Galectin-3 is a lectin important in animal development and regulatory processes and is found selectively localized at the implantation site of the mouse embryo. To better understand the role of galectin-3 at the maternal-fetal interface, a binding partner was isolated and characterized. Homogenates of uteroplacental tissue were incubated with immobilized recombinant galectin-3, and specifically bound proteins were eluted using lactose. The principal protein, p400, had an $M_r$ of 400,000 in SDS-PAGE. Physical properties of p400 and amino acid sequences of seven tryptic peptides were similar to cubilin from rats, humans, and dogs, identifying p400 as the murine ortholog of cubilin. This was further supported by the tissue distribution observed only in yolk sac, kidney, and ileum with monospecific antisera for p400. Cubilin occurred in yolk sac epithelium throughout pregnancy, but galectin-3 was there only during the last week. Unexpectedly, cubilin was found only in perforin-containing granules of uterine natural killer (uNK) cells, although galectin-3 occurred throughout the cell cytoplasm. In situ hybridization revealed cubilin mRNA in yolk sac epithelium but not uNK cells, implying that yolk sac-derived cubilin is endocytosed by uNK cells via galectin-3. This is consistent with cubilin being an endogenous partner of galectin-3 at the maternal-fetal interface and suggests an important role for cubilin in uNK cell function.

Galectin-3 (gal-3)$^1$ is one of at least 10 members of a lectin family, which share a conserved carbohydrate recognition domain and which exert their varied biological effects through interaction with complementary β-galactoside ligands of glycoprotein or glycolipid “counterreceptors.” Since galectins are divalent and are able to form multivalent aggregates, they can interact with complementary family, which share a conserved carbohydrate recognition domain and regulatory processes and is found selectively localized at the implantation site of the mouse embryo. Galectin-3 (gal-3)1 is one of at least 10 members of a lectin family, which share a conserved carbohydrate recognition domain and which exert their varied biological effects through interaction with complementary β-galactoside ligands of glycoprotein or glycolipid “counterreceptors.” Since galectins are divalent and are able to form multivalent aggregates, they can interact with complementary family, which share a conserved carbohydrate recognition domains and regulatory processes and is found selectively localized at the implantation site of the mouse embryo.

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**Cubilin, a Binding Partner for Galectin-3 in the Murine Utero-Placental Complex**

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‡ The abbreviations used are: gal-3, galectin-3; rgal-3, recombinant gal-3; PBS, phosphate-buffered saline; 2-ME, 2-mercaptoethanol; PMSF, phenylmethylsulfonyl fluoride; uNK cells, uterine natural killer cells.

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transduction. Isolation of several membrane or matrix counter-receptors by affinity chromatography has revealed that galectins are linked to a variety of important processes including neoplastic transformation, cell adhesion, tumor invasiveness and metastasis, cellular proliferation, and localized immunomodulation (see Ref. 1). gal-3 and its mRNA are expressed in trophoblast cells of placenta as well as in the granular uNK cells of the metrial gland and decidualized endometrium of the murine implantation site (2–4). That finding, along with the additional observation that gal-3 is absent from nondecidualized endometrium between implantation sites and is not present in uteri of nonpregnant females (3), strongly suggests that this lectin has a pregnancy-related function at the maternal-fetal interface. Although a role for gal-3 at the maternal-fetal interface is not yet known, it may contribute in some fashion to cell adhesion, proliferation, or immunomodulation, all of which occur in other contexts. Therefore, to gain more insight into a role for this lectin at the maternal-fetal interface, we isolated and characterized a counterreceptor for gal-3 from tissues of the murine utero-placental complex that was identified as cubilin. This large protein is produced by epithelial cells of the kidney, small intestine, and yolk sac, and it can function as coreceptor for endocytosis of many important biological molecules in these tissues. Consequently, given the proximity of yolk sac to the maternal-fetal interface, the association of cubilin with gal-3 is especially intriguing, as is its association with the granules of uNK cells in the context of cell adhesion, proliferation, or immunomodulation.

**EXPERIMENTAL PROCEDURES**

Animals—Outbred Swiss-Webster mice were used for all studies. Virgin females (10–12 weeks of age; Charles River, Wilmington, MA) were selected at random stages of the estrous cycle, paired with fertile males, and checked daily for the presence of a vaginal plug (designated as day 1 of pregnancy).

Preparation of Lactosyl-Sepharose Affinity Matrix—Sepharose 4B was suspended in an equal volume of 500 mM Na$_2$CO$_3$, filtered in a Buchner funnel, and washed with 5 volumes of 500 mM Na$_2$CO$_3$. This was resuspended in an equal volume of 500 mM Na$_2$CO$_3$, divinylsulfone (10% of the gel volume) was added to activate the matrix; and the slurry was stirred for 70 min gently at 20 °C, washed as above, resuspended in an equal volume of 10% lactose in 500 mM Na$_2$CO$_3$, and stirred for 15 h gently at 20 °C. The lactosyl-Sepharose matrix was washed as above, followed by 5 volumes of distilled H$_2$O and 5 volumes of PBS (pH 7.2). The gel was resuspended in an equal volume of PBS, packed in a 200-ml column (5 × 30 cm), and stored at 4 °C. All subsequent procedures were done at 4 °C unless otherwise indicated.

Isolation of gal-3—Escherichia coli strain JA221 was transformed with murine CBP35 cDNA in a pIN IIIospA2 vector (gift of Dr. J. L. Wang (Michigan State University)) (5) to produce rgal-3. A 10-ml culture, grown overnight in Luria-Bertani broth, was used to inoculate 2 liters of Terrific Broth (12 g/liter tryptone, 24 g/liter yeast extract, 2.31 g/liter KH$_2$PO$_4$, 12.54 g/liter K$_2$HPO$_4$, 4 ml/liter glycerol) containing 100 μg/ml ampicillin. This was allowed to grow with shaking at 37 °C

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for 4 h, and then protein production was induced with the addition of 50 mM isopropyl-1-thio-β-D-galactopyranoside, and cells were cultured with shaking at 20 °C for 16–24 h. Cells were harvested by centrifugation (4000 × g, 20 min); washed in ice-cold PBS; and lysed for 30 min on ice in a buffer (10 mM Tris-HCl, pH 7.2, 0.1 mM EDTA, 1% Triton X-100) for isolation of gal-3-binding proteins. The detergent extract of placental-fetal membranes was determined by immunohistochemistry (using an Amicon stirred cell (PM-10 membrane; Amicon, Beverly, MA). Protein amounts were determined by Bradford assay (Bio-Rad Protein Assay).

Preparation of rgal-3 Affinity Matrix—The affinity resin was prepared by hydrating cyanogen bromide-Sepharose with 0.001 N HCl (15 min), washed with 10 volumes of PBS, and the lysate, containing rgal-3, was applied. Nonbinding material was removed with 5 volumes of TET buffer (50 mM Tris-HCl, pH 7.6, 2 mM 2-ME, 300 mM sucrose), followed by 5 volumes of sucrose buffer without 2-ME. rgal-3 was eluted with lactose elution buffer (300 mM lactose, a disaccharide specific for the carbohydrate recognition domain of gal-3, 50 mM Tris-HCl, pH 7.6, 100 mM sodium acetate, pH 4.0, 500 mM NaCl, and coupling buffer). It was equilibrated with 5 volumes of TET buffer (50 mM Tris-HCl, pH 7.8, 1 mM EDTA, 1% Triton X-100) for isolation of gal-3-binding proteins.

Preparation of Placental-Fetal Membrane Homogenate—Animals were killed by CO2 asphyxiation followed by cervical dislocation at various times of pregnancy, and uteri were removed and opened along the antimesometrial margin. Placentas and associated fetal membranes were separated from uterine implantation sites, and fetuses were discarded; maternal and fetal components were frozen separately in liquid nitrogen and stored at −80 °C. Tissues were homogenized (PT 10/35; Brinkman, Westbury, NY) at 1.0 g of tissue/2 ml of buffer A (PBS with 4.0 mM 2-ME, 1 mM PMSF) with Complete Mini Protease Inhibitor Mixture (Roche Molecular Biochemicals) at 1 tablet/10 ml of solution with 1 ml EDTA and 300 mM lactose. The homogenate was centrifuged (10,000 × g, 20 min at 4 °C), and the supernatant was discarded. The resulting pellet was homogenized with the equivalent of 1.5 g of tissue/1 ml of buffer B (20 mM Na2PO4, pH 7.2, 1.0 mM NaCl, 1 mM PMSF, and Complete Mini Protease Inhibitor Mixture) and centrifuged (10,000 × g, 20 min). This pellet was homogenized in buffer C (50 mM Tris-HCl, pH 7.5, 1 mM EDTA, and Complete Mini Protease Inhibitor Mixture) at the equivalent of 1.5 g of tissue/1 ml of buffer C with 1% Triton X-100 at the equivalent of 1.5 g of tissue/1 ml and centrifuged (10,000 × g, 20 min). Protein content of the final supernatant (i.e. the detergent extract of placental-fetal membranes) was determined by the BCA method (Pierce). In a tissue survey experiment, extracts were similarly prepared from fetal membranes, placenta with and without fetal membranes, decidualized and nondecidualized uteri, and artificially induced deciduomas, kidney, ileum, smooth muscle (large bowel), heart, lung, spleen, brain, and liver.

Isolation of gal-3-binding Proteins from Placental-Fetal Membranes—gal-3-binding proteins were isolated by affinity chromatography on rgal-3-Sepharose and eluted with lactose. In initial experiments, placental-fetal membrane proteins were 125I-labeled (ISOBOEADS; Pierce) and applied to an rgal-3 matrix. Nonbinding material was eluted by extensive washing with TET buffer containing 300 mM sucrose, specifically bound proteins were eluted using TET in 300 mM lactose, and 200-μl fractions were collected. gal-3-binding proteins were run on SDS-PAGE (4–15%) under reducing conditions (Ready Gels; Bio-Rad) and autoradiographed. Preparative amounts of p400 were isolated batchwise, using a slurry of placental-fetal membrane and rgal-3, mixed for 4 h, placed in a chromatography column (0.7 × 10 cm) and washed with 100 column volumes of TET. The matrix was resuspended in an equal volume of TET containing 300 mM lactose and rotated in a 12 × 75-mm Falcon tube overnight. The slurry was put in a 500-μl microcentrifuge tube with a pinhole in the bottom and containing a glass wool plug, fitted in a 12 × 75-mm Falcon tube and centrifuged for 1 min at 188 × g. The quality of each filtrate was checked by SDS-PAGE on a 4–15% gel under reducing conditions and visualized with Coomassie Blue stain. Typically, 50 μg of gal-3-binding proteins were recovered from placental-fetal membrane extracts per 50 placental-fetal membrane units. Precipitation of Anti-p400—gal-3-binding proteins (137 μg), eluted from a rgal-3 column, were electrophoresed on a 5% acrylamide gel in nonreducing conditions and stained with Coomassie Blue. The M, 400,000 band was excised, frozen, homogenized in PBS, and emulsified in an equal volume of Freund’s complete adjuvant. Half of the emulsion was injected intradermally at multiple sites in a rabbit, and the remaining emulsion was injected intramuscularly in the quadriceps muscles. Tests for reactivity were done at 6, 7, and 11 weeks; a booster (30 μg of homogenized p400, but without adjuvant emulsification) was given intramuscularly at 8 weeks. The rabbit was exsanguinated by cardiac puncture at 12 weeks, blood was allowed to clot for 1 h at 20 °C, and the clot contracted overnight. Antiserum was collected, frozen in aliquots, and stored at −80 °C. Specificity was demonstrated by Western blotting of gal-3-binding proteins (1 μg) and placental-fetal membrane proteins (20 μg). Samples were run on 4–15% polyacrylamide gels (Ready gels) under nonreducing conditions and transferred to nitrocellulose. Blots were blocked in 3% nonfat dry milk in TBS (10 mM Tris-HCl, pH 7.5, 200 mM NaCl) for 1–2 h at 20 °C, followed by incubation overnight in primary antiserum at 1:2,500 (i.e. p400 antiserum, preimmune serum, or a commercial polyclonal antibody against placental membranes). Supernatants were decidualized and nondecidualized endometrium, and placental-fetal membranes, decidualized and nondecidualized endometrium, and placental units. Blots were washed (4 × 5 min) in TBS, incubated in secondary antibody (1:20,000 Immunopure goat anti-rabbit IgG horseradish peroxidase-conjugated; Pierce) in 3% milk in TBS for 30 min, washed in TBS, incubated for 5 min in substrate (Super Signal West Pico chemiluminescence substrate; Pierce), and exposed to film. Some blots were incubated in secondary antibody alone as a negative control. For use in immunohistochemistry, anti-p400 was made monospecific by absorption with acetone mouse liver powder (i.e. liver absorbed anti-p400) to remove the M, 70,000 immunoreactive species found in most tissues. 300 μl of anti-p400 (1:50 in TBST plus Complete Mini Protease Inhibitor Mixture) and 5 μg of liver powder were mixed for 30 min and centrifuged at 20,000 × g for 15 min. Supernatant was then collected, and the process was repeated twice. This treatment effectively removed anti-p70 from antiserum; aliquots of liver absorbed, monospecific anti-p400 were stored at −20 °C.

Deglycosylation of p400—The extent of glycosylation of p400 was examined with endoglycosidases using a protocol modified from manufacturer’s instructions (Glycoflex Deglycosylation Kit; Frozyme, San Leandro, CA). Briefly, 3 μg of gal-3-binding proteins isolated from homogenates of decidualized membranes against 250 μg of liver phosphatase buffer, pH 7.0, SDS was added to 0.1% final concentration, and proteins were denatured at 65 °C for 5 min. The sample was cooled to 20 °C; Triton X-100 was added to 0.75% final concentration; 1 μl of peptide N-glycosidase F (500 units/ml), endo-O-glycosidase (1.25 units/ml), or sialidase A (5 units/ml) was added; and samples were incubated at 37 °C. Aliquots were removed at various times up to 72 h and assayed for nonreducing SDS-PAGE (5%) and Western analysis with anti-p400.

Assay for Intramolecular and Intramolecular Disulfide Bonds—gal-3-binding proteins were trichloroacetic acid-precipitated, washed with ice-cold 90% acetone, and dried. The pellet was dissolved in 0.01 N NaOH, denatured at 65 °C for 10 min in nonreducing Laemmli buffer, then mixed against 0.4% acrylamide tube gel, and electrophoresed at 100 V for 3 h. The tube gel was extruded, incubated in reducing buffer (loading buffer plus 20 mM dithiothreitol for 15 min), and loaded onto a 4% preparative slab gel run at 100 V for 1 h in a second dimension. The gel was either silver-stained or transferred to nitrocellulose and probed with anti-p400 for Western analysis.

Amino Acid Sequence Analysis of p400—Amino acid sequencing of p400 was performed by Prof. C. Slaughter (Howard Hughes Medical Institute, University of Texas Medical School, Dallas, TX). Peptides, separated by reverse phase high pressure liquid chromatography on a 150-mm RP300 column (PerkinElmer Life Sciences), were subjected to automated Edman degradation using a model 477A amino acid sequencer (Applied Biosystems, Foster City, CA). Sequences of p400 peptides were compared for alignment with proteins of the National Center for Biotechnology Information database.

Tissue Collection for Immunohistochemistry and in Situ Hybridization—Tissue was prepared from uterine horns of various days of pregnancy, fixed in 4% paraformaldehyde in PBS overnight, and embedded (Paraplast Plus, Sherwood Medical Laboratories, St. Louis, MO), and 5-μm serial sections were mounted on Superfrost Plus slides (Fisher).

In some experiments, a ligature was made at one or both utero-tubal...
junct.ions on day 1 of pregnancy to create a unilateral or bilateral sterile pseudopregnant horn (3), using a standardized stimulus (i.e. an 11-mm cut along the antimesometrial margin of the ovarian end of the uterine horn) delivered on day 4; the resulting deciduomata were harvested on days 8–14 and processed as for normal implantation sites.

Immunostaining—Tissue sections were deparaffinized in xylene, rehydrated in a gradient series of ethanol, treated in 0.3% hydrogen peroxide/methanol (20 min), washed in PBS plus 0.1% bovine serum albumin (fraction V; Fisher), and placed 10 min in boiling 10 mM citrate buffer, pH 6. Slides were cooled, washed 30 min, and blocked in 1% bovine serum albumin plus 1% normal goat serum in PBS; this was followed by incubation with primary antibody (3 h at 20 °C). Anti-p400 and preimmune rabbit sera (negative control) were used at 1:24,000. Sections were incubated in goat anti-rabbit IgG biotinylated secondary antibody (30 min at 20 °C; Vectastain Elite ABC kit, Vector, Burlingame, CA), washed with PBS, and covered with the ABC reagent (30 min at 20 °C). Slides were washed in PBS, developed in 100 mM Tris-HCl, pH 7.2, with 3,3'-diaminobenzidine and H2O2, counterstained with Mayer's hematoxylin, dehydrated, and coverslipped. Anti-perforin (rat IgG2a anti-mouse; Alexis, San Diego, CA) or the isotypic control (rat IgG2a anti-lyt-53 (6-7.2)) was used at 1:400; anti-gal-3 (rat IgG2a anti-Mac-2 (M3/38)) or the isotypic control (anti-lyt) was prepared and used at 1:1600 (3). Slides were washed in PBS and incubated in goat anti-rat horseradish peroxidase secondary antibody (Cappel, Durham, NC) for 90 min, washed, and 3,3'-diaminobenzidine-developed as for the anti-p400.

Dual Label Fluorescence Immunocytochemistry—Slides were treated as above, through the primary antibody incubation step (anti-p400 1:4,000, anti-perforin 1:400, or anti-gal-3 1:800), washed in PBS, and incubated with secondary antibodies (Alexa Fluor 488 goat anti-rat IgG and Alexa Fluor 594 goat anti-rabbit IgG; Molecular Probes, Inc., Eugene, OR) for 45 min at 37 °C. Slides were washed in PBS three times and then in PBS at pH 8.5 mounted in MOWIOL (Calbiochem) solution under glass coverslips and viewed with a Zeiss Axiovert microscope using standard epifluorescence.

In Situ Hybridization—Mouse cubilin (GenBank™ accession number AF 197159) PCR primers were used to prepare a 394-bp cDNA with T7 promoter (see Table I, p. 12004, in Ref. 7) and verified by DNA sequencing. A 940-bp gal-3 cDNA (8) clone, pMac2.3, was made (4). T7 promoter (see Table I, p. 12004, in Ref. 7) and verified by DNA sequencing. A 940-bp gal-3 cDNA (8) clone, pMac2.3, was made (4).

Electrophoretic mobility was selected for further study, because as the major gal-3-binding protein, it may be important for gal-3 function in the implantation complex.

Specificity of Anti-p400—A batch method provided sufficient p400 to produce an antisera for studies of its chemical and immunohistochemical properties. The specificity of anti-p400 was examined as follows: gal-3-binding proteins and placental-fetal membrane extracts were subjected to PAGE, blotted to nitrocellulose, and probed with anti-p400 (Fig. 3); an immunoreactive band (lane 1) extrapolating to about M, 400,000 is found, with a slower moving band extrapolating to about M, 800,000. Immunoactive proteins in placental-fetal membranes are found (lane 2) at an M, of 400,000 and an M, of 70,000. These did not react with preimmune serum (lanes 3 and 4), a nonrelevant antiserum, or secondary antibody alone. After liver powder adsorption, the antiserum was monospecific for p400.

Chemical Characteristics of p400—Electrophoretic mobility of p400 was run under nonreducing and reducing conditions to see if it contains intermolecular or intramolecular disulfide bonds. One-dimensional analysis, visualized with silver staining (Fig. 4A), shows reduced p400 runs slower than the nonreduced form; Western blot analysis (Fig. 4C) confirms both are p400. This is supported by two-dimensional gel analyses (Fig. 4, B and D), in which the p400 is above a theoretical diagonal
on the gel, showing that migration was slower under nonreducing conditions. These results are only compatible with the presence of intramolecular disulfide bonds in a 400 molecule, so p400 is a monomer whose tertiary structure is stabilized by disulfide bonds. Finally, anti-p400 always reacted much more intensely with nonreduced p400 than an equal amount of reduced protein. Since nonreduced p400 was used as immunogen to make this antiserum, loss in Western sensitivity to p400 after reduction probably corresponds to epitope loss from p400.

The p400 was exposed to glycosidases and subjected to Western analysis to determine whether it is glycosylated. After 18 h of incubation (Fig. 5), the electrophoretic mobility of p400 increased about 10% (lane 6) compared with untreated (lanes 1 and 8) and mock-treated samples (lane 7). Thus, p400 is a glycoprotein with significant amounts of N-linked carbohydrate. Incubations of 48 and 72 h or treatment with O-glycosidases or sialidases, produced no additional detectable changes.2

Amino acid sequences of seven peptides of p400 (p400-1 through p400-7) are shown in Fig. 6A. The p400 peptides bear a close resemblance to segments of a full-length protein of rat (AAC 71661), human (AAC 82612), and dog (AAF 14258); i.e. of the 73 amino acids from p400, 72.6% are identical versus those in rat, 69.9% versus those in human, and 67% versus those in dog. 12 of the remaining 21 nonidentical p400 rat-cubilin amino acid pairs are conservative substitutions. In addition, for the only overlap with the recently published fragment of mouse cubilin (AAF 61487), all nine amino acids of p400-7 are identical. Alignment of these seven peptide fragments with rat cubilin is also shown to map them relative to the full-length rat sequence (Fig. 6B). Because these highly similar peptides are
Comparison of Spatiotemporal Patterns of Expression of gal-3 and Cubilin—The cellular sites of synthesis of these proteins and their mRNAs were examined on adjacent histologic sections, using both monoclonal antibody against gal-3 and radiolabeled gal-3 antisense cRNA as probes. On day 5 of pregnancy, decidualizing endometrium and uNK cells were labeled with both the antibody and the cRNA, and by day 12 trophoblast as well as decidual and uNK cells were observed to be expressing both the gal-3 mRNA and protein, as previously reported (4). However, while there was no evidence of labeling of the visceral yolk sac epithelium with either the gal-3 antibody or cRNA up to day 12 of pregnancy, patchy labeling was observed with both probes on day 12. Throughout the remainder of pregnancy, an increasing proportion of epithelial cells of the visceral yolk sac were labeled with both gal-3 antibody and cRNA probes, as shown for day 15 (Fig. 8, B1 and B3).

Results of probing sections of day 16 yolk sac simultaneously with anti-p400 and anti-gal-3 are shown in Fig. 9, A1–A3. The p400 was observed mainly in the apical portion of the cells (Fig. 9, A1), while gal-3 appeared to be distributed throughout the cytoplasm (Fig. 9, A2); superimposition of these images suggests an overlap of these proteins within the apical cytoplasm (Fig. 9, A3). Although anti-gal-3 labels uNK cells (Fig. 9, C2), it is localized mainly in the cytoplasm and possibly the plasma membrane; it does not appear to be directly associated with p400 within perforin-positive granules (Fig. 9, C1–C3).

Finally, to determine if p400 is also present in uNK cells associated with artificially stimulated deciduomata, which do not have an adjacent yolk sac, deciduomata sections were probed with anti-perforin and anti-p400. Granules were labeled with anti-perforin as well as anti-p400. In situ hybridization work on adjacent sections demonstrated that the cells did not contain cubilin mRNA.2

DISCUSSION

This study demonstrates that a monomeric protein, p400, ~400 kDa, can be isolated from homogenates of murine utero-placental complex by affinity chromatography with immobilized rgal-3. Peptide fragments of p400 have amino acid sequences similar to those in cubilin of other species. Although the complete amino acid sequence of murine cubilin is unknown, its sequence has been deduced from full-length cDNA clones of several other species (rat, AAC 71661; human, A00 82612; and dog, AAF 14258). Also, p400 is similar in size to cubilin, which is a 460-kDa peripheral membrane protein with 13% of its mass accounted for by carbohydrate. Like cubilin, p400 is heavily glycosylated and has intramolecular disulfide bonds. Finally, p400 is found only in the yolk sac, kidney, and small intestine, and thus its tissue distribution is identical to that of cubilin (12). Consequently, we conclude that p400 is the mouse ortholog of cubilin.

Cubilin has eight N-terminal epidermal growth factor-like domains followed by 27 "CUB" domains (12), characterized as conserved stretches of amino acids separated by nonconserved regions of variable length. CUB domains are predicted to result from a barrel-like structure with two layers of five-stranded β-sheets, stabilized by two disulfides from four conserved cysteines, and with β-turns in a surface-exposed position, similar to antigen binding regions of IgG (13). CUB domains 5–8 are the binding site for intrinsic factor-vitamin B12 complex, and domains 13 and 14 bind the “receptor-associated protein,” a chaperone-like protein that protects multiple ligand binding sites of processed low density lipoprotein receptor family proteins (15, 16). Other proteins known to bind cubilin with high affinity include megalin, a 600-kDa intrinsic membrane protein, which functions as a multiligand receptor and is localized in clatherin-coated pits of absorptive epithelium in kidney and...
yolk sac (12, 16–19); IgG κ light chain (20); holoparticle high density lipoprotein (21); transferrin (22); and apolipoprotein A-I (23). That cubilin comprises several “specific” binding sites for these and presumably other molecules accounts for its capacity as a multiligand receptor or carrier and explains its ability, possibly in association with megalin, to facilitate endocytosis of a variety of proteins (7, 12, 14). With the identification of p400 as cubilin, it was expected

Fig. 8. Localization of cubilin and gal-3 proteins and their mRNA in yolk sac cells from day 15 implantation sites. Adjacent sections are used for cubilin protein and mRNA (A1–A3); cubilin controls (A4–A6), gal-3 protein and mRNA (B1–B3); and gal-3 controls (B4–B6). A1, probed with anti-cubilin (arrow); A4, probed with preimmune serum as negative control; A2 and A3, probed with antisense [35S]cubilin riboprobes and detected by autoradiography (light field and dark field, respectively). A5 and A6, probed with sense [35S]cubilin riboprobes as negative controls (light field and dark field, respectively). B1, probed with monoclonal anti-gal-3 (arrow); B4, probed with nonrelevant monoclonal antibody 53-6.72 as a negative isotypic control; B2 and B3 (arrows), probed with antisense [35S]gal-3 riboprobes and detected by autoradiography (light field and dark field, respectively). B5 and B6, probed with sense [35S]gal-3 riboprobes as negative controls (light field and dark field, respectively). Note the abundant localization of cubilin mRNA (A2 and A3, arrows) and gal-3 mRNA (B2 and B3, arrows) in yolk sac cells. Bar (A1), 80 μm (applies to all panels).
FIG. 9. Dual localization of cubilin, perforin, and gal-3 protein in day 16 implantation sites. A1–A7, yolk sac; B1–B7 and C1–C7, metrial gland cells. A1, B1, and C1, cubilin; A2 and C2, gal-3; B2, perforin; A3 and C3, overlay of cubilin and gal-3 expression; B3, overlay of cubilin and
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that anti-p400 would label yolk sac epithelium (16–18). Consistent with observations on the ontogeny of cubilin in rats (17), our in situ hybridization studies in mice show that the gene is transcribed in extraembryonic endoderm of visceral yolk sac as early as day 6 of pregnancy through term. The importance of cubilin in the yolk sac has become clear with a demonstration that it was the antigenic target of “teratogenic antibodies” in previous studies (16, 24–26). In this earlier work, it was found that antibodies raised against yolk sac or kidney and administered to pregnant rats (27) or anti-p400 against mouse cubilin administered to pregnant mice2 during the period of organogenesis resulted in severe fetal abnormalities and death. Although the mechanism responsible for the pathological effect of the antibodies is unknown, they localized in yolk sac epithelium, possibly inhibiting the endocytic apparatus in those cells (25–27). Since rodent yolk sac is the major portal for maternal-fetal exchange prior to development of chorioallantoic circulation (28), it is presumed that disruption of transport of nutrients, such as maternal serum proteins, vitamin B12, and cholesterol-containing lipoproteins, during the critical phase of organogenesis could have severe fetal abnormalities and death.

Having isolated cubilin as a gal-3 binding partner and having demonstrated its expression in yolk sac epithelium, it became important to determine whether there was overlap in the tissue distributions of these two molecules and thus the potential for them to interact in vivo. Probing sections of the implantation site with anti-gal-3 and with antisense gal-3 cRNA confirmed earlier observations of its expression in trophoblast cells, decidualized endometrial cells, and uNK cells throughout the course of pregnancy (4). Although neither the gal-3 protein nor its mRNA were detected in yolk sac epithelium before day 12, both were present in increasing amounts throughout the last week of pregnancy, and thus it is possible that during that time cubilin and gal-3 do interact in this region. The prospect of a mechanism by which gal-3 might alter the cubilin-dependent uptake of specific ligands by yolk sac during the last week of pregnancy is potentially very important. However, that interesting question remains to be answered by future experiments.

The observation that cubilin accumulates in perforin-positive granules of the uNK cells was completely unexpected. These cells, considered to be a subset of NK lymphocytes, accumulate in large numbers in the decidua basalis and the metrial gland of the rodent implantation site (30, 31). Their cytolytic granules contain perforin and granzyme, typical of cytolytic lymphocytes (32). However, compared with peripheral NK cells, uNK cells are notoriously poor killers of YAC cell targets in vitro, especially after recovery from the uterus in the latter half of pregnancy (33). While the uNK cells are more potent when stimulated with interleukin-2 (34), normally they do not mount a damaging immune reaction against the semiallogeneic products of conception. Although the importance of uNK cells to pregnancy seems to be that they influence developing placental vascularature by production of proteases, matrix, or biologically active factors (30, 35, 36), the question of why they do not mount an effective immune response against placenta or extraembryonic membranes may be just as important.
to our ultimate understanding of the biology of the implantation site.

Since the lytic granules of NK cells are dual function organelles, which combine the function of secretory and prelysosomal compartments (37), the finding of immunoreactive cubilin in the cytoplasmic granules of uNK cells raises several interesting questions. First, what is the source of cubilin in uNK cells? Since its mRNA was not detected in these cells, the gene appears not to be transcribed. Although adjacent yolk sac is a likely source, the observation that it is present in uNK cells in artificially induced deciduomata, which are not associated with the developing yolk sac, is compatible with cubilin also being taken up from the systemic circulation. Additionally important and interesting questions include the following. What is the mechanism of cubilin uptake by the uNK cells? Once cubilin is in uNK cells, what is its vectoring pathway to the granules? What is the final molecular form of immunoreactive cubilin in the granules? What is the nature of its association with perforin or granzyme in the granules? What is the function of cubilin in uNK granules? Finally, what are the details of the relationship of gal-3 to cubilin within uNK cells; i.e. does gal-3 facilitate a cubilin function or vice versa? Cubilin uptake by uNK cells might be of major significance if it reflects some mechanism for sampling or processing fetal antigens or for altering immune function by modifying the ability of granules to lyse potential targets.

The original objective of this work, to isolate a binding partner for gal-3 in the uteroplacental complex as a step toward elucidating the role of the lectin in pregnancy, was achieved with the identification of cubilin. The finding that it co-localized with the lectin in yolk sac epithelium in the last week of pregnancy is intriguing and may suggest the existence of unappreciated mechanisms governing maternal-fetal exchanges. The additional finding that cubilin accumulates in uNK cells may also have far reaching implications for the localized modulation of the immune system that occurs at the implantation site. These observations provide important new directions for research of the maternal-fetal interface.

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