

LOCALIZATION OF A DEVELOPMENTALLY REGULATED PROTEIN IN PORCINE FOLLICLES IDENTIFIED BY A MONOCLONAL ANTIBODY

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Objective. In the present study we employed a monoclonal antibody (Mab 3D8) obtained against a rat ovarian antigen and identified a 76 kDa protein in porcine ovarian follicles.

Methods. The localization of this antigen was studied by light and electron-microscopic immunocytochemistry and further characterized by polyacrylamide gel electrophoresis and immunoblotting on nitrocellulose membranes.

Results. We found that the antigen recognized by Mab 3D8 is localized in granulosa cells (GCs) and oocytes. The expression of the 76 kDa protein apparently depends on the developmental stage. A particularly strong reaction was observed in cumulus cells and oocytes in early and late antral follicles. In granulosa cells the reaction product was localized in rough endoplasmic reticulum (RER), cis and trans faces of the Golgi stack, the outer nuclear envelope and in numerous transport vesicles budding from the endoplasmic reticulum. In the oocyte the reaction product was localized in structures related to specific endocytosis – small pits at the cell surface, two subsets of endosomes, endocytic carrier vesicles and the prelysosomal compartment (PLC).

Conclusions. The results obtained suggest that porcine oocytes possess the cellular structures which allow them to bind and internalize this protein, which is most probably produced by granulosa cells. During oocyte development the intracellular site of accumulation of the 76 kDa protein varies, which implies that it is under developmental control.

Key words: Monoclonal antibody – Immunocytochemistry – Endocytosis- Porcine ovarian follicle

During follicular development specific factors influencing oocyte maturation might be transmitted from granulosa cells to the oocyte. The granulosa cell-oocyte communication represents a route by which signals generated in granulosa cells regulate a number of oocyte functions such as growth (BUCCIONE et al. 1987) and meiotic progression (EPPIG and DOWNS 1988). Granulosa cells are competent to send the appropriate “signal” to the growing oocyte and during oogenesis, oocyte protein phosphorylation is constantly influenced by follicle cells in a developmentally regulated fashion (COLONNA et al. 1989).

Using rat granulosa cells and oocytes as immunogen and the hybridoma technology we accumulated a library of monoclonal antibodies against rat ovarian antigens. Our interest was directed toward granulosa cell secreted proteins because each protein excreted from the granulosa cells is likely to participate in oogenesis, either by interacting with the oocytes or by creating special conditions in the fluid microenvironment. The production of Mabs against rat ovarian antigen was described previously (RUSSINOVA et al. 1994). A monoclonal antibody from a hybridoma line 3D8 reacted immunocytochemical-

ly with an antigen found both in the granulosa cells and oocytes in rat ovarian follicles (RUSSINOVA 1999). This monoclonal antibody (3D8) was found to react intensely with porcine granulosa cells and oocytes in a developmentally dependent manner.

The aim of the present work was the light- and electron-microscopic localization, as well as the partial characterization of a porcine ovarian 76 kDa antigen, associated with follicular development, using Mab 3D8 as immunocytochemical marker.

Materials and methods

Monoclonal antibody preparation. The preparation and characterization of the specificity of the antibody, used in this study, have been described previously (RUSSINOVA et al. 1994). Briefly, Balb/c mice were immunized intraperitoneally with 5×10^6 granulosa cells and with an average of about 300 oocytes five times at intervals of 2 weeks. The granulosa cells and oocytes were collected from rat ovaries after pregnant mare serum gonadotropin (PMSG) stimulation. Fusion was performed using a P3x63-Ag8.653 myeloma line. On immunocytochemical screening of the hybridoma supernatants a line (3D8) was found to react with an antigen, localized both in oocytes and granulosa cells in rat developing follicles (RUSSINOVA 1999). This Mab 3D8 was of IgM type. In an extensive screening of cell lines and a variety of tissues Mab 3D8 was found to react in a similar staining pattern with porcine granulosa cells and oocytes. In addition, Mab 3D8 did not react with other tissues – kidney, lung, heart and liver.

Cell culture and medium collection. Ovaries were obtained from 4 to 6-month old pigs less than 20 minutes after slaughter and are immediately placed on ice in a buffered salt solution containing penicillin, streptomycin and mycostatin. Granulosa cells were isolated from antral follicles by the non-enzymatic needle puncture method (CHANNING and LEDWITZ-RIGBY 1975). Cells were seeded in tissue culture dishes at a density $1 \times 10^6 \text{ ml}^{-1}$ in Dulbecco's modified Eagle's medium (DMEM, Gibco BRL, Gaithersburg, MD) supplemented with 50 IU ml^{-1} penicillin, 50 $\text{g} \cdot \text{ml}^{-1}$ streptomycin 2.5 $\text{g} \cdot \text{ml}^{-1}$ fungizone and 5 % fetal calf serum (FCS, Sigma, St Louis, MO). The cells were cultured for 48 h. For the next 24 h GCs were cultured in serum-free medi-

um. The culture medium was collected and centrifuged at 1000 x g for 30 min. The conditioned medium was concentrated 10-fold using a Speed Vac concentrator (Savant, USA). The amount of protein present in concentrated conditioned medium was generally between 0.7 and 1 mg/ml as estimated by the method of BRADFORD (1976). This medium containing granulosa cell-secreted proteins was designated as GSPs.

Immunocytochemistry. Avidin-biotin technique (ABC). Paraffin sections from pig ovaries fixed in the Bouin's solution were processed for immunocytochemistry using avidin-biotin-peroxidase technique of HSU et al. (1981). In this procedure methanol hydrogen peroxide solution was used to block endogenous peroxidase activity and normal rabbit serum was used to block nonspecific binding of the secondary antibody (biotinylated rabbit anti-mouse total Ig). Sections were incubated with Mab 3D8 (hybridoma supernatant) for 18 h at 4 °C then rinsed with phosphate buffered saline (PBS) and incubated for 60 min with biotinylated anti-mouse total Ig (Vector-Burlingam) diluted 1:250 in PBS. After rinsing in PBS avidin-biotin-peroxidase conjugate (Vector-Burlingam) diluted 1:250 in PBS was applied for 60 min. Visualization of binding sites was accomplished with 3,3'-diaminobenzidine tetrahydrochloride (DAB) in 0.05M Tris-HCL-buffered saline (pH 7.6) 0.01 % H_2O_2 , dehydrated, and coverslipped.

Immunofluorescence microscopy. Granulosa cells were grown on glass coverslips, washed rapidly twice with cold PBS (pH 7.2) and incubated in methanol at -20 °C for 6 min. After methanol treatment cells were rinsed with PBS containing 0.1 % Tween-20. Primary antibody was added for 1 h at room temperature. After rinsing the primary antibody off in three changes of PBS, the secondary FITC conjugated antibody against mouse IgM (Cappel/Organon Teknika Corp, West Chester, PA) diluted 1:50 in PBS was applied for 30 min. After extensive washing the coverslips were mounted on microscope slides in 90% glycerol in PBS and analyzed in a Zeiss epifluorescence microscope equipped with interference filters for FITC. Photomicrographs were recorded using a Zeiss MC-100 camera system and Kodak T-MAX 400 film.

Controls for light microscope observations. Control sections from pig ovaries and granulosa cell

grown on glass coverslips were incubated either with normal mouse serum or with Mab 1F2 (IgM) which recognizes a 44 kDa antigen, specific for rat germ cells (RUSSINOVA et al. 2000). Further controls were performed with Mab 3D8 preabsorbed either with porcine GC and oocyte protein extract or rat GC and oocyte protein extract obtained from the cells utilized for immunization (RUSSINOVA et al. 1994).

Immunoelectron microscopy. Electronmicroscopic immunocytochemical studies were performed on 30 mm thick frozen sections from pig ovaries fixed with a solution containing 4 % paraformaldehyde, 0.1 % glutaraldehyde and 0.15 % picric acid in 0.1 M phosphate buffer (pH 7.4). The ABC technique was applied as described above. Following visualization of the peroxidase activity by means of diaminobenzidine (0.2 mg/ml) and H₂O₂ (0.02 %), the sections were treated with 1 % OsO₄ in phosphate buffer (pH 7.4) and embedded in Durcupan by invert capsule method. Ultrathin sections were observed without additional counterstaining in a Zeiss EM-109. Controls were performed as follows: (a) the first antibody was omitted; (b) the ABC procedure was omitted; (c) incubation with control Mabs (5G5 and 2H7 – IgM subclass) instead of the primary antibody; (d) preincubation of the Mab 3D8 with porcine granulosa cell and oocyte protein extract; (e) preincubation of the Mab 3D8 with rat granulosa cell and oocyte protein extract obtained from the cells utilized for immunization.

Polyacrylamide Gel Electrophoresis (SDS-PAGE). Preparation of protein extract and SDS-PAGE. Pig ovaries were decapsulated and granulosa cells and oocytes were isolated from follicles by the needle puncture method (CHANNING and LEDWITZ-RIGBY 1975). Atretic follicles were discarded. The cell pellet was homogenized on ice in PBS containing 1 % Triton X-100 and following protease inhibitors: 1 mM PMSF and 5 mg/ml each of benzamidin HCl, aprotinin and leupeptin. Following extraction the mixture was centrifuged at 20 000 x g for 30 min at 4°C and the supernatant was collected.

The protein extract and concentrated conditioned medium (GSPs) were suspended in sample buffer in a boiling water bath for 5 min. SDS-PAGE was run on a 12.5 % separating gel (pH 8.7) with 5 % stacking gel (pH 6.8) according to LAEMMLI (1970). In parallel lane rat granulosa cell and oocyte protein

extract obtained from the cells utilized for immunization was run. Molecular mass was calculated from the position of markers that were subjected to electrophoresis in parallel lanes and stained with Coomassie Blue.

Immunoblotting. Proteins separated by SDS-PAGE were transferred onto nitrocellulose membranes. The transferred proteins were stained using Ponceau S (Sigma, France) as described by HARLOW and LANE (1988), destained by washing with Tris-buffered saline (TBS, 10 mM Tris-HCl, pH 8, 100 mM NaCl) and the blots were cut vertically in 5mm wide strips. To prevent nonspecific protein binding the nitrocellulose strips were blocked for 2 h at 37°C with 3% bovine serum albumin (BSA). The blocked nitrocellulose membranes were then incubated with hybridoma supernatant (appropriate dilution with TBS-1%BSA) overnight at 4 °C and for 1 h at room temperature. After washing with TBS containing 0.05 % Tween 20, the antigen-antibody complex was visualized with the avidin-biotin peroxidase method. Parallel strips treated with non-immune serum or control antibody (Mab 1F2 - IgM) served as negative controls. Additional negative controls were performed after preincubation of the Mab 3D8 either with porcine granulosa cell and oocyte protein extract or rat granulosa cell and oocyte protein extract obtained from the cells utilized for immunization.

Results

Immunocytochemistry. Immunoreactivity in pig ovaries was found in the cytoplasm of granulosa cells at all stages of follicular development. However, the intensity of immunostaining increased with the degree of follicular development. Initially, in young growing follicles, all GC layers were labeled faintly. As growth progressed the reaction became stronger both in the innermost layers of granulosa cells and in the compact region of stratified cuboidal epithelial cells (cumulus oophorus) that are separated from the oocyte by zona pellucida (Fig.1A). The strongest reactivity was observed in the perinuclear region of the oocyte in late antral follicle.

In atretic follicles the reaction was observed only in the cytoplasm of granulosa cells, but the strength of the reaction decreases with the degree of atresia.

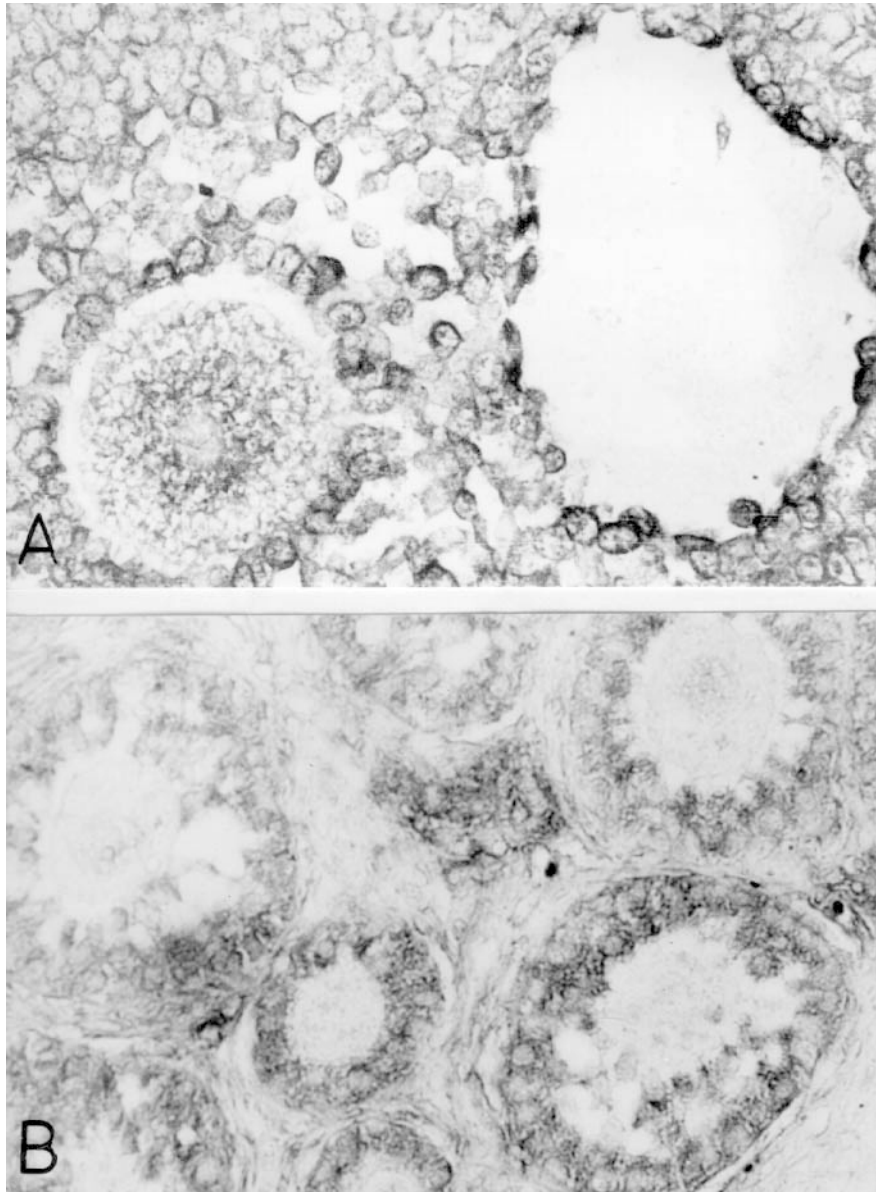


Fig 1

ABC staining of sections from pig ovaries. The labeling was observed in the innermost layer of GCs and in the oocytes in a late antral follicle (A). In atretic follicles the reaction was observed only in GCs (B). A and B x 250

The oocytes in atretic follicles were totally devoid of staining product (Fig.1B). Control experiments omitting the primary antibody or using the control Mab (IGM) and preabsorbed Mab 3D8 were negative (not shown).

In cultured granulosa cells immunofluorescence revealed a fine reticular network, that spread throughout the cytoplasm and labeling of the nuclear mem-

brane (Fig.2). The cell surface and the nuclear matrix were not labeled.

The subcellular localization of the reaction product was examined by immunoelectron microscopy. In granulosa cells the reaction occurred on the membranes of the rough endoplasmic reticulum (RER) (Fig. 3A) and on vesicles budding from the endoplasmic reticulum. The Golgi stacks

in GCs of innermost layers and cumulus were larger in diameter than the juxtannuclear – disposed Golgi elements in peripherally located GCs. The labeling apparently involves an abundant tubulovesicular system on both the cis and trans surfaces (fig. 3B).

In oocytes the reaction product was observed in several different organelles, whereby its localization apparently depends on the stage of development. Moreover, the differential localization of the reaction product appears to represent the different stages of endocytosis. The individual steps of endocytosis are illustrated on Fig.4. During the early antral stage the reaction product in oocytes was found on the outside of indented regions of the oocyte membrane. The tracer formed small clusters closely associated with the plasma membrane. More prominently indented pits were present, in which the reaction product was found in the membranes at the opening of pit, moving closer together prior to the formation of vesicles and endosomes. Figure 4A shows representative profile of a labeled pit, which could be recognized by its characteristic shape and coating with electron dense material, corresponding to the morphological appearance of clathrin coated pit. We distinguished two general subsets of endosomes – endosomes containing a central vacuolar element and endosomes, whose central vacuole was filled with many internal vacuoles (Fig.4B). The final destination of the antigen recognised by Mab 3D8 we observed in structures with a specific morphological appearance (Fig. 4C), which we tentatively defined as the prelysosomal compartment (PLC). Similar structures are seen in normal rat kidney cells (Griffiths et al. 1990a) and also referred to as the PLC. The PLC is located perinuclearly and is very complex. It consists of labeled vesicular structures and a large number of “fibres” or “thin sheets” packed closely together (Fig.5 A, B, C, D). In the same oocyte the degree of immunolabeling was often variable in the PLC, with some parts displaying numerous labeled vesicular/tubular structures and others with little labeling. The labeled PLC is observed in the late antral stage. It should be noted that at this stage significant amounts of reaction product remain associated with endosomes and endosome vesicles. In late preovulatory stage, identified on the basis of morphological cri-

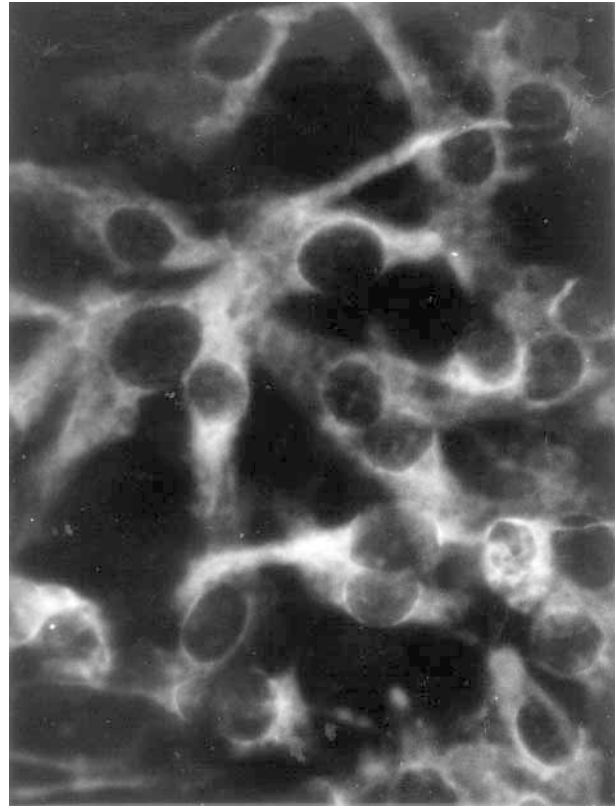


Fig 2
Immunofluorescence of GC culture using Mab 3D8 and FITS conjugated secondary antibody against mouse IgM. Fine reticular network throughout cytoplasm and labeling of nuclear membrane were observed. X 630

teria (cumulus mass is reduced to a population of dissociated cells), the reaction product was observed almost exclusively in the PLC in all of the examined oocytes. The oocytes in atretic follicles were devoid of immunoreaction. All control experiments were negative (not shown).

Antigen identification. Granulosa cell and oocyte protein extract and conditioned medium from granulosa cell culture containing granulosa cell secreted proteins (GSPs) were subjected to SDS-PAGE and immunoblotting using the ABC procedure. A single band of an apparent molecular mass of 76 kDa was detected on nitrocellulose blot of porcine granulosa cell and oocyte protein extract (Fig.6, column 3). No bands were detected on blot of GSPs (Fig.6, column 6). Control were in all cases negative (not shown).

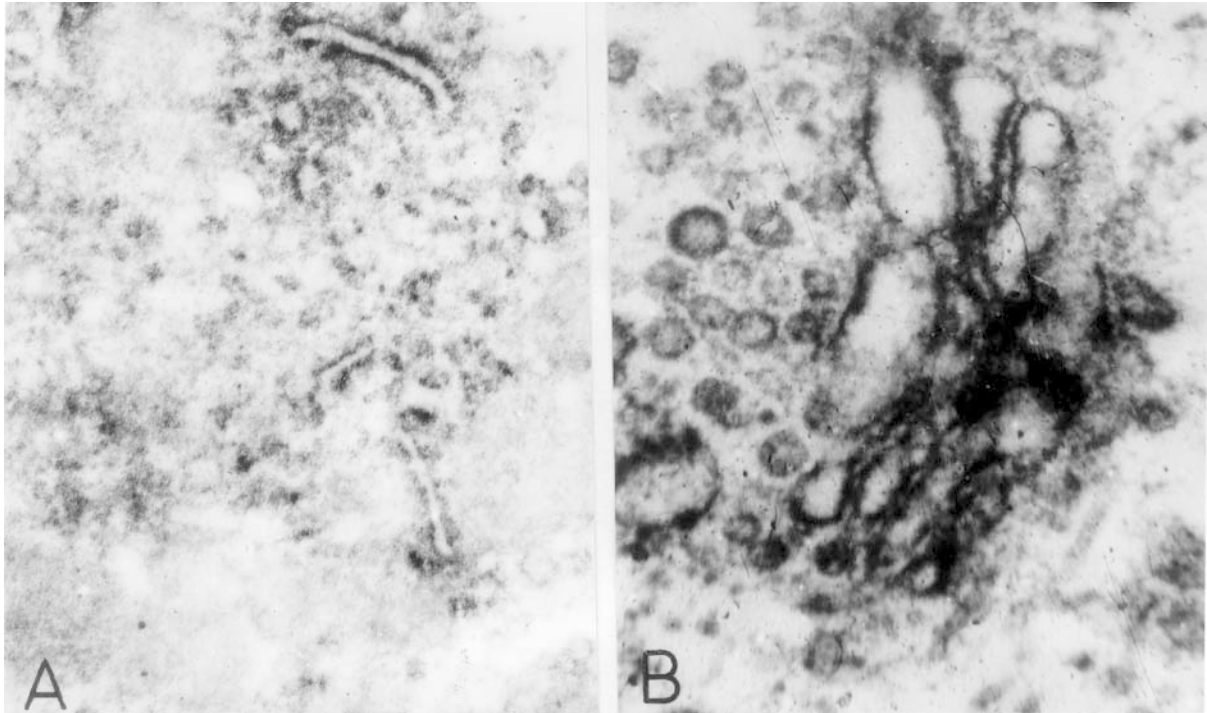


Fig 3

Electron micrographs showing portions of cumulus cells in an early antral follicle. Immunoperoxidase labeling was observed on the membrane of RER (A) and cis and trans side of the Golgi stack (B). A x 7000; B x 2000

Discussion

In the present study we utilized a monoclonal antibody prepared against a rat ovarian antigen and identify a 76 kDa protein in the porcine follicles. This protein was established on blot of rat granulosa cell and oocyte protein extract obtained from the cells utilized for immunization.

We observed an especially strong immunoreaction in cumulus cells and oocytes in early antral and late antral follicles, less pronounced labeling in the oocytes of preovulatory follicles, and no reaction was detected in the oocyte of involuting follicles.

It is known that the cumulus-oocyte complex in antral follicle is characterized by a large number of cytoplasmic processes that can be detected in zona pellucida and a significant exchange of molecules between the cumulus cells and the oocyte occur (GILULA et al. 1978). This communication is terminated near the time of ovulation. This temporal pattern of the termination of communication between the cumulus and the oocyte indicates that communication

provides a mechanism for regulating the maturation of the oocyte during follicular development (GILULA et al. 1978). The biochemical mechanisms involved in the breakdown of communication remain unknown.

Our immunoelectronmicroscopical observations of granulosa cells revealed that the reaction product is localized in RER, cis and trans faces of the Golgi stack, NE and numerous transport vesicles budding from the ER. This picture is in accordance with the current views on the exocytic pathway (BALCH et al. 1994).

On the basis of the localization of the antigen, recognized by the Mab 3D8, it can be expected that it is produced by the granulosa cell. The degree of follicular maturation and the position of GCs within the follicle appear to modulate their capacity for production of the 76 kDa antigen.

Using immunofluorescence we observed the 76 kDa protein in granulosa cells cultured in serum-free medium. However, we failed to identify the 76 kDa band on blot of GSPs. This can be explained by one

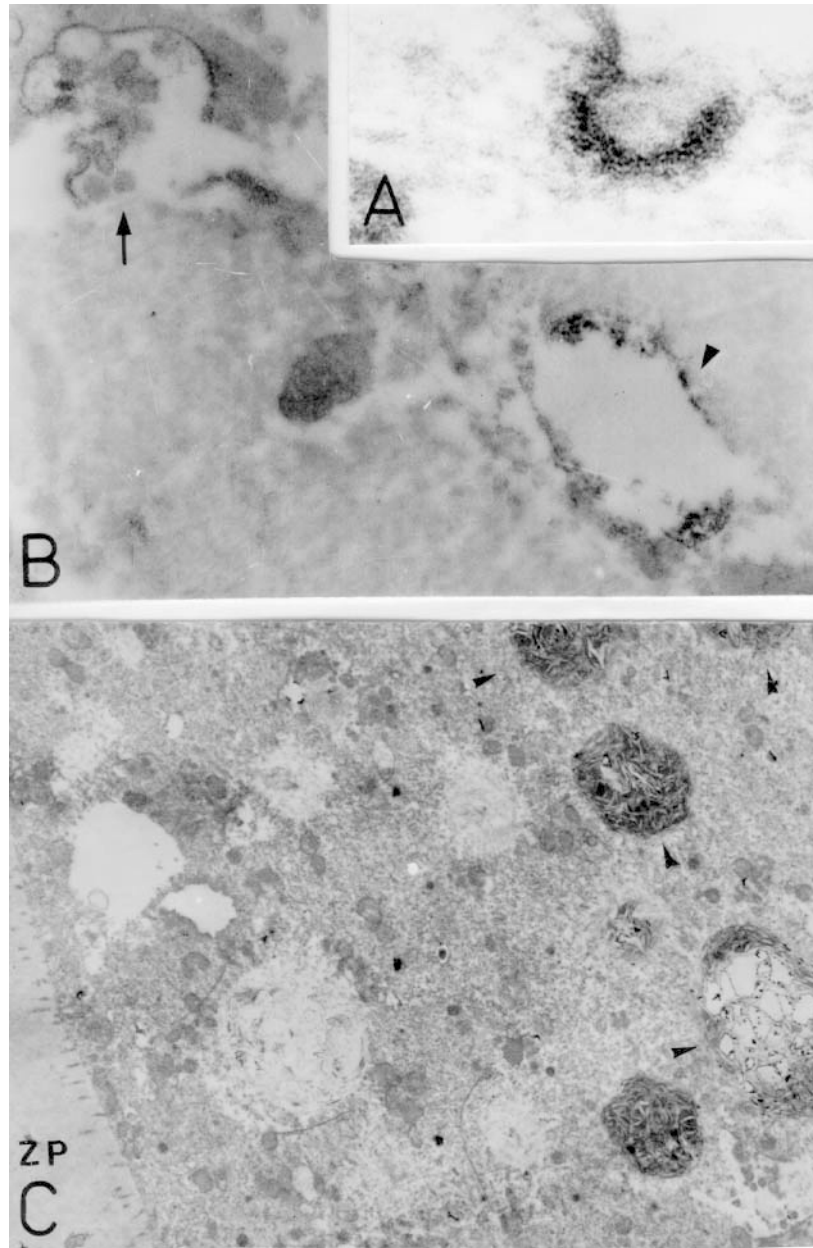


Fig 4

Immunoelectron micrographs of portions of oocytes showing different organelles involved in the endocytosis. (A)- labeled coated pit at the oocyte cell surface in early antral stage. (B)-two subset of labeled endosomes. Arrow – endosome consisting of a central vacuolar element. Arrowhead – endosome whose central vacuole carried many internal vacuoles. (C) – low magnification image of perinuclear region of the oocyte (late antral follicle) with reaction product in structures which we refer to as the prelysosomal compartment (arrowheads). ZP, zona pellucida. A x 30000; B x 12000; C x 7000

of a number of possibilities: The disruption of intracellular communication in culture between granulosa cells, between granulosa cells and oocytes, and

the changes in the cytoskeletal structure may alter the secretory activity of the granulosa cells, as it has been suggested by CARNEGIE and TSANG (1988). An-

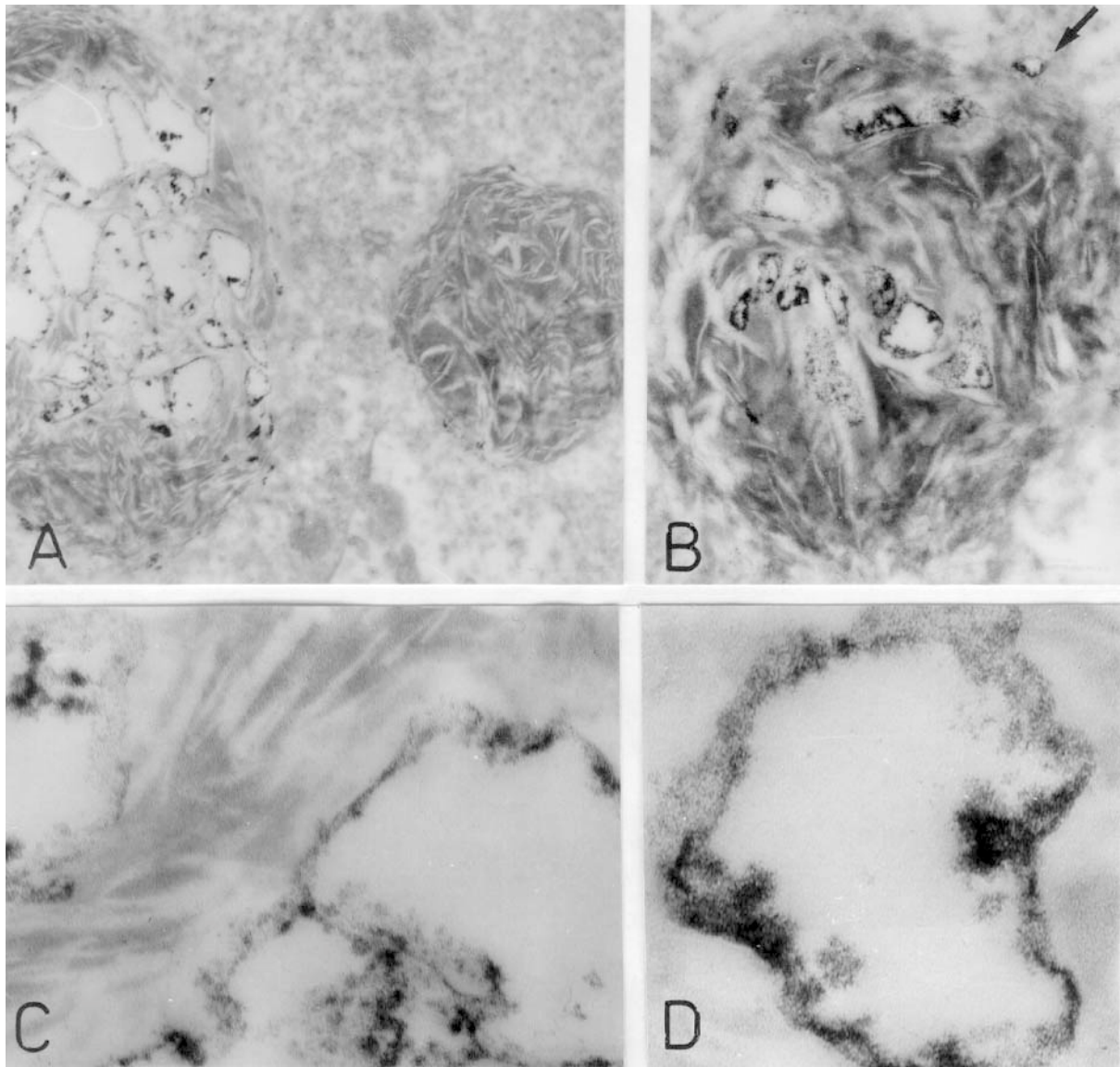


Fig 5

Immunoelectron micrographs – higher magnification of prelysosomal compartment (PLC) in a late antral follicle. Arrowhead in B points at a labeled endocytic vesicle with irregular shape located at the periphery of PLC. Higher magnification of matrices of the PLC (A,C and D). A x 12000; B x 30000; C and D x 90000

other possibility is that the 76 kDa antigen is degraded within the ER, as it has been reported about many proteins, by a proteolytic system that has not been fully characterized (KLAUSNER and SIFIA 1990). A third possible explanation of this result is that GCs acquire the characteristics of luteal cells in culture, a process which has been termed “spontaneous luteinization” (LUCK et al. 1990; LAVRANOS et al.

1994). Finally, at this stage of our study we cannot rule out the possibility, that the concentration of the 76 kDa protein in the serum-free medium is as low as to preclude its detection with the methods employed.

Our immunoelectron microscopic study yield evidence that the oocytes have the ability to internalize the 76 kDa granulosa-cell-secreted protein via an

endocytic pathway. We find that in oocytes at different stages the reaction product is localized in different organelles, whereby its localization apparently depends on the oocyte developmental stage. We assume that the intracellular site of 76 kDa protein accumulation varies during oocyte development.

We observed the reaction product in organelles related to specific endocytosis – small pits at the cell surface, early endosomes, endocytic carrier vesicles and structures closely resembling the prelysosomal compartment. These morphological data correlate nicely with the morphological features of the receptor-mediated endocytic pathway described for other cell types (GRIFFITHS et al. 1988, 1989, 1990a,b). Receptor-mediated endocytosis is accepted as a general mechanism used by many cells for uptake of biologically important molecules. It is a constitutive endocytic process that uses clathrin-coated pits as vehicle for internalization. We are aware that the colocalization of clathrin-coated structures with the 76 kDa antigen would yield more conclusive evidence for our interpretation. However, this will be the aim of our future investigations.

In the present study using Mab 3D8 we established that the final destination of the 76 kDa protein is in structures which we refer to as the PLC. We suppose that endocytosis of the 76 kDa protein proceeds in at last two discrete steps. Firstly, the internalized molecules are delivered to the early endosomal compartment. The endocytosed material is then translocated to a perinuclear location, namely into the PLC, which may represent a later endosomal stage. The PLC observed by us have a specific morphological appearance which is very similar to the late endosomes described by GRIFFITHS et al.(1988). A similar structure filled with tubulo-vesicular membranes could also be detected in other cell types (GRIFFITHS et al. 1990a,b) and is one of the major stations along the endocytic pathway (GRIFFITHS et al. 1989).

SEGRETAIN et al.(1992) described receptor-mediated endocytosis in all classes of mammalian male germ cells. The authors suggested that the endocytic processes are most probably required for male germ cell division, differentiation and metabolism. Receptor-mediated endocytosis of yolk protein precursors (vitellogenin) has been described by WALL and MELEKA (1985) in the oocytes of most nonmammalian vertebrates. The same authors identified two distinct pop-

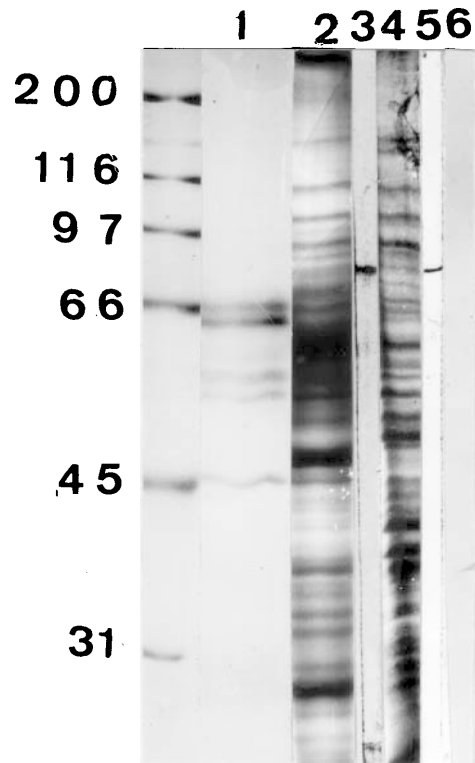


Fig 6

Characterization of the 3D8 antigen. Column 1 – protein analysis by SDS- PAGE of conditioned medium from porcine GC culture (GSPs). Column 2 – protein analysis of porcine granulosa cell and oocyte protein extract. Column 3 – the nitrocellulose replica of the same gel as shown in column 2, labeled with Mab 3D8. A band with molecular size of approximately 76 kDa was stained. Column 4 shows protein analysis of rat GCs and oocyte protein extract. Column 5 – nitrocellulose replica of the same gel (column 4), labeled with Mab 3D8. No band was detected on blot of GSPs (column 6). The position of molecular mass markers in kilodaltons are indicated on the left.

ulations of lysosomal organelles in vitellogenic oocytes. They suggest that endocytic vesicles, containing vitellogenin, can at a later stage fuse with modified lysosomes.

Studies on the regulation of vitellogenin uptake by OPRESKO and KARP (1987) led to the idea that oocytes seem to be unique in their way of regulating receptor-mediated endocytosis. The information derived from this system will be generally useful in understanding of cell surface dynamics.

In conclusion, Mab 3D8 recognizes a unique 76 kDa protein in porcine ovarian follicles. On the basis of

the results obtained in the present study it can be suggested that the porcine oocyte possess the endocytic structures which allow them to bind and internalize this protein, which is most probably produced by the granulosa cells. The expression of the 76 kD protein varies during follicular development. To our knowledge, there is no information to data about such staining pattern given by Mab 3D8. The physiological function of the 76 kDa protein is still unknown. It is possible that this protein participates in the processes governing the follicular development.

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