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Ovocalyxin-32, a Novel Chicken Eggshell Matrix Protein

ISOLATION, AMINO ACID SEQUENCING, CLONING, AND IMMUNOCYTOCHEMICAL LOCALIZATION*

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The eggshell is a highly ordered structure resulting from the deposition of calcium carbonate concomitantly with an organic matrix upon the eggshell membranes. Mineralization takes place in an acellular uterine fluid, which contains the ionic and matrix precursors of the eggshell. We have identified a novel 32-kDa protein, ovocalyxin-32, which is expressed at high levels in the uterine and isthmus regions of the oviduct, and concentrated in the eggshell. Sequencing of peptides derived from the purified protein allowed expressed sequence tag sequences to be identified that were assembled to yield a full-length composite sequence whose conceptual translation product contained the complete amino acid sequence of ovocalyxin-32. Data base searches revealed that ovocalyxin-32 has limited identity (32%) to two unrelated proteins: latexin, a carboxypeptidase inhibitor expressed in the rat cerebral cortex and mast cells, and a skin protein, which is encoded by a retinoic acid receptor-responsive gene, *TIG1*. High level expression of ovocalyxin-32 was limited to the isthmus and uterus tissue, where immunocytochemistry at the light and electron microscope levels demonstrated that ovocalyxin-32 is secreted by surface epithelial cells. In the eggshell, ovocalyxin-32 localizes to the outer palisade layer, the vertical crystal layer, and the cuticle of the eggshell, in agreement with its demonstration by Western blotting at high levels in the uterine fluid during the termination phase of eggshell formation. Ovocalyxin-32 is therefore identified as a novel protein synthesized in the distal oviduct where hen eggshell formation occurs.

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 that vary in concentrations during the sequen-

tial process of shell formation (1–3). Its formation constitutes one of the most rapidly mineralizing biological processes known, in which ~6 g of shell is deposited in 20 h resulting in a highly ordered ultrastructure (1–3), consisting of a mineral phase (95%) composed of calcitic calcium carbonate (98% of the mineral phase) and an organic phase (3.5%). Calcification of the shell takes place upon organic cores associated with the fibers of the outer shell membrane, giving rise to the mammillary (inner) and palisade (outer) layers. The latter consists of columns of rhombohedral calcite with preferred orientation (4). Such a degree of structural and crystallographic organization may result from competition in crystal growth between adjacent nucleation sites (5) or from control by matrix components of calcite crystal shape, size, and orientation (6). Identification of the individual matrix components is a prerequisite for testing the second hypothesis. The matrix comprises proteins, glycoproteins, and proteoglycans (3, 7, 8). Distinct matrix proteins have been identified after decalcification and solubilization of the eggshell extract (9, 10). Three of these have previously been identified in the egg white: ovalbumin (11), ovotransferrin (12), and lysozyme (13). Osteopontin, a phosphorylated glycoprotein present in bone and other hard tissues, is also present in the eggshell (14). Its expression by cells of the uterine surface epithelium is greatly up-regulated in response to mechanical strain when an egg enters the uterus (15). Eggshell-specific proteins termed ovocleidins have been identified by N-terminal amino acid sequencing and immunochemistry (16, 17). One of these, ovocleidin-116, has been cloned and corresponds to the core protein of an eggshell dermatan sulfate proteoglycan (17). Ovocleidin-17 is a C-type, lectin-like protein of 17 kDa, which also occurs as a glycosylated form (ovocleidin-23) (18, 19).

Despite these studies, specific roles for individual matrix components in the eggshell remain undefined. There has been a lack of precise molecular detail regarding the uterine-specific, eggshell matrix proteins: information that would be valuable in allowing comparisons with similar proteins in other calcifying tissues. In previous work, we reported that uterine fluid collected at the terminal phase of eggshell formation could dramatically delay the precipitation of calcium carbonate from a metastable solution of calcium chloride and sodium bicarbonate (20). Samples of this uterine fluid, containing an abundant 32-kDa protein, inhibited the spontaneous precipitation of calcium carbonate in this crystal growth assay. The present study reports the isolation and amino acid sequencing of this protein, as well as its corresponding cDNA sequence, protein localization, and tissue expression.

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The nucleotide sequence(s) reported in this paper has been submitted to the GenBank™/EBI Data Bank with accession number(s) AJ307060.

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EXPERIMENTAL PROCEDURES

Uterine Fluid Sampling

Brown egg-laying hens (ISA BROWN, 30–60 weeks of age) were housed individually in cages located in a windowless air-conditioned poultry house. Throughout the study period, they were subjected to a cycle of 14-h light:10-h darkness and were fed *ad libitum* on a layers' diet as recommended by the Institut National de la Recherche Agronomique (21).

Cages were equipped with a computerized system to record the daily egg-laying event (oviposition). Eggs were expelled at precise stages of calcification with an intravenous injection of prostaglandin (PGF₂, 50 µg/hen) at 6–9 h (initial stage), 18–19 h (active growth phase), or 22–23 h (terminal phase of shell calcification) after the preceding oviposition (post-oviposition (p.o.)¹) (20). At this time, uterine fluid was collected in a plastic test tube placed at the entrance of the everted vagina. Aliquots of uterine fluid were then diluted in sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) buffer (1:1; 0.0625 M Tris-HCl, pH 6.8, 2% SDS, 5% 2-mercaptoethanol, 25% glycerol, 0.01% bromophenol blue). Some of these were used immediately to determine protein concentration. The remainder were rapidly frozen in liquid nitrogen and stored at –20 °C.

Preparation of Tissue Extracts

Eggshell Matrix—Proteins associated with the eggshell matrix were extracted according to the procedure described previously (10, 13, 17, 20). Briefly, 54 eggs were collected from hens at 6 months of age. The eggshells were rinsed first with running tap water, followed by 154 mM NaCl containing protease inhibitors (2.5 mM benzamidine-HCl, 50 mM amino-*n*-caproic acid, 0.5 mM *N*-ethylmaleimide, 1 mM phenylmethylsulfonyl fluoride).

The samples of powdered eggshell were first treated with 4 M guanidine hydrochloride (GdnHCl), 0.05 M Tris-HCl, pH 7.4, for 4 days. The supernatant was dialyzed, desalted, and concentrated in a stirring cell (Amicon Cell, cut-off 5 kDa; Amicon Bioseparation, Millipore S.A., Saint-Quentin/Yvelines, France). This sample is referred to as the extramineral shell extract.

The pellet resulting from centrifugation of the preceding extraction was demineralized with 0.5 M EDTA, 0.05 M Tris-HCl, pH 7.4, then extracted in 4 M GdnHCl, 0.05 M Tris-HCl, pH 7.4. The supernatant was collected desalted and concentrated as described above. This sample is referred to as the intramineral shell extract.

Bone and Cartilage Extraction—Proteins from tibiotarsal bones and from the upper proliferating zone of the growth plate of broiler chickens (4 weeks old) were extracted by established procedures (22). Samples (1 g) were first extracted in 4 M GdnHCl, 0.05 M Tris-HCl, pH 7.4, containing the protease inhibitor mixture to produce the extramineral cartilage and bone extracts. The pellets obtained during the preceding extraction were completely demineralized by extraction with 0.5 M EDTA, 4 M GdnHCl, 0.05 M Tris-HCl, pH 7.4, with protease inhibitors to produce the intramineral cartilage and bone extracts.

Other Tissues—Laying hens (4 hens, aged 7 months) were sacrificed by lethal intravenous injection of pentobarbital at 2–4 or 16–17 h p.o. Various tissues (magnum, white and red isthmus, uterus, liver, kidney, duodenum, and skeletal muscle) were collected (0.5–2 g) and homogenized in 10 ml of Tris buffer (50 mM Tris-HCl, 77 mM NaCl, pH 7.4, protease inhibitors). Each sample was centrifuged for 30 min at 10,000 × *g*, and the supernatant was stored at –20 °C. Blood was collected from the same birds immediately before sacrifice. After centrifugation at 2,500 × *g*, the plasma was frozen for storage.

Antibody Preparation

Two preparative SDS-PAGE gradient gels (3 mm width) were loaded with uterine fluid samples harvested at the terminal phase of shell calcification (8 mg of protein). After electrophoresis, the gels were stained with Coomassie Blue and then thoroughly rinsed with demineralized water. The gel containing the 32-kDa band was excised and ground into a fine powder with a Spex freezer mill. The powder was suspended in 154 mM NaCl, mixed 1:1 with Freund's complete adju-

vant, and injected into 2 rabbits. The animals were boosted seven times, at 3-week intervals, with the same antigen preparation in Freund's incomplete adjuvant to obtain a satisfactory titer.

Electrophoresis and Electroblothing

The protein concentration of each sample was determined by the Bradford procedure (23) (Pierce micromethod) using ovalbumin as the standard. SDS-PAGE was performed on a 4–20% gradient gel (24). Tissue samples were diluted 1:1 in SDS-PAGE buffer and boiled for 5 min before being loaded onto the gels. Proteins were stained with Coomassie Blue, or electroblotted (0.8 mA/cm²) onto either: (i) polyvinylidene fluoride membrane (Hyperbond, Beckman Instruments Inc., Fullerton, CA) in 10 mM CAPS buffer, pH 11, 10% methanol, for microsequencing, or (ii) nitrocellulose membrane (Bio-Rad, Ivry sur Seine, France) in 25 mM Tris, 192 mM glycine, 10% methanol for Western blot analysis.

Western Blotting

Nitrocellulose membranes were washed in PBS-Tween (0.01 M phosphate buffer, 0.0027 M potassium chloride, 0.137 M sodium chloride, pH 7.4, 0.1% Tween 20) and blocked for 1 h in 5% nonfat dry milk in PBS-Tween. The membranes were then washed twice in PBS-Tween (5 min) and incubated for 90 min with the rabbit antiserum raised against the 32-kDa protein (1:5,000 in PBS-Tween, 1% bovine serum albumin (BSA)). After three washes in PBS-Tween (5 min), the membranes were incubated for 1 h with 1:20,000 anti-rabbit Ig, peroxidase linked species-specific whole antibody (NA 934, Amersham Pharmacia Biotech) in PBS-Tween, 1% BSA. The membranes were washed four times in PBS-Tween, and then twice in PBS. The enhanced chemiluminescence method (ECL) (Amersham Pharmacia Biotech) was used to reveal immunoreactive bands. The exposure times used were 2, 10, 30, and 300 s.

Purification and Sequencing of Ovocalyxin-32

Intact eggs (89) were treated to selectively dissolve the shell exterior by immersion in 1 N HCl at room temperature for 3 min. This time was found to be optimal for relatively selective extraction of OCX-32 in small scale time trials (data not shown). This limited dissolution of the very superficial layer of shell produced an extract enriched in OCX-32 from which the protein was purified by liquid chromatography. This solution was dialyzed against three changes of 25 mM sodium acetate, pH 4.0, and passed through a column of CM-Sepharose FF previously equilibrated with 50 mM sodium acetate, pH 4.0. The column was washed with 50 mM sodium acetate, pH 4.0, and a gradient to 2 M NaCl, 50 mM sodium acetate, pH 4.0, was developed. Fractions were analyzed by SDS-PAGE, and those eluting above 1.4 M NaCl were found to contain a single 32-kDa band (OCX-32). These fractions were pooled, dialyzed against water, and freeze-dried.

The purified protein was reduced and carboxymethylated under denaturing conditions (18) and precipitated twice with 10 volumes of ethanol. Cyanogen bromide (CNBr) cleavage was performed in 70% formic acid by addition of a small crystal of CNBr and incubation of the mixture at 23 °C for 24 h in the dark. The resulting fragments were separated by reversed phase HPLC using a C18 column (18). The fragments contained in the largest peak were further cleaved with lysyl endopeptidase and the peptides separated by HPLC. Sequencing was performed with an Applied Biosystems model 492 sequencer.

N-terminal microsequencing of OCX-32 was performed with uterine fluid collected at the terminal phase or with eggshell extract, separated by SDS-PAGE, and electrotransferred onto polyvinylidene fluoride membranes. The membranes were stained with 0.1% Coomassie Brilliant Blue, 40% methanol, and 1% acetic acid for a few minutes and destained in 50% methanol until the bands became clearly visible. The 32-kDa protein of interest was excised, loaded into a protein sequencer, and subjected to N-terminal sequencing by Edman degradation in an LF 3000 Protein Sequencer (Beckman/Porton, Beckman Instruments Inc.).

Reverse Transcriptase-mediated PCR and Northern Blotting

Total RNA was prepared by using an RNA InstaPure kit (Eurogentec) to extract tissue from the different parts of the oviduct (magnum, white and red isthmus, uterus) and from liver, kidney, and duodenum. Hens were sampled at two stages of egg formation: before (3 h p.o.) and during (16 h p.o.) eggshell calcification. Reverse transcription was performed with 5 µg of RNA, using random primers and 200 units of Superscript II reverse transcriptase (RNase H⁻, Life Technologies, Inc.) at 42 °C for 60 min. PCR amplification was performed to amplify nucleotides 101–614 of the OCX-32 cDNA sequence, using the following

¹ The abbreviations used are: p.o., post-oviposition; OCX-32, ovocalyxin-32; PAGE, polyacrylamide gel electrophoresis; GdnHCl, guanidine hydrochloride; PBS, phosphate-buffered saline; ECL, enhanced chemiluminescence; SSC, sodium citrate sodium chloride buffer; TBS, Tris-buffered saline; CAPS, 3-(cyclohexylamino)propanesulfonic acid; BSA, bovine serum albumin; HPLC, high performance liquid chromatography; EST, expressed sequence tag; PCR, polymerase chain reaction; bp, base pair(s); kb, kilobase pair(s).

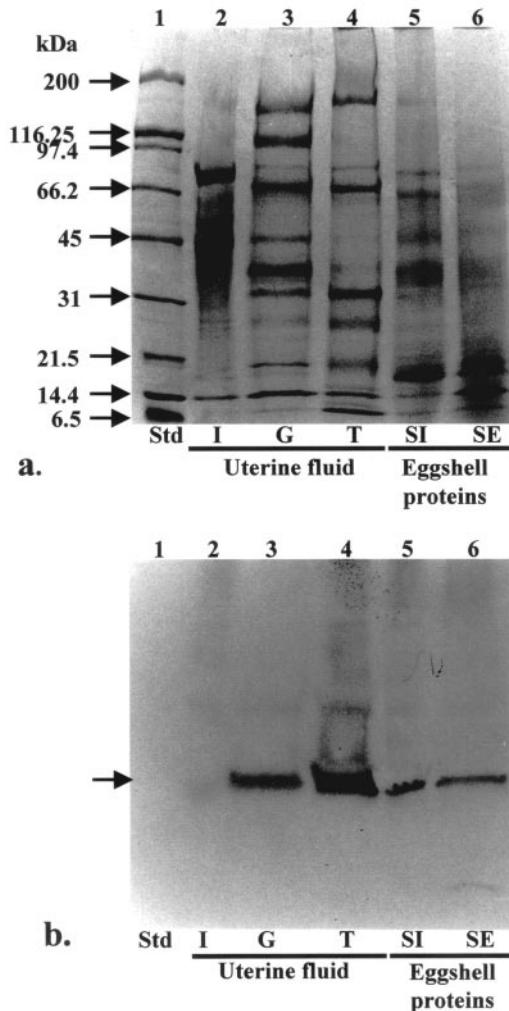


FIG. 1. SDS-PAGE and Western blot analysis for 32-kDa protein in uterine fluid at various stages of shell calcification and in intramineral and extramineral eggshell extracts. All samples were 20 μ g. Lanes were loaded as follows: lane 1, molecular size markers (size on left); lanes 2–4, uterine fluid at different stages of shell formation (I, initial stage; G, active growth phase; T, terminal phase); lanes 5 and 6, shell extract (SI, intramineral eggshell extract; SE, extramineral eggshell extract). a, gel stained with Coomassie Blue. b, Western blotting after electrotransfer to nitrocellulose. Blot probed with antiserum raised to the 32-kDa protein (1:5,000). The 32-kDa band is indicated by an arrow.

primers: upper, 5'-GGACAGCACTGCACTACATCAA; lower, 5'-GGAA-TTTCGTGGAGCAAGACAA, for 22 cycles at 60 °C. The sequence of this 514-bp PCR product was verified by automated sequencing protocols, and radiolabeled (32 P) by random priming at room temperature to serve as a cDNA probe for Northern blotting. RNA from various tissues (20 μ g) was separated on 1% agarose gel containing formaldehyde and transferred to Hybond N membrane by standard protocols (25). The membrane was hybridized with the labeled probe overnight at 42 °C and washed (42 °C: 2 \times sodium citrate sodium chloride buffer (SSC), 0.1% SDS, 15 min; 0.5 \times SSC, 0.1% SDS, twice for 15 min each; 55 °C: 0.5 \times SSC, 0.1% SDS). Labeled bands were visualized with x-ray film and quantified with a Storm PhosphorImager. The size of the OCX-32 transcript was calculated to be 1.3 kb.

Two-way analysis of variance was used to study the effect of stage of eggshell formation and tissue on level of OCX-32 expression using the SYSTAT software program (Systat, Inc., Evanston, IL).

Immunohistochemical Localization of Ovocalyxin-32

Tissues used for this study were obtained from 8 hens with a hard-shelled egg in the uterus or with a forming egg in the white or red isthmus at the time of sacrifice. Tissue samples from the magnum, white isthmus, red isthmus, and uterus were immediately fixed in 10% buffered neutral formalin for 24 h. Samples from five eggshells were

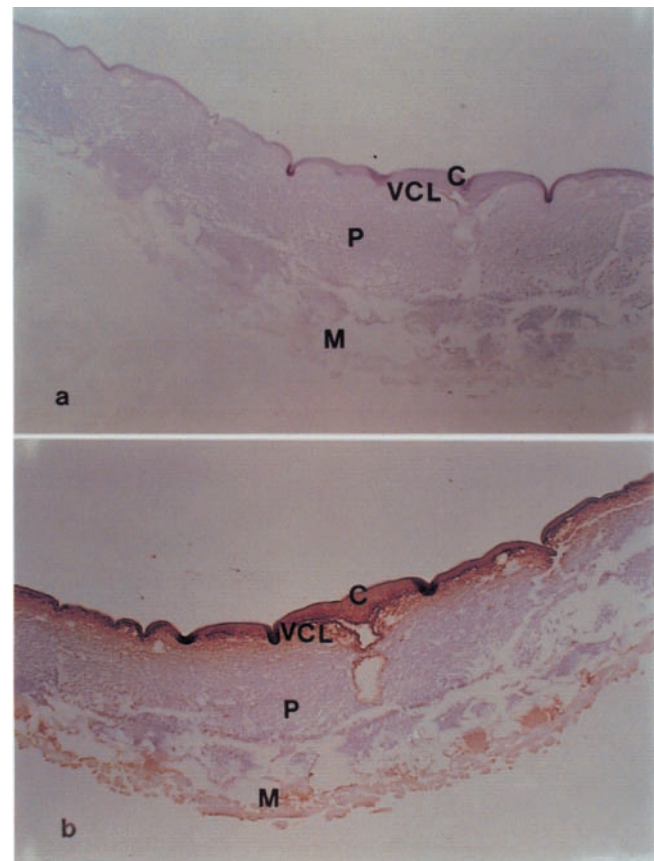


FIG. 2. Immunohistochemistry to localize the 32-kDa protein in eggshell matrix. Shell was decalcified/fixated and paraffin-embedded prior to sectioning (see "Experimental Procedures"). Sections were incubated with either normal rabbit serum (a, negative control, 1/3000) or antiserum raised to 32-kDa protein (b, 1/3000). a, normal rabbit serum control (magnification, \times 50). b, positive immunostaining (brown) of the eggshell matrix with anti-32-kDa antiserum (magnification, \times 50). M, membranes and mammillary layers; P, palisade layer; VCL, vertical crystal layer; C, cuticle.

decalcified at room temperature over a period of \sim 1 week in equal portions of 4% formaldehyde in 0.1 M sodium cacodylate buffer plus 150 mM EDTA, pH 7.7. The decalcified eggshells were subsequently fixed in 10% buffered neutral formalin for 24 h.

Following fixation, samples were dehydrated through an ascending graded series of alcohol, cleared in two changes of histological clearing agent and infiltrated with two changes of polywax (code Z7882920, TCS Microbiology, Buckingham, United Kingdom). Each sample was then embedded (edge-on in the case of shell) into polywax blocks. Transverse sections (3–4 μ m in thickness) of the embedded material were obtained using a Leitz rotary microtome and mounted on glass slides. Sections were dewaxed, and endogenous peroxidase was blocked using a solution of 0.5% hydrogen peroxide in methanol for 30 min. Sections were washed in demineralized water and then again in Tris-buffered saline (TBS) containing 0.05% Tween 20, before incubation with 1% normal swine serum in TBS for 30 min. Sections were incubated overnight with rabbit antiserum to the 32-kDa protein (1/3,000 in 0.1% BSA, 0.01% sodium azide, TBS) at 4 °C. After another three rinses in TBS (5 min each), the sections were incubated for 40 min with biotinylated swine-anti-rabbit IgG 1/200. Following this, they were rinsed again (three 5-min rinses in TBS) and then incubated with StreptABCComplex as per the manufacturer's instructions (StreptABCComplex/HRP kit, code K0377, Dako, Cambridgeshire, United Kingdom). Rinsing was repeated in TBS (three times for 5 min each), followed by the addition of diaminobenzidine chromogen. The reaction was terminated by rinsing in distilled water. Sections were counterstained with hematoxylin, dehydrated, cleared, and mounted. The presence of a brown staining reaction indicated a positive result, with a negative result showing only the blue coloration of the counter stain. Controls to check for the presence of nonspecific reactions were performed with no primary antibody or with normal rabbit serum substituted for the primary antibody.

FIG. 3. Colloidal gold immunolocalization of 32-kDa protein in eggshells. *A* and *B*, scanning electron micrographs of cross-fractured eggshell and shell membranes. The mineralized eggshell rests on the shell membranes (*SM*) and consists of a mammillary layer (*ML*), palisade layer (*PL*), and surface cuticle (*CUT*). The vertical crystal layer (*VCL*) is the outermost portion of the palisade layer found just below the cuticle. Abundant spherical holes/vesicles (*arrows*) are found throughout the palisade layer. Areas illustrated in subsequent panels are indicated by *boxes*. *C–E*, immunocytochemical preparations of decalcified eggshell viewed by transmission electron microscopy after incubation with ovocalyxin-32 antibody and secondary antibody-colloidal gold conjugate. Prominently labeled vesicular structures in the crown region of the mammillary layer (*arrowheads*) and in the palisade layer (*arrows*) are indicated in *D* and *E*. *Asterisks* indicate the unlabeled fibrous/flocculent matrix (*D* and *E*), which partially resembles the labeled fibrous organic matrix of the vertical crystal layer (*C*).

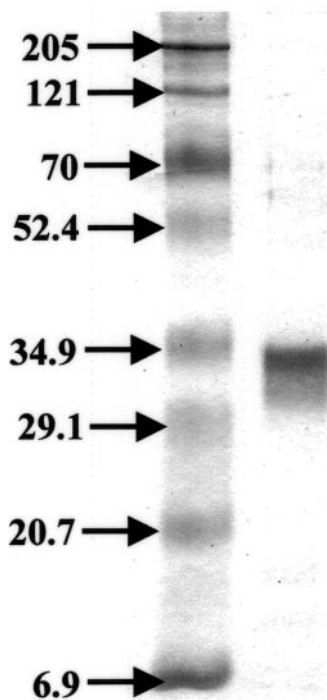
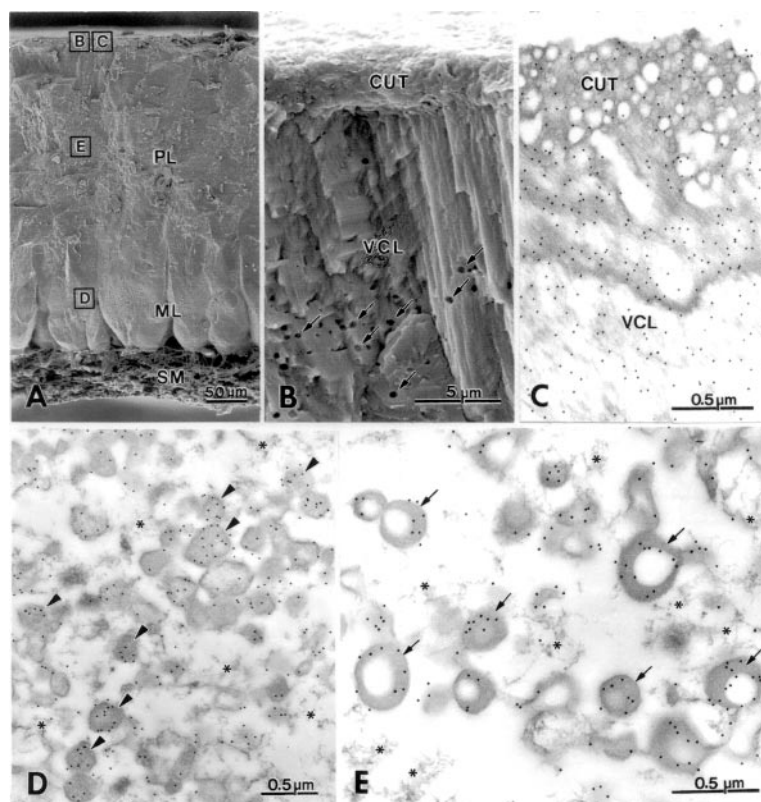


FIG. 4. SDS-PAGE analysis of purified ovocalyxin-32. Eggshell matrix proteins were selectively extracted from the external shell by treatment of intact eggs with 1 N HCl (see “Experimental Procedures”) sample that was subjected to amino acid sequencing (see “Experimental Procedures”). *Lane 1*, markers; *lane 2*, purified ovocalyxin-32 (10 μ g). Protein markers (Bio-Rad) are indicated by *arrowheads* on the left. Their descending sizes (kDa) are: 205, 121, 70, 52.4, 34.9, 29.1, 20.7, and 6.9.

Microscopic Analyses and Colloidal Gold 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 (26).

TABLE I
Sequencing results for the N terminus and internal peptides of ovocalyxin-32

N-terminal microsequencing was carried out after electrotransfer to polyvinylidene difluoride membranes of the 32-kDa electrophoretic band from uterine fluid collected at the terminal phase (three repetitions) and from partially purified eggshell matrix extract. Furthermore, N-terminal sequencing was performed with purified OCX-32. Internal sequences were obtained after cleavage of purified ovocalyxin-32 with CNBr (CB) or lysyl endopeptidase (K). Positions 5 and 13 of the N-terminal sequence displayed microheterogeneity as indicated. Parentheses denote uncertain residues; X, unidentified residues.

N-terminal sequence	
Uterine fluid	ERLPGPQVPGVMRXLN
Eggshell extract	ERLPWPQVPGVMHPLNPKHREAV
Purified OCX-32	ERLPWPQVPGVMHPLNPSHREA
	R K
CB5	ERLPWPQVPGVM
CB1	PQVPG (VM)
CB2	ERLP
Internal peptide sequences	
CB3	GLAIVGSSH (I)
CB4	YLV
	ERLPW
K1	X(Q) IRK
K2	(G)NF
K3	KSPXVVHAK
K4	DNAVAFK
K5	X(Q) IRXDNAVAFK
K6a	(Q) IQEED (HR) FYE (YLQ)
K6b	KQIQEEDHRFYE (YLQ)
K7a	KQSTEHTGYLLAQVSS (V)K
K7b	QSTEHTGYLLAQVSS (V)K
K8	YLVVTLGHP IRVK
K9	FIVLLHEIPTQLNVX (H)
K10	KPITANYIPDS (N) GNIA (HDH)

Survey sections of embedded tissue were viewed by light microscopy, and selected regions 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 using protein A-colloidal gold complex (26) followed by conventional staining with uranyl acetate and lead citrate. Incubated grids were examined in a JEOL JEM 2000FX

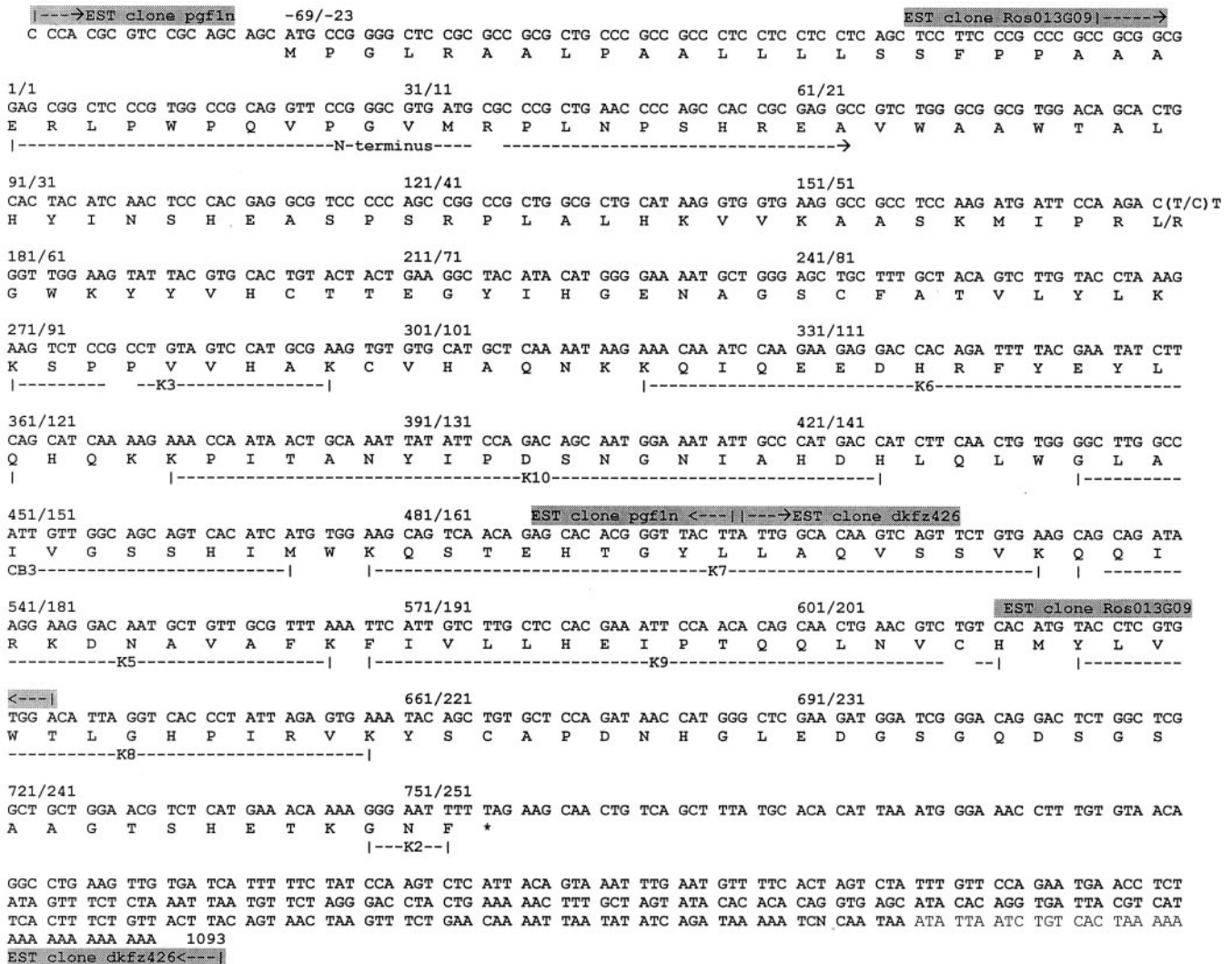


FIG. 5. Ovocalyxin-32 cDNA sequence and alignment with N-terminal protein sequence and peptide sequences derived from the 32-kDa eggshell matrix protein. The full-length composite sequence derived from overlapping EST clones pgfln.pk001.a10, pgfln.pk010.g11, Ros013G09, and dkfz426, and the conceptual translational product from its open reading frame are indicated. The N-terminal and internal peptide sequences obtained for OCX-32 protein (Table I) could all be found within the hypothetical translation product as indicated. Uncertainties in the direct sequencing are presented as *gaps* in the peptide depictions. The N terminus of the mature protein is the starting point for numbering of the protein and corresponding cDNA, and the putative signal peptide is *numbered backwards* from this point.

operated at 80 kV. Control incubations consisted of identical section processing steps but omitting the primary antibody. Other samples of eggshell were fractured, critical-point or air-dried, and examined at 10 kV in a JEOL JSM 840A scanning electron microscope.

RESULTS

Characterization of an Abundant 32-kDa Eggshell Matrix Protein—Analysis of uterine fluid collected at different stages of eggshell formation revealed a changing pattern in the protein species that were detected by Coomassie Blue staining of SDS-PAGE gels (Fig. 1a). The stages investigated corresponded to the initiation of calcification (6–9 h), the active calcification growth phase (18–19 h), and the completion of eggshell formation (22–23 h), respectively, as timed from the preceding oviposition (p.o.). Of particular interest were the protein species present in the uterine fluid collected at the terminal phase of calcification, which displayed a very intense 32-kDa band (Fig. 1a, lane 4). This protein was also visible in uterine fluid harvested from the active calcification phase, but was not seen in the initial phase of calcification. Polyclonal antibodies against this 32-kDa protein were raised in rabbits (see “Experimental Procedures”) and subsequently used to investigate its presence within the uterine fluid and in eggshell

extracts (Fig. 1b). This antiserum detected a single immunoreactive 32-kDa band in the uterine fluid samples collected at the active and terminal phases of shell formation (Fig. 1b, lanes 3 and 4), verifying its specificity. Immunoreactivity was not observed in uterine fluid collected at the initial stage unless the ECL exposure time was lengthened 5-fold, at which point a faint 32-kDa band was observed (data not shown).

Eggshell matrix was analyzed by Western blotting to determine whether the 32-kDa protein became incorporated into the eggshell structure. Matrix proteins were extracted in two steps in order to distinguish between an extramineral (Fig. 1, lane 6) and intramineral (Fig. 1, lane 5) localization. SDS-PAGE analysis of such samples showed that eggshell mineral possesses a complex array of internal and external proteins (Fig. 1a). There was a predominance of OC-17 (17 kDa) and the presence of higher molecular mass bands in the intramineral extract (lane 5). Lysozyme was abundant (15 kDa) in the extramineral extract (lane 6). The 32-kDa band was detected in both the extramineral and intramineral eggshell extracts (Fig. 1b, lanes 5 and 6).

The distribution and localization of the 32-kDa protein within decalcified eggshells was further investigated by immu-

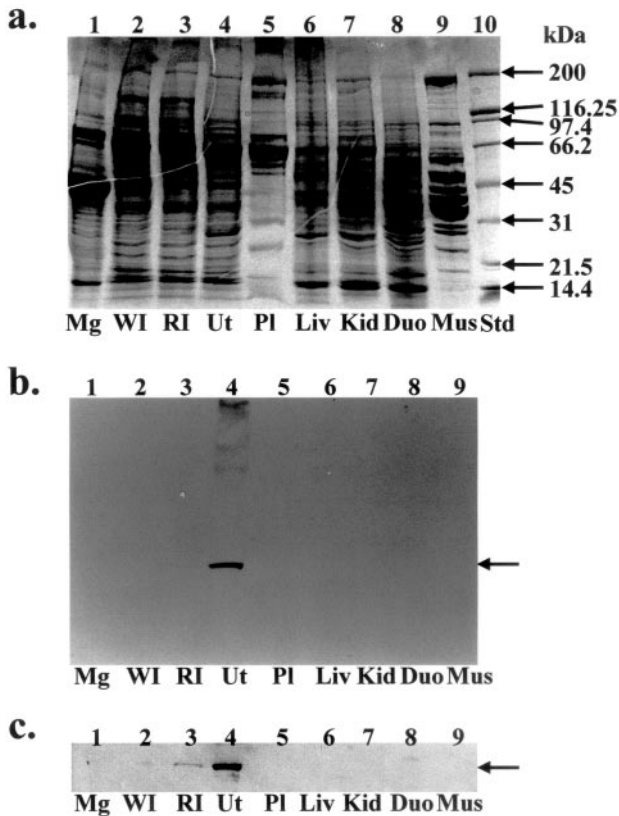


FIG. 6. SDS-PAGE analysis and Western blotting for OCX-32 in oviduct and other hen tissues. All samples were 20 μ g of protein. Lanes were loaded as follows: lane 1, magnum (*Mg*); lane 2, white isthmus (*WI*); lane 3, red isthmus (*RI*); lane 4, uterus (*Ut*); lane 5, plasma (*Pl*); lane 6, liver (*Liv*); lane 7, kidney (*Kid*); lane 8, duodenum (*Duo*); lane 9, muscle (*Mus*); lane 10, molecular size markers (size indicated on right). *a*, gel stained with Coomassie Blue. *b* and *c*, Western blotting after electrotransfer to nitrocellulose. The blot was probed with the antiserum raised to the 32-kDa protein (1:5000) and exposed 30 s (*b*) or 300 s (*c*). The 32-kDa band is indicated by an arrow.

nohistochemistry (Fig. 2). A strong positive staining reaction was observed in the cuticle, vertical crystal layer (*VCL*), and outer palisade layer (Fig. 2*b*). The interface between the mammillary layer and the palisade columns showed an inconsistent patchy reaction. These observations suggested that the 32-kDa protein was preferentially found in the upper layers of the eggshell. The structure of the eggshell is revealed by scanning electron microscopy in Fig. 3 (*A* and *B*). The mineralized eggshell rests on the shell membranes (*SM*) and consists of a mammillary layer (*ML*), palisade layer (*PL*), and surface cuticle (*CUT*). The vertical crystal layer (*VCL*) is the outermost portion of the palisade layer found just below the cuticle. Abundant spherical holes/vesicles (arrows) are found throughout the palisade layer. Immunocytochemical preparations of decalcified eggshell were viewed by transmission electron microscopy after incubation with OCX-32 antibody and secondary antibody-colloidal gold conjugate. Immunolabeling for ovocalyxin-32 was most prominent in the cuticle (*CUT*), the vertical crystal layer (*VCL*), and in vesicular structures of the palisade layer (arrows) and crown region of the mammillary layer (arrowheads), which corresponds to the fusion region of the cones to form the compact palisade layer. In the uppermost region of the palisade layer (corresponding to the vertical crystal layer), gold particles were mainly associated with the vertically oriented, fibrous organic matrix (Fig. 3*C*). Similar fibrous/flocculent matrix (asterisks) between vesicular structures deeper in the palisade layer (Fig. 3*E*) and in the mammillary layer crowns (Fig. 3*D*) was not labeled. In control incubations of

sections not exposed to primary anti-OCX-32 antibody but otherwise treated identically, only background labeling was observed, in which gold particles were few and randomly distributed across the tissue sections and had no specific association with any tissue structures (data not shown).

Sequencing of Ovocalyxin-32—In agreement with the immunolocalization results (Figs. 2 and 3), SDS-PAGE analysis of eggshell extracts obtained by limited dissolution of the outer shell with 1 N HCl indicated that surface-resident OCX-32 was abundant and relatively pure (data not shown). A purification scheme was developed to obtain sufficient OCX-32 for sequencing. The purity of ovocalyxin-32 was assessed by Coomassie Blue staining after SDS-PAGE (Fig. 4), which revealed a single band. N-terminal microsequencing of this purified protein yielded almost identical results to the sequence obtained for the 32-kDa band from terminal phase uterine fluid, with a sole exception at the fifth position (Trp in lieu of Gly; Table I).

Data base searching (TblastN) with sequences of peptides purified after cleavage by CNBr and endolysyl peptidase (Table I) allowed one expressed sequence tag sequence (EST) to be identified (DKFZ426 14H11R1) that contained the sequences of peptides CB4, K2, K5, K8, and K9. Further searching (BlastN) with this EST sequence identified three overlapping ESTs (pgf1n.pk001.a10, pgf1n.pk010.g11, and FM ROS013G09-T3-1) that could be assembled with the first one to obtain a full-length cDNA sequence (1180 nucleotides) containing all of the amino acid sequencing results. The resulting nucleotide sequence, the predicted protein sequence, and the locations of the sequenced peptides from Table I are shown in Fig. 5. The context of the first start codon at nucleotide -69 is compatible with the Kozak consensus sequence for initiation of transcription in higher eukaryotes (27). A signal peptide cleavage site is predicted between residues -1 and 1, corresponding to the experimentally observed N terminus of the mature protein (www.cbs.dtu.dk/services/SignalP) (28). All of the peptide sequences were represented in the complete predicted sequence. The predicted amino acid sequences from the pgf1n.pk001.a10/pgf1n.pk010.g11 ESTs (derived from fat cells) and the FM ROS013G09-T3-1 (derived from 5-day chick embryo) were non-identical at four positions (9: Q/P; 60: R/L; 98: A/G; 156: H/Y, respectively). These possible residues were distinguished at positions 9, 98, and 156 by direct sequencing of the N terminus and peptides K3 and CB3. Position 60 remains uncertain and is so indicated in Fig. 5. The mature protein sequence from this conceptual translation product is 28.4 kDa, similar to the experimentally determined value of 32 kDa. BLAST searching of the public data base with the amino acid and nucleotide sequences indicated that this protein was novel. Therefore, it was named ovocalyxin-32 (OCX-32). Purified OCX-32 exhibited a broad electrophoretic mobility in SDS-PAGE, which is suggestive of protein modification (Fig. 4). However, sequence analysis indicated that motifs directing N-glycosylation (Prosite, www.expasy.ch/tools/scnpsit1.html) and mucin type GalNAc O-glycosylation (www.cbs.dtu.dk/services/NetOGlyc) were not present. Purified OCX-32 did not stain with Alcian Blue following SDS-PAGE, suggesting that it is not glycanated (data not shown). Prosite analysis predicted phosphorylation of a number of residues by PK-C or CK2 kinase, but direct sequencing of Ser-25 and Ser-181, which were among the potential phosphorylation sites, produced no evidence to substantiate this prediction.

Tissue Origin of Ovocalyxin-32—The egg is sequentially formed as it traverses the oviduct of the hen. The egg white constituents are secreted by the magnum, and the shell membranes assemble on the surface of the egg white from precursors secreted in the white isthmus. Eggshell calcification is

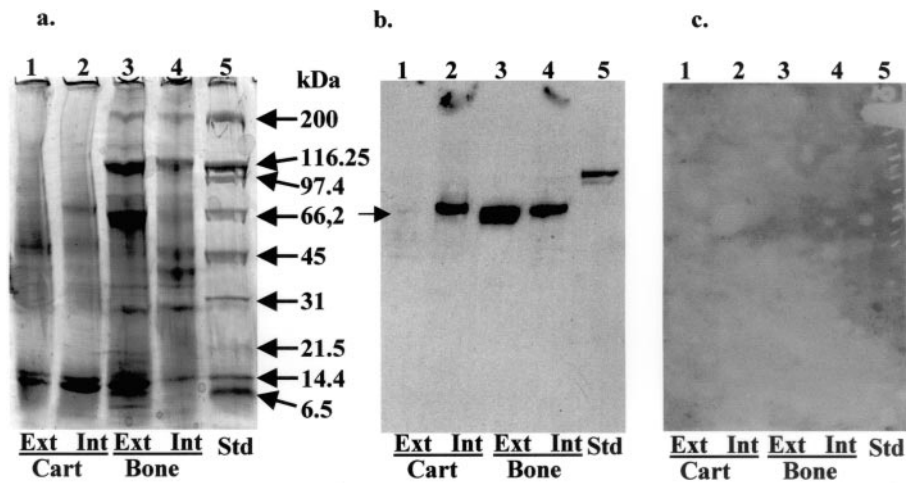


FIG. 7. SDS-PAGE and Western analysis of cartilage and bone extracts. Replicate samples of extramineral (*Ext*) and intramineral (*Int*) extracts of cartilage (*Cart*) and bone were subjected to electrophoresis and transblotting for analysis (all samples are 20 μ g of protein). The size of molecular mass markers is indicated on the right of panel *a*. *a*, control gel stained with Coomassie Blue to demonstrate total protein. *b*, Western blotting for serum albumin. The blot was probed with antiserum raised to chicken serum albumin protein (Accurate Chemical and Scientific Corp., 1:5000), with 45 s of ECL exposure. The 66-kDa band is indicated by an arrow. *c*, Western blotting for OCX-32. The blot was probed with antiserum raised to the 32-kDa protein (1:5000), with 300 s of ECL exposure.

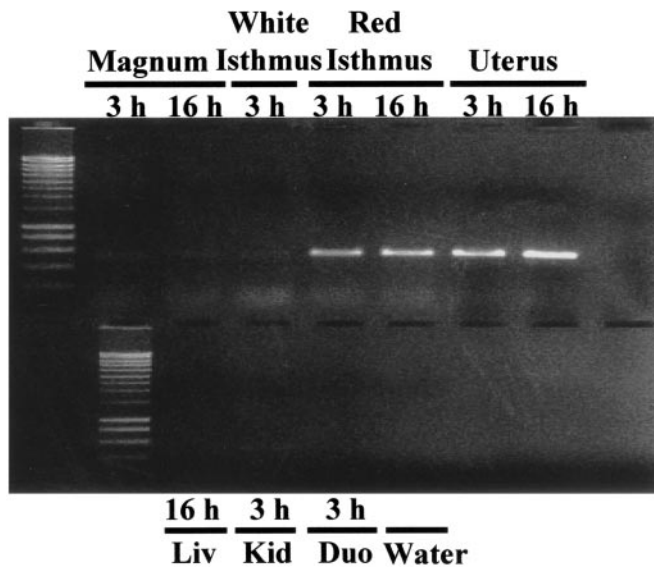


FIG. 8. Reverse transcriptase-PCR to detect expression of ovocalyxin-32 mRNA. Total RNA was extracted from the different parts of the oviduct (magnum, white and red isthmus, uterus) and from liver (*Liv*), kidney (*Kid*), and duodenum (*Duo*), sampled before (3 h p.o.) and during eggshell formation (16 h p.o.). Following reverse transcription, PCR was performed using primers designed to amplify a 514-bp portion of the OCX-32 sequence. When amplification was carried out for 22 cycles, expression was limited to the isthmus and uterus. Standards (200-bp Smart ladder) are on the left.

initiated in the red isthmus and then largely deposited while the egg remains in the uterus. Of these different segments of the oviduct, OCX-32 was only detected in the uterus by Western blotting with specific antiserum. OCX-32 was observed when the uterine tissue was collected during the active stage of shell deposition (Fig. 6, lane 4) and 3–4 h after the preceding ovulation (data not shown). When the exposure time during the ECL development procedure was increased 10-fold, a 32-kDa immunoband was also observed barely in the white isthmus and distinctly in the red isthmus (Fig. 6c). Western analysis of other tissues (blood plasma, liver, kidney, duodenum, muscle; Fig. 6) and egg white (data not shown) were negative for OCX-32, even under the most sensitive Western blotting conditions.

Bone and cartilage from 4-week-old chick was assessed by Western blotting in order to clearly discern whether OCX-32

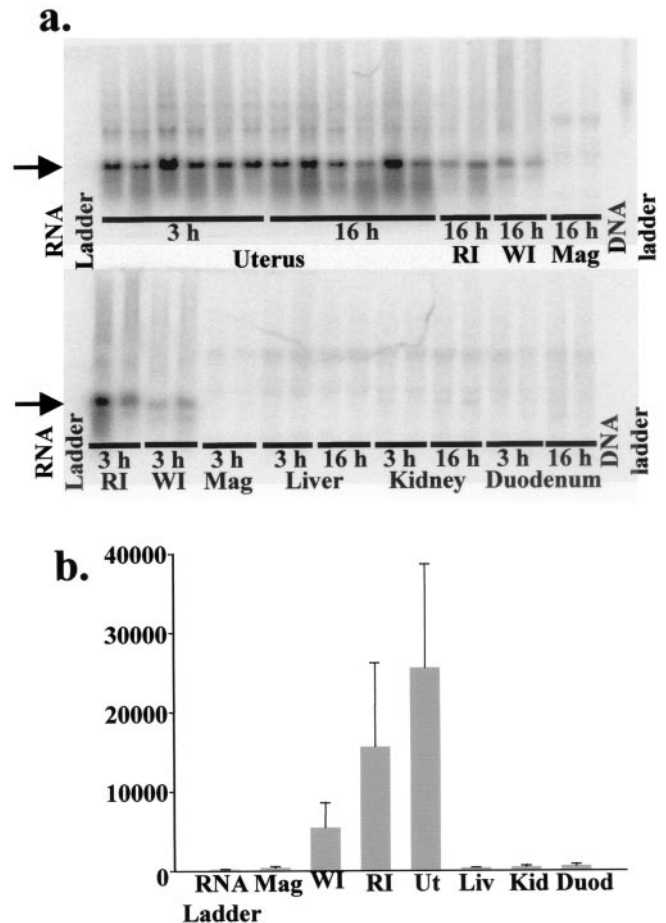


FIG. 9. Expression pattern of ovocalyxin-32 mRNA. Total RNA was prepared from oviduct (magnum (*Mag*), white isthmus (*WI*), red isthmus (*RI*), and uterus) and from liver, kidney, and duodenum for analysis by Northern blotting. Each lane represents RNA samples prepared from different hens. Tissues were collected 3 h p.o. (egg white deposition) and 16 h p.o. (eggshell deposition) (*a*). OCX-32 cDNA (514 bp) obtained by PCR (see Fig. 7) was 32 P-labeled to prepare a suitable probe (*b*). Expression was highest in the uterus, then in red isthmus. It was observed also in the white isthmus but was absent from the proximal oviduct and other tissues. The 1.3-kb message is indicated by an arrow. No significant difference between the uterine message levels at 3 and 16 h p.o. was observed, and these samples were therefore pooled for presentation in *b*.

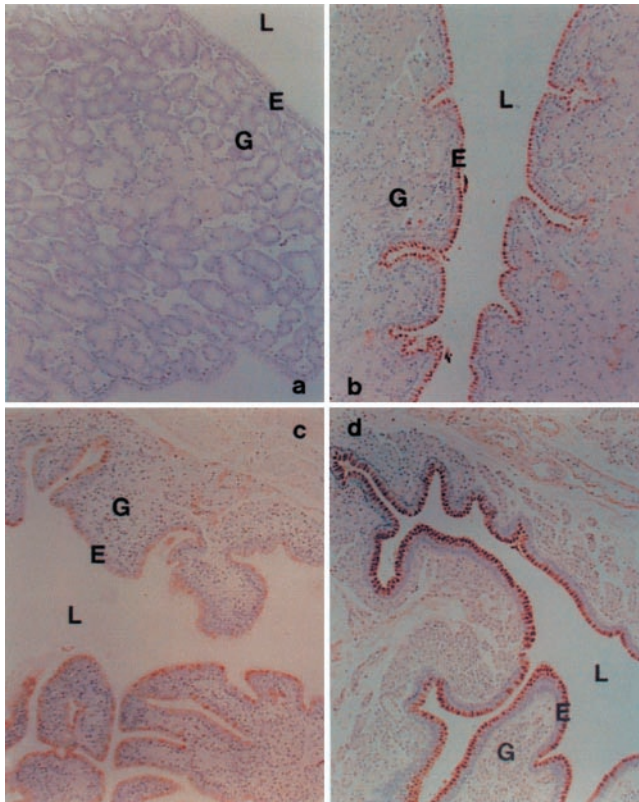


FIG. 10. Localization of the 32-kDa protein within the surface epithelial cells of oviductal tissue. *a*, immunostaining of magnum cells with anti-32-kDa antiserum (magnification, $\times 25$). *b*, immunostaining of white isthmus cells with anti-32-kDa antiserum (magnification, $\times 25$). *c*, immunostaining of red isthmus cells with anti-32-kDa antiserum (magnification, $\times 5$). *d*, immunostaining of uterus cells with anti-32-kDa antiserum (magnification, $\times 25$). *L*, lumen; *E*, mucosal epithelium; *G*, tubular glands.

protein could be detected in another mineralizing tissue (Fig. 7). This stage of bone formation corresponds to active growth and the major expression of proteins involved in the mineralization process. Fig. 7c shows that these tissue samples were totally negative for OCX-32, whereas serum albumin (Fig. 7b) is readily demonstrated in the same samples of both bone and cartilage.

The tissue origin of ovocalyxin-32 was further examined by determining the expression of OCX-32 message using reverse transcriptase-PCR and Northern blotting. Amplification by PCR (22 cycles) demonstrated that expression was limited to the uterine and red isthmus portions of the oviduct (Fig. 8). On the other hand, proximal oviduct and other tissues tested (liver, kidney, and duodenum) were negative. More sensitive evaluation by amplification for 35 cycles indicated that proximal oviduct (white isthmus, magnum), liver, kidney, and duodenum did express low levels of OCX-32 message (data not shown). These message levels were not, however, associated with production of detectable OCX-32 protein (Figs. 1 and 6), even at extended ECL exposure times (data not shown). This pattern of OCX-32 message expression in the distal oviduct was confirmed quantitatively by Northern blotting (Fig. 9). A 1.3-kb message was detected, in agreement with the putative full-length cDNA (1.2 kb). Expression was highest in the uterus, high in the red isthmus, and 5-fold lower in the white isthmus. It was undetectable in the magnum and in other tissues (liver, kidney, duodenum) (Fig. 9b). Variation in uterine message expression during the circadian cycle of eggshell calcification was assessed by quantifying the level of OCX-32 mRNA in tissues collected 3 h p.o. (egg in magnum during egg white

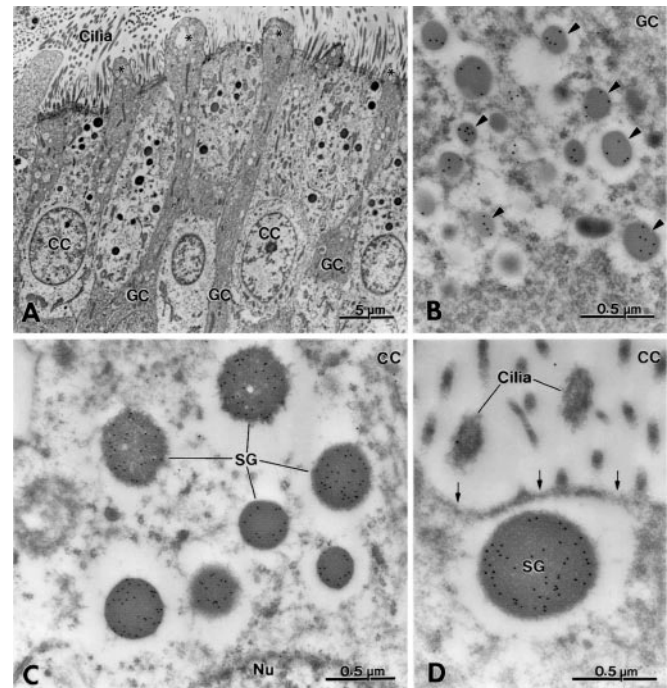


FIG. 11. Transmission electron microscopy of uterine epithelium after colloidal-gold immunocytochemistry for ovocalyxin-32. *A*, shell gland epithelium. Ciliated cells (*CC*) alternate with granular cells (*GC*). Whereas ciliated epithelial cells possess abundant cilia at their apical end, granular cells have an apical cytoplasmic extension (*asterisks*) that protrudes significantly into the lumen of the shell gland. *B–D*, immunocytochemical labeling for ovocalyxin-32. Abundant gold particles are observed over secretory granules of both granular (*B*) and ciliated (*C* and *D*) cells. Secretory granules of granular cells (*B*, *arrowheads*) are typically smaller in size and less labeled than those of the ciliated cells (*C* and *D*). *D*, slightly higher magnification of an immunolabeled secretory granule adjacent to the apical surface of the cell (*arrows*) presumably just prior to secretion. *SG*, secretory granule; *Nu*, nucleus.

secretion) and 16 h p.o. (egg in uterus during eggshell deposition) ($n = 6$ at each stage). Two-way analysis of variance was performed using SYSTAT software. No significant difference in ovocalyxin-32 mRNA expression was observed between these stages of eggshell formation.

Localization of Ovocalyxin-32 in the Uterus—The wall of the oviduct contains a mucosa of tubular glands lined by a surface epithelium. Immunocytochemistry revealed that the uterine epithelial surface was positive for the presence of OCX-32, whereas the internal tubular gland cells were negative (Fig. 10d). The magnum, in comparison, was uniformly negative (Fig. 10a). The surface epithelium of the white isthmus and red isthmus displayed positive staining, but at a lower intensity (Fig. 10, *c* and *d*). No staining was observed in the absence of primary antibody (replaced with normal rabbit serum). Immunostaining intensity did not appear to vary between tissues collected from birds sacrificed with a hard shell in the uterus or with an egg in the isthmus.

Ovocalyxin-32 localization was investigated at higher resolution by transmission electron microscopy after colloidal gold immunocytochemistry. Shell gland epithelium consists primarily of two cell types, both of which contain abundant secretory granules (Fig. 11A). Lightly stained ciliated cells (*CC*) alternate with more darkly stained granular cells (*GC*). Whereas ciliated epithelial cells possess abundant cilia at their apical end, granular cells have an apical cytoplasmic extension (*asterisks*) that protrudes significantly into the lumen of the shell gland. Following immunocytochemical labeling for ovocalyxin-32, abundant gold particles are observed over secretory granules of both

FIG. 12. Similarity of ovocalyxin-32 amino acid sequence to two related human proteins, latexin and tazarotene-induced gene 1 (TIG1). Multiple sequence alignment was performed with a progressive alignment approach (ClustalW, www.ebi.ac.uk/clustalw/) and shaded with Boxshade (www.ch.embnet.org/software/BOX_form.html). Human latexin, GenBank[®] accession no. AAF82807; human TIG1, GenBank[®] accession no. NM 002888.

OCX-32	1	-----MPCLRAALPAALLLTSSFFPAAERLPWFQVQVGMVRPNPNSHREAV
Hum. latexin	1	-----MCLPPTNYPAS
Hum. TIG1	1	MQPRRQRLPAPWSGPRCPPTAELLALALLLAAVAAAPAGSGGDEDEGQPOAGVPRLLQ
OCX-32	47	WAAWTAALHYINSERSSPRLADHKVVKAAASKLIPRLGKQYVFCITGEGYTHG-----
Hum. latexin	12	RAALVQNYINYNQCTEPRWFEQKVKOASMEDLPGRCHKYHKAFAVEEILQK-----
Hum. TIG1	61	QKRAALHEFNERSGSPSARVLAEVQERAWINERPCCKVHVVESTERYNPESLLQEGE
OCX-32	100	ENAGSDFATVLYLKK---SPFVVAKCVHAKQKQIQEEDHREYFYLQHKKPEETANYIF
Hum. latexin	65	QVKVNCDAEVLYPSTGQEAAPVNF--FTFESETGKNPDEEDNPEYQRLKSKPELEAGNIF
Hum. TIG1	121	GRLGKCSARVFKNQ--KPRETINVTCTELIEKRRKQEDYLLAKQKQKRNPELIVSIF
OCX-32	157	DSNGNIAHDHQLWGLAIVGSSHIMWKQSTEHCTYLACQSSVKQQRKDNAAVAFKIVL
Hum. latexin	124	DNFGNIAEVLVHLAVVCCYIHWNSTEDTWKVKQVQVQVORNDDFIELDITL
Hum. TIG1	179	DNHCHIDPSERLWDLAEVSSSYMVMEMTEVHYVLAQVLSVQVRRRT-----
OCX-32	217	LHEIPVQDENVCHYLVWTLGHPIVVKYSCAPDNHGLEGSGQDSGSAAGTSHETKGNF
Hum. latexin	184	LHNIAACEIIPWQVQVWHPQYGTQVKHNSRLPKEVQL
Hum. TIG1		-----

granular (Fig. 11B) and ciliated (Fig. 11, C and D) cells. Secretory granules of granular cells (*arrowheads*) are typically smaller in size and less labeled than those of the ciliated cells (SG). Fig. 11D demonstrates slightly higher magnification of an immunolabeled secretory granule (SG) adjacent to the apical surface of the cell (*arrows*) presumably just prior to secretion.

DISCUSSION

The eggshell is laid down upon the eggshell membranes in the distal part of the oviduct in association with an organic matrix composed of proteins, glycoproteins, and proteoglycans (7, 8). This matrix is believed to modify the calcification process and influence the final microstructure of the eggshell (3). A prerequisite to investigate this hypothesis is the characterization of the individual matrix components.

Numerous matrix proteins have already been identified in the eggshell. These can be classified according to the tissue(s) in which they were first identified and with which they are most prominently associated. One group of identified eggshell matrix proteins corresponds to major proteins of the egg white. Ovalbumin was the first egg white protein described in the matrix of the eggshell (11). More recently, the presence of lysozyme and ovotransferrin has been reported (12, 13). A second group includes proteins such as osteopontin, which are also found in other calcified tissues such as bones and teeth (14). Finally, a third group of proteins comprises those specific to the uterine tissue and to the eggshell, which have only been observed in extracts of eggshell or its precursor milieu, the uterine fluid. Two such eggshell-specific proteins have so far been identified: ovocleidin-17 (OC-17) (16, 18) and ovocleidin-116 (OC-116) (17). Ovocleidin-116 corresponds to the core protein of an eggshell dermatan sulfate proteoglycan (29). Other proteoglycans that are believed to influence eggshell mineralization include keratan sulfate proteoglycans, which are present at the site of mineral nucleation (30). Their protein moiety has not yet been characterized.

In the current study, a new eggshell-specific matrix protein was identified with a molecular mass of 32 kDa. Significant levels of this protein were only observed in the uterine fluid collected during the active and terminal phases of shell deposition. Sequence determination of the protein and its corresponding cDNA indicated that it has not yet been characterized, and it is therefore termed OCX-32 (Latin *ovum* (egg) and *calyx* (shell)). OCX-32 was purified in amounts sufficient for peptide sequencing after CNBr or lysyl endopeptidase fragmentation. The internal sequences thus obtained were used for data base searches and were instrumental in identifying overlapping ESTs that included all peptide sequences and apparently represented cDNA coding for the entire protein. Data

base searching with the predicted protein sequence yielded two distinct mammalian proteins, each with ~30% identity to OCX-32 after alignment (Fig. 12). One of them is latexin, a carboxypeptidase inhibitor, the expression of which seems to be restricted to a subset of neurons and a few non-neural tissues, as, for instance, peritoneal mast cell, in the rat (31–33). The other one is the putative translation product of tazarotene-induced gene 1 (TIG1), a retinoic acid receptor-responsive gene of human skin (34, 35). These human proteins have only a 30% identity to each other, so their evolutionary and functional relationship to OCX-32 is not clear.

The specificity of ovocalyxin-32 as a uterine and eggshell matrix protein was established by immunocytochemistry using polyclonal antibodies raised against the 32-kDa electrophoretic band from uterine fluid. The antibodies reacted with the 32-kDa band in Western blotting of eggshell extracts and uterine fluid samples, revealing that the uterine fluid contained ovocalyxin-32 as an eggshell matrix precursor. The antibodies also demonstrated that ovocalyxin-32 was specific to the uterus and distal oviduct (isthmus). No immunoreactivity was observed in the proximal oviduct responsible for egg white protein production and secretion (magnum), or in the egg white albumen. Ovocalyxin-32 was also absent from all other tissues analyzed, including extracts from cartilage and bone extracts. These observations demonstrate that this protein does not belong to the egg white- or bone-associated classes of matrix proteins. Finally, ovocalyxin-32 was also absent from the blood plasma, suggesting that it is synthesized *in situ* within the cells of the uterus and, at lower levels, the isthmus.

The tissue origin of OCX-32 mRNA was investigated by reverse transcriptase-PCR and Northern blotting analysis, which indicated that high levels of this message could only be detected in the distal oviduct in full agreement with the Western blotting results. According to the Western blot data, there is an enhanced secretion of OCX-32 protein at the terminal stage of eggshell formation, which was not, however, associated with a significant change in levels of its message throughout the laying cycle. Since the isthmus and uterine regions of the oviduct are distinguished from the proximal oviduct by their critical role in the elaboration and mineralization of the eggshell, a functional role for OCX-32 in shell formation is implied.

Immunocytochemistry of decalcified eggshell and of different parts of the oviduct confirm that ovocalyxin-32 is an eggshell-specific matrix protein secreted by the surface epithelial cells of the uterus. Two types of uterine cells, those of the surface epithelium and of the underlying tubular gland, are involved in secretion of the eggshell matrix proteins. Ovocleidin-116 and the corresponding dermatan sulfate proteoglycans (17, 36), along with osteopontin (14), are all secreted by the surface

epithelium. In contrast, ovocleidin-17 (16), keratan sulfate proteoglycan, and collagen X (30) are secreted by the tubular gland cells that also are the source of the calcium and bicarbonate ions of the uterine fluid (3).

Matrix components are differentially distributed within the calcified eggshell. Following demineralization, transmission electron microscopy reveals that the matrix of the palisade region is organized as flocculent sheets of organic material interspersed among vesicular structures with electron lucent cores. We have suggested previously that these circular features correspond to the walls of the holes that are found throughout the palisade region (Fig. 11B). Certain matrix proteins are predominantly associated with the sheets of matrix (ovocleidin-17 (Ref. 16), osteopontin (Ref. 3)), whereas ovocleidin-116 is associated with the electron-dense periphery of the vesicular structures (17). The results of this study reveal that OCX-32 is associated with the vesicles of the mammillary crowns and the palisade region. Therefore, in the palisade region, OCX-32 co-localizes with OC-116 and the associated dermatan sulfate proteoglycan that is found here (17, 36, 37). However, in contrast to other characterized matrix proteins, OCX-32 is also associated with the outermost layer of eggshell, specifically the vertical crystal layer and the cuticle (Fig. 2). With the exception of lysozyme (13), no other matrix protein has been localized to this region. In addition, immunohistochemical localization in the eggshell and Western blotting of uterine fluid collected at various stages of eggshell formation suggest a higher level of the ovocalyxin-32 at completion of eggshell formation. These observations suggest a possible role for OCX-32 in the processes that lead to termination of shell formation, and which morphologically manifest themselves as a change in the direction of calcite crystal growth (1, 2, 3). The terminal phase of eggshell formation occurs in a uterine fluid that is supersaturated in calcium and bicarbonate; the mechanism by which calcite deposition from this milieu is inhibited has not yet been elucidated. The uterine fluid at this stage also contains high levels of organic phosphorus, and there is an accumulation of phosphorus in the outer shell (20, 3), leading to the hypothesis that termination of eggshell formation is induced by secretion of an inhibitory phosphoprotein. However, our data provide no direct evidence that OCX-32 is a phosphoprotein, so further studies are necessary to determine its role in eggshell formation.

Keratan sulfate proteoglycans (38) and the egg white proteins (12, 13) of the eggshell are secreted by the isthmus region and are implicated in the initial nucleation of calcite crystals upon the outer shell membranes. The dermatan sulfate proteoglycan OC-116, ovocleidin-17, ovocalyxin-32, and osteopontin are secreted by the uterus, and are predominantly present in the uterine fluid when collected during the active phase of eggshell deposition. These proteins are observed throughout the calcified eggshell and are therefore possible candidates for influencing mineralization, but their role as structural elements of the matrix, or regulators of crystal growth, is not yet known. The experimental evidence for a functional role of ovocalyxin-32 is partial. First, the protein is present in the uterine fluid during the phase of active calcification, but mainly during the terminal phases of eggshell deposition, in agreement with its presence in the outermost part of the shell. The uterine fluid when collected during these phases has been shown to strongly alter the morphology of calcite crystals and to reduce the rate of calcite crystal growth *in vitro* (39). OCX-32 precipitates with calcium carbonate *in vitro* from freshly collected uterine fluid and becomes concentrated in the mineral pellet (20). However, this uterine fluid contains a diverse population of other proteins in addition to ovocalyxin-32. Ovocalyxin-32 is therefore one of the possible candidates that may

affect the process of mineralization and completion of the eggshell. Purification of the protein, evaluation of its effect on crystal morphology, and the quantification of ovocalyxin-32 in eggshells of variable strength are approaches currently being developed to test this hypothesis.

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