Anticoagulant heparan sulfate proteoglycans endow the vascular endothelium with antithrombotic properties, but their role outside the vascular bed is unknown. Granulosa cells form an avascular compartment in the ovarian follicle, in which a heparin-like activity has been described. At ovulation extravascular coagulation occurs around ovolatory follicles, and after expulsion of the oocyte, a fibrin clot forms in the antral cavity. Granulosa cells synthesize two major heparan sulfate proteoglycans, whose anticoagulant nature has not been investigated. The purpose of this study was to characterize anticoagulant heparan sulfate proteoglycans synthesized by rat ovarian granulosa cells. Affinity purified 35S-labeled anticoagulant heparan sulfate glycosaminoglycans represent 6.5% of the total heparan sulfate synthesized, and they contain 13% 3-O-sulfated disaccharides that are markers of the antithrombin-binding site of heparin. The biological activity of granulosa cell heparan sulfate proteoglycans was demonstrated by their ability to bind antithrombin and to accelerate the formation of thrombin-antithrombin complexes. The impact of hormonal stimulation on granulosa cell anticoagulant heparan sulfate proteoglycans was studied using 125I-antithrombin binding assays. Follicle-stimulating hormone induced a redistribution of anticoagulant heparan sulfate proteoglycans from the granulosa cell layer to the culture medium, indicating that their distribution could be modulated according to the stage of follicular development. These results suggest that anticoagulant heparan sulfate might be critically located in the follicle to maintain fluidity around the oocyte until its expulsion at ovulation.
Granulosa Cell Antiocoagulant Heparan Sulfate Proteoglycans

**Materials**

- Purified rat FSH was a kind gift from Prof. M. L. Aubert (Division of Biol. Croissance et Reproduction, Geneva University, Switzerland). Human chorionic gonadotropin was from Serono. Diethylstilbestrol was obtained from Asta Medica (Switzerland). Bovine vitronectin, chicken serum, and tissue culture reagents were purchased from Life Technologies, Inc. Cloned rat epididymal fat pad microvascular endothelial cells and a subclone of murine fibroblastic L-cells that produce aHSPGs were kindly provided by Prof. R. D. Rosenberg (Massachusetts Institute of Technology, Cambridge). Purified human thrombin was a generous gift from Dr. J. Fenton (New York State Department of Health). Purified human AT was obtained from Cutter Biological. diethylstilbestrol was as previously published with a specific activity of 5 × 10⁶ cpm/mg (27). Flavobacterium heparinase (EC 4.2.2.8) was purchased from Seikagaku (Tokyo) and chondroitinase ABC from Sigma. Porcine mucosal heparin was from Biosynth Inc (Chicago, IL) and had anti-anticoagulant activity of 169 USP units/mg (28). Heparitinase (EC 4.1.2.8) was purchased from Boehringer Mannheim. Carrier-free Na₂[³⁵S]SO₄ (1500 Ci/mmol) was purchased from DuPont NEN. DEAE-Sephadex and concanavalin-A-Sepharose were from Pharma Biotech Inc. Phe-Pro-Arg chloromethyl potassium was obtained from Calbiochem. All other chemicals used were of the highest grade available.

**Animals**

In vivo stimulation of follicular development was performed according to published procedures (31, 32). Briefly, immature 21-day-old female Sprague-Dawley rats, purchased from Iffa-Credo (L’Arbresle, France), were treated daily by subcutaneous injections of 1 mg of diethylstilbestrol in sesame oil for 4 days to induce granulosa cell proliferation and were sacrificed by decapitation at day 25.

**Granulosa Cell Preparation**

Ovaries were dissected and placed in McCoy’s medium containing 100 units/ml penicillin, and 100 μg/ml streptomycin. Using a dissecting microscope, granulosa cells were released by puncturing the large follicles protruding from the ovary surface with 30-gauge needles according to published procedures (33, 34). After removal of remnant ovarian tissue, granulosa cells were pelleted by centrifugation (70 × g, 20 min) and resuspended in medium, and an aliquot was diluted 1:1 with trypsin to determine viability and cell number in a hemocytometer. One ovary yielded about 1 × 10⁶ viable granulosa cells.

**Granulosa Cells Culture**

Precocating of Culture Dishes—Fetal bovine serum desensitizes granulosa cells to stimulation by gonadotropins (35). Serum-free conditions have therefore been established for granulosa cell cultures, which are used for FSH bioassay (36). In the absence of serum, granulosa cells attach to the culture dish but remain rounded and do not condition that can alter their proteoglycan synthesis (37). To allow granulosa cells to stimulation by gonadotropins (35). Serum-free conditions for granulosa cell cultures, which are used for FSH bioassay (36). In the absence of serum, granulosa cells attach to the culture dish but remain rounded and do not

**Culture Conditions**—Granulosa cells were cultured for 48 h in McCoy’s medium supplemented with 100 units/ml penicillin, 100 μg/ml streptomycin, 2 mM L-glutamine, 0.03% isobutylmethylxanthine, and 2.6 μM 19-hydroxandrostendione (supplemented McCoy’s medium) in humidified 5% CO₂, 95% air at 37°C. For AT-binding studies, granulosa cells were seeded into vitronectin-coated wells at a density of 0.5 × 10⁶ cells/cm² in 96-well plates (Costar) in supplemented McCoy’s media with or without 50 ng/ml FSH and incubated for 48 h. For metabolic labeling with Na₂[³⁵S]SO₄, granulosa cells were seeded into chicken serum-coated wells and incubated for 48 h in McCoy’s medium without MgSO₄ and antibiotics (labeling medium) supplemented with 400 μM Na₂SO₄ and Na₂[³⁵S]SO₄ at a final concentration of 0.25 Ci/mmol as described below.

**Preparative Purification of ³⁵S-HS Chains**

Granulosa cells obtained from 100 rats (about 200 × 10⁶ cells) were metabolically labeled in labeling medium containing 80 μM Na₂[³⁵S]SO₄ (1.25 Ci/mmol) for 48 h as described above; conditioned media and trypsintes were pooled, and ³⁵S-HS was purified in parallel for analysis of HS and chondroitin/dermatan sulfate (CS) distribution, and the purification was followed by liquid scintillation counting. Total ³⁵S-labeled GAG chains were isolated by ion exchange chromatography on DEAE-Sephalocel. The labeled sample was loaded in 150 mM NaCl, 50 mM Tris, pH 7.4; the gel was washed with 10-column volumes of the same buffer followed by 2-column volumes of 150 mM NaCl, 50 mM sodium acetate, pH 5.0, and ³⁵S-GAGs were eluted with 1 M NaCl, 50 mM Tris, pH 7.4. The ³⁵S-GAGs were cleaved from proteoglycans (conditioned media) or peptide stubs (trypsinate) by β-elimination, proteins removed by phenol extraction, and GAGs concentrated by ethanol precipitation (39). The relative content of HS and CS was determined analytically in purified ³⁵S-GAGs as ethanol-soluble ³⁵S counts generated by the respective activities of chondroitinase ABC (0.1 unit/ml, 37°C) and Flavobacterium heparinase (0.5 units/ml, 43°C) as described (39). Digestion by both enzymes quantitatively degraded over 95% of the ³⁵S-GAGs (data not shown). Alternatively, ³⁵S-HS chains were isolated after degradation of CS with chondroitinase ABC (0.1 unit/ml) at 37°C for 6 h, followed by phenol extraction and ethanol precipitation. Completion of CS degradation was ascertained by a second incubation with chondroitinase ABC, which did not release additional degradation products (data not shown).

**Isolation of aHS by Affinity on Immobilized AT**

aHS were isolated from anticoagulantly inactive HEPES (iHS) by AT affinity on concanavalin A-Sepharose as described (40). Briefly, aHS-AT complexes were formed by incubating total HEPES chains with 2.5 μM AT in 150 mM NaCl, 10 mM Tris-Cl, pH 7.4, containing 10 μM dextran sulfate (M, 8000), 002% Triton X-100, and 1 mM of each CaCl₂, MgCl₂, and MnCl₂, for 1 h. The complexes were isolated by suspension of concanavalin A-Sepharose equilibrated in the same buffer; aHS-AT complexes were bound to concanavalin A, and iHS were removed by washing. aHS were eluted from the gel by dissociation of the aHS-AT complexes in buffer containing 1 M NaCl, and aHS and iHS content (aHS and iHS) were quantified by scintillation counting.

**Metabolic Labeling of Granulosa Cells**

Granulosa cells were seeded in chicken serum-coated wells in labeling medium at 0.25 × 10⁶ cells/cm² and incubated for 2 h at 37°C to allow cell attachment, and subsequently incubated for 24 h in labeling medium containing 400 μM Na₂[³⁵S]SO₄ (0.25 Ci/mmol) and supplemented with 50 ng/ml FSH (except in nonstimulated cultures as stated below). The spent medium was collected, and the cells were labeled for an additional 24 h with fresh labeling medium. The spent media were freed from floating cells by centrifugation, boiled at 100°C for 5 min, filtered through a 0.45-μm filter, and kept frozen at −20°C until used. The pooled spent media and cell layer-associated GAGs were released by treatment with 0.05% trypsin, 0.5 mM EDTA as described (9).
Granulosa Cell Anticoagulant Heparan Sulfate Proteoglycans

Using iHS the assay background was determined to be less than 0.2% of input radioactivity.

Molecular Size Determination of HSPG, aHS, and iHS

Sepharose CL-4B—HSPG size distribution was analyzed on a Sepharose CL-4B column (0.9 x 63 cm) eluted in 50 mM Tris, pH 7.4, containing 5 mM guanidine HCl and 1 mM phenylmethylsulfonyl fluoride. The column was calibrated with dextran blue (Pharmacia, Kav = 0) and Na1,PSO4 (Kav = 0.1), and heparin was eluted at Kav 0.75. Seprose 6—The size distribution of granulosa cell total HS, aHS, and iHS was analyzed by gel filtration on a Superose 6 column using a FPLC system (Pharmacia). The column was run in phosphate-buffered saline, pH 7.2, and was loaded with about 15,000 cpm of labeled GAGs. 0.5-ml fractions were collected and GAGs quantified by scintillation counting. The column was calibrated using dextran sulfate M, 500,000 (Kav = 0) and free Na1,PSO4 (Kav = 1).

PAGE—GAGs were electrophoresed on polyacrylamide gradient (5-15%) gels without SDS, in buffer containing 0.1 M NaCl (41, 42) and stained using silver-enhanced azure A (41). GAG molecular weight standards were heparin (M, 16,400), chondroitin sulfate A (M, 21,600), heparan sulfate I (M, 15,000), and heparan sulfate III (M, 7,000) (43). 1 µg of unlabeled GAGs or 35,000 cpm of 35S-HS chains were loaded per lane, and migration profiles of 35S-HS were recorded with a PhosphorImager analyzer (Molecular Dynamics, CA). Modal Rf of GAGs were measured by densitometry scanning of the stained gel or of PhosphorImager analysis of 35S counts. Granulosa cell HS molecular weight was determined by extrapolation from the linear regression of the standard GAGs Rf and log molecular weight.

Glucosamine Content and Specific Activity of 35S-HS

The molar content in glucosamine of purified 35S-HS was determined by HPLC. The samples were hydrolyzed in 2 M HCl for 4 h at 100° C, derivatized with ortho-phthalaldehyde, and analyzed in a C18 HPLC amino acid analyzer. 35S was measured in aliquots of the same samples by scintillation counting, and the specific activity of 35S-HS was calculated in dpm/35S/pmol sulfate.

Disaccharide Analysis

Granulosa cell aHS and iHS were degraded to disaccharides by deacetylation by hydrazinolysis followed by high pH (4.5) nitrous acid and then low pH (1.5) nitrous acid treatments as described previously (1, 44). The disaccharide of sulfated and heparan sulfate was analyzed by reverse-phase ion pairing HPLC on a C18 column (0.46 x 24 cm, Vydac); 0.5-ml fractions were collected, and radioactivity was quantified in a scintillation counter. Similar to the procedure of Guo and Conrad (45), the samples were eluted at 0.5 ml/min with 1 M tetrabutylammonium chloride to preserve their sensitivity to hormone stimulation. To verify that the size of HSPGs synthesized is not affected by the absence of serum in the culture medium, we analyzed the size distribution of HSPGs released by granulosa cells. Granulosa cells were metabolically labeled for 48 h with Na1,PSO4 (0.25 Ci/mmol) as described. 35S-HSPGs were purified from conditioned media by DEAE-Sephadex chromatography, and CS were degraded by chondroitinase ABC treatment. 35S-HSPGs, loaded on a Sepharose CL-4B gel filtration column, eluted as a major peak of HSPGs at Kav 0.4–0.6 and a minor peak of degradation products at Kav 0.8 (Fig. 1). This size distribution is comparable with that reported by Yanagishita and Hascall (22) for HSPGs from granulosa cells cultured in medium con-

125I-AT Ligand Binding Assay

Granulosa cells were seeded in 96-well plates and kept in supplemented McCoys's medium for 48 h prior to the assay. 125I-AT cell binding assay was performed in triplicate wells as described (6, 27). The protein content of control wells was measured using the BCA protein assay (Pierce) and used to normalize the values of bound 125I-AT that were expressed as cpm/mg protein.

Phospholipase C Treatments

Cultured granulosa cells were treated with phospholipase C to release GPI-anchored aHSPGs from the cell surface according to Yagagishita (25, 26). Briefly, the cells were cultured for 48 h as described above and subsequently incubated with 0.4 units/ml phospholipase C in culture medium for 30 min at 37 °C. The supernatant was collected, cleared of floating cells and debris by centrifugation, and assayed for aHSPGs by 125I-AT binding assay. In parallel, cell-bound aHSPGs were assayed before and after phospholipase C treatment by 125I-AT cell binding assay. The amounts of bound cpm found on intact granulosa cells was comparable with the sum of the counts bound to phospholipase-treated cells and to the counts bound to enzyme supernatant, indicating that aHSPGs were quantitatively recovered in this procedure (data not shown).

Statistical Analysis

Comparison of data obtained from cells grown in absence or in presence of FSH was done by Student's paired t test. p < 0.05 was considered significant.

RESULTS

Molecular Size Distribution of Granulosa Cell 35S-HSPGs—Primary rat granulosa cells were cultured in serum-free medium to preserve their sensitivity to hormone stimulation. To verify that the size of HSPGs synthesized is not affected by the absence of serum in the culture medium, we analyzed the size distribution of HSPGs released by granulosa cells. Granulosa cells were metabolically labeled for 48 h with Na1,PSO4 (0.25 Ci/mmol) as described. 35S-HSPGs were purified from conditioned media by DEAE-Sephadex chromatography, and CS were degraded by chondroitinase ABC treatment. 35S-HSPGs, loaded on a Sepharose CL-4B gel filtration column, eluted as a major peak of HSPGs at Kav 0.4–0.6 and a minor peak of degradation products at Kav 0.8 (Fig. 1). This size distribution is comparable with that reported by Yanagishita and Hascall (22) for HSPGs from granulosa cells cultured in medium con-
taining 10% fetal bovine serum, indicating that our serum-free culture conditions do not affect the general size of HSPGs produced by granulosa cells.

Isolation of Granulosa Cell aHS and iHS—AHS and iHS chains were purified from granulosa cell layers and culture media and subsequently fractionated into aHS and iHS according to their affinity for AT as described under "Experimental Procedures." The AHS content of granulosa cell HS chains was of 6.5 ± 0.4% (mean ± S.D., n = 3) of the total HS chains, and iHS constituted 93.5 ± 0.4% of total HS, while the assay background was less than 0.2%. Comparable values were obtained for a control culture condition containing 10% fetal bovine serum, indicating that our serum-free culture conditions do not affect the general size of HSPGs produced by granulosa cells.

Size Distribution of Granulosa Cell 35S-Labeled aHS and iHS—We have examined the molecular size distribution of total 35S-labeled HS, aHS, and iHS from granulosa cells by gel filtration on Superose 6. Fig. 2 shows that total HS, aHS, and iHS have a similar elution profile, with a major peak eluting at Kav 0.52, at a higher molecular weight than heparin which is present in the gel (not shown). Migration profiles were recorded by densitometry scanning, and the modal molecular weight of granulosa cell HS was extrapolated from the linear regression of the RF and the log molecular weight of GAGs standards (log Mm = −1.998 RF + 5.508, r = −0.914). Accordingly, the molecular weight scale shows the migration positions of GAGs extrapolated from this standard curve.

Degree of Sulfation of Granulosa Cell HS—The extent of sulfation of granulosa cell HS chains was measured by determining the molar content in glucosamine of 35S-labeled HS. The ratio of SO3/glucosamine was calculated using the Na2[35S]SO4 specific activity used for labeling granulosa cells, and we obtained ratios of 0.7 and 1.0 SO3/glucosamine in two independent preparations, which gave an average Mm of 58,900 ± 8,400 (42).

2 S. Tumova and K. J. Bame, personal communication.
previous section) are constituted of about 130 disaccharides. Consequently, we derived HS concentrations from glucosamine determinations using a ratio of 130 mol of glucosamine per mol of HS chain. Accordingly, the concentrations of granulosa cell aHS used to accelerate the formation of thrombin-AT complexes illustrated below were calculated to be 0.86 nM (49 ng/ml) for aHS and 7.5 nM (426 ng/ml) for iHS.

Disaccharide Composition of Granulosa Cell aHS and iHS—aHS and iHS were subjected to chemical degradation, and their disaccharide composition was analyzed by ion pairing reverse phase HPLC. 35S-Labeled granulosa cell disaccharides were loaded on the column together with purified 3H-labeled heparin plexes illustrated below were calculated to be 0.86 nM (49 ng/ml) for aHS and 7.5 nM (426 ng/ml) for iHS.

Specific Binding of aHS to AT: Analysis of aHS-AT Complexes by Affinity Coelectrophoresis—The complexes formed between aHS and AT were visualized by affinity coelectrophoresis (Fig. 4). 35S-labeled aHS and iHS from granulosa cells and aHS from endothelial cells were loaded on an agarose gel containing various amounts of AT cast in the gel. The binding of aHS to AT retarded their migration and is observed in aHS from granulosa cells and endothelial cells but not in iHS. Granulosa cell aHS formed complexes with AT at 30 and at 500 nM, with similar degrees of retardation as endothelial cell aHS, suggesting that the affinity of aHS from both cell types to AT is similar. Moreover, aHS from both cell types behaved homogeneously in the presence of AT, without smearing of the bands between the retarded and nonretarded positions, suggesting that all AT-binding species bound with high affinity.

Functional Activity of Granulosa Cell aHS in Accelerating the Formation of Complexes between Thrombin and AT—The biological activity of granulosa cell aHS, namely the activation of AT increasing its reactivity toward thrombin, was demonstrated in a purified system by SDS-PAGE. Thrombin (50 nM) was incubated for various times with 125I-AT (2 nM, 0.5 × 10⁶ cpm/ml) in the absence or presence of GAGs, and the products of the reaction were analyzed by SDS-PAGE. At time t = 0, AT migrates as a single band of M₅₈,000 (Fig. 5A, lane 1). In control samples, incubated in the absence of GAGs, the formation of high molecular weight thrombin-AT complex occurs slowly, and only trace amounts are visible after 10 min of incubation (Fig. 5A, lanes 2–4). When thrombin and AT are incubated in the presence of granulosa cell aHS (0.86 nM, 49 ng/ml), the appearance of thrombin-AT complex is accelerated; the complex is present after 2 min (14%), and its amount is moderately increased after 10 min (16%) (Fig. 5A, lanes 5–7). Together with the formation of high molecular weight complex, an additional low molecular weight band appears, migrating under native AT, which corresponds to the cleaved form of AT (modified AT) (49). In contrast, granulosa cell iHS (7.5 nM, 426 ng/ml) do not accelerate the formation of thrombin-AT complex (Fig. 5A, lanes 8–10), and the pattern observed is identical to that of control samples without GAG added. In order to illustrate the anticoagulant activity of granulosa cell HS, we have incubated thrombin and AT with the aHS and iHS obtained from 123,000 granulosa cells, and therefore, the amounts of iHS (8.5 ng) used in these experiments were about 10-fold higher than that of aHS (1.0 ng). To estimate the anticoagulant
activity of granulosa cell aHS, we quantified the fraction of 
\( 125^I \)-AT present as thrombin-AT complex formed after 2 min of incubation by densitometry (Fig. 5B). In control conditions, 
without GAG added, no thrombin-AT complex was detectable (lane 1), whereas in the presence of heparin (120 ng/ml, 7.3 nM, 
14 \times 10^{-3} \text{ USP units/ml}) 6% of the AT was complexed to 
thrombin (lane 2). When granulosa cell aHS (49 ng/ml, 0.86 nm) 
was added to the reaction mixture 14% of the protease inhibitor 
was present as high molecular weight complex (lane 3), but in 
the presence of granulosa cell iHS (426 ng/ml, 7.5 nM) complexed 
\( 125^I \)-AT was at the limit of detection (0.14%) (lane 4). These data 
demonstrate that granulosa cell aHS are biologically active and 
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Having demonstrated the synthesis of aHSPGs by rat ovar-
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stimulations on granulosa cell HSPGs. First, we analyzed the 
total \( ^{35} \text{S} \)-labeled GAGs synthesized by granulosa cells and com-
pared their composition in HS/CS under basal conditions and 
after stimulation with FSH. Second, we used analytical detect-
on of aHSPGs by \( 125^I \)-AT binding assays, on cell surfaces and 
and soluble aHSPGs released in the culture media, to further 
analyze the effect of FSH on granulosa cell aHSPGs.

HS/CS Composition of Cell-bound and Soluble Granulosa 
Cell GAGs: Effect of FSH Stimulation—We compared the dis-
tribution of total \( ^{35} \text{S} \)-labeled cell-bound and soluble HS and CS 
in granulosa cells cultured for 48 h in the absence or presence 
of FSH. The results from two independent preparations of 
granulosa cell \( ^{35} \text{S} \)-GAGs show that cell-surface \( ^{35} \text{S} \)-HS repre-
sent 33.5 \pm 2.9% (mean \pm S.D.) of the total \( ^{35} \text{S} \)-GAGs under 
basal conditions and that this proportion does not vary after 
FSH stimulation (31.3 \pm 2.1%). In contrast, \( ^{35} \text{S} \)-HS constitute 
22.2 \pm 4.5% of the soluble \( ^{35} \text{S} \)-GAGs under basal conditions 
and are increased to 32.8 \pm 3.2% after stimulation by FSH. 
These data indicate that FSH induces granulosa cells to in-
crease the proportion of HSPGs present in the total pool of 
proteoglycans they release into their culture medium. To spe-
cifically examine the effects of FSH stimulation on granulosa 
cell aHSPGs, we used analytical \( 125^I \)-AT-binding assays.

Specificity of \( 125^I \)-AT Binding to Granulosa Cell aHSPGs— 
Analytical detection of granulosa cell aHSPGs was achieved on 
granulosa cell monolayers and in granulosa cell conditioned 
media. Cell-surface aHSPGs and nitrocellulose-immobilized 
soluble aHSPGs released by granulosa cells in culture were 
revealed by \( 125^I \)-AT- and ligand-binding assays, respec-
tively (6, 27). We have observed that aHSPGs are present on 
cell surfaces and in the culture media of rat primary granulosa 
cells, in amounts comparable with endothelial cells. In a typical 
experiment, we obtained 35,600 \pm 4,000 cpn/\( 10^6 \) cells bound to 
cell-surface aHSPGs and 9,860 \pm 1,530 cpn/\( 10^6 \) cells bound 
to soluble aHSPGs (mean \pm S.D.). In parallel incubations a control 
endothelial cell line gave values similar to granulosa cells 
(13,140 \pm 1,030 and 10,950 \pm 390 for cell-surface and soluble 
aHSPGs, respectively) while a fibroblastic cell line that does 
not produce aHSPGs gave background values.

The specificity of \( 125^I \)-AT binding on granulosa cell aHSPGs 
was verified by incubations of granulosa cell monolayers with 
\( 125^I \)-AT in the presence of competitors. Average values of three 
independent experiments showed that excess unlabeled AT (1 
\mu M) competed with \( 125^I \)-AT and reduced \( 125^I \)-AT binding by 
86 \pm 8% (mean \pm S.D.), while heparin (10 \mu g/ml) reduced it by 
84 \pm 8%. Pretreatment of the cell layers with heparitinase (0.5 
units/ml) for 60 min at 37 \degree C prior to the incubation with 
\( 125^I \)-AT prevented its binding, and only 12 \pm 8% residual bind-
ing was observed. In contrast, a similar treatment with chon-
droitinase ABC (0.1units/ml) did not alter \( 125^I \)-AT binding 
(82 \pm 17%). These values are very similar to those obtained for 
endothelial cells (6). 

Thus, granulosa cell aHSPGs can be measured by \( 125^I \)-AT 
binding to surface-associated aHSPGs and to soluble aHSPGs. 
These assays were used to study the effects of hormonal stimu-
lution of granulosa cells on their aHSPGs production.

Stimulation of Granulosa Cells by FSH: Effects on aHSPGs— 
Granulosa cells were cultured with or without 50 ng/ml FSH 
for 48 h. At the end of the incubation, aHSPGs were evaluated 
in conditioned media by \( 125^I \)-AT ligand-binding assay and on 
the cell monolayers by \( 125^I \)-AT cell-binding assay. Alterna-
tively, control wells for each condition were used to measure 
the levels of estradiol secreted in the media and the cell’s 
protein contents. The results of eight independent experiments 
are presented in Fig. 6. The effectiveness of FSH stimulation 
was verified by measuring the increase in estradiol secretion. 
The results show that granulosa cells respond to FSH stimu-
lation by increasing their estradiol secretion (Fig. 6A); in the 
influence of FSH on granulosa cells increase the amounts of soluble 
aHSPGs they release in the medium (Fig. 6B), and they 
comitantly decrease their cell surface-bound aHSPGs (Fig. 
6C). Statistical analysis showed highly significant differences 
between stimulated and nonstimulated conditions for soluble 
and cell-bound aHSPGs (Student paired t test, p < 0.01). When 
the partition between cell-bound and soluble aHSPGs was 
examined using normalized values, about 77% aHSPGs were present 
on the cell surfaces and 23% in the media, under basal 
conditions. In contrast, in FSH-stimulated conditions approxi-
respectively), despite the fact that the total aHSPGs were de-
synthesized by ovarian granulosa cells, which constitute an
cells were incubated with phospholipase C.
Partitioning of aHSPGs between cell-surface and culture media
were released with phospholipase C. We quantified cell-bound
stimulated conditions—Granulosa cell GPI-anchored HSPGs
of the cell surface aHSPGs and were quantitatively recovered
basal and FSH-stimulated conditions (16
lipase C from granulosa cell surfaces did not vary between
sense of FSH. The proportion of aHSPGs released by phos-
was released from the cell
and of GPI-anchored aHSPGs present on granulosa cells in
and therefore to their differentiation stage.

The presence of aHSPGs has been reported in the vascular
bed (6, 50–54), and structural analysis of aHSPGs has been carried
for endothelial cells (1, 47), nonvascular cell types like
fibroblast and epithelial cell lines (8, 9), and for Reichert’s
embryonic membrane (2). We set out to characterize the aHS
synthesized by primary granulosa cells to unequivocally dem-
strate their presence in an extravascular compartment. We
used serum-free culture conditions, suitable to induce granu-
losa cell differentiation by FSH stimulation, and have shown
that under these conditions the size distribution of HSPGs is
similar to that reported by Yanagishita and Hascall (22) for
granulosa cells grown in the presence of serum and to those
reported for HSPGs from endothelial and fibroblastic cell lines
that synthesize aHSPGs (27, 47). We next isolated 35S-labeled
HS chains from granulosa cells stimulated by FSH, a condition
known to increase the biosynthesis of proteoglycans (17, 55).
Granulosa cell aHS GAG chains were fractionated according
to their AT affinity and represented 6.5% of the total HS
chains, a value comparable with that found in various types of
endothelial cells (1, 47, 48). aHSPGs, constituting about 1%
of bovine aortic endothelial cell HSPGs, were found to be abun-
dant in situ in the aortic wall as evidenced by 125I-AT binding
(1, 6). By analogy it seems that granulosa cells synthesize aHSPGs in
sufficient amounts to justify a biological activity in vivo.

The size distribution of aHS chains was similar to that of the
general HS population, and extensive size polydispersity was
apparent both on gel filtration and on PAGE. The modal M, of
granulosa cell HS was about 57,000, and this value was con-
firmed using two independent sets of GAG molecular weight
standards (42). This modal chain size is higher than the M, of
30,000 estimated by Yanagishita and Hascall (56) using gel
chromatography, and this discrepancy might be due to differ-
ences in the methods used for molecular weight determination.
However, chain sizes of 30,000 were also encompassed in the
population of chains resolved by PAGE. In parallel, we deter-
minalized that granulosa cell HS disaccharides contain about 0.85
sulfate/disaccharide, and we calculated that granulosa cell HS
contain approximately 130 disaccharides per GAG chain. For
comparison, mouse L cells were shown to produce HS contain-
ing about 0.45 sulfate/disaccharide with a modal M, of 53,000.
Moreover, Reichert’s membrane HS, which are constituted pre-
dominantly of aHS chains, were estimated to contain a sulfate/
disaccharide ratio of 1, whereas basement membrane HS from
EHS cells were mostly nonsulfated with a sulfate/disaccharide
ratio of about 0.25 (2). Therefore, granulosa cells seem to pro-
duce relatively highly sulfated HS species (0.85 sulfate/disac-
charide), resembling those of Reichert’s membrane aHS, rather
than HS from established cell lines.

The pentasaccharide constituting the AT-binding site of hepa-
rin and aHS contains a cardinal 3-O-sulfated glucosamine
essential for AT binding. We found that granulosa cell aHS
contain markedly increased amounts of 3-O-sulfated disaccha-
drides (13%) as compared with mHS (1%). These values are com-
patible with those reported for other aHS from endothelial cells
(1, 47), from fibroblast and epithelial cell lines (8, 9), and from
Reichert’s embryonic membrane (2). In these cells, aHS 3-O-

avascular compartment in the ovarian follicle in vivo. The aHS
GAG chains synthesized by rat ovarian granulosa cells constit-
tute 6.5% of their total HS, and aHS biological activity was
shown by their ability to specifically complex AT and to ac-
celerate the inactivation of thrombin by AT. Stimulation of aHSPGs
with FSH altered the distribution of aHSPGs and favored the release of soluble aHSPGs in culture
media, suggesting that granulosa cells are able to regulate the
localization of their aHSPGs according to the hormonal context
and thus to their differentiation stage.

DISCUSSION
The data presented in this study provide compelling evidence
that considerable amounts of biologically active aHSPGs are
synthesized by ovarian granulosa cells, which constitute an

Fig. 7. Total cell-bound aHSPGs and GPI-anchored aHSPGs in
basal and FSH-stimulated conditions. aHSPGs were assayed on
granulosa cell surfaces before and after phospholipase C treatment by
125I-AT cell-binding assay. GPI-anchored aHSPGs, released by phos-
philase C, were recovered quantitatively and assayed by 125I-AT
ligand binding. Values presented are normalized to total cell-bound
aHSPGs in cultures performed in the absence of FSH and are mean
values from three independent experiments (error bars, ± 1 S.D.). Open
bars, unstimulated cells; hatched bars, cells stimulated by FSH (50
ng/ml).
of high affinity heparin (2.4 nM) was comparable with that of the heparin chains have high affinity for AT, the concentration of granulosa cell aHS appear to contain substantial amounts of 3-O-sulfated disaccharides.

Complex formation between aHS and AT retards the migration of aHS in coelectrophoresis according to the protease inhibitor concentration in the gel (46, 47). Granulosa cell aHS, like endothelial cell aHS, were markedly retarded by the presence of AT in the gel, showing the formation of aHS-AT complexes. Both types of aHS behaved homogeneously, suggesting that all molecules bind to AT with high affinity, in agreement with published data on endothelial cell aHS coelectrophoresis reporting a single class of high affinity binding sites for AT (47). For comparison, in the presence of fibronecrtin, heparin was found to become broadly smeared, as some heparin molecules were bound weakly and others were bound strongly (57).

Having shown that granulosa cell aHS form complexes with AT similar to those formed by endothelial cell aHS, we next demonstrated the functional anticoagulant activity of granulosa cell aHS by their ability to increase the reactivity of AT toward its target enzyme thrombin. Thrombin inhibition by AT results in a stoichiometric thrombin-AT complex in which both the enzyme and the inhibitor are inactivated. In addition, thrombin sometimes escapes inhibition by cleaving AT at its reactive site without being trapped in a complex, thereby generating modified inactive AT. Thus AT can be either an inhibitor or a substrate of thrombin, and the amount of cleaved inhibitor produced is markedly increased in the presence of heparin (49, 58, 59). We observed that, like heparin, granulosa cell aHS accelerate the formation of thrombin-AT complexes as well as the cleavage of AT in its modified form. In the presence of granulosa cell aHS, the amounts of AT recovered in thrombin-AT complexes (14%) exceeded that obtained in the presence of heparin (6%). The limited amounts of purified primary granulosa cell aHS available prevented us from performing additional experiments to determine more precisely their anticoagulant activity. However, we carried out a conservative estimate of the minimum anticoagulant activity of granulosa cell aHS using the known values for heparin. The concentration of granulosa cell aHS and heparin used in these experiments was 0.86 and 7.3 nM, respectively. Considering that about one-third of the heparin chains have high affinity for AT, the concentration of high affinity heparin (2.4 nM) was comparable with that of granulosa cell aHS. We therefore conclude that the anticoagulant activity of granulosa cell aHS is comparable with that of high affinity heparin (about 8 USP units/mg). Similar calculations showed that microvascular endothelial cell aHS have an anticoagulant activity of 1.4 USP units/mg (47). We have estimated the anticoagulant activity of aHS recovered from granulosa cell cultures to be at least 2.3 \times 10^{-3} USP units/10^6 cells (by comparison with heparin), a value certainly underestimated since we did not correct for losses during purification. For comparison, bovine aortic and rat microvascular endothelial cells were found to have about 1 \times 10^{-3} and 5 \times 10^{-3} USP units/10^6 cells, respectively (1, 47). In addition, Andrade-Gordon and collaborators (21) have reported a heparin-like activity of about 1 \times 10^{-3} USP units/10^6 cells in rat granulosa cells. These authors used granulosa cell layers as a source of HS chains, while we purified aHS from both the cell layers and the culture medium, which explains our higher estimate of granulosa cell aHS anticoagulant activity. This suggests that granulosa cells might release aHS into their culture media and is in agreement with the observation that porcine follicular fluid contains heparin-like activity (21). Altogether, it appears that ovarian granulosa cells are endowed with considerable anticoagulant potential.

We further examined the effects of stimulation by FSH on the distribution of granulosa cell HSPGs and aHSPGs between cell layer and culture medium. We first analyzed total 35S-labeled GAGs purified from granulosa cell surface and culture medium and found that the percentage of 35S-labeled HS as compared with CS released in the culture medium was higher (33%) after FSH stimulation than under basal conditions (22%). These results are in agreement with data from Yanagishita and collaborators (18, 33), who reported that granulosa cells release 90% dermatan sulfate proteoglycans and 10% HSPGs, while after stimulation by FSH and insulin-like growth factor 1 soluble HSPGs represent 29% of the soluble proteoglycans. FSH seems, therefore, to increase the proportion of HS in the GAGs accumulating in granulosa cell culture medium. In a second step, we analyzed the effects of FSH stimulation on aHSPGs present on granulosa cell monolayers and in their culture medium by 125I-AT binding. These experiments demonstrated that FSH induces a decrease in cell-bound aHSPGs and a concomitant increase of the aHSPGs present in the culture medium, suggesting that FSH increases the amounts of aHSPGs released from the cell surfaces into the culture medium. This observation in vitro would correspond in vivo to a release of aHSPGs toward the follicular fluid during the FSH-induced maturation of the ovarian follicle.

Levels of aHSPG expression can be modulated in vitro and vary according to the circumstances in vivo. Increased expression of HSPGs by transforming growth factor-β in cultured endothelial cells (60), and overexpression of the HSPG ruydican in transfected fibroblastic cells (40), both result in a decrease in the percentage of aHSPGs. Homocysteine by altering the redox potential seems to specifically alter aHSPG production by endothelial cells (61). Moreover, the characterization of a cell mutant specifically defective in aHSPGs suggests the presence of a regulatory component responsible for the assembly of the specific AT-binding oligosaccharide sequence in HSPGs (9, 62). Together, these data indicate that the expression of aHSPGs could be specifically regulated, independent of the general synthesis of HSPGs. We demonstrated in a previous study (27) that the distribution of aHSPGs between cell surface and culture medium varies between fibroblastic and endothelial cells, but this is the first report to show the modulation of aHSPGs distribution according to the functional state of primary cells.

Detailed metabolic studies by Yanagishita and Hascall (26, 56) demonstrated that granulosa cells recycle their cell surface HSPGs mainly by internalization. Under these conditions GPI-anchored HSPGs are exclusively internalized, whereas 70% of the membrane-spanning HSPGs are internalized, and 30% of these latter species are released into the culture medium supposedly by proteolytic cleavage of their core proteins (26). We tested whether or not aHS chains are present on GPI-anchored HSPGs and if the release of aHSPGs in culture medium induced by FSH involves GPI-anchored aHSPGs.

Under basal conditions about 16% of granulosa cell-bound aHSPGs were released by phospholipase C, demonstrating that aHS are attached to GPI-anchored HSPG core proteins in amounts comparable with those reported for the general population of HSPGs (25). The aHSPGs detected in granulosa cell culture media are presumably released from cell-surface aHSPGs. Phospholipase C released a similar proportion of aHSPGs from granulosa cells cultured under basal and FSH-stimulated conditions. These results indicate that the 40% decrease in total cell-bound aHSPGs observed in FSH-stimulated granulosa cells does not involve GPI-anchored aHSPGs but is probably due to a decrease in membrane-spanning
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...with the FSH receptor, seem promising candidates for such experiments.

Proteolytic enzymes might also be involved in the regulation of the release of aHSPGs from FSH-stimulated granulosa cells. The identity of these proteases is not yet established, and the presence of conserved basic amino acids in the C-terminal portion of the extracellular domains of HSPGs from the syndecan family suggests that tryptic proteases might be involved in this mechanism (64). In addition, plasmin has been shown to release bFGF-HSPG complexes from endothelial cells (65), and leupeptin (an inhibitor of thiol proteases and of some serine proteases including plasmin) was found to decrease the shedding of HSPGs from granulosa cells into the medium (23). We tested whether serine proteases were involved in the release of aHSPGs from granulosa cells under basal or FSH-stimulated conditions. We observed that the tryptic inhibitor aprotinin did not affect the release of aHSPGs in granulosa cell culture medium under basal or FSH-stimulated conditions (data not shown). These observations suggest that other types of proteases might be responsible for the release of aHSPGs. Experiments are currently underway in our laboratory to investigate this matter.

Data available concerning the localization of AT in tissues are limited and contradictory. Plasma AT is synthesized mainly in the liver, but small amounts of AT mRNA are also detected in the kidney and in endothelial cells (66). In the vessel wall, aHSPG-bound AT constitutes a natural anticoagulant mechanism protecting the vasculature against thrombosis (67). Immunocytochemical studies have shown that endogenous AT is detected only in association with vascular endothelial cells and their underlying matrix (6, 13, 51), thereby following the distribution of aHSPGs on the vascular endothelium of various tissues. On the other hand, kinetic radiotracer studies suggest that AT is distributed in vivo between three physiological pools, plasma, the vascular wall, and a third extravascular compartment (68), and AT has been found in follicular fluid (69, 70). As of now the presence of AT mRNA and the localization of AT in the ovary remain to be investigated. Ovulation occurs in parallel with local inflammation, and vascular permeabilization, extravasation of plasma proteins, and fibrin deposition are observed in the outer layers of ovulatory follicles (19, 71, 72). Extravascular coagulation has been shown to be regulated by vascular permeability (73), and after ovulation a fibrin clot forms in the remnant antral cavity (74). The presence of large quantities of aHSPGs in the inner compartment of preovulatory follicles could serve to localize activated AT to this locale, thereby preventing the formation of a fibrin clot within the antral cavity prior to the expulsion of the oocyte. Therefore, aHSPGs could be critically located in the inner follicle to maintain fluidity in the environment of the oocyte. Alternatively, aHSPGs could serve as cofactors to activate other serine protease inhibitors present in the follicle and thus participate in the control of the proteolytic events leading to the breakdown of the follicular wall at ovulation. Further studies are underway in our laboratory to elucidate the physiological functions of aHSPGs in the extravascular compartment formed by the ovarian follicle.

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