Identification of Link Protein during Follicle Development and Cumulus Cell Cultures in Rats

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ABSTRACT

Cumulus oocyte complex (COC) expansion is induced through hyaluronic acid production and accumulation of proteins of the inter-
-alpha-trypsin inhibitor family in the gonadotropin-stimulated cumulus cells. Link protein, a glycoprotein found in cartilage, interacts spe-
cifically with hyaluronic acid and stabilizes the binding of proteogly-
can monomers to hyaluronic acid to form aggregates. The aim of this study was to investigate the expression of immunoreactive link protein during follicle development in rats and in cumulus cells in culture by immunohistochemistry and Western blot as well as by specific enzyme-linked immunosorbent assay. Immunohistochemical analy-

sis revealed that the extracellular matrix of cumulus cells that were morphologically at a stage of COC expansion were markedly stained for link protein, whereas granulosa cells from immature follicles were not stained. Cumulus cells deposited link protein into the extracel-

lular matrix in an in vitro culture system. The staining intensity was negated by the treatment with hyaluronidase, suggesting that the link protein is bound to hyaluronic acid. We have identified a 42-kDa immunoreactive link protein in rat ovary during the preovulatory period and in COC extracts. Addition of FSH to the medium of cu-
mulus cells in culture supplemented with 10% FBS and oocyte-condi-
tioned medium resulted in an increased rate of link protein syn-
thesis. This work suggests that the cumulus cells synthesize the link protein that may stabilize the binding of inter-alpha-trypsin inhibitor or dermatan sulfate proteoglycan to hyaluronic acid to make up hyal-
uronic acid-rich matrix aggregate. (Endocrinology 140: 3835–3842, 1999)

GONADOTROPIN-INDUCED cumulus oocyte complex (COC) expansion, a process necessary for the release of matured mammalian oocyte into the oviduct, is believed to arise from the increased deposition of cumulus extracel-

lular matrix enriched by hyaluronic acid (1–3). The major part of the hyaluronic acid is involved in an aggregated structure that plays an important role in the maintenance of the hyaluronic acid-rich matrix aggregates (3). Hyaluronic acid accumulation is an important component of ovulation (4). We have investigated the morphological and biochemical characteristics of COC expansion (5).

A natural ligand for hyaluronic acid has been discovered and identified as a 240-kDa inter-alpha-trypsin inhibitor (6–11) and dermatan sulfate proteoglycan (12). Inter-alpha-trypsin inhibitor is comprised of three genetically different polypeptides: two heavy chains and one light chain (urinary trypsin inhibitor), which is one of the Kunitz-type protease inhibitors present in human serum and urine (13). Urinary trypsin inhibitor inhibits various serine proteases, such as trypsin, alpha-chymotrypsin, granulocyte elastase, plasmin, cathepsin G, and hyaluronidase (13). Several articles have been devoted to study of the molecular mechanisms responsible for the ini-
tiation, progression, and maintenance of hyaluronic acid-rich matrix on the cumulus cells, demonstrating the coincident appearance of hyaluronic acid and proteoglycans (inter-
-alpha-trypsin inhibitor and dermatan sulfate proteoglycan) during follicle development (5, 6, 9, 12, 14). In the last few years, several studies have been made of the molecular system that controls the initiation and progression of COC expansion. These findings are very similar to the biochemical evidence indicating that the aggregates involving hyaluronic acid and proteoglycans are important components of the extracellular matrix of cartilage (15). Camaioni et al. (12) reported that the dermatan sulfate proteoglycan and the approximately 46-

kDa protein synthesized by the cumulus cells form similar ternary complexes that are necessary for retaining hyaluronic acid in the COC matrix and hence are required for successful COC expansion. The approximately 46-kDa protein has the same molecular size as the link protein that interacts with hyaluronic acid and hyaluronic acid-binding proteoglycans to form stable ternary complexes in a variety of extracellular matrixes.

Link protein is a glycoprotein found in all hyaline cartilage examined to date (15). It interacts specifically with a de-
caccharide segment of hyaluronic acid (12, 16–19). It also stabilizes the binding of proteoglycan monomers to hyal-
uronic acid to form aggregates and appears to interact with both proteoglycan and hyaluronic acid (20–22). It has been established that proteins of the inter-alpha-trypsin inhibitor family associate with hyaluronic acid directly, and so does hyal-
uronic acid-binding protein, which is comprised of carti-
lage proteoglycan core protein and link protein (5). Link protein binds simultaneously to the hyaluronic acid-binding region of the proteoglycan molecule and hyaluronic acid (23). We (5) and others (12) thus speculate that link protein may stabilize the binding between hyaluronic acid and inter-alpha-trypsin inhibitor or other extracellular matrix proteins, such as dermatan sulfate proteoglycan, and influence the spacing...
of the monomers along the hyaluronic acid filament during follicle development. However, there is no information on the expression and localization of the link protein molecule during follicle development in rats. For a better understanding of the physiology of COC expansion, analysis of the expression of link protein in individual cells would be required.

In the present study we investigated the presence and synthesis of link protein in rat COC. To this end we prepared specific antibodies raised against bovine nasal cartilage link protein and investigated the localization of immunoreactive-link protein in PMSG-treated hCG-stimulated mature rat ovaries immunohistochemically. In addition, we report herein the identification and synthesis of a protein with the antigenic and electrophoretic characteristics of link protein in cumulus cell cultures.

**Materials and Methods**

**Preparation of link protein**

The isolation of hyaluronic acid-binding protein derived from bovine nasal cartilage has been described in detail previously (24, 25). The hyaluronic acid-binding protein was purified by affinity chromatography on hyaluronic acid covalently coupled to Sepharose. A purified preparation was specific for bovine nasal cartilage link protein, but not for aggrecan, and had a 50% maximal binding at a dilution of 1:10,000 in a specific enzyme-linked immunosorbent assay (ELISA). A 45-kDa link protein peak (by Western blot) was obtained. The link protein purified from hyaluronic acid-binding protein does not contain aggrecan, which was confirmed by Western blot with specific monoclonal antibodies raised against cartilage aggrecan (data not shown).

**Preparations of polyclonal antibodies raised against link protein**

Polyclonal antibodies against bovine nasal cartilage link protein were prepared by intradermal injection of rabbits with 2 mg purified link protein emulsified in Freund’s complete adjuvant. Four weeks after the first injection, the rabbit was boosted with 1 mg protein in incomplete Freund’s adjuvant and then was boosted at 4-week intervals. The antisera contained specific for bovine nasal cartilage link protein, but not for aggrecan, and had a 50% maximal binding at a dilution of 1:10,000 in a specific ELISA. This antisera was reactive with both 42-kDa (ovary) and 45-kDa (cartilage) proteins in Western blot assay. Affinity-purified IgG was prepared by mixing 3 ml antiserum with 1 ml link protein-coupled Sepharose 4B overnight at 4 °C. After washing, the IgG was eluted with 100 mmol/liter glycine-HCl, pH 2.5. The pH of the eluted fractions was immediately raised, and the IgG was stored at −20 °C.

**Animals and tissue sections**

Twenty-five-day-old immature female Wistar rats and 10-week-old adult cyclic female Wistar rats were purchased from SLC (Shizuoka, Japan). Rats were housed in a temperature-controlled room with a 12-h light, 12-h dark schedule and were fed chow and water ad libitum. Female rats estrous cycles were monitored by daily vaginal cytology. Only animals exhibiting two or more consecutive 4-day estrous cycles were used in these experiments. We used animals taken during the morning of estrus. The estrous cycle of adult rats was confirmed by vaginal smear at 0900 h, and ovaries were obtained at 1500 h. Immature rats were treated with ip injection of 15 IU PMSG in 0.2 ml PBS, pH 7.4. Rats were treated with ip injection of 15 IU hCG 48 h later, and ovaries were removed 12 h later and used for further immunological (by Western blot) and immunohistochemical experiments.

**Isolation and culture of COC**

For culture of cumulus cells, rats were killed 48 h after PMSG injection, and the ovaries were transferred to HEPES-buffered HBSS (Sigma Chemical Co., St. Louis, MO) containing 1% (wt/vol) BSA. Large follicles were immediately punctured with a 27-gauge needle, and escaping COCs were harvested. Culture medium was prepared according to the description of Singh et al. (26) with few modifications and consisted of medium 199 with Earle’s salt (Life Technologies, Grand Island, NY) supplemented with 25 mm HEPES buffer (Life Technologies), 2.5 mm glucose, 3 mm glutamine (Life Technologies), 0.03 mm sodium pyruvate (Life Technologies), 2.5 mm sodium lactate, 5 mm glucose (Yoneyama Chemical Co., Tokyo, Japan), 100 U/ml penicillin, 100 μg/ml streptomycin, 0.25 mg/ml Amphotericin (Life Technologies; further referred to as medium), and 10% FBS (Life Technologies) in the presence or absence of 10 μg/ml of FSH-LH (Sankyo Co. Ltd., Tokyo, Japan). Intact COCs were incubated in medium containing 10% FBS and 10 μg/ml FSH for 12 h at 37 °C.

In a parallel experiment, oocytes were stripped of granulosa cells by pipetting with a glass capillary. Granulosa cells were recovered by centrifugation at 20 × g for 5 min. The supernatant was discarded, and the cells were resuspended in culture medium. Furthermore, oocyte-conditioned medium was obtained by culturing isolated oocytes as described previously (27). Granulosa cells were suspended in the medium containing oocyte-conditioned medium (10 μl) at a final concentration of about 5 × 10⁶ cells/100 μl supplemented with and without 10 μg/ml FSH. Fifteen oocytes were used to condition the 10 μl medium. Incubations were carried out for 12, 24, and 36 h at 37 °C in an atmosphere of 95% air and 5% CO₂.

**Immunodetection of link protein in extracts from whole ovaries and COC lysate**

For immunodetection, whole ovaries of both adult cyclic rats and mature PMSG-treated hCG-stimulated rats as well as cultured COCs were homogenized in lysis buffer (8 m urea and 50 mm sodium acetate at pH 5.8 containing 50 U/ml Streptomyces hyaluronidase (Calbiochem, La Jolla, CA), 0.1% Triton X-100, 1 mmol/liter phenylmethylsulfonyl fluoride, 1 μg/ml leupeptin, and 1 μg/ml pepstatin). Protein content of the preparation was determined by the method of Bradford (28), using BSA as the standard. Tissue extract and COC lysate (50 μg) were mixed with the SDS sample buffer (5% SDS and 10% glycerol), boiled for 5 min, separated by SDS-PAGE with 12% gel according to Laemmli’s method (29), and electrophoretically transferred onto polyvinylidene difluoride membranes (Immobilon, Millipore Corp., Bedford, MA). The membranes were blocked for 1 h in Tris-buffered saline (TBS) with 2% BSA and incubated for 2 h with a 1:500 dilution of polyclonal antibodies raised against link protein and then for 1 h with biotinylated goat antirabbit IgG as the second antibodies (2 μg/ml, 1 h, 23 °C, DAKO Corp., Copenhagen, Denmark), followed by avidin-peroxidase (2 μg/ml, 1 h, 23 °C, DAKO Corp.). Bands were visualized with the enhanced chemiluminescence detection system (Amersham, Tokyo, Japan). In all experiments, some strips were incubated with nonimmune rabbit IgG as a negative control.

**Immunohistochemistry**

Culture of cumulus cells for immunohistochemistry: Granulosa cells were seeded into Lab-Tek culture slides (Nunc, Copenhagen, Denmark) at a density of approximately 1 × 10⁴ cells/chamber in the medium containing oocyte-conditioned medium and 10% FBS supplemented with and without 10 μg/ml FSH-LH in triplicate and cultured in a humidified atmosphere of 5% CO₂ in air. After 12-h incubation, the cells were fixed with 4% paraformaldehyde in PBS and stained with polyclonal antibodies raised against link protein, as performed in tissue sections (see below).

Rat ovaries were fixed in 4% (wt/vol) paraformaldehyde in 0.1 M PBS. The tissues were fixed at 23 °C overnight and washed in 0.1 M phosphate buffer containing 0.25 m sucrose and 0.2 m glycine, pH 7.4, several times over a period of 16 h. After passing through a series of graded alcohol and xylene solutions, the tissues were embedded in paraffin by the standard procedures. Five-micron sections were taken on glass slides for immunostainings. In some cases the sections and the cells were treated with 50 U/ml...
Fig. 1. Detection of link protein in extracts from whole ovaries and in the COC lysate by Western blot. Western blot analysis of the following extracts was conducted for link protein detection. A, Left, The extracts from whole ovaries of both adult cyclic rats (lane 1) and mature PMSG-treated hCG-stimulated rats (lane 2) as well as the extracts from COCs isolated from rat stimulated ovaries (lane 3). The samples (50 µg) were analyzed by 12% SDS-PAGE. In the left panel (A), the 42-kDa band (lanes 1–3) and the 45-kDa purified cartilage link protein (lane 4, arrowhead) were developed with the aid of antilink protein antibody as described in Materials and Methods. A, Right, SDS-PAGE of sample purified from cartilage link protein under nonreducing conditions (45-kDa band, arrowhead). Gel was stained with Coomassie blue. Note one distinct band (45 kDa) in the nonreduced sample. B, COCs were incubated for 12 h with medium containing 10% FBS (+FBS, lane 1) or in serum-free medium (−FBS, lane 2). In a parallel experiment, COCs were incubated in the presence of medium containing 10% FBS and then treated with hyaluronidase (HAase, lane 3). Cell lysates were then subjected to Western blot analysis with the aid of antilink protein antibodies. C, The intensity of the bands in Western blot was measured by densitometry, and their ratios were compared. Data in three separate experiments were averaged. The link protein concentration of +FCS was taken as 100%. Molecular mass markers (111, 77, 48, 34, and 22 kDa) were run simultaneously in the left lane of Coomassie blue staining.

Elisa

ELISAs for link protein

For the determination of link protein antigen, cell culture conditions were continued for 12, 24, and 36 h in medium containing oocyte-conditioned medium supplemented with and without 10 µg/ml FSH-LH in triplicate, and one flask was kept for cell counting. After 12-, 24-, or 36-h incubation, the media were collected for ELISA (see below) and stored at −20 C.

Immobilization of polyclonal antibody against link protein (2 µg/ml) to microtiter plates was carried out in 50 mm sodium carbonate buffer, pH 9.5, overnight at 4 C. All subsequent additions in ELISA were performed for 2 h at 23 C in TBS containing 2% BSA. After each addition the plates were washed with TBS containing 0.05% Tween-20. Forty nanomoles per liter of biotinylated link protein (50 µl) were first allowed to mix with each culture medium (50 µl) and then added as a complex to an antilink protein antibody-coated well. Detection of bound biotinylated link protein was performed with avidin-peroxidase (2 µg/ml). The substrate was tetramethylbenzidine in acetate citrate buffer, pH 6.0. The inter- and intraassay coefficients of variation were 10.2% (n = 10) and 9.3% (n = 10), respectively. In the present experimental conditions, the lowest detectable level of link protein was 10 pmol/liter. The purified link protein was used for the assay standard. Fresh medium supplemented with 10% FBS was used as a negative control.

Statistical analysis

All statistical analysis was performed using StatView for Macintosh. The Mann-Whitney U test was used for the comparisons between different groups. P < 0.05 was considered significant.
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**Results**

Electrophoresis of the link protein purified from bovine nasal cartilage extracts was performed to compare the electrophoretic mobilities (Fig. 1). Examination of Coomassie blue-stained gels containing unreduced preparations revealed one band with an apparent molecular mass of 45 kDa.
(Fig. 1A, right panel). This protein corresponds in electrophoretic mobility to link protein from bovine nasal cartilage. Additional characterization of the cartilage link protein was performed using Western blotting studies. Western blot of the sample of purified link protein from bovine nasal cartilage after electrophoretic transfer and incubation with the antilink protein antibodies revealed a single band at 45 kDa (Fig. 1A, left panel, lane 4).

We conducted Western blot analysis of the extracts from whole ovaries of both 10-week-old adult rats and PMSG-treated hCG-stimulated rats and of the extracts from cultured COCs isolated from PMSG-treated ovaries for link protein detection (Fig. 1A). The antilink protein antibodies detect a single band of approximately 42 kDa, which seems to correspond to the link protein monomer, in the extracts from whole ovaries [rat adult cyclic ovary (lane 1) and rat PMSG-treated hCG-stimulated ovary (lane 2)] and COC lysates (lane 3). No bands were detected in either the extracts from...
ovaries or COC lysates when identically processed preimmune rabbit IgG was used in Western blot analysis (data not shown).

Culture of rat COCs in serum-free medium or treatment of the cells with various mucopolysaccharide degradation enzymes showed that antilink protein antibody reactivity was reduced after elimination of FBS (Fig. 1B, −FCS, lane 2) or digestion with hyaluronidase (HAase, lane 3). The concentration of link protein was quantitated by measuring the density of the bands, and the ratio of the reactivity was statistically analyzed from three independent experiments (Fig. 1C). The Western blot showed much lower relative link protein concentrations of −FCS and HAase than of +FCS. The link protein concentrations of −FCS and HAase were 32.7 ± 8.0% and 18.1 ± 11.7% of that of +FCS, respectively. Treatment of the purified link protein with Streptomyces hyaluronidase did not lead to an increase in the mobility of this band, indicating that link protein itself is insensitive to hyaluronidase (Kobayashi, H., et al., unpublished data, 1998). In addition, treatment of the cells with keratanase, heparinase, or heparitinase did not affect antibody binding (data not shown).

As the presence of link protein in rat ovary and COCs in culture was confirmed by Western blotting, we examined immunohistochemically distribution and localization of link protein in correlation with COC expansion in stimulated ovaries. As shown in Fig. 2, intense and diffuse link protein staining was found in most of the oocytes in ovaries taken from immature rats before PMSG treatment (Fig. 2A). There was no detectable reaction product in theca cells, and the granulosa cells of small immature follicles (primary and secondary follicles) were completely devoid of immunoreactivity. No immunostaining was observed when the primary antibody was substituted with nonimmune IgG (Fig. 2B).

Treatment of the section with hyaluronidase resulted in abolishment of almost all the immunostaining of the follicular compartment for link protein (Fig. 2C). The follicles of immature rat ovaries are induced to develop into large preovulatory (Graafian) follicles by 48 h after the administration of hCG. Rat cumulus cells were incubated for 12, 24, and 36 h, and the amount of link protein in the medium was determined by a specific ELISA (Fig. 3). ELISA data revealed that gonadotropin caused a significant increase in cumulus cell link protein production. These stimulatory effects were seen after as little as 12 h and were prominent after 24 h of incubation. The rate of secretion of link protein increased approximately 240% by 24 h (mean ± sd, 20.6 ± 4.4 ng/10⁴ cells 24 h in the presence of gonadotropin vs. 8.6 ± 2.4 ng/10⁴ cells 24 h in the absence of gonadotropin).

Isolated rat cumulus cells in the presence of oocyte-conditioned medium were incubated with gonadotropin for 12, 24, and 36 h, and the amount of link protein in the medium was determined by a specific ELISA (Fig. 3). ELISA data revealed that gonadotropin caused a significant increase in cumulus cell link protein production. These stimulatory effects were seen after as little as 12 h and were prominent after 24 h of incubation. The rate of secretion of link protein increased approximately 240% by 24 h (mean ± sd, 20.6 ± 4.4 ng/10⁴ cells 24 h in the presence of gonadotropin vs. 8.6 ± 2.4 ng/10⁴ cells 24 h in the absence of gonadotropin).

In addition, evidence for the presence of link protein in cumulus cell cultures was obtained using antilink protein antibodies in immunocytochemical studies. Immunocytochemical experiments further established the specificity of the immunolabeling. In an in vitro culture system in which cumulus cells were cultured in the presence of oocyte-conditioned medium, FBS, and gonadotropin, immunohistochemistry revealed that the addition of gonadotropin enhanced link protein expression in most cumulus cells (Fig. 2G). Link protein was deposited diffusely in the extracellular matrix and on the cell membrane. However, the cells expressed a small amount of link protein on the cell surface without gonadotropin (Fig. 2H). Gonadotropin-stimulated cumulus cells cultured in the absence of FBS deposited a small amount of link protein on the cell membrane (data not shown). Furthermore, when cumulus cells were cultured in the medium supplemented with FBS and gonadotropin in the absence of oocyte-conditioned medium, weak reactivity was observed in the extracellular matrix of cumulus cells (data not shown), suggesting that an oocyte factor is also required for link protein production from cumulus cells. The staining of cultured cumulus cells with antilink protein antibody was completely negated by the addition of hyaluronidase (Fig. 2I). Experiments using three independent cultured cumulus cells gave essentially identical results, demonstrating that gonadotropin-stimulated cells increased the deposition of link protein into the extracellular matrix compared with that in the control without the addition of gonadotropin or FBS.

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![Fig. 3](https://academic.oup.com/endo/article-abstract/140/8/3835/2991013)
In a parallel study, this experiment was performed three times. After assay in cell culture medium, a protein antigenically related to link protein was produced in all experiments. The concentration in the culture medium reached a plateau at 24 h and remained at a low level when cumulus cells were cultured in the medium supplemented without gonadotropin. The production was significantly enhanced when gonadotropin was added to the culture medium. However, quantitative differences between experiments were observed, but could not be explained by differences in the cumulus cell count and did not depend on their cell viability. Therefore, the data from all four experiments were not combined in Fig. 3.

Discussion

COC of preovulatory follicles undergoes a dramatic change after an ovulatory stimulus. The process of COC expansion is required for ovulation (1–3). Cumulus cells synthesize and secrete large amounts of hyaluronic acid (4) and proteoglycans (12) into their extracellular matrix. The indication in earlier studies that cumulus cells contain a large amount of hyaluronic acid and proteoglycans as well as the approximately 46-kDa protein similar to link protein and the presence of link proteins in several connective tissues as well as in cartilaginous and noncartilaginous tissues rich in glycosaminoglycans, including hyaluronic acid (30), were the rationale for the present experiments.

The antilink protein polyclonal antibody produced by us detected a specific 42-kDa band on a Western blot of the stimulated ovaries and COC lysate as well as the 45-kDa cartilage link protein. The presence in cumulus cell extracts of the 42-kDa protein that reacted with the antilink protein antibody to cartilage link protein indicated that the 42-kDa protein shares an epitope with bovine nasal cartilage link protein. Extracts of rat COC cultures were found to contain a protein with molecular mass and electrophoretic behavior similar to those of bovine nasal cartilage link proteins (20, 21, 24, 31). As previously reported (24, 32), at least three link proteins in several connective tissues as well as in cartilaginous and noncartilaginous tissues rich in glycosaminoglycans, including hyaluronic acid (30), were the rationale for the present experiments.

In conclusion, we describe the presence and synthesis of link protein in rat ovaries during the preovulatory period that were morphologically at a stage of COC expansion and in COC cultures or cumulus cell cultures supplemented with oocyte-conditioned medium, using immunolocalization with a specific antilink protein antibody. With the immunohistochemical approach, it is possible to determine the specific tissue compartments that express link protein antigen during follicular development. The results of this study showed a periovulatory increase in cumulus cell and mural granulosa cell link protein staining of gonadotropin-treated animals.

ELISA analysis in cumulus cell cultures also indicated a synthesis of immunoreactive link protein. We demonstrated that gonadotropin significantly stimulates cumulus cell link protein production in an in vitro culture system. Protein with characteristics similar to those of the bovine nasal cartilage link protein was identified in the cumulus cell culture medium, indicating that it is exported from the cells in the absence of FBS.

The presence and synthesis of link protein in cumulus cell cultures are of importance because this is the first time, to our knowledge, that immunoreactive link protein has been definitively demonstrated in cumulus cells. The cartilage and noncartilage link proteins and proteoglycan core protein are reported to induce stabilization between hyaluronic acid and also bind to each other (12, 16–19). It is possible that link protein is involved in the stabilization of hyaluronic acid-rich aggregates. It would appear that the link protein is involved in interaction with proteins of the inter-α-trypsin inhibitor family or other extracellular matrix proteins. The important findings are that cultured cumulus cells deposited link protein mainly into the extracellular matrix and that link protein is released from the tissues and cultured cumulus cells by hyaluronidase digestion, which suggest that link protein is not anchored to the cell membranes lining the extracellular space and is bound to hyaluronic acid. Link protein itself is not sensitive to hyaluronidase (Kobayashi, H., et al., unpublished data, 1998). This suggests that the release was dependent on the degradation of hyaluronic acid, rather than being brought about by proteolytic activity. The interaction among hyaluronic acid, link protein, and extracellular matrix proteins, including inter-α-trypsin inhibitor and dermatan sulfate proteoglycan, on the cumulus cell surface may induce and strengthen COC expansion and lead to ovarian follicle maturation.

We also demonstrated that immunoreactivity for link protein has been localized primarily to oocyte regardless of the animal age and hormonal condition. It is possible that oocyte-derived link protein functions to stabilize an aggregate structure of the hyaluronic acid-rich matrix on the oocyte. Alternatively, link protein may have a function other than the stabilization of proteoglycan aggregates. Although link protein is abundantly expressed in cartilage (34), the tissue distribution of the link protein is not limited to the cartilaginous tissue, and expression of link protein messenger RNA has been found clearly in brain, aorta, embryonic retina, lens epithelium, and mesonephros by Northern blot analysis (35, 36). These results suggest that the role of the link protein-producing cells may be related to the sustaining supply or transportation of link protein to extracellular matrix. However, the exact role of link protein in the oocyte is as yet unknown.

In conclusion, we describe the presence and synthesis of immunoreactive link protein in stimulated rat ovary. Gonadotropin increases link protein production in cumulus cell cultures. It was established that cumulus cells significantly produce hyaluronic acid in the presence of appropriate gonadotropin (37) and FBS and that inter-α-trypsin inhibitor is accumulated in follicular fluids from plasma through in-
creased vascular permeability just before ovulation (9). The link protein may stabilize the interaction between hyaluronic acid and extracellular matrix proteins, such as inter-α-trypsin inhibitor on cumulus cells, by triggering COC expansion and may function as a part of this complex pathway. These results provide an intriguing area of investigation regarding the molecular mechanism of the hyaluronic acid-link protein-inter-α-trypsin inhibitor system in ovarian follicle development. Further work is needed to characterize cumulus cell-associated link protein, to clarify possible relationships between COC link protein and cartilage link proteins, and to determine its function in COC expansion. The distribution of link protein by in situ hybridization during different stages of ovarian follicle development is underway.

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