Localization of osteopontin in oviduct tissue and eggshell during different stages of the avian egg laying cycle

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Abstract

The avian eggshell is an acellular bioceramic containing organic and inorganic phases that are sequentially assembled during the time the egg moves along the oviduct. As it has been demonstrated in other mineralized tissues, mineralization of the eggshell is regulated by extracellular matrix proteins especially the anionic side chains of proteoglycans. Among them, osteopontin has been found in the avian eggshell and oviduct. However, its precise localization in the eggshell or in different oviduct regions during eggshell formation, nor its function have been established. By using anti-osteopontin antibody (OPN 1), we studied its immunolocalization in the isthmus, red isthmus and shell gland of the oviduct, and in the eggshell during formation. In the eggshell, osteopontin was localized in the core of the non-mineralized shell membrane fibers, in the base of the mammillae and in the outermost part of the palisade. In the oviduct, OPN 1 was localized in the ciliated epithelial but not in the tubular gland cells of the isthmus, in the ciliated epithelial cells of the red isthmus, and in the non-ciliated epithelial cells of the shell gland. The occurrence of osteopontin in each of the oviduct regions, coincided with the concomitant presence of the egg in such region. Considering the reported inhibitory function of osteopontin in other mineralized systems, together with its main occurrence in the non-mineralized parts of the eggshell and at the outermost part of the shell, suggests that this molecule could be part of the mechanism regulating the eggshell calcification.

Keywords: Osteopontin; Eggshell; Biomineralization; Oviduct; Biomineralization

1. Introduction

The avian eggshell is an acellular bioceramic containing organic and inorganic phases (Arias et al., 1993; Heuer et al., 1992). It is composed of a bilayered membrane and calcified extracellular matrix which are sequentially assembled during the 22 h the egg moves along the oviduct (Fernandez et al., 1997). From the inside to the outside, eggshells are composed of shell membranes, mammillary layer, palisade and cuticle (Arias et al., 1993).

The avian oviduct is a tubular organ responsible for forming the egg by the secretion of the components surrounding the yolk. It is divisible into six different regions: infundibulum, magnum, isthmus, red isthmus or tubular shell gland, shell gland or uterus, and vagina (Solomon, 1991). Isthmus, red isthmus, and shell gland are involved in eggshell formation. These regions are covered by an epithelium containing ciliated and non-ciliated cells (Breen and De Bruyn, 1969; Draper et al., 1972; Wyburn et al., 1973).

Comparative analysis of different models of biological mineralization, such as shells, bones, and teeth, have shown that particular organic components play a role in the control of crystallization (Mann, 2001). Eggshells are fabricated by combining particular extracellular matrix macromolecules with a crystalline calcite filler while the egg is moving along the oviduct to produce a mineral-organic composite (Arias and Fernandez, 2001). This fabrication is regulated by a precise spatio-temporal arrangement of sequentially deposited macromolecules

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(Fernandez et al., 1997, 2001). Among other molecules described elsewhere (Gautron et al., 1997, 2001; Hincke, 1995; Hincke et al., 1995, 2000; Lakshminarayanan et al., 2002; Mann, 1999; Mann and Siedler, 1999; Mann et al., 2002; Miksik et al., 2003; Nys et al., 1999; Panheleux et al., 2000), which role in the calcification process has not yet been well established, the eggshell organic phase contains collagen and proteoglycans (Arias et al., 1991a, 1992, 1997; Carrino et al., 1996, 1997; Fernandez et al., 1997; Nakano et al., 2001, 2002).

Together with a previous report indicating the occurrence of types I and V collagen in the shell membranes (Wong et al., 1984), we have showed that they contain type X collagen, which once secreted by the isthmus tubular gland cells (Arias et al., 1991a,b; Wang et al., 2002), constitutes the shell membrane fibers core (Fernandez et al., 2001). The mammillary layer is constituted by mammillae, also known as calcium reserve bodies, which are discrete aggregations of organic matter where nucleation of calcite takes place. They contain mammillan, a highly sulfated keratan proteoglycan (Arias et al., 1992; Fernandez et al., 1997, 2001). The shell matrix, that is the organic part obtained after a complete decalcification of the calcitic palisade, contains hyaluronic acid (Nakano et al., 2001) and ovoglycan, a unique dermatan sulfate proteoglycan (Arias et al., 1992; Carrino et al., 1996, 1997; Dennis et al., 2000; Fernandez et al., 1997, 2001; Hincke et al., 1999). Finally, the cuticle covers the entire shell and is composed of glycoproteins, hydroxyapatite crystals, and contains most of the pigments in the colored eggs (Dennis et al., 1996; Fraser et al., 1999; Nys et al., 1991). In addition, osteopontin has been found in the eggshell and oviduct, and suggested to be part of the array of macromolecules contributing to the regulation of eggshell mineralization (Lavelin et al., 1998, 2000; Pines et al., 1994).

Osteopontin (OPN) which was originally isolated from rat bone, is a highly phosphorylated sialoprotein that is a prominent component of the mineralized extracellular matrices of bones and teeth (Sodek et al., 2000). OPN contains a polyaspartic acid sequence and sites of Ser/Thr phosphorylation that mediate hydroxyapatite binding, and two highly conserved RGD motif in the chicken OPN, that mediates cell attachment/signaling (Sodek et al., 2000). Additionally, OPN can show other post-translational modifications such as glycosylation and sulfation. Osteopontin gene is expressed in skeletal embryonic tissue and in adult tissues (Weinreb et al., 1990), in normal and transformed bone cells (Kasugai et al., 1991; Kubota et al., 1989; Rodan et al., 1989), and in some cells of the bone marrow (Mark et al., 1987; Nagata et al., 1989; Nomura et al., 1988; Yoon et al., 1987). In addition, osteopontin is found in certain non-osseous tissues such as kidney (Mark et al., 1987, 1988; Nemir et al., 1989), the sensory epithelium of the embryonic ear (Mark et al., 1987; Swanson et al., 1989), and the placental decidua (Nomura et al., 1988). Expression of OPN in different tissues suggests the involvement of this protein in a variety of processes including development, wound healing, immunological response, tumorigenesis, bone resorption, and calcification (Sodek et al., 2000). The suggestion that osteopontin is involved in bone calcification has been based on its tissue distribution, its affinity to calcium, its immunolocalization to electron-dense regions of mineralization (Ikeda et al., 1992), and the regulation of its gene expression by calcitrophic substances such as vitamin D_3 (Noda et al., 1990; Prince and Butler, 1987) and parathyroid hormone (Noda and Rodan, 1989).

In the hen oviduct, it has been found that osteopontin gene expression was limited to the shell gland, while its precise localization in the eggshell matrix was not well established, although there was some evidence of a greater osteopontin immunoreactivity at the outer part of the shell. However, these previous works did not focus on the localization of osteopontin in the isthmus and red isthmus of the oviduct when the egg was in those regions or in the eggshell during its formation.

To have a further insight on the possible role of osteopontin in eggshell formation and calcification, we studied its immunolocalization in different regions of the oviduct and eggshell during the egg laying cycle.

2. Material and methods

2.1. Osteopontin localization

Eggs and oviducts were obtained from 2-year-old White Leghorn laying hens with artificial light provided for 16 h a day and food and water ad libitum.

Oviducts were surgically removed from euthanized hens at 4.75, 5.5, 12, and 18 h post-oviposition (p.o.), that is the time elapsed after the last egg was laid. Three regions of the oviduct involved in the formation of the eggshell were studied: isthmus, red isthmus (tubular shell gland), and shell gland proper (uterus). For immunofluorescence studies samples (1 cm^2) were washed with phosphate-buffered saline (PBS), fixed for 12 h in 2% paraformaldehyde, 0.2% glutaraldehyde in PBS 100 mM, rinsed in PBS, transferred to Tissue Tek OCT Compound (Miles, Elkart, IN), frozen in liquid nitrogen, and sectioned (10–15 μm thick) with a cryomicrotome (Lipshaw, Detroit, MI). For immunogold studies, samples were fixed as before and, after washing in PBS, they were dehydrated in a graded acetone series and embedded in Poly Bed 812 (Polysciences, Warrington, PA). Ultrathin sections (70–90 nm) were cut in a Porter-Blum MT2-B ultramicrotome.

For immuno-ultrastructural studies, pieces of eggshells in different stages of formation, obtained at the same sampling times than the oviduct tissue, were fixed

in 2% paraformaldehyde plus 0.2% glutaraldehyde in PBS 100 mM, for 24 h. Samples were then encased in gel of 2% agar in water to protect the specimen from shear forces during the remaining processing. Samples were decalcified by stirring for 48 h in 25 ml of fixative plus 25 ml of 10% formic acid plus 5% sodium citrate, changing the solution after 24 h. The samples were washed three times in PBS and then dehydrated in a graded acetone series and embedded in Poly Bed 812 (Polysciences, Warrington, PA). Ultrathin sections (70–90 nm) were cut with a Porter-Blum MT2-B ultramicrotome. For histological identification some ultrathin sections were stained with Toluidine Blue and examined with bright field optics.

For immunogold studies, decalcified eggshell and oviduct sections were treated as follows. Thin sections were blocked for 15 min in 0.1% bovine serum albumin (BSA) TBS–Triton (20 mM Tris, pH 7.4), in 150 mM NaCl, 20 mM NaN_3, and 50 mM Triton X-100. Sections were subsequently incubated overnight at 4 °C with the primary antibody and then incubated for 3 h in gold conjugated goat anti-rabbit IgG antibody (Sigma, St. Louis) 1:50 in TBS–Triton. Observations were made with a Zeiss EM-109 electron microscope.

For immunofluorescence studies, sections were rehydrated with PBS for 5 min and blocked for 5 min with 3% BSA/PBS. Sections were then incubated for 1 h at room temperature with the primary antibody, rinsed twice with PBS, blocked with BSA/PBS for 5 min, and incubated for 1 h with fluorescein isothiocyanate (FITC) conjugated goat anti-rabbit (Sigma) diluted 1:500 in 1% BSA/PBS. Slides were washed twice with PBS and mounted. The sections were examined on a Nikon Microphot microscope equipped with a 100-W mercury lamp and epi-illuminator, and a photomicrographic system.

2.2. Primary antibody

Anti-osteopontin polyclonal antibody (OPN 1) was used as primary antibody. OPN 1 recognizes the whole non-translational modified chicken osteopontin molecule. The antibody to chicken osteopontin expressed in *Escherichia coli* was produced as previously described (Barak-Shalom et al., 1995; Knopov et al., 1995). Briefly, chicken osteopontin cDNA was subcloned into expression vector pGEX-2T (Pharmacia) using the *Bam*HI/*Eco*RI sites, creating an in-frame fusion with glutathione-*S*-transferase. The chimeric plasmid pGEX-OP was transferred into *E. coli* (NM 522 strain) grown in LB at 37 °C to midlog phase before addition of isopropyl-β-D-thiogalactopyranoside, final concentration 1 mM. After 6 h of growth, cells were pelleted and suspended in phosphate buffer, lysed, boiled for 10 min, and centrifuged for 10 min at 10 000*g*. The expressed chimeric protein was separated by SDS–PAGE electrophoresis, sliced out and eluted from the gel using Electro-eluter (Bio-Rad). The protein was injected into rabbits by a standard protocol, and the specificity of the antiserum was determined.

2.3. Scanning electron microscopy

Pieces of non-decalcified eggshells at different steps of formation were air-dried, mounted on stubs with double-side tape, and coated with gold. Scanning electron microscopy was performed on a TESLA BS 343A instrument operating at 15 kV.

3. Results

At 4.75 h post-oviposition, the egg was found in the last third of the isthmus, where shell membranes were forming (Fig. 1A). Immunofluorescence analysis of the isthmus showed a weak positive reaction to OPN 1 antibody in the lining epithelial cells, but negative in the isthmus tubular gland cells (Fig. 1B), while by immunogold, gold particles were observed in exocytic vesicles of the epithelial ciliated cells (Fig. 1C) and also in the core of the shell membrane fibers (Fig. 1D).

At 5.5 h post-oviposition, the egg was located in the red isthmus, where mammillae were forming (Fig. 2A). Mammillae showed positive immunofluoresce reactivity (Fig. 2B), which was specifically located by immunogold in their basal region (Fig. 2C) where the shell membrane fibers also showed reactivity only in the fiber core (Fig. 2D). A positive immunofluorescence reactivity with OPN 1 antibody was observed only in the epithelial cells lining the lumen of the red isthmus (Fig. 3A), while by immunogold gold particles were observed inside cytoplasmic vesicles of the epithelial ciliated cells (Fig. 3B). A weak immunofluorescence reactivity was also observed in lining epithelium of the oviduct shell gland (Fig. 3C) where gold particles were found in cytoplasmic vesicles of non-ciliated epithelial cells (Fig. 3D).

At 12 h post-oviposition, when the egg was in the shell gland and the mineralized palisade was starting to be form (Figs. 4A and B), the forming eggshell showed a positive immunofluorescence reactivity to OPN in the shell matrix and in the mammillae (Fig. 4C). However, a low density of gold particles was observed in the shell matrix (Fig. 4D). At this time, the red isthmus epithelium showed a very weak immunofluorescence reactivity (Fig. 5A) which is located in cytoplasmic vesicles of ciliated cells (Fig. 5B). However, a more intense reactivity was found in the shell gland epithelium (Fig. 5C) and it is specifically located inside cytoplasmic vesicles of the epithelial non-ciliated cells (Fig. 5D).

At 18 h post-oviposition, when the egg was still in the shell gland and the palisade was almost complete (Figs. 6A and B), the shell matrix also showed a

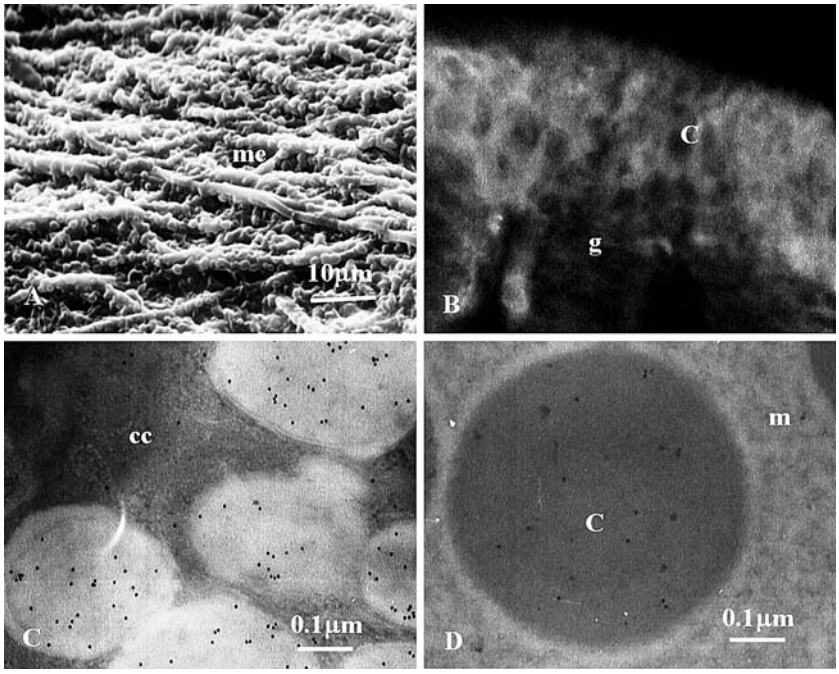

Fig. 1. Reactivity to anti-osteopontin antibody in the forming eggshell and oviduct at 4.75 h post-oviposition. (A) Scanning electron microscopy of eggshell membranes showing the absence of mammillae. (B) Positive immunofluorescence reactivity in the epithelial cells (e) and not in the tubular gland cells (g) of the isthmus; 400×. (C) Immunogold positive reaction in exocytic vesicles of the isthmus epithelial ciliated cells (cc). (D) Immunogold positive reaction inside the just formed core (c) but not in the mantle (m) of the shell membrane fibers located in the lumen of the isthmus tubular gland.

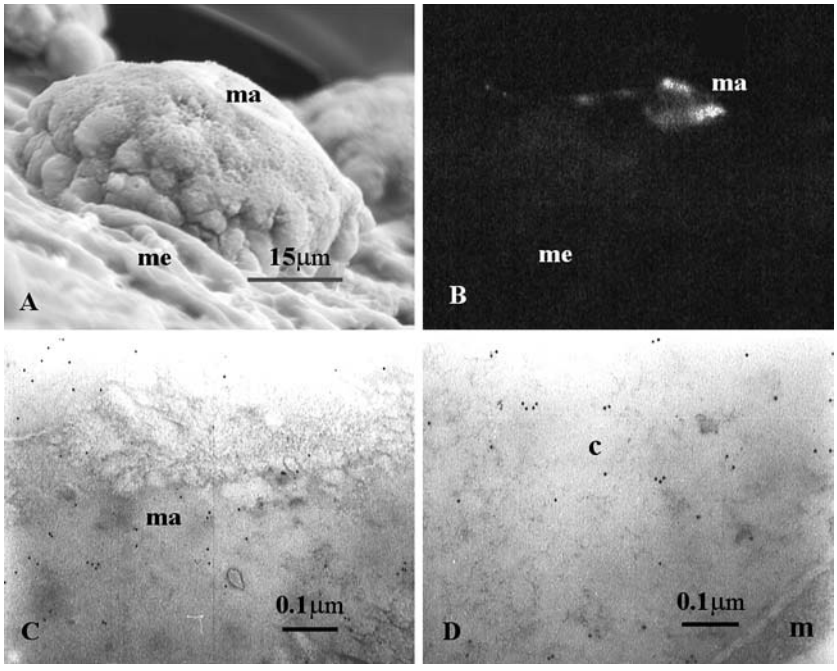

Fig. 2. Reactivity to anti-osteopontin antibody in the forming eggshell at 5.5 h post-oviposition. (A) Scanning electron microscopy of mammillae (ma) located at the outer side of shell membranes (me). (B) Positive immunofluorescence reactivity in the mammillae (ma) and not in the shell membranes (me); 400×. (C) Immunogold positive reaction in the basal region of the mammillae (ma). (D) Immunogold positive reaction in the core of a shell membrane fiber (c) and not in the surrounding mantle (m).

positive immunofluorescence reactivity (Fig. 6C) which, by immunogold, showed that gold particles were more numerous in its outside region (Fig. 6D). At this time, the red isthmus showed a very weak immunofluorescence reactivity to OPN (Fig. 7A), specifically located in cytoplasmic vesicles of the

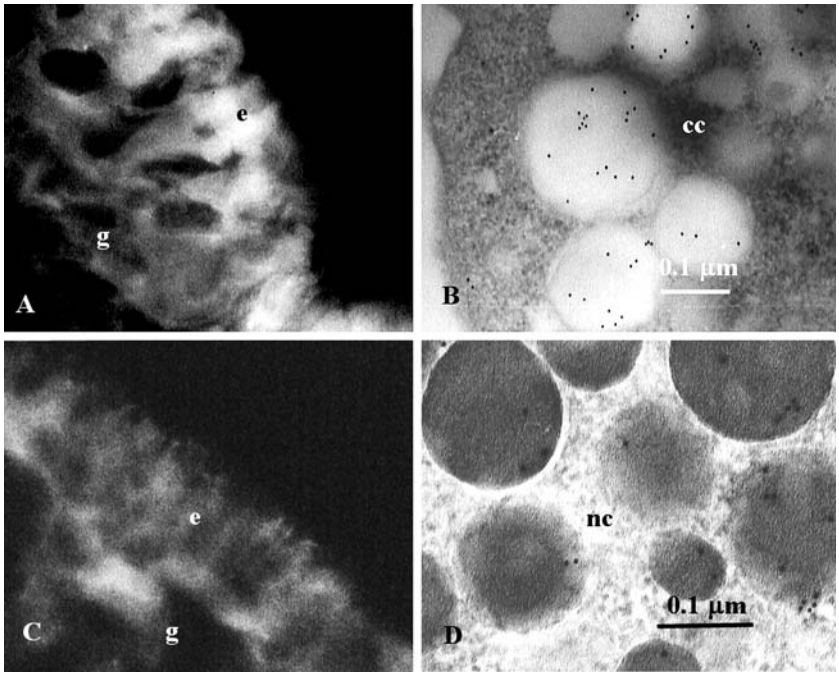

Fig. 3. Reactivity to anti-osteopontin antibody in the oviduct at 5.5 h post-oviposition. (A) Positive immunofluorescence reactivity in the lining epithelial cells of the red isthmus (e) and not in the tubular gland cells (g); 400×. (B) Immunogold positive reaction in cytoplasmic vesicles of the red isthmus ciliated epithelial cells (cc). (C) Discrete immunofluorescene reactivity in the shell gland epithelial cells (e) but not in the tubular gland cells (g). (D) Immunogold positive reaction in cytoplasmic vesicles of the shell gland non-ciliated epithelial cells (nc).

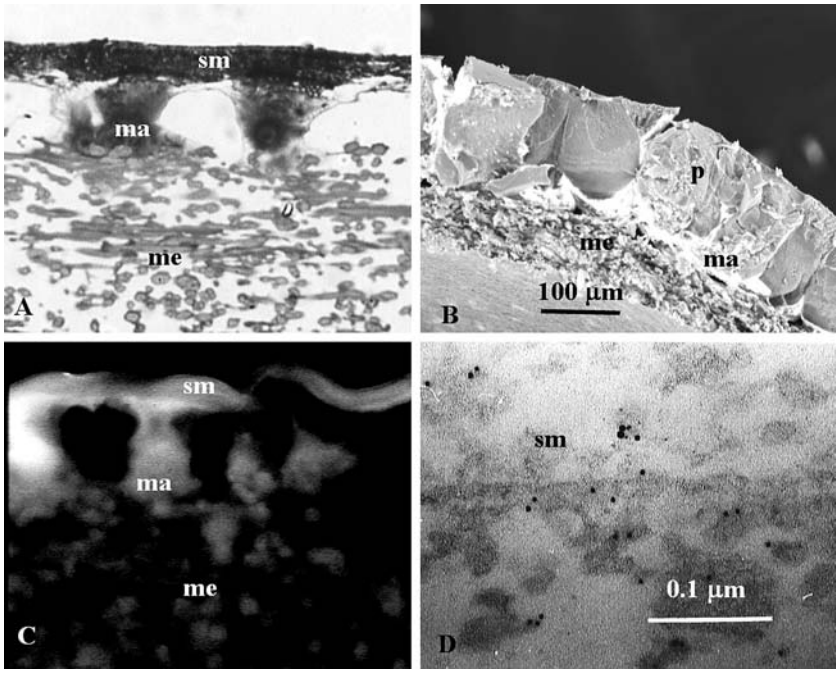

Fig. 4. Reactivity to anti-osteopontin antibody in the forming eggshell at 12 h post-oviposition. (A) Cross-section of a decalcified eggshell stained with Toluidine Blue showing shell membranes (me), mammillae (ma) and shell matrix (sm); 400×. (B) Scanning electron microscopy of eggshell showing shell membranes (me), mammillae (ma) and the calcified region or palisade (p). (C) Positive immunofluorescence reactivity in the shell matrix (sm) and mammillae (ma) but not in the membranes (me); 400×. (D) Weak positive immunogold reactivity in the shell matrix (sm).

ciliated epithelial cells (Fig. 7B). However, the shell gland epithelium showed a strong reactivity (Fig. 7C) which is specifically located in vesicles of its non-ciliated cells (Fig. 7D).

4. Discussion

The occurrence of osteopontin in the chicken oviduct and eggshell has been previously observed (Lavelin et al.,

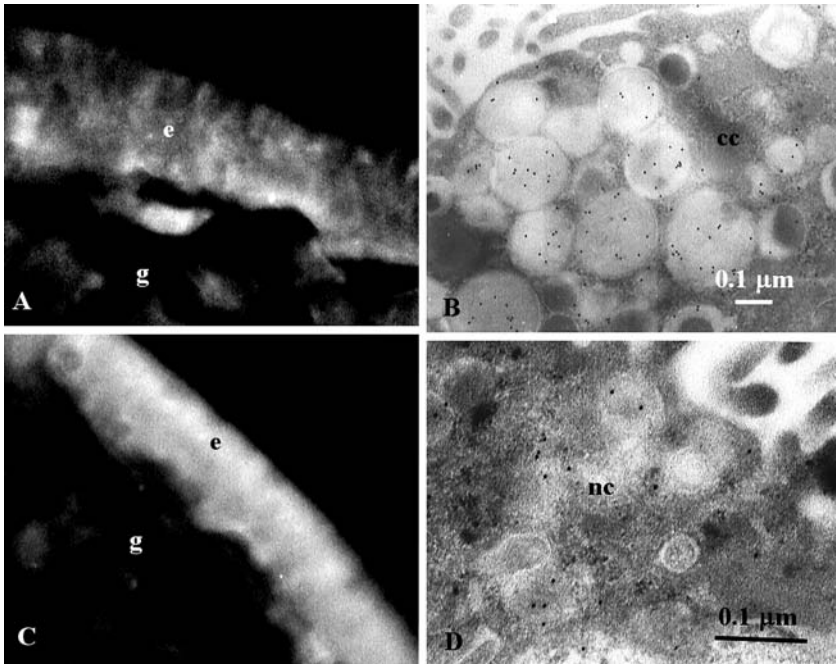

Fig. 5. Reactivity to anti-osteopontin antibody in the oviduct at 12 h post-oviposition. (A) Weak immunofluorescence reaction in the red isthmus epithelium (e) but not in the tubular gland cells (g); 400×. (B) Scarse gold particles in cytoplasmic vesicles of the red isthmus ciliated epithelial cells (cc). (C) Strong immunofluorescence reactivity in the shell gland epithelium (e) but not the tubular gland cells (g); 400×. (D) Positive immunogold reaction inside cytoplasmic vesicles of the shell gland non-ciliated epithelial cells (nc).

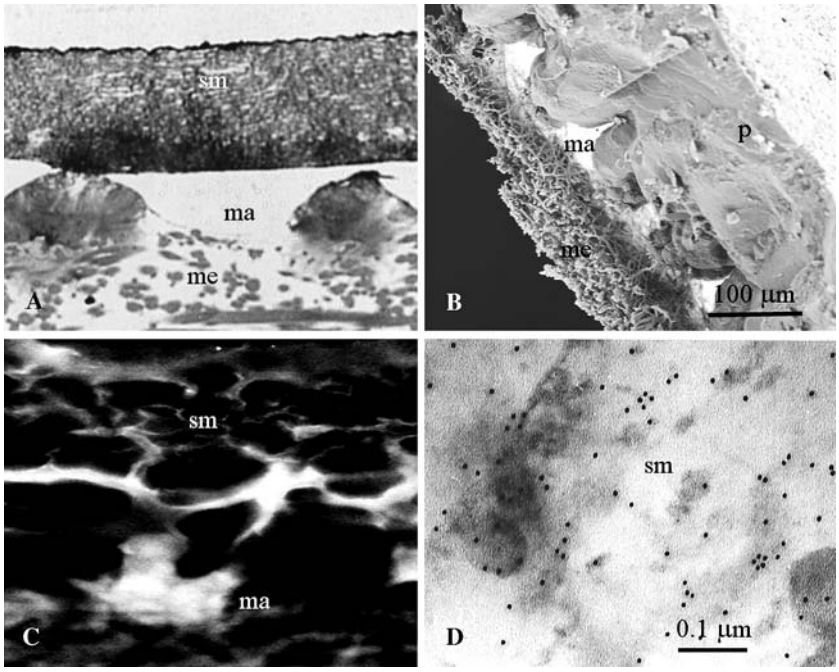

Fig. 6. Reactivity to anti-osteopontin antibody in the almost complete formed eggshell at 18 h post-oviposition. (A) Cross-section of a decalcified eggshell stained with Toluidine Blue showing shell membranes (me), mammillae (ma) and shell matrix (sm); 400×. (B) Scanning electron microscopy of eggshell showing shell membranes (me), mammillae (ma) and the calcified region or palisade (p). (C) Positive immunofluorescence reactivity in the shell matrix (sm) and mammillae (ma); 400×. (D) Strong positive immunogold reactivity in the outer part of the shell matrix (sm).

1998; Pines et al., 1994). In the chicken oviduct, osteopontin occurence and gene expression was only detected in the shell gland epithelium, when the egg is inside this region. It is produced in the form of two waves, first by the basal cells and then by the apical epithelial cells, induced by the mechanical strain applied by the presence of the egg in this region (Lavelin et al., 1998, 2000; Pines et al., 1994). However, these authors did not study these

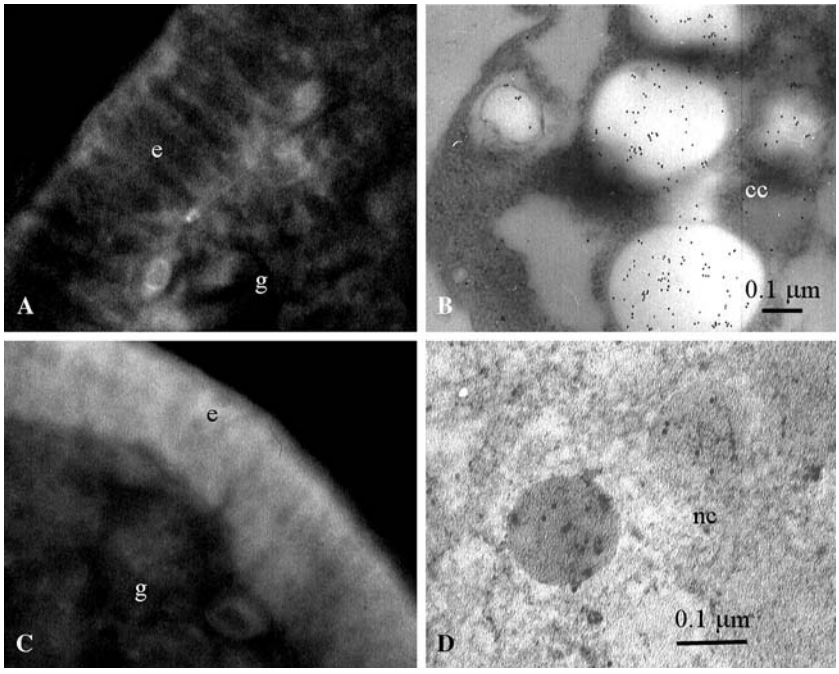

Fig. 7. Reactivity to anti-osteopontin antibody in the oviduct at 18 h post-oviposition. (A) Weak immunofluorescence reactivity in the red isthmus epithelium (e) and not in tubular gland cells (g); 400×. (B) Immunogold particles in cytoplasmic vesicles of the red isthmus ciliated epithelial cells (cc). (C) Strong immunofluorescence reactivity in the shell gland epithelium (e) but not in the tubular gland cells (g); 400×. (D) Positive immunogold reaction in vesicles of the shell gland non-ciliated epithelial cells (nc).

phenomena in the isthmus during the short period of time when the shell membranes are precisely formed (they studied 0.5, 2, 6, 12, and 18 h p.o.), or in the red isthmus where mammillae are formed, or the ultrastructural localization of osteopontin in the eggshell during its formation.

The shell membranes are formed in the isthmus between 4 and 5 h p.o. (Fernandez et al., 1997), and consist of fibers of type X collagen located in the core of each fiber (Fernandez et al., 2001). It is secreted by the tubular gland cells of the isthmus (Arias et al., 1991b; Wang et al., 2002). Here, we showed that osteopontin is detected in the isthmus at the time when the shell membranes are forming, and it is localized in the lining epithelial cells and it is also found in the core of the shell membrane fibers. The simultaneous expression of type X collagen and osteopontin has been demonstrated in hypertrophic chondrocytes during chicken endochondral ossification (Knopov et al., 1997; Pines et al., 1998). However, the concomitant occurrence of osteopontin and type X collagen in the shell membranes is even more interesting, because shell membranes do not mineralize, and both molecules have been suggested to inhibit mineralization (Arias et al., 1997; Giachetti and Steitz, 2000; Goldberg et al., 2001).

Between 5.25 and 5.5 h post-oviposition, the egg is in the red isthmus, where discrete aggregations termed mammillae start to form in close relation to the most external fibers of the shell membrane. The surface of these mammillae contain mammillan, a keratan sulfate proteoglycan secreted by the tubular gland cells of the red isthmus, which has a putative role in the nucletion of the first calcite crystals (Fernandez et al., 1997, 2001). Keratan sulfate proteoglycans show less calcium affinity than other proteoglycans due to the fact that they contain galactose instead of an alduronic acid (L-iduronic or D-glucuronic) as part of their disaccharide building blocks. The occurrence of keratan sulfate in the outermost region of the mammillae (calcium reserve bodies) is consistant with its role in nucleating calcite crystals during eggshell formation. However, its less calcium affinity, as compared to dermatan sulfate occurring in the palisade region, could be related with the facilitation of calcium withdrawal which takes place only in such region during chicken embryo development. Here, we found that osteopontin is detected in the lining ciliated cells of the red isthmus and is deposited only in the basal region of the mammillae and not on their surface. The base of the mammillae does not mineralize suggesting that osteopontin could contribute to the compartmentalization of the calcium reserve bodies. Concomitant occurrence of keratan sulfate and osteopontin has recently been found within the organic crystal sheaths of the bone structure (Hoshi et al., 2001).

At 18 h post-oviposition, when the shell is already formed, the shell gland non-ciliated epithelial cells cease the production of ovoglycan, which is involved in controlling calcite growth (Fernandez et al., 2001). However, at the same time the same cells showed now reactivity to osteopontin which is eventually found in

the outermost region of the palisade. The occurrence of osteopontin in the palisade shell matrix increases with the egg laying cycle, where its outermost region is intermixed with the cuticle. The cuticle is composed of other unknown glycoproteins, most of the pigments in colored eggs, and phosphate even as part of phosphoproteins or of hydroxyapatite crystals (Dennis et al., 1996; Fraser et al., 1999; Nys et al., 1991). Although it has been suggested that the mechanism of interaction of osteopontin to hydroxyapatite crystals is mediated by the aspartic- and glutamic-rich domains of the molecule, the effect of its post-translational modifications (i.e., phosphorylation) must be considered (Sodek et al., 2000). In fact, studies on some dentin phosphorylated proteins have shown that dephosphorylation or modification of carboxylate groups resulted in an increased binding to hydroxyapatite (Fujusawa et al., 1986), and dephosphorylation of porcine bone osteopontin abolishes its ability to inhibit hydroxyapatite formation (Hunter et al., 1994). In addition, dephosphorylation of eggshell osteopontin correlates with abolition of the inhibitory activity for calcium carbonate precipitation (Hincke and St. Maurice, 2000).

The outermost part of the eggshell, the cuticle, contains hydroxyapatite crystals whose occurrence has been involved in the eggshell calcification arrest (Dennis et al., 1996; Nys et al., 1991). Although, we do not know the post-translational modification status of the eggshell osteopontin, the strong reactivity to anti-osteopontin antibody in the outermost layers of the shell suggest that this molecule could be part of the mechanisms forming hydroxyapatite crystals thus controlling the eggshell calcification arrest.

Eggshells are fabricated by combining specific components of extracellular matrix molecules with a crystalline inorganic filler to produce a multilayered bioceramic. The observation that particular macromolecules are deposited in a defined temporo-spatial order provides a basis for understanding how this structure is precisely assembled.

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