringer Mannheim). Five micrograms poly(A) RNA per sample was denatured with glyoxal, separated on 1% agarose gel, and blotted to nitrocellulose. The results are presented as photographs, without densitometric quantitation. The probes were labeled with [^{32}P]dCTP by random priming. Hybridization probes were *Y81(DDC-4)*, *integrin-associated protein (IAP)*, and *Gas-1* [9], SGP-2 [13], and stromelysin [10].

RESULTS

Involution of the CL following Parturition

After reaching peak progesterone secretion, and before parturition, the CL commits to a programmed cell death of luteal cells by apoptosis, the central phenomenon of luteolysis. Figure 1, a and b, illustrates the striking changes in appearance of identically prepared rat CL tissue sections at Day 14 of pregnancy and 3 days after parturition, respectively. A TUNEL or in situ terminal transferase-catalyzed 3′-end label was employed to demonstrate cells that, in the process of apoptosis, had undergone significant nuclear DNA fragmentation [14]. Figure 1, c–f, shows an example of the TUNEL reaction as applied to CL samples. Only few positive, apoptotic cells were recognizable until 1 day after parturition (Fig. 1, c–e). By 3 days postpartum, more than 50% of the nuclei were positive (Fig. 1f). From the nuclear form and dimensions, positive cells seemed to be mainly luteal cells. Thus, although luteolysis is well established at Day 20 of pregnancy, few positive nuclei were apparent, with hardly more visible at 1 day after parturition. This study of DNA fragmentation was repeated with an independent set of involuting ovarian tissue samples, using both agarose gel electrophoresis to recognize a ladder of fragmented DNA (data not shown) and a different sort of TUNEL reaction performed with FITC-labeled-dUTP (Boehringer Mannheim). All three approaches revealed similar kinetics of DNA fragmentation.

Sections of rat mammary gland tissue in lactation at 3 days after parturition (Fig. 1g), and in involution, 3 days after forced weaning (Fig. 1h), illustrate the results of a TUNEL reaction showing the synchronous character of the apoptosis occurring in the involuting mammary gland.

Gene Expression during Pregnancy and Postpartum Involution

Northern blots were employed to examine the relative levels of expression for a set of genes believed, on the basis of investigation of mammary gland and prostate involution, to be relevant. Figure 2 shows a direct comparison of expression patterns for three new, apoptosis-associated genes previously reported [9]—*Y81*, *Gas-1* [16, 17], and *IAP* [18]—together with SGP-2 [13] and stromelysin [10]. Initial experiments showed that extensive RNA degradation ensued if CL were dissected prior to lysis and homogenization; hence the whole ovaries were homogenized for RNA. Collection of the data presented was repeated twice using these RNA samples and twice with separate tissue pools. The highest expression of *Y81* at Day 20 of pregnancy was confirmed in 4 experiments. Although a poly(A) enrichment was carried out, as in previous studies with involuting organs [8, 9], γ-actin and other housekeeping genes proved poor correlates of RNA amounts for a loading control. This is the case because in the course of apoptosis, the distribution of cell types in the populations changes. Thus, the acridine orange-stained gel was photographed and

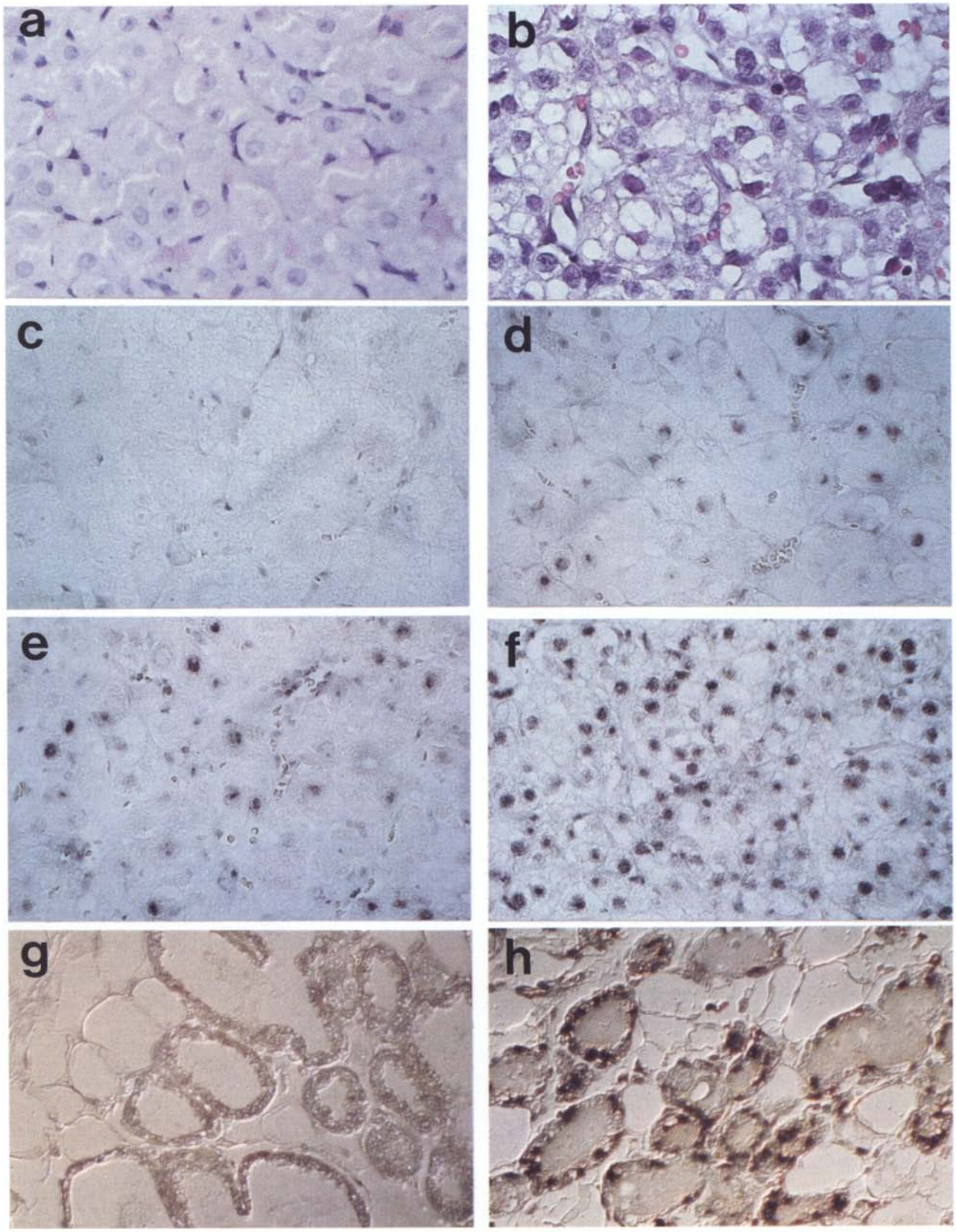

FIG. 1. The morphology of the rat CL: **a**, **b**) Micrographs of 14-day pregnant and 3-day postpartum paraffin sections, stained with hematoxylin and eosin. **c–f**) Results of the TUNEL DNA end-label reaction at 14 (**c**) and 20 (**d**) days pregnant, and 1 (**e**) and 3 (**f**) days postpartum, respectively. This assay was repeated using independent tissue samples of involuting ovary (CL). As a comparable control for the TUNEL reaction, lactating (**g**) and 3-day involuting (**h**) rat mammary gland sections are presented. Magnification ×350 (reproduced at 97%).

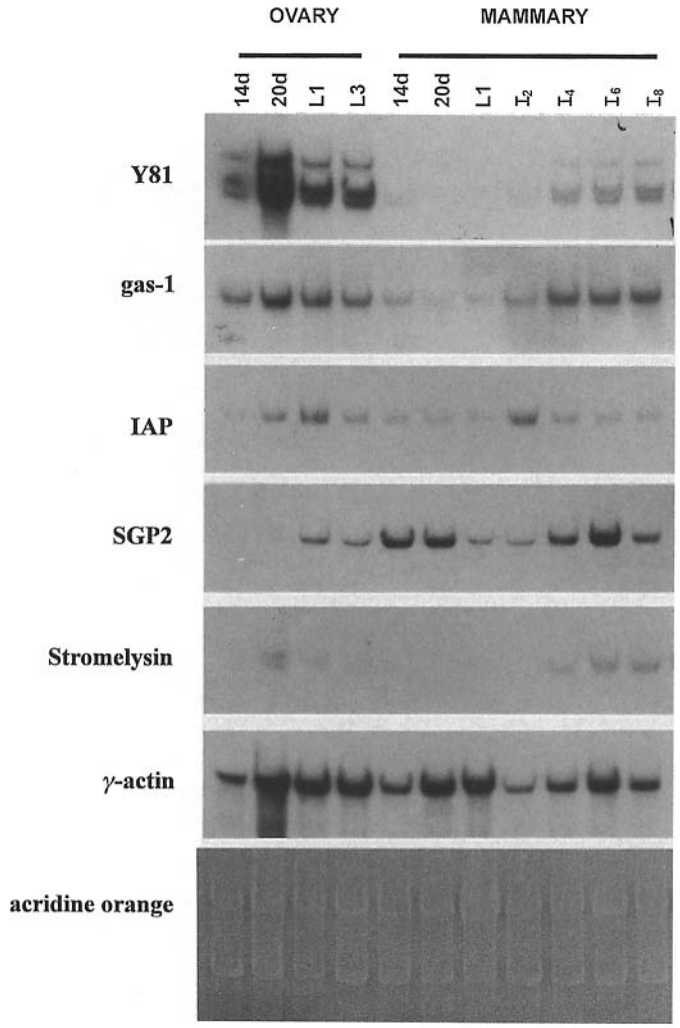

FIG. 2. The expression of *Y81* and various markers for apoptosis is shown for the ovary and the mammary gland at 14 and 20 days of pregnancy and 1 and 3 days postpartum. Northern blots of poly(A)-enriched RNAs are presented. Acridine orange-stained bands of residual ribosomal RNA indicate the relative loading as contrasted to the intensity of γ-actin bands. Samples were pooled from 3 animals for ovarian tissue and for mammary glands. A separate series of experiments in which similar results were obtained was performed with independent pools of 2 animals each.

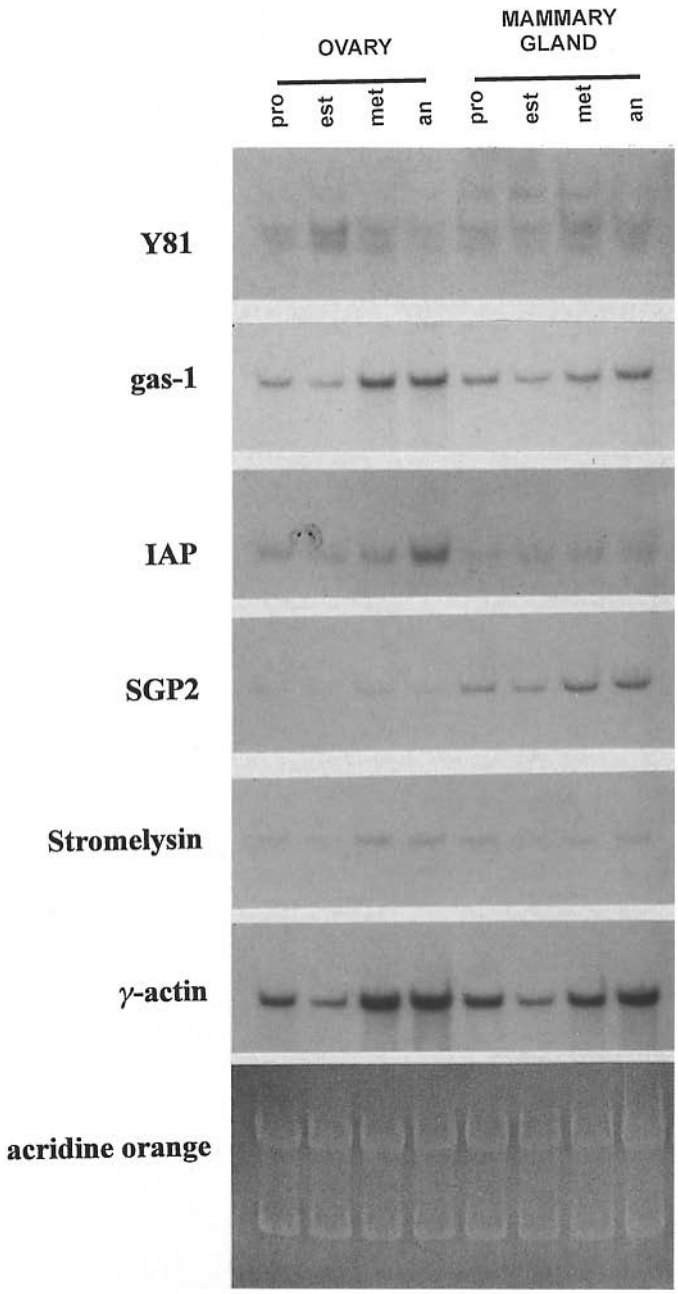

FIG. 3. A Northern blot shows expression of *Y81* and various apoptosis markers at different stages during the estrous cycle. Estrus status was monitored for at least 8 days, daily, with vaginal smears. Results with poly(A)-enriched RNAs are presented as for Figure 2. The RNA samples were prepared from mammary glands and ovaries of 4 animals for proestrus, 3 for estrus, 2 for metestrus, and 4 for anestrus. The results were reproduced in a separate experiment (not shown) with independent pools with the exception of metestrus.

is presented below the γ-actin hybridization result. The results presented are composites of films exposed from two identical blots of the same RNA samples prepared at the same time. As previously recognized [9], the ovary is the richest source for *Y81* even in midpregnancy, presumably owing to residual atretic follicles, which were also detected using in situ hybridization. *Y81* expression in the normal mammary gland or prostate is undetectable, as is the case with all other organs tested except the uterus [9]. During mammary gland involution, *Y81* levels increased > 10-fold. In the ovary, an equivalent rise was apparent, to a very high level seen at Day 20 of pregnancy (Fig. 2).

Gas-1, also exhibiting basal expression in the ovary, showed an increased ovarian expression in the Day 20 pregnant sample as well. The dramatic up-regulation, however, was seen in the involuting mammary gland (Fig. 2). As with *Y81*, *Gas-1* expression in vivo was restricted and was highest in organs of the reproductive system undergoing cyclic programmed cell death [9].

IAP showed peak activity in the ovary sample taken 1 day postpartum, and in the mammary gland 2 days after forced weaning (Fig. 2). Its expression is widespread in vivo but is highest in reproductive organs [9]. SGP-2, a widely used marker for apoptosis in various tissues [13], was up-regulated in a similar manner to *IAP*, significantly in postpartum ovary samples, and was elevated both in pregnancy and in involution with the mammary gland samples (Fig. 2). Stromelysin was expressed in the involuting ovary at levels much like those in the involuting mammary gland, in agreement with its role in an ongoing tissue remodeling process.

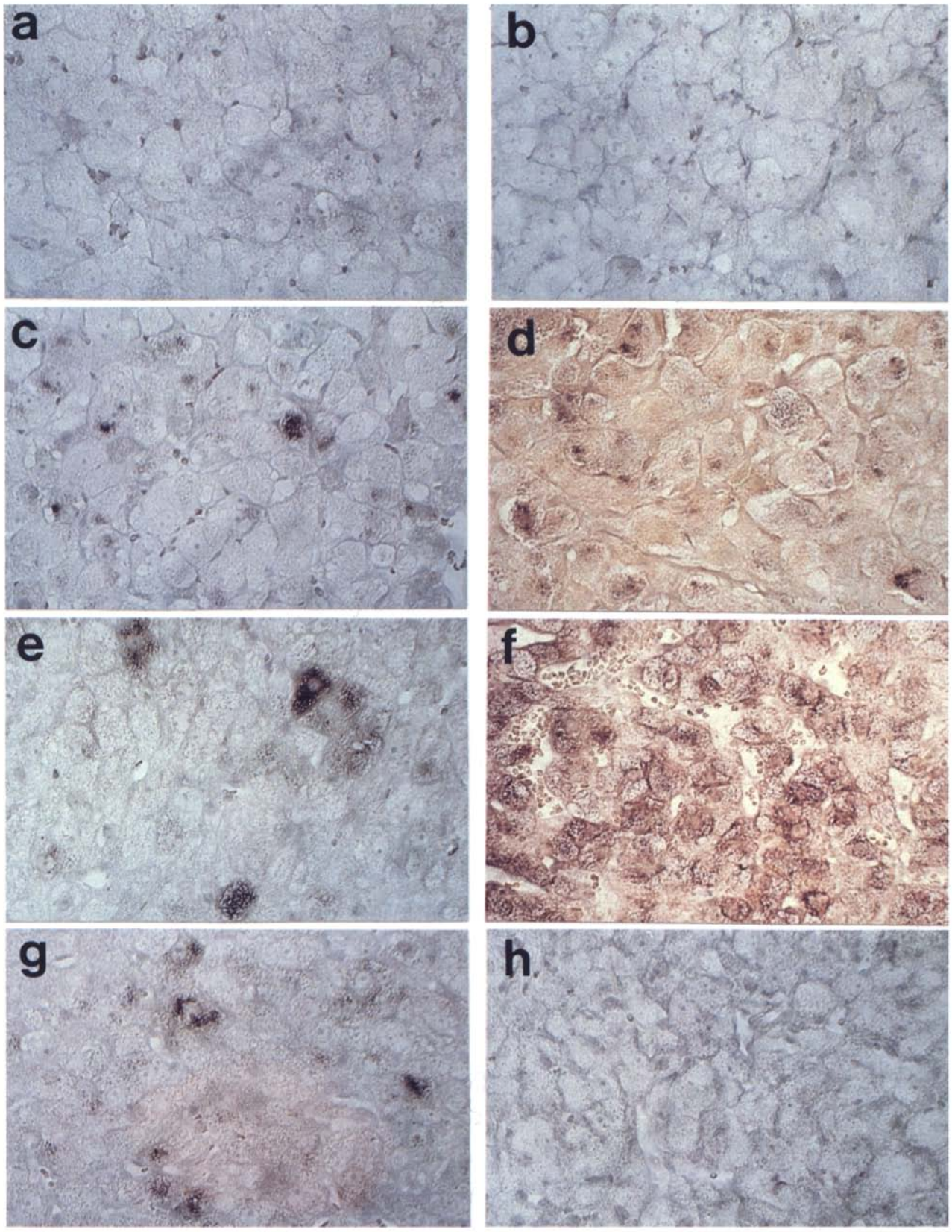

FIG. 4. In situ hybridization with *Y81*-(**a**, **b**, **c**, **e**, **g**, and **h**) and *Gas-1*-specific probes (**d** and **f**), sense (**b** and **h**), or antisense (all others), labeled, hybridized, and detected as described in *Materials and Methods*. Sections presented are pregnant 14 day (**a** and **b**) and 20 day (**c** and **d**) or postpartum 1 day (**e**) or 3 day (**f–h**). Procedures for obtaining these results were repeated twice using the same tissue blocks and probes. In addition, an entirely independent experiment was performed 6 mo later using independent involuting ovary samples and new probes.

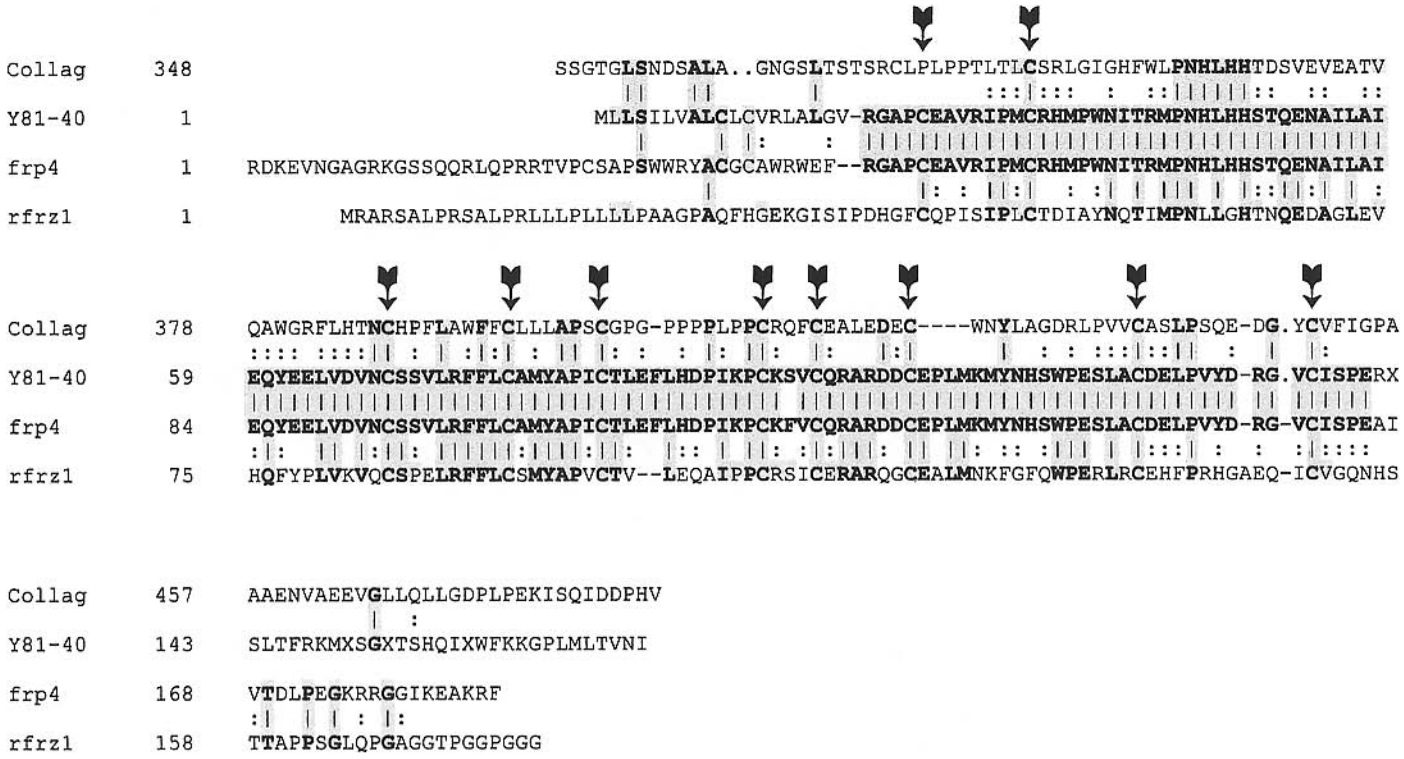

FIG. 5. Sequence comparison of *Y81* to *frizzled* family. Nucleotides indicated in bold are identical to *Y81*, comparing rat *frizzled-1* (*rfrz1*), the transmembranal receptor, and *frp4*, the secreted form, with α1 (XVIII) collagen and *Y81* clone 40. Conserved cysteines are indicated with arrows.

Developmental Gene Expression

Samples of ovary and mammary gland poly(A)-enriched RNA were obtained from animals monitored for estrous cycle status and were investigated using Northern blots (Fig. 3). While *Y81* exhibited a modest increase during the course of estrus, *Gas-1* and *IAP* showed significantly elevated expression in ovarian samples taken at metestrus and anestrus (Fig. 3), after progesterone secretion had collapsed. Hence, these latter express at times of frank luteal cell apoptosis. As seen above during pregnancy (Fig. 2), SGP-2 seems a relatively unsatisfactory apoptosis marker for the CL. Stromelysin, the tissue remodeling marker, indicated remodeling activity known to be present in the second half of the estrous cycle (Fig. 3). In the mammary gland samples, however, little regulation was apparent except in the case of SGP-2. This result presumably reflects the relatively small fraction of cells affected in estrus. This experiment was reproduced once using separate tissue pools, except for metestrus samples, which could be prepared only once from the material available.

In Situ Analysis of Expression

Because both *Y81* and *Gas-1* exhibited an early peak of expression prior to parturition, we investigated the localization of positive cells using in situ hybridization with FITC-labeled riboprobes. Detection was performed with an alkaline phosphatase-conjugated antibody. As illustrated in Figure 4c, *Y81*-positive cells were few on Day 20 of pregnancy, but their expression was extremely intense. At this point (Fig. 4d), *Gas-1* expression was general across the section, but at a much lower level. Even at 1 day postpartum (Fig. 4e), *Y81*-positive cells are were widely spaced. By 3 days postpartum, *Y81* expression was general, but individual, intensely labeled cells dominated (Fig. 4g), while *Gas-1* had reached high levels of expression uniformly in luteal cells across the tissue section (Fig. 4f). The difference in expression between *Y81* and *Gas-1* is responsible for the different background colors: *Y81* hybridizations were developed in only 1 h, while *Gas-1* development was carried out overnight. Thus, the intense expression seen with positive *Y81* cells constituted a very much higher expression. The pattern of expression exhibited by *Y81* was surprising and was unique among the genes we have studied. This experiment was repeated twice using the same tissue samples and probes, and again several months later using independent involuting ovarian samples and probes. Similar results were obtained each time.

Y81 Is a Frizzled Relative

The original *Y81* isolated using the polymerase chain reaction differential display method was only approximately 400 base pairs in length [9]. This isolate had no homology to other sequences in the EMBL GeneBank. Extensive sequencing was performed with *Y81* clone 40, and the resulting sequence was reexamined in an EMBL GeneBank data search. Based on this more extensive sequence, a clear homology to the *frizzled* family of developmental polarity genes could be recognized [19, 20]. The highest homology was to the mouse, rat, and human *frizzled* gene (*Hsfriz*: 74% homology over 350 base pairs). Figure 5 shows the homology at the amino acid level as determined in the SwissProt database between the translated *Y81* clone 40 and the extracellular human α1(XVIII) collagen [21], the mouse secreted *frizzled* ([22]; *frp4*) exhibiting the highest homology to *Y81*, and the rat transmembranal *frizzled* ([20]; *rfrz1*). The amino acid identity between *Y81* and the various *frizzled* relatives is approximately 50% over a region of 115 amino acids, with the exception of *frp4* [22], which is 98%

identical over a region of 124 amino acids. The amino acid homology is in the extracellular region of the *frizzled* protein referred to as the cysteine-rich domain [20, 23], which is also the ligand-binding site [23]. Figure 5 designates with arrows the 10 conserved cysteines characteristic of the *frizzled* family. Apart from the homologous cysteine-rich domain, *Y81* clone 40 is an entirely novel sequence.

DISCUSSION

Two peculiarities make the ovary especially interesting in terms of the sequence of events in programmed cell death: 1) the very large number of cycles that are planned into the lifetime of the organ and 2) the fact that luteolysis formally begins before parturition as defined by up-regulation of certain apoptosis-associated genes, while DNA fragmentation peaks later (Fig. 1). The regular cycling, rising, and/or falling levels of regulating hormones suggest a resonating system of almost mathematical precision. Death of granulosa or luteal cells in the CL is a natural fate at the end of each cyclical period.

The expression of tissue remodeling genes as evidenced by stromelysin-1 is low in CL involution but is maximal before parturition (Fig. 2). In this respect, tissue remodeling of the CL has an earlier onset than in the involuting mammary gland, which exhibits peak expression at 6 days after forced weaning. Strange et al. [11] and Talhouk et al. [12] have established the intimate collaboration between tissue remodeling proteases and the events of programmed cell death in the mammary gland. The role of SGP-2 in programmed cell death is still unclear, but its expression is one of the most consistent correlates of apoptosis [11, 13, 24–26]. SGP-2 is strongly up-regulated during CL involution but peaks after parturition (Fig. 2).

The new isolates detected in our coincidence analysis of apoptosis-associated genes are all well up-regulated between Day 20 of pregnancy and 3 days postpartum. CL expression of *Gas-1* rises early in luteolysis, while *IAP* peaks late in ovarian CL but early in mammary gland involution. Most interesting is the novel gene *Y81*, which shows peak expression early in luteolysis, before parturition (Fig. 2). In the estrous cycle, *Y81* is expressed maximally prior to luteolysis (still at the progesterone-secreting stage), while the other novel genes peak with luteolysis (Fig. 3).

With in situ hybridization, the striking difference between *Y81* and *Gas-1* lies in the distribution of positively expressing cells. Although a section through the CL seems to show a field of identical luteal cells, in late pregnancy the dramatically *Y81*-expressing cells are widely spaced, while the whole field consists of modestly *Gas-1*-expressing cells (Fig. 4). In view of the analogy to *frizzled* and *Wnt* genes discussed below, a possible polarity function or indexing role might account for distributed, strongly positive individual cells. In development, it is quite likely that index cells of particular lineages are formed first, directing later the functional differentiation of their neighbors. However, at present no correlates exist to suggest a distinguishable differentiation of individuals among a field of luteal cells.

Barely a few months after the homology of *Y81* with the *frizzled* gene family [19, 20, 22] was recognized, Bhanot et al. [23] demonstrated that one member of the family, *frizzled-2*, is the receptor for the Drosophila *Wingless* gene product. With this identification, a certain degree of understanding has been achieved in explaining the players in several parallel pathways known to regulate Drosophila development [27]. These pathways in Drosophila manifested themselves in a complex of different polarity mutants affecting especially cuticle and omnitidial development [28]. This pathway is highly conserved, as evidenced by function elicited by the Drosophila *Wingless* gene ectopically expressed in *Xenopus* [29].

Differential regulation of *Wnt* genes has been recognized in the natural development of the mammary gland [30]. In the mouse mammary gland, one has long been aware of the involvement of the *Wingless* homologue called *Wnt-1*, and its family members, in the development of carcinoma [31]. The manifestation of mutations in this pathway is apparent as disturbances in proliferation that can lead to uncontrolled growth.

How does this pathway, i.e., *Wnt-1* ligand acting on a *frizzled-2* receptor [23], relate to a possible role for *Y81* in involution and apoptosis? Firstly, *Y81* exhibits three transcripts, or even possibly three gene family members; clone 40 contains 3′ specific sequences that define the largest mRNA transcript. In vitro translation has shown that clone 40 *Y81* RNA yields a protein product. Secondly, *Y81* shares only approximately 120 amino acids of the cysteine-rich domain with other *frizzled* isolates [20]. In fact, the extensive unique sequence stretch following the cysteine-rich domain of *Y81* apparently does not encode transmembrane domains seen in most other *frizzled* family members (data not shown). Thirdly, it remains to be noted that other *frizzled* relatives exist that encode a cysteine-rich domain with very high *Y81* homology but lack the hydrophobic transmembranal regions, for example, the secreted *frizzled* relatives [22]. All in all, the role of *Y81*, and an explanation for its up-regulation in programmed cell death, remain a topic for speculation. Since the *frizzled* ligand-binding site, the cysteine-rich domain, is the limit of clear-cut homology, we can only speculate that *Y81* might compete for ligand in a functional *frizzled* signal transduction pathway.

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