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Maxwell T. Hincke, Joël Gautron, Charles P. W. Tsang, Marc D. Mckee, Yves Y. Nys. Molecular cloning and ultrastructural localization of the core protein of an eggshell matrix proteoglycan, ovocleidin-116. *Journal of Biological Chemistry*, 1999, 274 (46), pp.32915-32923. 10.1074/jbc.274.46.32915 . hal-02696733

HAL Id: hal-02696733

<https://hal.inrae.fr/hal-02696733>

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Molecular Cloning and Ultrastructural Localization of the Core Protein of an Eggshell Matrix Proteoglycan, Ovocleidin-116*

(Received for publication, June 16, 1999)

Maxwell T. Hincke†§, Joël Gautron¶, Charles P. W. Tsang||, Marc D. McKee**‡‡, and Yves Nys¶

From the ||Centre for Food and Animal Research, Agriculture Canada, Central Experimental Farm, Ottawa K1A 0C6, Canada, the **Department of Anatomy and Cell Biology, and Faculty of Dentistry, McGill University, Montreal, Quebec H3A 2B2, Canada, the ¶Station de Recherches Avicoles, Institut National de la Recherche Agronomique, Nouzilly, France 37380, and the ‡Department of Cellular and Molecular Medicine, University of Ottawa, Ottawa, Ontario K1H 8M5, Canada

The role of avian eggshell matrix proteins in shell formation is poorly understood. This calcitic biomaterial forms in a uterine fluid where the protein composition varies during the initial, calcification, and terminal phases of eggshell deposition. A specific antibody was raised to a 116-kDa protein, which is most abundant in uterine fluid during active eggshell calcification. This antiserum was used to expression screen a bacteriophage cDNA library prepared using mRNA extracted from pooled uterine tissue harvested at the midpoint of eggshell calcification. Plasmids containing inserts of differing 5'-lengths were isolated with a maximum cDNA sequence of 2.4 kilobases. Northern blotting and reverse transcriptase-polymerase chain reaction demonstrated that the 2.35-kilobase message was expressed in a uterine-specific manner. The hypothetical translational product from the open reading frame corresponded to a novel 80-kDa protein, which we have named ovocleidin-116. After removal of the predicted signal peptide, its N-terminal sequence corresponded almost exactly with that determined from direct microsequencing of the 116-kDa uterine protein (this work) and with that previously determined for the core protein of a 120-kDa eggshell dermatan sulfate proteoglycan (Corrino, D. A., Rodriguez, J. P., and Caplan, A. I. (1997) *Connect. Tissue Res.* 36, 175–193). Ultrastructural colloidal gold immunocytochemistry of ovocleidin-116 demonstrated its presence in the organic matrix, in small vesicles found throughout the mineralized palisade layer, and the calcium reserve assembly of the mammillary layer. Ovocleidin-116 thus is a candidate molecule for the regulation of calcite growth during eggshell calcification.

The avian eggshell forms in the uterine (shell gland) region of the oviduct in an acellular milieu that is supersaturated with respect to calcium and bicarbonate and which contains a variety of proteins whose concentrations vary during the sequential process of shell formation (1–4). The eggshell matrix proteins that have been identified can be subdivided into at least three groups. Osteopontin, also found in bone matrix, is an eggshell component, as are a number of egg white proteins (ovalbumin, ovotransferrin, and lysozyme) (5–7). The third group are proteins that N-terminal amino acid sequencing and immunochemistry indicate are eggshell-specific. One of these, ovocleidin-17, exhibits similarity in its N-terminal sequence to a variety of snake venom lectins and to the C-type lectin-like domain of certain proteoglycans (8).¹ The proteins are initially secreted by epithelial and glandular cells into the uterine fluid, whose protein composition varies remarkably during the initial, active calcification and terminal phases of eggshell mineralization (9). Sequential deposition of these matrix proteins into the forming eggshell results in their different localization patterns as the inner (mammillary) and outer (palisade) layers of the mineralized shell are assembled (10).

Partially purified eggshell matrix and uterine fluid proteins can dramatically delay the precipitation of calcium carbonate from a metastable solution of calcium chloride and sodium bicarbonate (9, 11). We have demonstrated that such partially purified mixtures contain eggshell osteopontin and that dephosphorylation dramatically reduces this inhibitory activity.² In addition, the carbohydrate moieties of dermatan sulfate and keratan sulfate proteoglycans have been demonstrated immunochemically in eggshell matrix (12, 13). *In vitro*, fractions containing partially purified eggshell dermatan sulfate proteoglycan alter the morphology and decrease the size of growing calcite crystals (14–16). However, despite these studies, the direct roles for specific matrix components in eggshell formation remain undefined. There has been a lack of precise molecular detail regarding the uterine-specific eggshell matrix proteins, information that would allow comparisons with similar proteins in other calcifying tissues. In the present study, we report the cloning, characterization, and ultrastructural localization of a 116-kDa protein that is a major constituent of the uterine fluid during the calcification stage of shell formation. Furthermore, sequence comparisons reveal that it corresponds to the core protein of a unique, partially characterized dermatan sulfate proteoglycan that possesses little homology to proteoglycans previously isolated from bone or cartilage.

* This work was supported by the Natural Sciences and Engineering Research Council (to M. T. H.) and the Ontario Egg Producers' Marketing Board (to M. T. H. and Y. N.) and the Medical Research Council (M. D. M.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

The nucleotide sequence(s) reported in this paper has been submitted to the GenBank™/EBI Data Bank with accession number(s) AF148716.

‡‡ A member of the Medical Research Council group in Periodontal Physiology and a scholar of the Fonds de la Recherche en Santé du Québec.

§ Some of this work was accomplished with Institut National de la Recherche Agronomique support during the sabbatical stay at Station de Recherches Avicoles, Institut National de la Recherche Agronomique, Nouzilly, France. To whom correspondence should be addressed: Dept. of Cellular and Molecular Medicine, University of Ottawa, 451 Smyth Rd., Ottawa, Ontario K1H 8M5, Canada. Tel.: (613) 562-5800 (ext. 8193); Fax: (613) 562-5434; E-mail: mhincke@uottawa.ca.

¹ M. T. Hincke, manuscript in preparation.

² M. T. Hincke and M. St. Maurice, submitted for publication.

MATERIALS AND METHODS

Uterine Fluid Sampling—Egg-laying hens (ISA BROWN) were housed individually in cages located in a windowless, air conditioned poultry house. They were subjected to a cycle of 14 h of light:10 h of darkness and were fed *ad libitum* on a layers' diet as recommended by the Institut National de la Recherche Agronomique (France). Cages were equipped with a computerized system to record the precise time of daily egg laying (oviposition). Ovulation was considered to occur 0.5 h after oviposition (11). Eggs were expelled with an intravenous injection of 50 µg/hen of prostaglandin (prostaglandin F_{2α}) at 6–9 h (initial stage), 18–19 h (active calcification phase), and 22–23 h (terminal phase of shell calcification) after the preceding oviposition (11). Uterine fluid was collected immediately after the egg expulsion by gravimetry into a plastic test tube placed at the entrance of the everted vagina. Aliquots of uterine fluid were immediately diluted 1:1 in sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE)³ buffer (0.0625 M Tris-HCl, pH 6.8, 2% SDS, 5% β-mercaptoethanol, 25% glycerol, 0.01% bromophenol blue). A portion of the fluid was kept to measure protein concentration, and the remainder was rapidly frozen in liquid nitrogen and stored at –20 °C.

Extraction and Solubilization of Proteins from Eggshell Matrix—Protease inhibitors were used throughout all extraction procedures (17). The first extraction protocol was based upon methods developed to extract matrix proteins from bone and cartilage (17) and has been previously described (9, 11). Briefly, eggshell powder was extracted with 4 M guanidine hydrochloride, 0.05 M Tris-HCl, pH 7.4, for 4 days. The resulting supernatant was dialyzed, desalted, and then concentrated by ultrafiltration (Amicon Cell, cutoff 5 kDa). This soluble material is referred to as the extramembral eggshell extract. The pellet was demineralized with 0.5 M EDTA, 0.05 M Tris, pH 7.4, extracted with 4 M guanidine hydrochloride, 0.05 M Tris, pH 7.4, and then centrifuged. The resulting supernatant was desalted and concentrated as described above and is referred to as the intramembral eggshell extract.

The second extraction protocol was an adaption of one previously published (13). A membrane extract was prepared by treating ground shell membranes with 4 M guanidine hydrochloride. The supernatant was desalted by dialysis and concentrated in an Amicon cell. This sample is referred to as the membrane extract. The ground shell was dialyzed against 50% acetic acid to demineralize. After further dialysis against demineralized water, the soluble material was concentrated as before and is referred to as the matrix soluble 1 fraction. The insoluble shell material was extracted as above for the membranes and is referred to as the matrix insoluble 1 fraction.

Extraction of Bone Proteins—Matrix proteins from tibiotarsal bones and from the upper proliferating zone of the cartilage growth plate of broiler chickens (4 weeks old) were extracted by established procedures (17) and are referred to as cartilage- or bone-EDTA extracts.

Tissue Preparation for SDS-PAGE and Western Blotting—Laying hens were sacrificed 2–4 h or 16–17 h after oviposition. Various tissues (0.5–2 g of magnum, white and red isthmus, uterus, liver, kidney, duodenum, and muscle) were homogenized in 10 ml of Tris buffer (50 mM Tris-HCl, 77 mM NaCl, pH 7.4, containing protease inhibitors). Homogenates were centrifuged (10,000 × g, 30 min) before freezing in liquid nitrogen for storage at –20 °C. Blood was collected from the same birds just before sacrifice and centrifuged at 2500 × g to obtain a sample of plasma. The protein concentration of all samples and extracts was determined by a Pierce micromethod (18) using ovalbumin as the protein standard.

Electrophoresis and Electrophoretic Blotting—Tissue samples were diluted 1:1 in SDS-PAGE buffer and boiled for 5 min before separation on gels. SDS-PAGE was performed on a 4–20% gradient gel (19, 20). Proteins were stained with Coomassie Blue or electroblotted (0.8 mA/cm²) for 1 h onto polyvinylidene difluoride membranes (Hybond, Beckman) in 10 mM CAPS buffer, pH 11, 10% methanol for N-terminal amino acid microsequencing or to nitrocellulose membranes (Bio-Rad) in 25 mM Tris, 192 mM glycine, 10% methanol for Western blot analysis.

Antibody Preparation—Two preparative SDS-PAGE gradient gels (3 mm width) were prepared. About 8 mg of uterine fluid protein collected at the active phase of shell calcification were distributed into the sample lanes of both gels and electrophoresed. Gels were stained with Coomassie Blue, destained, and then thoroughly rinsed with demineralized water. The 116-kDa band was excised from both gels and ground

to a fine powder with a Spex freezer mill. The powder was suspended in sterile 154 mM NaCl solution, mixed 1:1 with Freund's complete adjuvant, and injected into two rabbits. The animals were boosted after 3 weeks with the same antigen preparation in Freund's incomplete adjuvant. Animals were boosted 6 times to obtain a satisfactory titer. This antiserum is termed α-116.

Western Blotting—Proteins were electrotransferred to nitrocellulose membrane, which were washed in PBS-Tween (0.01 M phosphate buffer, 0.0027 M potassium chloride, 0, 137 M sodium chloride, pH 7.4, Tween 20 0.1%), and then blocked for 1 h in 5% nonfat dry milk in PBS-Tween. The membranes were washed in PBS-Tween (2 × 5 min) and then incubated for 1.5 h with diluted antiserum (1:5000–1:10,000 in PBS-Tween, 1% bovine serum albumin). After 3 washes in PBS-Tween, the membranes were incubated for 1 h with 1:10,000 anti-rabbit Ig, peroxidase-linked species-specific whole antibody (NA 934, Amersham Pharmacia Biotech) in PBS-Tween, 1% bovine serum albumin. Membranes were washed again (4 times) in PBS-Tween and then twice in PBS. The enhanced chemiluminescence (ECL) method was used according to the manufacturer's instructions to reveal immunoreactive bands.

Partial Purification of Eggshell Matrix Proteins—Normal eggs from White Leghorn hens were cracked open and extensively washed with distilled water. Shell membranes were removed following brief treatment with 1 N HCl at room temperature as described previously (10). Ground shell (140 g) was decalcified in a solution of 0.65 M EDTA, pH 7.5, 10 mM 2-mercaptoethanol, 0.05% NaN₃. The resulting suspension was centrifuged and dialyzed *versus* 10 mM Tris-HCl, pH 7.5, 1 mM 2-mercaptoethanol, 0.05% NaN₃ and loaded onto a previously equilibrated column of Trisacryl-DEAE. After washing the column extensively, all retained proteins were eluted with 250 ml of 1 M NaCl in the equilibration buffer. This solution was applied to a column of hydroxyapatite (8 ml of resin) that had been previously equilibrated with 5 mM potassium phosphate, pH 7.5, 250 mM NaCl, 1 mM 2-mercaptoethanol, 0.05% NaN₃. A linear gradient to 0.4 M potassium phosphate was developed, which eluted 3 peaks (A₂₈₀). These were analyzed by SDS-PAGE and pooled for dialysis *versus* water and lyophilization. The second peak (fraction 27–35) contained ovocleidin-17 and osteopontin in addition to several other bands that were found by microsequencing to be derived from OC-116 (see Fig. 5).

Microsequencing—Sequencing was performed as described previously (8). Proteins from uterine fluid sample (25 µg of protein) harvested from the calcification stage of shell formation were separated by SDS-PAGE on a 7% gel and then transferred to polyvinylidene difluoride (Bio-Rad Laboratories). Protein bands were visualized by Coomassie Blue staining. The 116-kDa band (which ran as 130 kDa on the 7% gel) was excised and washed extensively with sterile water. N-terminal microsequencing was performed at the Service de Sequence de Peptide de l'Est du Quebec, Laval, Quebec and revealed the following sequence: (T/V)PV(S/G)LPAR(A/I)(R/V)GN(D/C)PGQHQLLK.

cDNA Library Preparation and Expression Screening—Pooled RNA was prepared by the guanidinium isothiocyanate extraction method (21) from shell gland mucosal tissue harvested at the middle phase of eggshell calcification from 4 White Leghorn hens. Messenger RNA was purified by affinity chromatography on oligo(dT)-Sephacrose, and utilized to prepare a vector-ligated, directional bacteriophage cDNA library using the Stratagene ZAP-cDNA synthesis kit and Gigapack II packaging system exactly according to the manufacturer's protocols. The phage library was screened by standard methods using antiserum raised to the 116-kDa eggshell matrix protein (1/1000) and anti-rabbit IgG-alkaline phosphatase conjugate (1/3000) as a secondary antibody with nitro blue tetrazolium/5-bromo-4-chloro-3-indolyl phosphate. Protein synthesis was induced with nitrocellulose circles (Amersham Pharmacia Biotech Hybond C) pretreated with 10 mM isopropyl-1-thio-β-D-galactopyranoside. Phages with positive inserts were purified to homogeneity by three rounds of screening and rescued into Bluescript plasmid using helper phage, according to the kit instructions. Plasmid DNA was isolated by alkaline lysis miniprep, and the cDNA inserts were sequenced by automated protocols at the sequencing service, Station de Pathologie Aviaire et de Parasitologie, INRA-Center de Tours, Nouzilly, France.

Reverse Transcriptase-PCR, Northern Blotting, and Southern Blotting—RNA was extracted from oviduct tissues harvested at different stages of eggshell calcification (2 birds/stage) (RNA InstaPure, Eurogentech). Northern blotting was performed by established methods (22) following migration of 15 µg of total RNA on a 1% agarose gel containing formaldehyde and transfer of RNA to Hybond N membrane. A *Bst*II restriction fragment (OC-116 nucleotides 234–959) was radiolabeled by random priming. Following overnight hybridization with this labeled probe at 42 °C, the membrane was washed (42 °C: 2× SSC, 0.1%

³ The abbreviations used are: PAGE, polyacrylamide gel electrophoresis; CAPS, 3-(cyclohexylamino)propanesulfonic acid; OC-116, ovocleidin-116; PBS, phosphate-buffered saline; PCR, polymerase chain reaction.

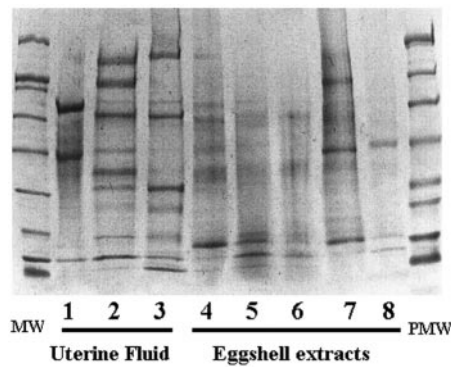


FIG. 1. **SDS-PAGE of uterine fluid and eggshell matrix proteins.** Samples were subjected to SDS-PAGE and stained with Coomassie Blue ("Materials and Methods"). All samples were loaded at 20 μ g of protein except the membrane samples (1.3 μ g). Uterine fluid samples were harvested at different stages of eggshell formation: 1, initial; 2, active calcification; 3, terminal. Eggshell extract samples were as follows: 4, intramineral; 5, extramineral; 6, matrix soluble 1; 7, matrix soluble 2; 8, membranes. Molecular mass markers were from Bio-Rad: MW, unstained broad (kDa, from top), 200, 116, 97.4, 66.2, 45, 31, 21.5, 14.4, and 6.6; PMW, prestained broad (kDa, from top), 210, 120, 84, 47.8, 33.2, 29, 20.1, and 7.7.

SDS, 15 min; 0.5 $\times$ SSC, 0.1% SDS, twice for 15 min; 55 $^{\circ}$ C: 0.5 $\times$ SSC, 0.1% SDS). Labeled bands were visualized with x-ray film and quantified with a Storm PhosphorImager. Southern blotting of chicken genomic DNA (prepared from White Leghorn liver (22)) digested with *Eco*RI and *Bam*HI was also performed using the OC-116 *Bst*EII restriction fragment as a probe. Reverse transcription was performed using random priming and Superscript II RNase H-reverse transcriptase (Life Technologies, Inc.). PCR primers to selectively amplify nucleotides 536–1227 had the following sequences: upper, 5'-TGGAGATGGCGATGGTG-3'; and lower, 5'-TGGCTGGAAGGCTATGC-3'.

Microscopic Analyses and Ultrastructural Immunocytochemistry—Samples of hen uterus and eggshell were fixed in aldehyde immediately after tissue harvest. These were washed, dehydrated in ethanol, and embedded in LR White acrylic resin as described previously (23). Survey sections of embedded tissue were viewed by light microscopy, and selected regions were trimmed for thin sectioning and transmission electron microscopy. Grid-mounted tissue sections were processed for immunocytochemistry by incubation with primary antibodies, and immunolabeling patterns were visualized after conventional staining with uranyl acetate and lead citrate using protein A-colloidal gold complex (23). Incubated grids were examined in a JEOL JEM 1200FX operated at 60 kV. Other samples of eggshell were fractured, critical-point dried, and examined at 3 kV in a JEOL JSM 6400V field emission scanning electron microscope.

RESULTS

OC-116 Protein in Hen Tissues and Eggshell—The protein composition of hen uterine fluid varies dramatically between the initial, active calcification and terminal stages of shell formation (Fig. 1). Particularly evident during the active calcification stage are bands at 180, 116, 66, 45, 36, 32, 21, and 14 kDa. In the present study, polyclonal antibodies were raised in rabbit toward the prominent 116-kDa protein. Western blotting with this antiserum showed immunoreactive bands of 180, 116, 66, and 45 kDa in uterine fluid only at this stage of shell calcification (Fig. 2). Only eggshell matrix extracted by decalcification from the calcified shell (intramineral eggshell extract and matrix soluble 1 fraction) showed similar immunoreactive bands (Fig. 2). Western blot analysis revealed that the 180- and 116-kDa bands were only seen in extracts of uterine tissue and not in other regions of the oviduct or any other tissue (Fig. 3). However, these bands could hardly be detected in uterine tissue harvested at the initial stage of shell formation (data not shown). No immunoreactivity was detected in intra- or extramineral extracts of cartilage or bone or in hen egg white (data

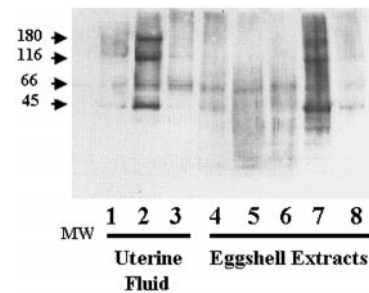


FIG. 2. **Western blotting to detect the 116-kDa protein in uterine fluid and eggshell extracts.** Samples were subjected to SDS-PAGE and electrotransferred to nitrocellulose membrane ("Materials and Methods"). Primary antiserum raised to the 116-kDa protein was utilized at 1:5000 dilution. All samples were 20 μ g of protein except uterine fluid (calcification, 17 μ g; and terminal, 19 μ g) and membranes (1.3 μ g). Uterine fluid samples were harvested at different stages of eggshell formation: 1, initial; 2, active calcification; 3, terminal. Eggshell extract samples were as follows: 4, intramineral; 5, extramineral; 6, matrix soluble 1; 7, matrix soluble 2; 8, membranes. The calculated masses (kDa) of the immunoreactive bands in lane 2 are indicated on the left (arrows).

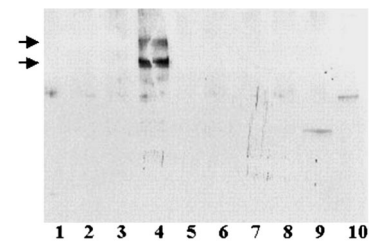


FIG. 3. **Western blotting to detect the 116-kDa protein in oviduct and other hen tissues.** Extracts from different tissues (all 20 μ g of protein) were subjected to SDS-PAGE and electrotransferred to nitrocellulose membrane ("Materials and Methods"). Primary antiserum raised to the 116-kDa protein was utilized at a 1:5000 dilution. Lanes were loaded as follows: 1, magnum; 2, white isthmus; 3, red isthmus; 4, uterus; 5, plasma; 6, liver; 7, kidney; 8, duodenum; 9, muscle. Lane 10 contained molecular weight markers (Bio-Rad, prestained). The arrows indicate the immunoreactive bands seen only in the uterus (145–166 and 99–118 kDa).

not shown). These results suggested that the antiserum raised to the 116-kDa band recognized a protein that was specifically synthesized and secreted by the uterine cells during the active stage of eggshell calcification, which subsequently became incorporated into the calcifying matrix.

Expression Cloning—To clone the coding sequences for the 116-/180-kDa immunoreactive bands, an expression screening approach was taken. A cDNA library prepared with mRNA purified from hen uterine tissue harvested at the midpoint of eggshell calcification was utilized. Expression screening this cDNA library with α -116 yielded a number of clones with inserts of variable length that differed in their 5'-sequence. These were assembled to produce a single composite cDNA sequence. In total, 24 independently purified clones were sequenced, two of which were full-length. All phage inserts that were immunoreactive with α -116 were derived from this sequence, confirming the specificity of the antiserum in this context. The total composite cDNA sequence was 2407 bases in length with a poly(A) tail. The total composite cDNA sequence is seen in Fig. 4.

Analysis of DNA Sequence—No significant similarity was found with any nucleotide sequence in the NIH GenBank or EST data bases.⁴ The context of the first start codon at

⁴ The nucleotide sequence for ovocleidin-116 has been deposited in the GenBank data base under GenBank Accession number AF148716.

	1 AAGCAGCACAGGAGCCAGCCAGG																									
24 1	ATG	CGC	GCA	ACC	CTC	TTC	TGC	CTC	TGC	CTC	TGC	CTT	CTG	GGC	ACC	GTG	CTG	CCC	ACG	CCC						
	Met	arg	ala	thr	leu	phe	cys	leu	cys	leu	cys	leu	leu	gly	thr	val	leu	pro	thr	pro						
84 21	GTG	TCA	CTT	CCA	GCC	AGG	GCA	AGA	GGA	AAC	TGC	CCT	GGA	CAG	CAC	CAG	ATA	CTG	CTG	AAA						
	val	ser	leu	pro	ala	arg	ala	arg	gly	asn	cys	pro	gly	gln	his	gln	ile	leu	leu	lys						
144 41	GGT	TGC	AAT	ACC	AAA	CAT	GGC	TTC	TAC	ATC	TTC	CAG	TAC	ATT	TAC	TCC	CAC	CTC	ATG	CAG						
	gly	cys	asn	thr	lys	his	gly	phe	tyr	ile	phe	gln	tyr	ile	tyr	ser	his	leu	met	glu						
204 61	AAA	AAT	CAG	ACA	CAG	GTC	AAG	AAA	GAA	GAA	GGT	GAC	CAC	CAG	GGC	ACA	ATC	CAT	GGC	CAC						
	lys	asn	gln	thr	gln	val	lys	lys	glu	glu	gly	asp	his	gln	gly	thr	ile	his	gly	his						
264 81	TGG	CTG	GGT	AAG	GTG	GAT	GGG	GAG	GCC	CCA	GGG	CAA	GGT	GTT	GGA	AGC	AGC	CAT	GTG	CCG						
	trp	leu	gly	lys	val	asp	gly	glu	ala	pro	gly	gln	gly	val	gly	ser	ser	his	val	pro						
324 101	GAG	GAC	AAG	GAC	AGC	CCC	AAG	CCC	CAC	AGC	CAC	ATC	ACT	CCA	GCA	AGC	AAA	GGG	GAA	GGC						
	glu	asp	lys	asp	ser	pro	lys	pro	his	ser	his	ile	thr	pro	ala	ser	lys	gly	glu	gly						
384 121	CGT	GCT	CTG	CGC	CCC	GGT	ATT	GGT	GAC	AGC	AAC	AGT	GTG	TAT	CCC	ACC	TCA	ACC	AGT	GTG						
	arg	ala	leu	arg	pro	gly	ile	gly	asp	ser	asn	ser	val	tyr	pro	thr	ser	thr	ser	val						
444 141	GAA	GGC	AGT	GGC	GAC	ATG	GGT	TCG	ATT	TTG	CTG	GGG	GAG	ATC	ATC	AAT	GGC	GAA	GAT	GGC						
	glu	gly	ser	gly	asp	met	gly	ser	ile	leu	leu	gly	glu	ile	ile	asn	gly	glu	asp	gly						
504 161	CTC	CCC	CAC	AGC	ACC	CAC	CCT	GGT	GGA	CCT	CAT	GGA	GAT	GGC	GAT	GGT	GGT	AAC	GGT	GTC						
	leu	pro	his	ser	thr	his	pro	gly	gly	pro	his	gly	asp	gly	asp	gly	gly	asn	gly	val						
564 181	CTG	GTG	GAT	GGG	GCT	GTG	ACT	GCA	GGG	AGG	GAG	AGG	GCC	TCT	GGT	TCC	AAA	GGG	GCT	GGG						
	leu	val	asp	gly	ala	val	thr	ala	gly	arg	glu	arg	ala	ser	gly	ser	lys	gly	ala	gly						
624 201	AGT	GAG	GGT	GGT	AGC	CAT	GCC	ACT	GTG	CCC	GAC	CAA	GGG	CAA	GCA	GGC	ACC	ATG	GGG	ACA						
	ser	glu	gly	gly	ser	his	ala	thr	val	pro	asp	gln	gly	gln	ala	gly	thr	met	gly	thr						
684 221	GGA	GAC	TCT	GCC	ATC	ACC	TCT	GTC	ACA	GAC	TCT	GCC	ATC	ACC	TCT	GTC	ACC	AAG	AAG	GAG						
	gly	asp	ser	ala	ile	thr	ser	val	thr	asp	ser	ala	ile	thr	ser	val	thr	lys	lys	glu						
744 241	GAT	GTC	CAT	GTG	GAC	ACA	GAA	GGC	ATA	GAT	GAG	TTC	GCA	TAC	ATC	CCT	G									

FIG. 4. **Nucleotide and hypothetical protein sequence for OC-116.** The composite nucleotide (*upper*) and amino acid (*lower*) sequences are indicated. The putative signal peptide (1–18) is *underlined*, and the residues that have been directly microsequenced are in *red* and *boxed* (amino acids 19–40, 588–610, 616–627). Potential sites of *N*-glycosylation are *shaded green*, and Ser and Thr residues that are possible targets for phosphorylation by protein kinase C, protein kinase A, or casein kinase II are *underlined* and *shaded yellow*. Ser-Gly sequences that may be *O*-xylosylated and therefore sites of glycanation are *double underlined* and also *shaded mauve* if in an acidic context.

nucleotide 24 is compatible with the Kozak consensus sequence for initiation of transcription in higher eukaryotes (24). An open reading frame of 2343 base pairs was found; the corresponding hypothetical 80-kDa protein sequence is indicated in Fig. 4. A signal peptide cleavage site is predicted between residues 18 and 19 (25). The putative signal peptide (1–18) is underlined in Fig. 4. The proposed N terminus of the mature protein corresponds to the results of direct microsequencing of the 116-kDa band (see "Materials and Methods," (T/V)PV(S/G)LPAR(A/T)(R/V)GN(D/C)PGQHILLK) and is virtually identical to that recently reported for a 120-/200-kDa eggshell dermatan sulfate proteoglycan (PVSLPARARGNCPGQHILLKG CNTK) (26).

Additionally, we have independently confirmed a portion of the OC-116 internal amino acid sequence. In the course of microsequencing eggshell matrix proteins that co-purify with

ovocleidin-17 during hydroxyapatite chromatography ("Materials and Methods"), we obtained amino acid sequences that are derived from internal regions of OC-116 (Fig. 5). The N terminus of band 5 (25.2 kDa) is VWPGAAPAGVVGVAR-PAPSKAY, which corresponds to OC-116 amino acids 588–610 (predicted maximum size, 20.4 kDa). Band 6 (22.4 kDa) has a N terminus VAIGKSTDVPRD, which corresponds to OC-116 amino acids 616–627 (predicted maximum size, 17.6 kDa). These protein sequencing results confirm the sequence fidelity of the OC-116 C-terminal region, which is predicted from the composite cDNA assemblage. In each case, the molecular mass estimated by SDS-PAGE is about 5 kDa larger than that calculated from the predicted OC-116 sequence, suggesting that the C-terminal region of OC-116 is post-translationally modified.

Prosit analysis of the OC-116 protein sequence suggests

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1164 GGT GTC CAA AGT GGC AAG GGG AGA AGC GGA CAG AGG GCA CTG GGG AAG CAT AGC CTT CCA
381 gly val gln ser gly lys gly arg ser gly gln arg ala leu gly lys his ser leu pro

1224 GCC ACC ATG ACA ACT CGG GGT GGC AGA GGA ACA GCC TCC TCA GGG CTG ACT ACA GGG GAC
401 ala thr met thr thr arg gly gly arg gly thr ala ser ser gly leu thr thr gly asp

1284 TGC AGC ACT GCT GCC AGC ACA CCG TCC AGG AAA GGG AGC CAT GTG GTC ACT GCA GGG CAA
421 cys ser thr ala ala ser thr pro ser arg lys gly ser his val val thr ala gly gln

1344 GGA GAG AGC GGT GAG GTG GGG ACA GCA GGC CCA GAG AGA CAG AGG TCT AGG GTC CAG CAA
441 gly glu ser gly glu val gly thr ala gly pro glu arg gln arg ser arg val gln gln

1404 GAG GTG GCA CCA GCA CGT GGT GTG GTG GGA GGA ATG GTG GTC CCT GAG GGG CAC AGG GCT
461 glu val ala pro ala arg gly val val gly gly met val val pro glu gly his arg ala

1464 AGA GTC CAG CAA GAG GTG GCA CCA TCA CGT GGT GTG GTG GGA GGA ATG GTG GTT CCT GAG
481 arg val gln gln glu val ala pro ser arg gly val val gly gly met val val pro glu

1524 GGG CAC AGA GCC AGG ACC CAG CCA GAG GTG GCA TCA GCA CCC AGC ACT GTG GGG AAA GCA
501 gly his arg ala arg thr gln pro glu val ala ser ala pro ser thr val gly lys ala

1584 GCC CCA GAG AGG CAC AGG AAC AGG GCT CAG CAA GAA GTA GCA CCA GTG CCC AGC ATG GTG
521 ala pro glu arg his arg asn arg ala gln gln glu val ala pro val pro ser met val

1644 GTG GAA ACA GTG GCC CCA GAG AGG CAC AGG GCC AGG GTG CGG CCA GAG TCA GCC AGG CTG
541 val glu thr val ala pro glu arg his arg ala arg val arg pro glu ser ala arg leu

1704 GGA CAA GCA GCG AGG CCA GAG GTG GCA CCA GCA CCC AGC ACA GGG GGG AGA ATT GTG GCC
561 gly gln ala ala arg pro glu val ala pro ala pro ser thr gly gly arg ile val ala

1764 CCA GGG GGG CAC CGG GCC AGG GTT TGG CCA GGG GCA GCA CCG GCA CCT GGA GTG GTG GGG
581 pro gly gly his arg ala arg val trp pro gly ala ala pro ala pro gly val val gly

1824 GTG GCC AGG CCA GCT CCC AGC AAA GCC TAT AAT GGG GAC AAA AGG GTT GCC ATA GGA AAG
601 val ala arg pro ala pro ser lys ala tyr asn gly asp lys arg val ala ile gly lys

1884 TCC ACT GAT GTG CCA AGG GAT CCC TGG GTG TGG GGC AGC GCC CAC CCC CAG GCC CAG CAC
621 ser thr asp val pro arg asp pro trp val trp gly ser ala his pro gln ala gln his

1944 ACC AGG GGC AGC ACG GTG GCA GGA GGC TTT GCC CAC CTG CAC AGA GGG CAA AGG TTG GGG
641 thr arg gly ser thr val ala gly gly phe ala his leu his arg gly gln arg leu gly

2004 GGC CTG ACA GAG ATG GAG CAC TCC AGG CAG GTG GAG CAG GTG AGG CAC GCT GAC CGG CTG
661 gly leu thr glu met glu his ser arg gln val glu gln val arg his ala asp arg leu

2064 CGC CTG CAT GAG CGG CCG TGT ATG GCC TCA GCG GGG TGG GCG GCC CCC TGC AGC CTC CCG
681 arg leu his glu arg pro cys met ala ser ala gly trp ala ala pro cys ser leu pro

2124 CAG TCC ACA CTG ACC CCT GGA GCG CCG ACA GCA GCC AGT CTT CTG AGG GGC GGT GGG GCA
701 gln ser thr leu thr pro gly ala pro thr ala ala ser leu leu arg gly gly gly ala

2184 GCC ACA GCG ACA GCC GTG AGG AGG ATG GCG AGG TCA GAG GGT ATC CCT ACG GCC GAC AGT
721 ala thr ala thr ala val arg arg met ala arg ser glu gly ile pro thr ala asp ser

2244 CCC TCT GAG GAG TGC CCT CAG AAG GGT CAG GGT GAT GCA GCA CCC TCT CGG TGG CGT AGG
741 pro ser glu glu cys pro gln lys gly gln gly asp ala ala pro ser arg trp arg arg

2304 GTA GCA TTG TTT CCG TGG AAA GAT GTG GGG AGA AAA GCC CTC CAA TCC TCA TCG TGC TTC
761 val ala leu phe pro trp lys asp val gly arg lys ala leu gln ser ser ser cys phe

2364 TGAAGAAATAAAGGCATTCTGTGCTACTAAAAAATAAAAAAAAAA 2407

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FIG. 4—continued

that there are potentially 2 sites for *N*-glycosylation and 21 sites for phosphorylation by protein kinases A and G, protein kinase C, and casein kinase II (as indicated on Fig. 4). There are 6 Ser-Gly sequences, of which 4 are in an acidic context and therefore are potential glycanation sites (27, 28). Secondary structure predictions for OC-116 are presented in Fig. 6. The only highly hydrophobic region of the molecule is at the N terminus, corresponding to the proposed signal peptide. There is a great propensity for β -sheet, particularly between amino acids 450 and 600, a region of the sequence that protein matrix analysis (Fig. 7) indicates is highly repetitive. This is because of the 8-fold repeat of a consensus sequence PER-HRARVQQEVAPARGVVGGMVV (Table I). The sequence repeat is most pronounced between residues 451–474 and 475–498, which exhibits 55% identity with a sequence found in a

hydroxyproline-rich glycoprotein from the multilayered cell wall of *Chlamydomonas reinhardtii* (29).

Expression of OC-116 Message: Reverse Transcriptase-PCR and Northern Blotting—PCR primers were designed from the composite OC-116 cDNA sequence to investigate the tissue specificity of OC-116 expression. Fig. 8 indicates that OC-116 was expressed in a highly uterine-specific manner and could not be detected in the proximal oviduct (*i.e.* white isthmus and red isthmus) or liver. Northern blotting confirmed the uterine specificity of expression of the 2.35-kilobase OC-116 message, in contrast to magnum (Fig. 9), duodenum, liver, and kidney where no message was detected (not shown). Comparisons between uterine RNA extracts from two CFAR (Center for Food and Agriculture Research, Ottawa, Canada) strains of White Leghorn laying hens indicated that each expressed high levels

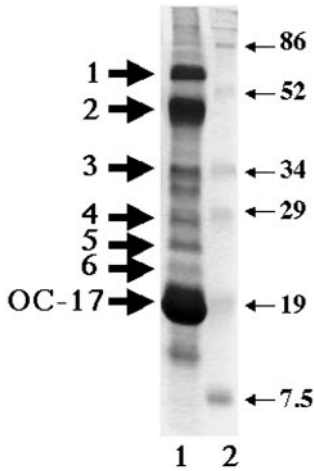
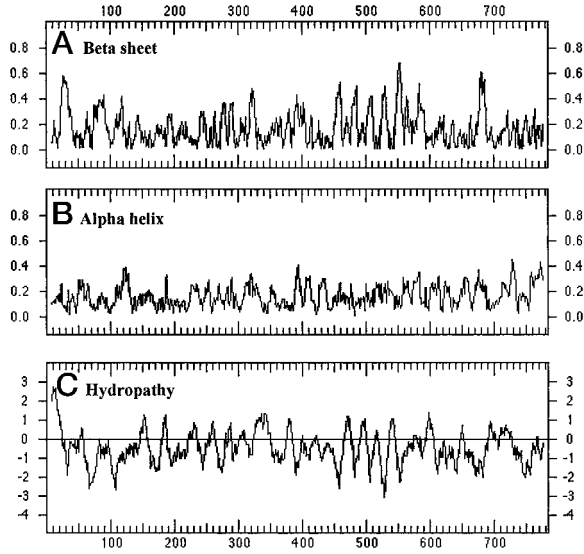


FIG. 5. Microsequencing of partially purified eggshell matrix proteins. Eggshell matrix proteins co-purifying with ovocleidin-17 on hydroxyapatite chromatography (fraction 27–35, “Materials and Methods”) (stained here with Coomassie Blue) were analyzed by microsequencing (lane 1) after transfer to polyvinylidene difluoride from a duplicate 15% gel. Chicken osteopontin residues 17–26 (WPVSKSRQHA) were unambiguously obtained for bands 1, 2, and 3. This corresponds to the N terminus of mature osteopontin. Of the remaining bands, unambiguous results were obtained for 5 (VWPGAA-PAPGVGVARPAAPSKAY, OC-116, amino acids 588–610) and 6 (VAIGKSTDVPRD, OC-116, amino acids 616–627). Protein standards are in lane 2 (Bio-Rad prestained broad).

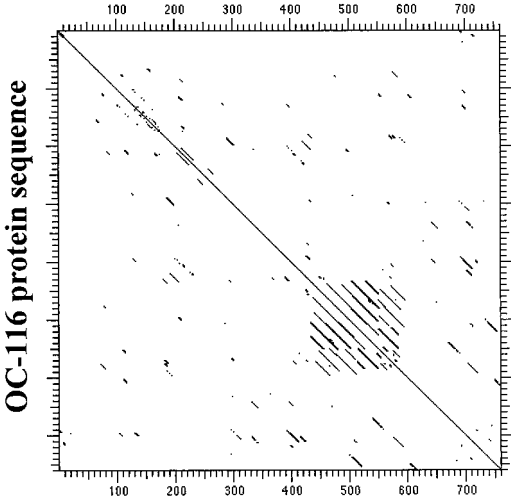


OC-116 Protein Sequence

FIG. 6. Predicted secondary structure of OC-116. The hypothetical protein sequence was evaluated for secondary structure using DNA Strider 1.3 software. A, β -sheet; B, α -helix; C, hydropathy (Kyte Doolittle parameters).

of OC-116 message at the middle of the calcification stage of shell formation (Fig. 9). Strains 1 and 8R differ in that the latter has been selected to lay poorer quality eggshell; it is intriguing that 1.8-fold higher expression levels were found in mRNA derived from this strain, but further study will be necessary to determine if this inverse correlation with shell strength is significant. Northern blotting, followed by quantitation of the 2.35-kilobase OC-116 message by PhosphorImager analysis, indicated that similar levels of OC-116 message were expressed at 3–4 h postoviposition (prior to entry of the egg into the uterus) and at 16–17 h (during eggshell calcification) in ISA BROWN hens ($n = 6$, $p = 0.4$) (not shown).

Genomic DNA Analysis—A Southern blot of chicken genomic



OC-116 protein sequence

FIG. 7. Protein matrix plot for OC-116 protein sequence. Lines parallel to the diagonal are because of homology between one region of the protein and another. This evaluation was performed with DNA Strider 1.3 software.

TABLE I

Repetitive sequence element in OC-116

Identities are bold.

428	PSRKGSVV	TAGQGESGEVGTAG
451	PERQSRVQ	QEVAPARGVVGGMVV
475	PEGHRARVQ	QEVAPSRGVVGGMVV
499	PEGHRARTQ	PEVASAPSTVGKAA
522	PERHRNRAQ	QEVAPVPSMVVETVA
546	PERHRARVR	PESARLGQAAR
566	PEVAPAPSTGG	RIVAPGGHRARVW
590	PGAAPAPGV	VGVARPAPSKAY
Hydroxyproline-rich glycoprotein ^a 4	PEEQRARVQ	LRVRPLEGQPG

^a Accession no. M58496.

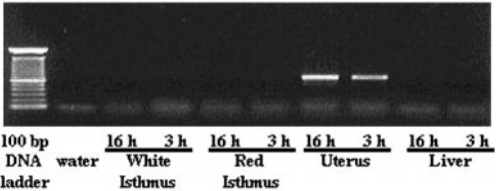


FIG. 8. Reverse transcriptase-PCR demonstration of uterine specificity of OC-116 expression. Primers designed to specifically amplify nucleotide 536–1227 of the composite OC-116 cDNA were utilized for 30 cycles of PCR using standard conditions (“Materials and Methods”). RNA was prepared from tissues harvested from ISA BROWN hens at different times following the expulsion of the previous egg (oviposition): 3 h, prior to entry of the egg into the uterus; 16 h, midpoint of eggshell active calcification.

DNA cut with *Eco*RI and *Bam*HI and probed with the *Bst*XII OC-116 restriction fragment revealed that in each case a single band could be detected (Fig. 10). A single band was also seen following digestion with *Not*I or *Sal*I (not shown). This result suggests that in all probability there is a single gene for OC-116.

Colloidal Gold Immunocytochemistry—The α -116 antiserum was used to localize the 116/180-kDa protein in uterine tissue and in the mineralized eggshell. Ciliated (clear) and granular (nonciliated) cells constitute the epithelium that lines the shell gland lumen, as demonstrated in Fig. 11A by transmission electron microscopy. Colloidal gold immunocytochemistry revealed that the secretion granules in the granular cells are intensely immunopositive (Fig. 11B), whereas those of the cil-

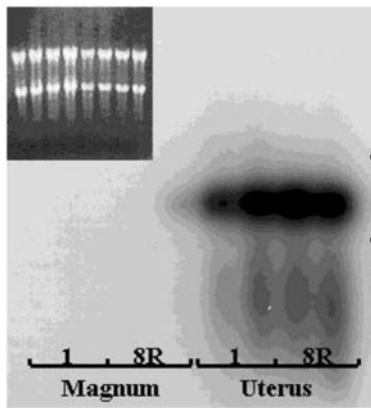


FIG. 9. **OC-116 expression in uterus and magnum (Northern blotting).** Total RNA was extracted from oviduct tissues harvested at the midpoint of eggshell calcification from 4 White Leghorn hens (CFAR strains 1 and 8R) and transferred to nylon hybridization membrane following agarose gel electrophoresis ("Materials and Methods"). A *BST*II restriction enzyme fragment from OC-116 cDNA (nucleotides 234–959) was radiolabeled by random priming and utilized as a probe. The positions of the ethidium bromide-stained 18 and 28 S RNA species (see *inset*) are indicated with *black horizontal bars* on the autoradiogram.

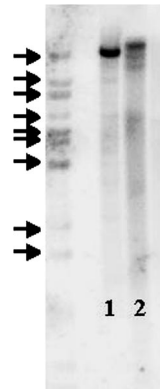


FIG. 10. **Genomic DNA analysis.** Total chicken liver DNA (15 μ g) was digested exhaustively with *Eco*RI (lane 1) or *Bam*HI (lane 2) and transferred to nylon hybridization membrane following agarose gel electrophoresis ("Materials and Methods") for Southern blotting. A single band at 16.0 (*Eco*RI) or 14.1 (*Bam*HI) kilobases was seen after probing with a random primed *BST*II restriction fragment. Standards are indicated from the top (kilobase): 14.1, 8.4, 7.2, 5.7, 4.8, 4.3, 3.7, 2.3, and 1.9.

iated cells exhibit only background staining (Fig. 11C). No staining of the tubular glands of the shell gland mucosa was detected. These results suggest that this protein is synthesized and secreted by the granular epithelial cells.

Within the eggshell, no immunogold labeling was detected in the outermost (cuticle) or innermost (shell membrane) layers. However, substantial staining was present in the palisade layer (Fig. 12A) and crown reserve portion of the mammillary layer (Fig. 12B). In both regions, labeling was predominately associated with the periphery of vesicular structures that may correspond to the walls of microvesicular holes in the calcitic eggshell whose origin remains unclear (see scanning electron microscopy in Fig. 13, A and B). This antiserum stains much less frequently the flocculent sheets of organic material, which are almost exclusively stained by antibodies specific for osteopontin.⁵

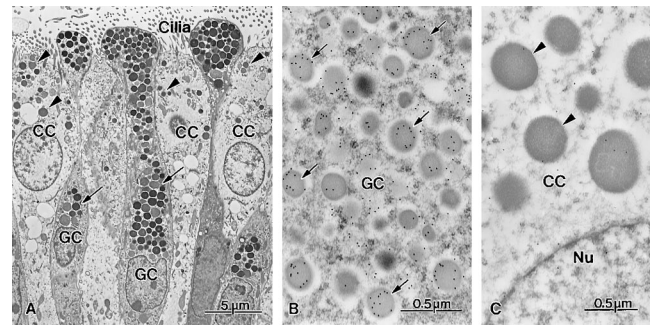


FIG. 11. **Transmission electron micrographs of shell gland epithelium for morphology and for colloidal gold immunocytochemical localization of OC-116 in epithelial cells.** A, a region of the shell gland epithelium containing alternating clear (ciliated) cells (CC) and granular (nonciliated) cells (GC). Clear cells are notable for their abundant apical cilia extending into the lumen of the shell gland, and they contain moderate numbers of secretory granules (arrowheads) dispersed throughout their cytoplasm. Granular cells, on the other hand, do not possess cilia and show numerous secretory granules (arrows) filling much of the supranuclear compartment of the cell. B, secretory granules (arrows) in granular cells (GC) label intensely with colloidal gold after postembedding immunocytochemical labeling for OC-116 using a polyclonal antibody and protein A-gold conjugate. C, ciliated cells (CC) incubated with OC-116 antibody as in B show only background labeling over secretory granules (arrowheads).

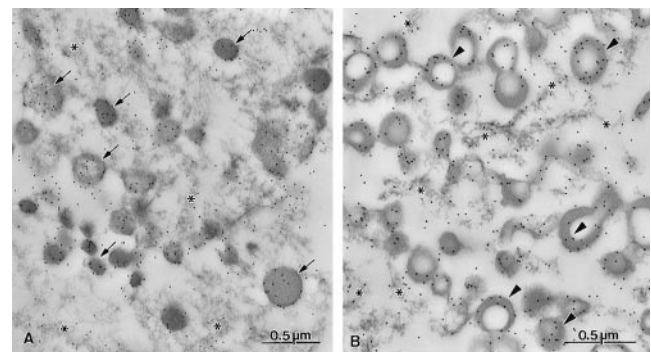


FIG. 12. **Transmission electron micrographs of decalcified eggshell after incubation with OC-116 antibody followed by protein A-gold complex.** A, eggshell matrix in the crown region of a mammillary body shows a loose, flocculent organic material (asterisks) containing discrete vesicular structures (arrows). Crown vesicles immunolabel strongly for OC-116, whereas between the vesicles, the less structured but prevalent matrix of this region shows weak to moderate labeling for this protein. B, in the palisades layer, immunolocalization of OC-116 likewise demonstrates this protein to be primarily associated with the organic material that forms crescent-shaped vesicles (arrowheads) surrounding the roughly spherical voids characteristic of this region. Moderate immunolabeling for OC-116 is observed over the flocculent eggshell matrix (asterisks) dispersed among these vesicles.

DISCUSSION

To our knowledge this is the first example of the molecular cloning of an eggshell-specific matrix protein. This work was undertaken in the context of our hypothesis that such matrix proteins interact with calcium carbonate during calcite nucleation or crystallization and thereby influence the properties of the resulting biomaterial during eggshell formation. This hypothesis is derived from our observations that partially purified mixtures of eggshell or uterine fluid proteins dramatically delay the precipitation of calcium carbonate from a metastable solution of calcium chloride and sodium bicarbonate (9, 11). In addition, partially purified eggshell dermatan sulfate proteoglycan alters the morphology and decreases the size of growing calcite crystals *in vitro* (12–16). A number of proteins, ranging from 14 to 180 kDa, are detected in uterine fluid harvested during the active calcification phase of shell formation. The

⁵ M. T. Hincke, L. C. Gerstenfeld, and M. D. McKee, manuscript in preparation.

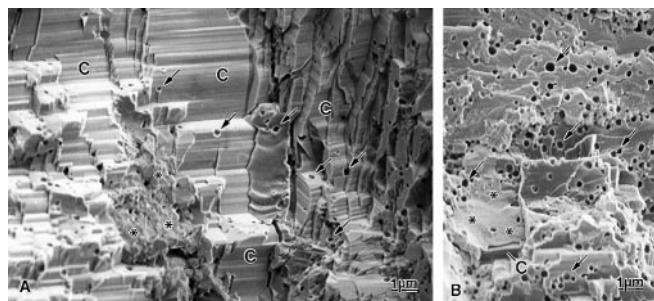


FIG. 13. Scanning electron micrographs illustrating the morphology of the mineral and organic phases of the eggshell palisades layer. A, eggshell fractured to reveal the interior structure of the palisades layer demonstrates irregular, cleaved vertical columns of calcite (C) with some intermingling sheets of organic material (asterisks) dispersed among the columns. Small spherical voids/vesicles (arrows) are observed at the fractured faces of the calcite mineral. B, a region of the palisades layer showing abundant voids/vesicles (arrows) at the fractured surface. In this field, a thin sheet of organic matrix can be observed (asterisks) draping over a segment of calcitic mineral (C).

14-kDa protein is lysozyme.⁶ Our preliminary data indicate that the 21-, 32-, and 36-kDa bands correspond to novel proteins that we have named ovocalyxins (ovocalyxin-21, -32, and -36).⁷

An antibody was raised to the 116-kDa protein, which is most abundant in hen uterine fluid during the active calcification phase of shell formation. Western blotting revealed that only the uterine portion of the oviduct, where shell calcification occurs, was immunopositive. Here, 180- and 116-kDa immunoreactive species were detected. Proteins of 180, 116, 66, and 45 kDa were subsequently detected in uterine fluid and eggshell matrix extracts. Despite this apparent lack of antibody specificity, a single family of inserts was isolated by expression screening a chicken uterus cDNA library. These sequences were assembled to obtain a full-length cDNA possessing an open reading frame coding for a protein of 780-amino acid residues. The predicted N terminus (after removal of a putative signal peptide) corresponds to our direct determination by microsequencing the N terminus of the 116-kDa band and to the N terminus previously obtained for a 200-kDa eggshell matrix proteoglycan that is converted to 120 kDa by chondroitinase ABC treatment (26). Taken together, these results suggest that we have cloned the core protein (80 kDa) of an avian eggshell proteoglycan. The potential for 2 *N*-glycosylated sites and up to 4 sites of glycanation are present in the hypothetical sequence. It is reasonable to propose that *N*- and/or *O*-linked oligosaccharides add another ~35 kDa to its apparent mass on SDS-PAGE and that chondroitin sulfate moieties account for another 60–80 kDa in the mature proteoglycan (180–200 kDa).

We also found that partially purified eggshell matrix fractions contain protein species that have N-terminal sequences corresponding to predicted internal OC-116 sequences. This observation supports the composite cDNA sequence that we have assembled by aligning overlapping sequencing results. In view of the antibody specificity, as demonstrated by Western blotting in uterine tissue, and unique results from expression screening, it is highly likely that the 66- and 45-kDa immunoreactive bands detected in uterine fluid and eggshell matrix correspond to fragments of partially degraded OC-116. Preliminary microsequencing results with these bands from uterine fluid revealed that each contains multiple N termini, and OC-116 sequences could not be unambiguously assigned. It remains to be determined whether these fragments possess bio-

logical activity in the context of eggshell formation. Temporally regulated and sequential proteolytic degradation of enamel matrix proteins is believed to be an essential element in enamel maturation where high levels of mineralization are achieved (30); further work will be necessary to determine the role and potentially obligatory nature of OC-116 proteolysis during eggshell calcification.

The results of this study indicate that OC-116 corresponds to the 120-/200-kDa eggshell dermatan sulfate proteoglycan, which is recognized by a monoclonal antibody that is specific for an epitope on the core protein of avian versican (26). Low stringency BLAST searching with the proposed OC-116 protein sequence generated restricted and poorly significant alignments to mammalian and chicken collagens (types I, II, VII, and IX), human perlecan (heparan sulfate proteoglycan), chicken aggrecan (chondroitin sulfate proteoglycan), chicken bone sialoprotein, and lustrin A (component of molluscan shell extracellular matrix). However, no homology with avian versican was apparent by BLAST searching. Additionally, BLAST 2 sequence comparisons between avian versican and OC-116 found no significant homology. However, it is possible that this epitope is made up of two or more noncontiguous sequences or a single sequence <20 amino acids in length; in these cases no homologies would be detected by software searching. In any event, it is evident that OC-116, an eggshell proteoglycan specific to the calcified matrix, is quite unlike proteoglycans that have been cloned from any other chicken or mammalian tissue. A number of the common structural characteristics (*i.e.* domains that are EGF-like, C-type lectin-like and complement regulatory protein-like) that are found found in aggrecan, PG-M/versican, neurocan, and brevican are absent in OC-116 (31).

Secondary structure predictions for OC-116 suggest that residues 440–600 are a region with a high propensity to form β -sheet. Carboxylate-rich acidic proteins that control oriented crystal nucleation and growth of calcite (32, 33) and octacalcium phosphate (34) are thought to do so via the β -sheet structure that polyaspartate sequences adopt. The interaction of phosphophoryn, the major noncollagenous protein of dentin, with hydroxyapatite crystals is proposed to be via the β -sheet structure that its aspartate residues adopt in contact with the mineral (35). Alternatively, the β -sheet structure has been implicated in the segregation and presentation of serine acceptor sites for *O*-xylosylation in bovine and chicken cartilage (36, 37). However, in contrast to these proposals, the repetitive domains in OC-116 that are predicted to display a β -sheet secondary structure only contain one potential *O*-xylosylation site (serine 443) and are devoid of aspartate residues. An alternative possibility is that this secondary structure serves to orient the highly conserved glutamate residues that align throughout the repetitive elements to influence calcite mineralization. A more speculative proposal is that OC-116 interacts with other matrix components via this domain.

Ultrastructural immunocytochemistry indicates that the 116-kDa protein is synthesized and secreted from the granular cells of the uterine epithelium and is incorporated into and widely distributed throughout the palisade region of the calcified eggshell. This localization agrees with the demonstration by light microscopic immunohistochemistry and immunofluorescence that a chondroitin sulfate glycosaminoglycan epitope is likewise distributed throughout the palisade layer of eggshell from chicken and five other species of domestic birds (12, 13, 16, 38). However, ultrastructural localization with the high resolution afforded by the colloidal gold approach additionally reveals that OC-116 is concentrated not only in the organic material diffusely distributed throughout the palisade layer but is prominent in the vesicular structures in this region of the

⁶ M. T. Hincke, J. Gautron, and Y. Nys, manuscript in preparation.

⁷ J. Gautron, M. T. Hincke, and Y. Nys, manuscript in preparation.

chicken shell. The relationship of these striking, spherical structures to eggshell formation, structure, or function is unknown. However, this localization pattern is different from that seen for osteopontin and ovocleidin-17, both of which are mainly associated with the sheets of organic flocculent material that diffusely permeates the palisade layer (39).⁵ *In vitro*, partially purified eggshell dermatan sulfate proteoglycan alters the morphology and decreases the size of growing calcite crystals (14–16). The important role that proteoglycans play in hard tissue formation is demonstrated by the cartilage phenotypes seen in recessive chondrodysplasias that result from the aggrecan mutations, cartilage matrix deficiency in mice, and nanomelia in chickens (31). Proteoglycans have the potential to function in biomineralization because their glycosaminoglycan units consist of repeating disaccharides with carboxylate and/or sulfate moieties. Although we propose that OC-116 plays an important role in the formation of the calcitic eggshell, it remains to be determined whether the underlying mechanisms by which OC-116 acts are similar to those by which proteoglycans promote cartilage calcification and collagen mineralization *in vitro* (40–43).

Acknowledgments—We are pleased to acknowledge the enthusiastic technical participation of Dr. Michelle Pelloile (cDNA sequencing) and Dr. Sylvie Bourassa (protein sequencing). We also wish to acknowledge the skilled and dedicated assistance of Michel Mangin and Jean-Didier Terlot-Brysinne (Y. Nys), and Sylvia Clarkin, Hong Tong, and Stephanie Sleeth (C. P. W. Tsang). M. T. H. is grateful to Karen Jardine and to Drs. Marco Di Fruscio and Douglas Gray (Ottawa Regional Cancer Laboratory) for advice, encouragement, and bench space during the preparation of the cDNA library. M. D. M. thanks Isabelle Turgeon (immunocytochemistry) and Sylvia Zalzal (scanning electron microscopy) for their expert technical assistance with this project, and Dr. Antonio Nanci for insightful comments.

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