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Expression of the Neural Cell Adhesion Molecule in Endocrine Cells of the Ovary*

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ABSTRACT. In the adult mammalian ovary morphogenesis and differentiation processes are under hormonal control and, thus, occur in a highly regulated way during the sexual cycle. Cell-cell interactions, such as cell adhesion and cell separation, are crucial during these events. Here we show that the ovarian endocrine cells, which are prototypes of steroid-producing cells, express neural cell adhesion molecules (NCAMs). The combined use of *in situ* hybridization histochemistry, immunocytochemistry at the light and electron microscope levels, S1 nuclease protection assays, and Western blotting revealed that in the ovary of the adult rat during the estrus cycle and pregnancy, NCAM mRNA and the 140-kDa isoform of this protein are expressed mainly in granulosa cells of growing preantral and antral follicles and in corpora lutea. Since the granulosa cells lining the forming antrum and the antral fluid were strongly immunoreactive, a role for NCAM in the formation of the follicular antrum is proposed. The expression of NCAM was also associated with luteal cells of the active corpus luteum, indicating a role for NCAM in the morphogenesis of this endocrine compartment. Moreover, thecal cells of large follicles and hypertrophic thecal cells of atretic follicles expressed NCAM, as did interstitial cells, which are derived from thecal cells of atretic follicles. We propose that the adhesion molecule, NCAM, is an important factor involved in the recognition and intercellular interaction of ovarian endocrine cells and, thus, participates in the regulation of the cyclic remodeling processes of the ovarian endocrine compartments. (*Endocrinology* **129:** 792–800, 1991)

CELL to cell adhesion is a complex process controlled by a variety of surface molecules. One of the best examined examples of adhesion molecules is the neural cell adhesion molecule (NCAM), which was first characterized in the nervous system (1–3). These molecules, glycoproteins belonging to the immunoglobulin (Ig) superfamily, exist in three major isoforms, with mol wt of approximately 180,000, 140,000, and 120,000 daltons (1–3). They have been shown to be transiently expressed during embryonic development in a variety of tissues (1–3), indicating the importance of NCAMs in morphoregulatory processes. The main differences between the NCAM isoforms, which are all derived by alternative splicing of a single NCAM gene (3), are found in their membrane-spanning and cytoplasmic domains, while the amino acid sequences of the extracellular domain are identical (4). The NCAM isoforms are expressed in different proportions by different cells. Specific posttranslational modifications of these molecules, such as phosporylation, sulfation, and polysialylation, influence the homophilic adhesive binding properties of the molecules (3).

In contrast to the embryo, in the adult only a few tissues undergo morphogenesis and differentiation under normal physiological conditions, namely those in the reproductive tract. Thus, in the ovary extensive remodeling during follicular growth (5), subsequent ovulation, and formation of the corpus luteum resemble processes occurring during embryogenesis. These events are under hormonal control. The notion that the formation/dissociation of specific cell-cell contacts is necessary in mediating hormonal influences has recently been proposed for growing follicles, as rat granulosa cells, but not other endocrine cells of the ovary are immunoreactive for the calcium-dependent cell adhesion molecule E-cadherin (6, 7). Ovarian endocrine cells, comparable to pure peptidergic endocrine cells (like pituitary cells), produce a number of peptidergic hormones (*e.g.* inhibin and relaxin) besides steroids (8). All peptide hormone-producing cells analyzed in previous studies express a specific isoform of NCAM (9, 10). These observations have led us to examine at mRNA and protein levels, whether NCAMs are expressed in the rat ovary and whether they could be involved in the complex morphogenetic events in the rat ovary.

Materials and Methods

Animals

Adult 3- to 5-month-old female rats (Sprague-Dawley; n = 18) as well as pregnant rats [gestation day (GD) 14 or 16; n =

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5] were purchased from Charles River (Sulzfeld, Germany) and housed under standard conditions with free access to food and water. Animals were decapitated under deep CO_2 anesthesia. Ovaries were removed and frozen immediately in liquid nitrogen (for S1 nuclease protection assay and Western blotting), fixed in 4% paraformaldehyde-PBS for 8 h (for cryostat sectioning and subsequent immunocytochemistry or preembedding immunocytochemistry at the electron microscope level), or immersed into Bouin's fixative overnight (for subsequent embedding into paraffin and immunocytochemistry at the light microscope level). In addition, for control of the cycle with the help of the histological appearance of the vaginal epithelium, vaginae of the nonpregnant rats were immersed into Bouin's fixative, embedded into paraffin, and processed for routine histological examination (including hematoxylin-eosin staining). The cerebellum, a tissue known to contain all three major NCAM isoforms, was frozen to use as a control in S1 assays and Western blots.

cDNA and cRNA probes used for S1 nuclease assays and in situ hybridization histochemistry

The mouse NCAM cDNA DW 22-H\E (generous gift of Dr. Wille, Cologne, Germany) used for cDNA probe synthesis is a M13 mp19 subclone of DW 22 (position 1640–2601) (11, 12). This probe covers parts of exons 10 and 11–13 (see Fig. 2). For S1 nuclease analysis the bacterial strain CMK603 was infected with M13 mp19, which contained NCAM cDNA. Uniformly labeled single stranded probe was prepared as previously described (13), using an oligo that hybridizes to nucleotides (nt)

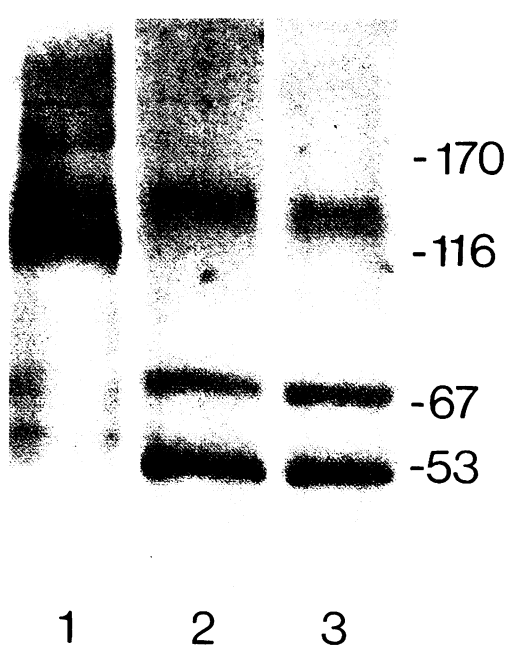

FIG. 1. Result of Western blot. Ovaries from pregnant rats (gestation day 16; lane 2) and ovaries from cycling rats (proestrus; lane 3) contain NCAM 140 and additional immunoreactive bands of approximately 70 and 50 kDa. Rat cerebellum shows NCAM 180, 140, and 120 (lane 1). The mol wt of markers (kilodaltons) are indicated at the *right*. Antiserum was obtained from Dr. G. Rougon (Marseille, France). Dilution, 1:1000.

2054–2070 (11). The labeled primer-extended NCAM subclone was digested with *Hin*dIII, yielding two fragments, which were separated on 5% polyacrylamide gels (containing 8.3 M urea). The 485-nt fragment was isolated and rerun on a 1% agarose gel. Subsequently, the DNA fragments were transferred electrophoretically to NA 45 DEAE-cellulose membrane. The yielded purified fragments were used for S1 nuclease protection assays, as described below. Figure 2 depicts the probe, which spans 19 nt of exon 10 and 11–13, with the insertion of two extra exons a18 (15 and 3 nt) between exons 12 and 13, and the protected fragments.

cRNA synthesis

As a template for cRNA synthesis we used the cDNA clone pM1.3 (generous gift of Dr. C. Goridis, Marseille, France) (14) in the pGEM-1 vector (Promega Biotec, Madison, WI). Synthesis of ^{35}S-labeled cRNA (SA, 8×10^8 cpm/μg) for *in situ* hybridization was carried out according to the T7-polymerase protocol of Promega Biotec using 107 μCi [α-^{35}S]CTP (SA, 37 Tera-Bequerel/mM) and NCAM cDNA clone pM1.3 linearized with *Sau*3A. The resulting cRNA probe contained 219 nt complementary to exons 12–14 (position 2044–2263) and 24 nt of vector sequence (see Fig. 2).

S1 nuclease protection assay

To detect specific NCAM mRNA in rat ovarian homogenates, we performed S1 nuclease protection assays (13). Ovaries were thawed and homogenized, and RNA was isolated, as described previously (15), by a modified guanidinium thiocyanate-CsCl method (16). A total of 20 μg RNA (determined photometrically) was hybridized with an excess of ^{32}P-labeled cDNA probe (5×10^4 cpm; SA, 1×10^8 cpm/μg) in 75% formamide, 400 mM NaCl, 1 mM EDTA, and 20 mM Tris-HCl (pH 7.4) (13) for 16 h at 58 C. Hybridization was terminated by digestion with 680 U S1 nuclease (Angewandte Gentechnologie Systeme, Heidelberg, Germany) for 2 h at 37 C. After phenol extraction and ether precipitation, samples were separated electrophoretically on 5% polyacrylamide gels (8.3 M urea). Gels were dried and exposed to x-ray film at −70 C using intensifying screens.

In situ hybridization histochemistry

Bouin-fixed ovaries were embedded into paraffin, and 5-μm thick sections on gelatin-coated glass slides were used for *in situ* hybridization histochemistry, as described previously (15). In brief, sections were deparaffinized (xylene, ethanol, chloroform, and ethanol) and air dried. Sections were then prehybridized at 50 C for 3 h with 1 ml hybridization solution [50% formamide, 0.75 M NaCl, 25 mM PIPES (pH 6.8), 25 mM EDTA, 5 × Denhardt's, 0.2% sodium dodecyl sulfate (SDS), 10 mM dithiotreitol, 250 μg/ml denaturated herring sperm DNA (Boehringer, Mannhein, Germany), and 10% dextran sulfate]. Subsequently sections were hybridized at 50 C overnight with 5 ng labeled cRNA probe (specific activity 8×10^8 cpm/μg) in 150 μl hybridization solution in a humidified chamber. Controls consisted of sections pretreated with 100 μg/ml RNAse-A (Boehringer) at 37 C for 30 min before hybridization. After hybridization, sections were treated for 30 min at 37 C in a

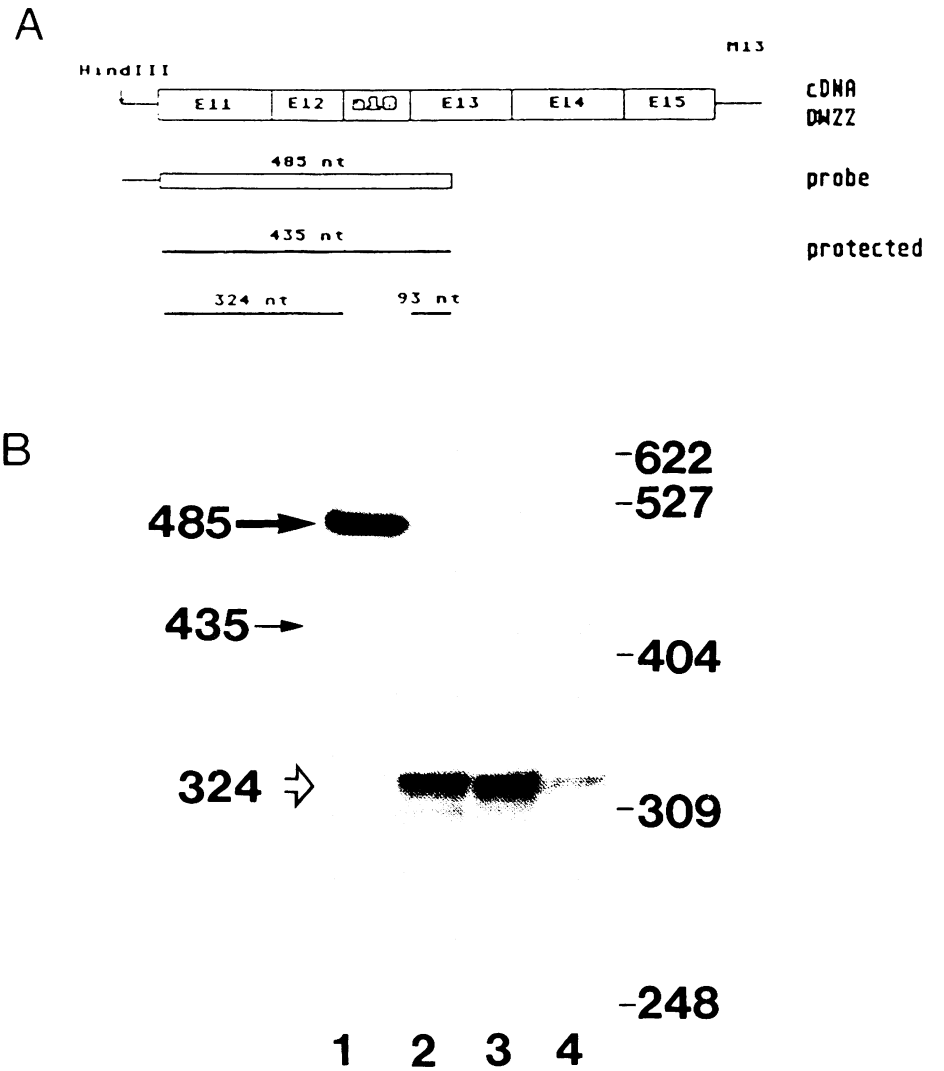

FIG. 2. Analysis of ovarian mRNA. A, Scheme of the NCAM cDNA probe used for S1 nuclease protection assays and the protected fragments. A cDNA probe, derived from cDNA clone DW 22 (11, 12) was used, spanning parts of exon 10 (19 nt of the 3′ end) and exons 11, 12, and 13, with the insertion of two extra exons a18 (15 and 3 nt) between exons 12 and 13, as specified previously (11). The protected fragment of 435 nt indicates NCAM mRNA-containing transcripts of the end of exon 10 and exons 11–13 with a18. If two protected fragments of 324 and 93 nt are yielded, the examined mRNA is devoid of extra exons a18, but is colinear to parts of exon 10 and exons 11–13. B, Results of S1 nuclease protection assay. The mRNA from rat ovaries (lanes 3 and 4) contain NCAM-specific mRNA. The 324-nt fragment is present (*open arrow*), indicating the absence of extra exons a18. Twenty micrograms of each RNA were examined. Lane 1, Probe (485 nt; *thick arrow*); lane 2, cerebellum containing NCAM mRNA with (435-nt fragment; *thin arrow*) and without extra exons a18 (324-nt fragment; *open arrow*; 93-nt fragment not shown). The ovary from a cycling (proestrous) rat (lane 3) and the ovary from a pregnant rat on gestation day 16 (lane 4) contain specific NCAM mRNA without a18 (fragment 324 nt). The sizes (nt) of markers (PBR 322 × *Hpa*II) are indicated at the *right*; the sizes of the probe and the protected fragments are shown at the *left*.

buffer containing 0.5 M NaCl, 10 mM Tris-HCl (pH 7.5), 1 mM EDTA, and 100 μg/ml RNAse-A. They were then incubated for 30 min at 37 C in the same buffer without RNAse-A and washed twice with 2 × SSC at 50 C for 15 min. Slides were rinsed in ascending alcohol solutions, air dried, and dipped in Ilford K2 emulsion diluted 1:1 with distilled water. They were exposed for 3–6 weeks at 4 C and developed with Kodak D19 (Eastman Kodak, Rochester, NY) at 16 C for 4 min. The sections were stained with Hemalaun and eosin and examined with a Zeiss Axioplan microscope (Oberkochen, Germany).

Western blotting

Western blotting was performed as described previously in detail (15, 17). In brief, frozen samples were thawed, homogenized in 62.5 mM Tris-HCl buffer (pH 6.8) containing 10% saccharose and 2% SDS, and sonicated before heating (100 C for 5 min). Protein content was determined using the BCA kit (Pierce, Weiskirchen, Germany), and samples (15 μg protein/lane) were separated electrophoretically on 7.5% SDS-polyacrylamide gel electrophoresis (SDS-PAGE). Proteins were blotted electrophoretically onto nitrocellulose and probed with a well characterized NCAM-specific rabbit antiserum (18) (generous gift from Dr. G. Rougon, Marseille, France), which is directed against the N-terminal end of NCAM (used at a 1:1000 dilution; overnight at 4 C). Immunoreaction was either detected with ^{125}I-labeled protein-A (15, 17) or by a nonradioactive detection. For the latter, the incubation of the nitrocellulose with NCAM antiserum was followed by incubation with biotinylated goat antirabbit IgG antiserum (1:500; 1 h at 20 C; Camon, Wiesbaden, Germany), followed by the avidin-biotin (ABC) method, according to the instructions of the kit (Vectastain ABC kit, Camon). Immunoreaction was made visible by incubation with 0.01% H_2O_2 in a 0.075% 3,3′- diaminobenzidine-tetrahydrochloride dihydrate solution (DAB; Aldrich, Milwaukee, WI; diluted in 0.05 M Tris-HCl, pH 7.6) and 0.03% $NiCl_2$.

Immunocytochemistry

Light microscope. The localization of NCAM in the ovary was assessed with the same antiserum as that used for Western blotting (18). In addition, a monoclonal antibody (MoAB 735; generous gift from Dr. D. Bitter-Suermann, Hannover, Ger-

many) (19), which is directed against homopolymeric $\alpha(2\text{–}8)N$-acetylneuraminic acid (polysialic acid; PSA) linked to NCAM, was used for immunocytochemistry. Both, the ABC method (20) and the peroxidase-antiperoxidase (PAP) method (21) were performed on cryostat sections and deparaffinized Bouin's-fixed paraffin sections. The monoclonal antibody, as expected from previous reports (22, 23), recognized the PSA epitope of NCAM in paraffin section. Since the NCAM antiserum did not recognize its (protein) epitope in paraffin section (most likely due to the known poor preservation of the protein core of the surface molecule NCAM in paraffin-processed tissue) (3), we also used cryostat sections. For this purpose, approximately 10- to 15-μm thick cryostat sections of paraformaldehyde-fixed ovaries were cut. For the PAP method, they were preincubated with 2% normal swine serum (NSS) in Tris-buffered saline (TBS; 0.05 M Tris and 150 mM NaCl; pH 7.6) and then incubated with the NCAM antiserum (diluted in TBS with 2% NSS; 1:1000) overnight at 4 C. Subsequently, they were incubated with a secondary swine antirabbit IgG antiserum (diluted in TBS with 2% NSS; 1:50; Dakopatts, Hamburg, Germany) and then incubated with rabbit PAP (Dakopatts; 1:100) in the PAP method (for details, see Ref.15). For the ABC method, preincubation with 0.01 M PBS (pH 7.3) containing 0.5% BSA, a biotinylated secondary antiserum (goat antirabbit IgG; 1:500 diluted in PBS with 1% BSA; Camon) and a commercial ABC kit (Vectastain, Camon) were used. Immunoreaction was visualized in either case with 0.01% H_2O_2 in a 0.075% DAB solution (in 0.05 M Tris-HCl, pH 7.6). Controls consisted of 1) omitting the first antiserum and incubations with respective buffers, 2) incubation with normal rabbit serum instead of the specific antiserum or antibody, and 3) incubation with a rabbit antisynaptophysin antiserum (1:1000; generous gift from Dr. Jahn, Martinsried, Germany).

For detection of the PSA epitope by the monoclonal antibody, we also used deparaffinized 5-μm thick sections of Bouin's-fixed ovaries, which were incubated with the monoclonal antibody (1:1000) overnight. For detection with the PAP method, a secondary rabbit antimouse IgG antiserum (1:50) followed by mouse PAP (1:100) were used (both from Sternberger-Meyer Immunocytochemicals, Inc., Jarretsville, MD). For detection with the ABC method, a biotinylated horse antimouse IgG antiserum (1:500; Camon) and a commercial ABC kit (Vectastain, Camon) were used. Immunoreaction was visualized with DAB. Besides omission of the antibody, controls consisted of incubation with two other commercial IgG mouse monoclonal antibodies (antityrosine hydroxylase and antiglial fibrillary acid; 1:1000; Boehringer). All sections were examined with a Zeiss Axioplan microscope.

Immunocytochemistry at the electron microscope level. The subcellular localization of NCAM in the ovary (corpus luteum) was examined with the help of the NCAM rabbit antiserum used for Western blotting and immunocytochemistry (18), following the methods previously described (23) with minor modifications. Vibratome-sections (100 μm) of 4% paraformaldehyde-fixed ovaries of pregnant (GD 14) and nonpregnant rats were cut and regions occupied by corpora lutea were selected and subsequently incubated with 0.5% Triton X-100 in TBS for 5 min. After a preincubation in 2% swine serum in TBS for 30 min, sections were incubated overnight at 4 C with the NCAM antiserum (1:1000 diluted in 2% swine serum in TBS). Incubation with a secondary antibody (swine antirabbit IgG at 1:50) and incubation with rabbit PAP followed, as described above. After incubation with DAB for 10 min, sections were treated with 1% OsO_4 (in Tris-HCl, pH 7.6) for 45 min. Sections were then embedded into Araldite. Thin sections were cut, stained with 2% uranyl acetate for 15 sec and lead citrate for 1 min, and then examined with a Zeiss EM 10 transmission electron microscope. For controls the incubation with the first antibody was omitted.

Results

By Western blotting, immunoreactive NCAM with a molecular mass of 140 kDa was identified in ovarian homogenates of pregnant and nonpregnant cycling rats (Fig. 1). Additional immunoreactive bands of approximately 70 and 50 kDa were seen, the nature of which was not analyzed in depth in this study (see Ref. 10 for details). No quantitative difference in immunoreactive NCAM became obvious between pregnant and nonpregnant animals (only proestrous ovary shown).

The results of the S1 nuclease assays revealed that NCAM-specific mRNA is present in the ovary of pregnant and nonpregnant rats. The hybridization with the probe mainly spanning exons 11–13, followed by digestion with S1 nuclease, yielded a 324- and a 93-nt fragment (93-nt fragment not shown), indicating that ovarian NCAM mRNA is characterized by the lack of extra exons a18 (Fig. 2). Greater amounts of NCAM-specific mRNA were observed in the ovaries of cycling animals (during the proestrous phase where follicular development is taking place) compared to the pregnant rat ovary (which mainly consists of corpora lutea; Fig. 2).

Immunocytochemistry at the light microscope level, using the same NCAM antiserum as that used for immunoblotting and a monoclonal antibody detecting NCAM-bound PSA, revealed that several endocrine ovarian compartments were immunoreactive. Follicular epithelial cells of small preantral follicles and to a greater degree granulosa cells of large follicles were NCAM immunoreactive. Distinctly stronger immunoreaction was associated with those granulosa cells that lined the antrum and surrounded the oocyte (Figs. 3 and 4). The NCAM antiserum, directed against a peptide sequence of the NCAM molecule also stained the follicular fluid of large follicles (Figs. 3 and 4). In contrast, the monoclonal antibody directed against the NCAM-specific PSA did not stain the follicular fluid, only granulosa cells. Immunoreaction was also associated with thecal cells of large antral follicles (Fig. 4). The NCAM antiserum and the monoclonal antibody also stained the luteal cells of the forming and active corpus luteum. The luteal cells from presumably older corpora lutea (stemming from

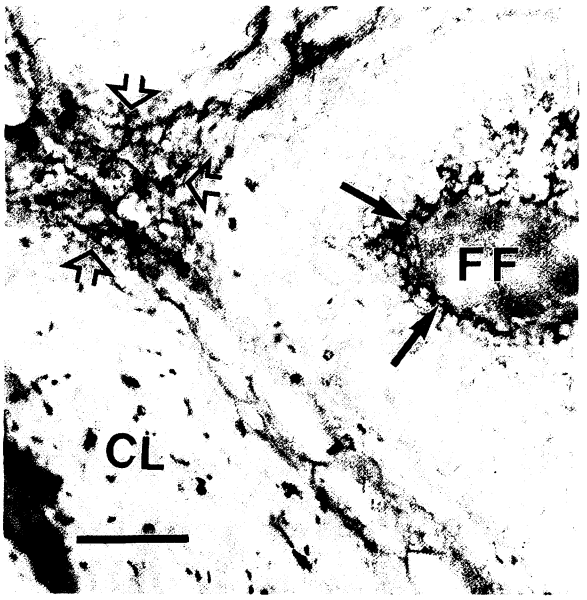

FIG. 3. While mural granulosa cells of an antral follicle only faintly stain for NCAM, strong NCAM-immunoreactive granulosa cells (*arrows*) border at the immunoreactive follicular fluid (FF). Note strongly stained interstitial cells (*open arrows*) and only faintly stained cells of a corpus luteum (CL). Cryostat section; PAP method; rabbit antiserum from Dr. G. Rougon (Marseille, France); dilution, 1:1000; *Bar* = 100 μm. Figures 3–6 show immunocytochemical analysis of NCAM at the light microscope level. In Figs. 4–6 the endocrine compartments also contain PSA linked to NCAM.

previous cycles, as judged by areas of degeneration, reduced blood support, and leucocyte infiltration) showed little or no immunoreaction. By contrast, hypertrophic thecal cells of atretic follicles (Fig. 5) and interstitial cells, which are derived from thecal cells of atretic follicles (Figs. 3, 4, and 6), were consistently strongly immunoreactive (Fig. 3). All controls performed were negative and synaptophysin, glial fibrillary acid protein, and tyrosine hydroxylase antibodies, respectively, did not stain the endocrine compartments of the ovary (not shown).

Preembedding immunocytochemistry at the electron microscope level performed on sections of corpora lutea of pregnant rats indicates specific and exclusive labeling of the cell surface for NCAM (Fig. 7).

Results of *in situ* hybridization histochemistry indicate that the NCAM-immunoreactive cell types also contained NCAM mRNA. The strongest labeling occurred in the granulosa and thecal cells of growing preantral and antral follicles (Figs. 8 and 9). Thecal cells of large follicles were labeled (Fig. 9). Active corpora lutea and interstitial cells (Figs. 8 and 9) were also consistently labeled. No labeling of RNAse-A-treated controls was observed (not shown).

Discussion

The present study examines for the first time expression of the cell surface molecule NCAM in the adult

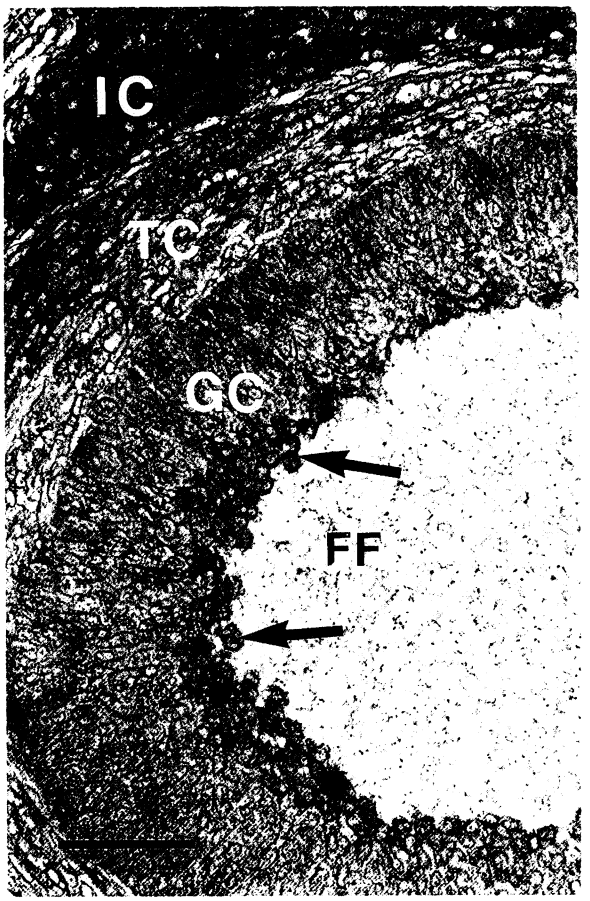

FIG. 4. Immunoreactive, strongly NCAM-positive interstitial cells (IC), thecal cells (TC), and granulosa cells (GC) of a large antral follicle are shown. *Arrows* point to distinctively stained granulosa cells found adjacent to the follicular antrum. Note that the follicular fluid is unstained (compare Fig. 3). Bouin fixation; paraffin section; ABC method; Mab 735 from Dr. D. Bitter-Suermann (Hannover, Germany); dilution 1:1000. *Bar* = 100 μm.

ovary. We report the presence and sites of expression of the 140-kDa isoform of NCAM and its mRNA in the ovary of the adult rat. The ovary is subjected to continuous remodeling processes and in this respect is not unlike embryonic tissue, where NCAM expression is associated with morphogenesis and differentiation (1–3). Expression of the adhesion molecule NCAM in the ovary was likewise, associated with morphological development and differentiation of endocrine compartments of the adult ovary. The spatial and temporal patterns of NCAM expression indicate that NCAM expression is associated with growing follicles, active corpora lutea, as well as degenerating (atretic) follicles and interstitial cells. Moreover, follicular fluid showed NCAM immunoreactivity. Similar to NCAM during embryonic morphogenesis, when the functional significance of the adhesion protein NCAM is well established, the sites of NCAM expression in the ovary indicate specific functions of NCAM in the ovary.

FIG. 5. Hypertrophic thecal cells of atretic follicles (AF), but not the remainders of the oocytes (*stars*) are NCAM immunoreactive. Note that the follicular epithelium of the small follicle (*arrow*) also reveals NCAM immunoreactivity. Bouin fixation; paraffin section; ABC method; Mab 735 from Dr. D. Bitter-Suermann; dilution, 1:1000. *Bar* = 100 μm.

We propose that NCAM may be involved in the complex processes of cell-cell adhesion and cell separation of granulosa cells in the growing follicle during antrum formation. It is well known that the development of a follicular antrum requires FSH and estrogens synthesized by granulosa cells (25). Moreover, several other factors (*e.g.* insulin-like growth factor, epidermal growth factor, and fibroblast growth factor) (26) have been reported to be involved in the regulation of follicular growth. The mechanism(s) by which a follicular antrum forms is at present not fully understood. It is thought that initial secretion of follicular fluid by granulosa cells is followed by accumulation of plasma derived fluids (25, 27, 28). It is well established that granulosa cells are connected with each other by cellular junctions, namely gap junctions and desmosome-like structures. However, they lack tight junctions and are separated by channels measuring up to 20 nm (27, 29, 30). It has been proposed that the formation of an antrum requires decreased adhesiveness of adjacent granulosa cells and filling of the intercellular spaces with fluid (31). While the need for fluid accumulation in the formation process of the antrum has recently been doubted, since antrum-like reorganization can occur in cultured preantral follicles (25), it is necessary that granulosa cells separate. In the present study we have localized NCAM mRNA and NCAM immunoreaction in granulosa cells and have observed stronger immunoreaction for NCAM (protein and PSA) in those granulosa cells that line the follicular antrum.

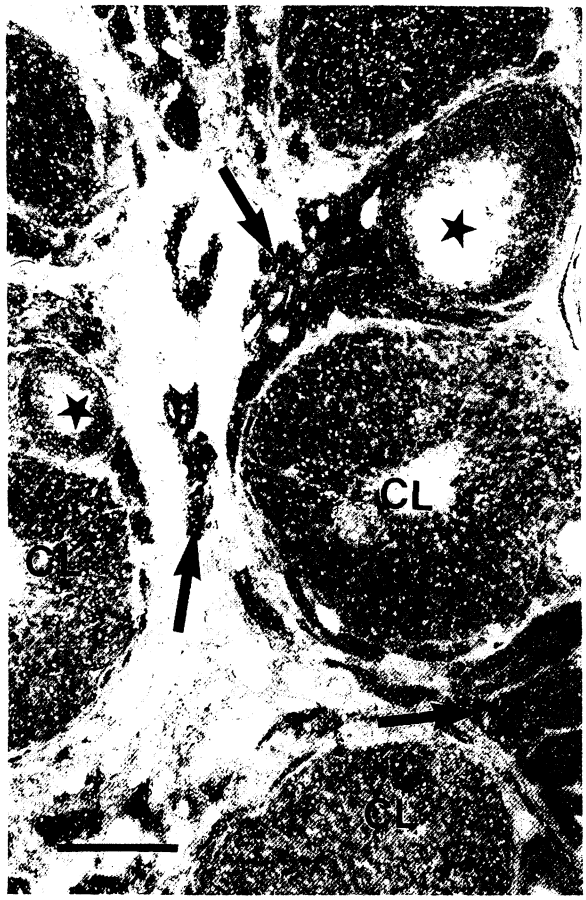

FIG. 6. NCAM immunoreactive luteal cells of several corpora lutea (CL), immunoreactive interstitial cells (*arrows*), and immunoreactive granulosa cells, but unstained follicular fluid of follicles (*stars*) are seen. Bouin fixation; paraffin section; ABC method; Mab 735 from Dr. D. Bitter-Suermann; dilution, 1:1000. *Bar* = 100 μm.

Thus, NCAM is formed by granulosa cells and appears to be involved in the cellular adhesion of granulosa cells. Since the monoclonal antibody MoAB 735 directed against NCAM-linked PSA failed to stain the follicular fluid, in contrast to the NCAM antiserum which strongly stained the follicular fluid, one may conclude that NCAMs without PSA subsequently are released into the follicular fluid. The binding of NCAM is a homophilic mechanism (1–3). One might speculate that release of NCAM forms into the follicular fluid could represent a way in which NCAM can reach and saturate NCAM-binding sites on granulosa cells. In this way, these cells could separate, and NCAM in the growing follicle would have a dual role: cell adhesion and cell separation. Several soluble forms of NCAMs have been described (32–34), one of those bears no PSA residues and has a mol wt of 65 kDa (32). Cunningham and colleagues (32) have reported that 140-kDa NCAM can give rise to this fragment by either spontaneous cleavage *in vitro* (at 37 C after 4–72 h) or protease treatment. Although this frag-

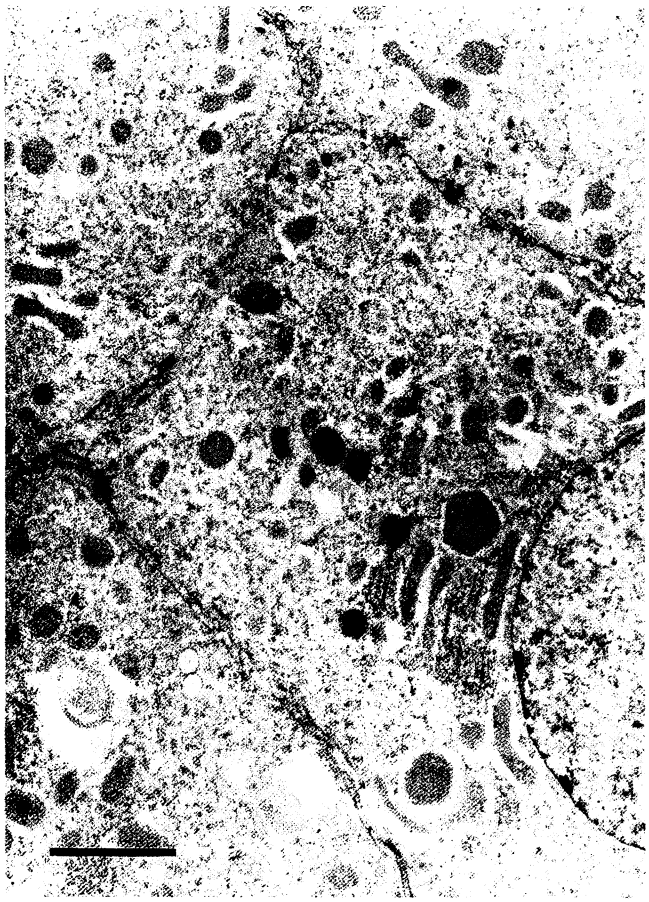

FIG. 7. Immunocytochemical analysis of NCAM at the electron microscope level. The surface of adjacent luteal cell of the corpus luteum of a pregnant rat (GD 16) is labeled, indicting the presence of NCAM. PAP method; rabbit antiserum from Dr. G. Rougon (Marseille, France); dilution, 1:1000. *Bar* = 2 μm.

ment was unable to bind, it was a specific inhibitor of the binding of NCAM to cells, and if convalently bound to solid matrix, it acquired the ability to bind to cells (32). Follicular fluid, besides proteins, proteoglycans, and ill defined substances, contains enzymes, including collagenase (29). It appears possible that 140-kDa NCAM could be cleaved either spontaneously or enzymatically within the follicle. Therefore, the ovarian NCAM form of approximately 70 kDa seen in Western blots of this study might be identical to the fragment described by others. Likewise, one may speculate that the aproximately 50-kDa immunoreactive band found in ovarian homogenates is another NCAM fragment (see Ref. 10). Although additional studies are required to prove the assumption of a soluble NCAM form(s), we suggest that soluble NCAM(s)/NCAM fragments could be involved in antrum formation and, thus, mediate the development of the mature follicle. Alternatively, the strong staining for NCAM-specific PSA of the granulosa cells bordering the antrum of the follicle could indicate reduced adhesiveness, since it is known that the binding properties of NCAM with high levels of PSA are reduced (1–3).

At this point it should be mentioned that the fact that granulosa cells bordering the antrum of follicles subjectively had stronger NCAM immunoreaction than mural granulosa cells is reminiscent of the well known functional heterogeneity of granulosa cells (*e.g.* mural granulosa cells have more receptors for LH, and those close to the antrum express more receptors for PRL) (29).

Interestingly, another type of cell adhesion protein, E-cadherin, a membrane integral glycoprotein, has recently been described on rat granulosa cells *in vitro* (6, 7). In contrast to NCAM, the cadherins mediate calcium-dependent cell adhesion. It was demonstrated that granulosa cells harvested from rat preantral follicles possess E-cadherin, but lose it after manipulations leading to disintegration (6, 7). Reacquisition of E-cadherin and reaggregation of cultured granulosa cells are associated and can be stimulated indirectly by FSH or directly by estradiol (6, 7). Taken together, expression of two completely different adhesion molecules, calcium-dependent E-cadherin and calcium-independent NCAM 140, on rat granulosa cells shed light for the first time on the intercellular dynamic events occurring during follicular growth and antrum formation, which are prerequisites for ovulation.

In this study we, furthermore, provide evidence that after ovulation the endocrine cells of the fast growing and hormonally active corpus luteum express NCAM. To the best of our knowledge, the only adhesion mechanism examined in the corpus luteum is the cell contact of luteal cell, but the presence of adhesion molecules, such as cadherin, has not been reported for this tissue (35). Luteal cells are derived from both thecal and granulosa cells (36), *i.e.* cells that already express NCAM before ovulation, and thus, it is not surprising that after ovulation we found them to express NCAM as well. It appears conceivable that NCAM is involved in the morphogenesis of the corpus luteum. Preexisting NCAM could contribute to cell-cell recognition and adhesion of the differentiating luteal cells and, thus, be one of the factors regulating the rapid development of this endocrine tissue. Interestingly, we observed that not all corpora lutea present in rat ovaries were NCAM immunoreactive, nor did all corpora lutea have NCAM mRNA. A corpus luteum in the rat can persist structurally for about 12–14 days (35), but its functional lifespan, *i.e.* its progesterone production, lasts only 3 days (36). After that time corpora lutea can only produce small amounts of progesterone. Thus, older corpora lutea, indicated by reduced size, the presence of areas of degeneration, leucocytic infiltration, and reduced vascular bed, apparently loose NCAM expression during the process of functional luteolysis.

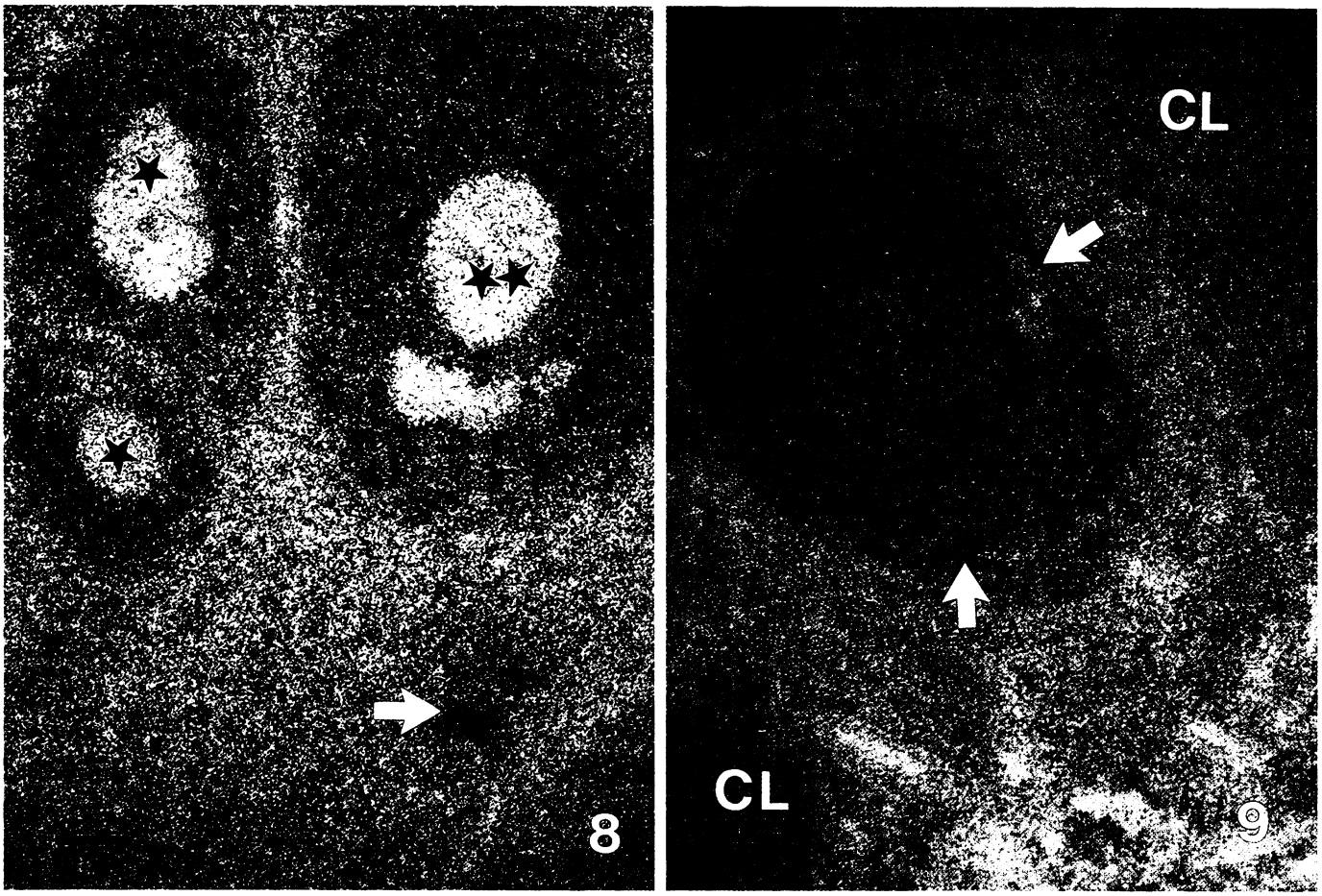

FIG. 8. In this figure and in Fig. 9, *in situ* hybridization histochemistry of NCAM is shown. The presence of NCAM mRNA is indicated by labeling of the granulosa cells of two preantral growing follicles of different sizes (*asterisks* over oocyte) and granulosa cells of an antral follicle (two *asterisks* over oocyte). The *arrow* points to a cluster of interstitial cells, also positive for NCAM mRNA. *Bar* = 40 μm.

FIG. 9. The theca (*arrows*) of a tangentially sectioned large follicle as well as its granulosa cells and luteal cells of corpora lutea (CL) possess NCAM mRNA. *Bar* = 20 μm.

The observation that NCAM is also expressed in thecal cells of large follicles and in the hypertrophic theca of atretic follicle indicates that during the process of atresia of follicles, the adhesion protein NCAM may play a regulatory role. The fact that interstitial cells express NCAM support this assumption, because interstitial cells are thought to be derived from thecal cells of degenerating (atretic) follicles (37). Interstitial cells in the ovary form cell clusters within the ovarian stroma, a behavior that could be related to the fact that these cells adhere to each other via NCAM.

Concerning the presence of adhesion molecules in the theca, we are aware of a single previous report on the presence of another adhesion protein, Thy-1 (38; reviews in Refs. 39 and 40). Thus, Thy-1 immunoreactivity has been reported to occur in the theca of growing ovarian follicles (38). Although the functional significance of Thy-1, a surface glycoprotein in rodent nervous tissue and thymocytes and a member of the Ig superfamily, is not well established, it has been suggested that it could be involved in cell surface recognition (39, 40).

Besides the proposed functional significance of NCAM in the ovarian specific remodeling processes, the fact should be emphasized that NCAM is present on endocrine ovarian cells. These cells are classically regarded as prototypes of steroid producers; however, their dual endocrine nature has been shown. Thus, it is now well established that several peptide hormones (including granulosa-derived inhibin, luteal cell-derived oxytocin and relaxin) (reviewed in Ref. 8) are produced by these cells. Our results for NCAM-positive steroid-producing cells in the ovary are in agreement and largely extend the studies by Langley *et al.* (9, 10), who have localized 140-kDa (endocrine) NCAM on a variety of pure peptide hormone-producing or catecholaminergic cells (*e.g.* adrenal medullary cells, anterior pituitary cells, and pancreatic islet cells). However, the fact that steroid-producing cells share the surface protein NCAM 140 with

peptide hormone-producing cells and neurons has to our knowledge not be shown and may reveal a closer relationship between these cells than hitherto suspected.

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