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Short Report

Sexual differentiation of the somatic gonad tissue in marine bivalve mollusks: esterase- and fibronectin-like recognition signals

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ABSTRACT Textbooks usually indicate that in many species sex determination starts with gonad differentiation, triggered during early embryonic development. Once a primary somatic gonad is formed, the gonad-derived inductive signals are used to maintain the sex differentiation of germ cells. In marine bivalves, sex is not determined until the germ cells are differentiated, whether that is in the larva or in the juvenile. As sex is often decided late in post-larval development, gonad differentiation starts in the adult state. It is generally accepted that bivalves have no great difficulty in transforming from hermaphroditism to gonochorism and *vice versa* (Mackie, 1984). Our results focus attention on the patterns of gene expression in the "common" gonad (i.e., ovotestis) of the simultaneous hermaphroditic scallop species, *Pecten maximus,* during maturation and reproduction periods. In this species, as in almost all marine bivalves, the connective-tissue cell types present in the testis and ovary of the "common" gonad are essentially identical. Using biochemical, immunochemical and molecular approaches, we demonstrate for the first time that the scallop ovotestis is characterized by germ-cell-associated somatic protein expression.

Identification of male-predominant polypeptides in the ovotestis of *P. maximus* led us to compare them with those detected in the gonad and the reproductive tract of *Mytilus galloprovincialis* ("male-associated polypeptide", MAP) and *Drosophila virilis* (esterase S). *Mytilus* MAP and *Drosophila* esterase S are characterized by a certain biochemical, immunochemical and structural similarity and they belong to a multigene carboxyl/cholinesterase family (see Mikhailov and Torrado, 1999; 2000; Torrado *et al*., 2000). Anti-MAP and anti-esterase S antibodies cross-reacted with the 38-kDa-polypeptide from the *P. maximus* gonad (Fig. 1 A,B) so that we designated this polypeptide as an esterase-like protein (Es-LP). In the male gonad compartment, Es-LP was detected (by immunohistochemistry and Western blot) in: (1) integument, follicle membranes, and duct epithelium (in a high concentration), (2) luminal fluid of gonad ducts and haemolymph sinuses (in a very high concentration), and (3) on mature sperm cells. In the female gonad compartment, a weak positive signal was observed in the integument and follicle membranes but not in either the duct luminal fluid or in sinuses. The results suggest that a predominant Es-LP expression in the male gonad tissue is sperm-dependent and essentially contributes to the luminal fluid protein environment in which sperm cells are terminally differentiated and matured.

Among female-predominant polypeptides of the *P. maximus* gonad, a protein with properties similar to those of mammalian fibronectin (Fn) was detected and given the name fibronectin-like protein (Fn-LP). Fn-LP could be extracted from the gonad tissue by urea- but not by Tris-containing buffers. Fn-LP seems to be composed of disulfide-bonded monomers: when reduced with mercaptoethanol or dithiothreitol it appears as a doublet (on electrophoretic gels and Western blot transfers) with a molecular weight of 180 and 200 kDa. This doublet revealed a positive cross-reactivity with antibodies against a highly purified human Fn and its N-terminal sequence (Fig. 1F). Note that Fn-LP is present in lower amounts in the male gonad compartment compared to a relatively high concentration in female gonad tissue. In the latter, a predominant accumulation of Fn-LP was detected (by immunofluorescence) in follicle membranes and extra-follicular connective-tissue structures. Fn-LP-positive signals were also observed in the integument and digestive tube walls of both male and female gonad compartments. The relatively high concentration of Fn-LP detected in the *P. maximus* female gonad portion could be linked to its secretion by follicle cells and its accumulation in the somatic gonad tissue. In the mammalian ovary, Fn concentrations are increased during maturation of the follicle (De Candia and Rodgers, 1999).

Sex-predominant expression of Es-LP and Fn-LP is characteristic not only in the mature *P. maximus* gonad but also in other bivalves, namely: (1) *M. galloprovincialis, M. edulis, Ruditapes phillipinarum,* and *Dosinia exoleta* (gonochoric species); (2) *Aequipecten opercularis* (simultaneous hermaphrodite species): (3) *Ostrea edulis* (rhythmical consecutive hermaphrodite species); (4) *Crassostrea virginica* and *C. gigas* (alternative consecutive hermaphodite species). In resting gonads (which do not contain mature sex cells and consist of atretic gonad tubules) of the species studied, these polypeptides were detected in very low concentrations in both male and female gonad tissue. Taken together, the results obtained favor our working hypothesis that signals coming from differentiating sperm cells or oocytes determine the activation of the germ-cell-associated protein overexpression in the somatic gonad of bivalve mollusks.

Experimental Procedures

Bivalve mollusks (*Pecten maximus, Aequipecten opercularis, Mytilus galloprovincialis, Mytilus edulis, Ruditapes phillipinarum*, *Dosinia exoleta, Ostrea edulis, Crassostrea virginica, and Crassostrea gigas*) were purchased from commercial suppliers in La Coruña (Galicia, NW Spain). Using a scalpel, mollusks were opened and a small portion of gonad tissue was microscopically

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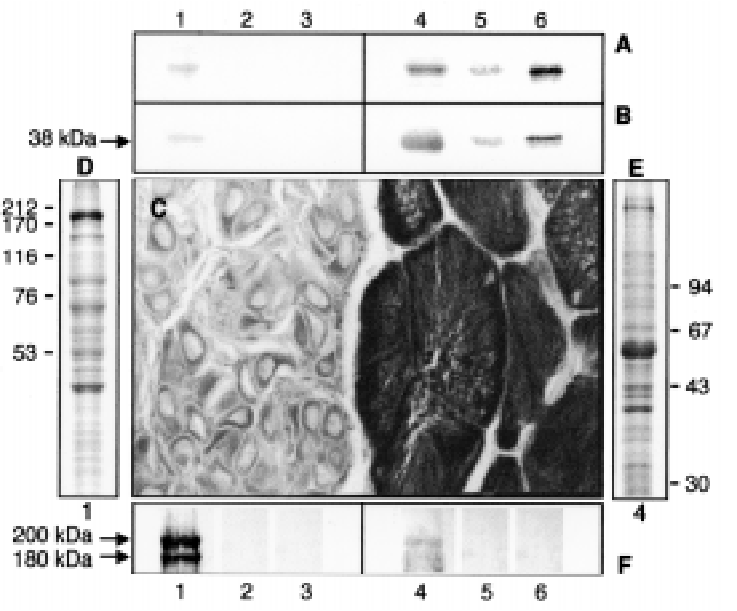

Fig. 1. Schematic illustration of the pattern of Es-LP and Fn-LP expression in the *P. maximus* ovotestis. *Histological cross section* **(C)** *through the mid-region of the ovotestis; mature sperm cells and oocytes can be seen filling all the follicles of the female (left) and male (right) gonad tissue, respectively. Electrophoretic patterns of total proteins extracted from female* **(D)** *and male* **(E)** *portions of the ovotestis. Positions of high and low MW calibration kit protein markers (Pharmacia) are indicated on the left and on the right, respectively. Representative Western blot analysis of Es-LP* **(A,B)** *and Fn-LP* **(F)** *expression levels in both female (left) and male (right) gonad portions using antibodies directed against: A, M. galloprovincialis MAP; B, D. virilis esterase S; and F, human fibronectin N-terminus. 1, female gonad tissue; 2, mature oocytes; 3, female gonad duct fluid; 4, male gonad tissue; 5, mature sperm cells; 6 , male gonad duct fluid. Apparent MW values of Es-LP and Fn-LP bands are marked (by arrows) on the left.*

examined to detect sperm cells or oocytes. Gonad tissue dissection and processing was performed as described previously (Torrado and Mikhailov, 1998, 2000). Total protein was extracted using the sequential extraction kit (Ready Prep, Bio-Rad), separated by SDS-PAGE on 8-12% acrylamide gels and electrotransferred to nitrocellulose or nylon membranes for subsequent immunodetection (Torrado and Mikhailov, 2000). Band intensities (on gels and blots) were quantified by densitometry using a model GS-700 densitometer (Bio-Rad) and software "Molecular Analysis" (Bio-Rad). Paraffin or frozen sections of the *P. maximus* gonad were stained by indirect immunofluorescence. In all cases, no specific immunofluorecent staining was observed when primary antibodies were omitted or replaced by non-immune gamma-globulins. For immunocytochemical and immunoblot analysis, we used the following rabbit polyclonal antibodies (PABs): 1. PABs raised against the *M. galloprovincialis* MAP; 2. PABs directed against the *D. virilis* esterase S-fused protein; 3. PABs raised against *D. virilis* ejaculatory bulb proteins; 4. PABs directed against porcine liver esterase (Polysciences), and 5. PABs directed against human fibronectin (Sigma). Primary mouse monoclonal antibodies (MAB) were: 1. MAB directed against human fibronectin (Chemicon); 2. MAB raised against human fibronectin gelatin binding domain (Chemicon); 3. MAB raised against human fibronectin C-terminus (Chemicon), and 4. MAB directed against human fibronectin N-teminus (Chemicon). Standard molecular techniques were used for DNA isolation and subsequent PCR amplification of conserved carboxylesterase (Torrado *et al.*, 2000) and fibronectin (De Candia and Rodgers, 1999) sequence blocks.

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