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LOCALIZATION OF ACTIVIN AND FOLLISTATIN PROTEINS IN THE *XENOPUS* OOCYTE

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Summary We found a binding protein for activin and follistatin in serum from female *Xenopus laevis* and identified it as vitellogenin, which is synthesized in the liver and transported into yolk platelets. Then, we investigated the localization of activin and follistatin proteins in early *Xenopus* oocytes (stage 6) by electron microscopic immunolabeling with gold colloidal particles. The protein molecules were found to be localized uniformly in oocyte yolk platelets, but not in other cytoplasmic organelles. These findings suggest a novel role of yolk platelets as a reservoir for inductive signals transported by vitellogenin in the differentiation and patterning of cells in *Xenopus* embryos.

Activins, members of the transforming growth factor ß (TGF-ß) superfamily, have potent mesoderm-inducing activity in early *Xenopus* embryos (1-3), and are known to occur as homodimers of two ßA subunits (activin A) and two ßB subunits (activin B) or a heterodimer of ßA and ßB subunits (activin AB) (4, 5). We have recently detected activin-like activity in unfertilized eggs and blastulae of *Xenopus laevis* and proposed that activin proteins are present maternally in *Xenopus* embryos (6). In fact, we have been able to identify three isoforms of activins A, AB and B in early embryos (stage 1-5) (7). They were demonstrated to exist at least in part as a complex with follistatin, an activin-binding protein (8) in the egg. Since mesoderm

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induction is initiated around the morula stage (stage 7), when both activin subunit genes have yet to be expressed, the maternal activin proteins may (play a crucial role in the initial step of mesoderm induction in early *Xenopus* embryogenesis. Therefore, to understand the molecular mechanism of mesoderm induction by activin, it is important to know how the maternal activin and follistatin proteins are delivered into the egg, and the cytoplasmic organelle(s) in which they accumulate. More recently, α_2-macroglobulin (α_2M) was shown to be a primary binding protein for activin and follistatin in mammalian serum (9, 10). Although the role of binding of these proteins in activin activity is currently uncertain, binding may provide a mechanism whereby these protein factors are transported to their functional location, as well as escaping proteolysis in the local environment. Therefore we attempted to search for such binding protein in *Xenopus* serum to reveal the mechanism responsible for delivery of maternal protein factors into *Xenopus* embryos.

Materials and Methods

Porcine activins and follistatin were prepared as described previously (11). SDS-PAGE was performed according to Laemmli (12) in 3.6% gels, and the proteins were transblotted onto Immobilon P membrane (Millipore). The membrane was blocked with 5% skim milk, washed and incubated with ^{125}I-activin A or ^{125}I-follistatin. The blots were exposed to Kodak XAR film for 1 day at -80°C. The NH_2-terminal sequence of protein bands transblotted onto the membrane was determined by microsequencing with an Applied Biosystems 477A protein sequencer. Rabbit polyclonal antibodies were raised against synthetic *Xenopus* activin β_A subunit (residues 95-107) and β_B subunit (residues 67-80) peptides, and *Xenopus* follistatin peptide (residues 122-135) (13). *Xenopus laevis* eggs were fixed with 4% paraformaldehyde in 0.1 M sodium cacodylate, pH 7.4, embedded in Lowicryl K4M (Electron Microscopy Science, USA), and sectioned. Lowicryl ultra thin sections were picked up on uncoated nickel grids and immunolabeled. The sections were then left untreated, or treated with 5% 2-mercaptoethanol in 0.1 M phosphate buffer, pH 7.5, for 30 min, washed with PBS, blocked with 2% skim milk, and incubated with affinity-purified antibody against activin or follistatin. The sections were incubated with goat anti-rabbit IgG-coated gold colloidal particles (15 nm) (Ultra Biosols, UK). After extensive washing with 0.05% Triton X-100 in 0.05 M PBS, the sections were examined directly using a JEOL-100C electron microscope (JEOL, Japan). Control sections were incubated with anti-peptide antibodies preabsorbed with synthetic peptides.

Results

Sera from male and female *Xenopus laevis* were subjected to ligand blotting analysis using ^{125}I-labeled ligands, activin A and follistatin. SDS-PAGE of male and female sera gave similar protein-staining patterns with a single exception: a 220-kDa band was seen in female serum but not in male serum (Fig. 1a). Incubation of the blots with ^{125}I-labeled ligands resulted in labeling of only the 220-kDa band from female serum (Fig. 1b). No or only weak labeling was observed on

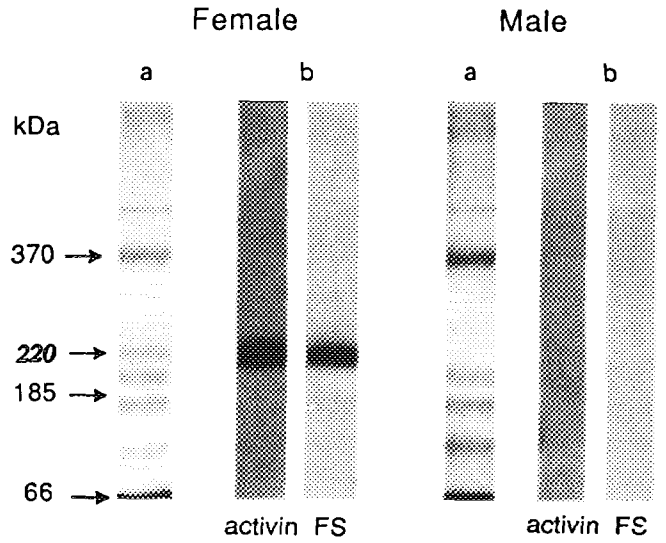

Fig. 1. SDS-PAGE (a) and ligand blotting analysis (b) of female and male *Xenopus* sera. SDS-PAGE was carried out under nonreducing conditions in 3.6% gels and the proteins were detected by staining with Coomassie Brilliant Blue (a). The proteins on the gels were transferred to Immobilon membranes, which were then incubated with ^{125}I-labeled activin or follistatin (FS) (b).

the bands from male serum. This result indicated that activin and follistatin bound preferentially to the 220-kDa band protein in female serum.

To identify the 220-kDa protein, we determined its NH_2-terminal amino acid sequence. The protein was shown to possess a single NH_2-terminal sequence: Glu- Lys- Ser- Gln- Tyr- Glu- Pro- Phe- Phe- Ser- Glu - X- Thr- Tyr- (X; unidentified residue), which was identical to the NH_2-terminal sequence of *Xenopus* vitellogenins B1 and B2 (14), indicating that both activin and follistatin have specific affinity for vitellogenin in female *Xenopus* serum.

Vitellogenin is synthesized in the liver and transported into yolk platelets. Therefore, it would be expected that maternal activin and follistatin proteins in the *Xenopus* embryo are distributed in the yolk platelets of the egg. Accordingly, we attempted to identify these proteins in yolk platelets by immunogold labeling. Sections of *Xenopus laevis* oocytes (stage 6) were incubated with anti-activin βA and βB subunit peptide antibodies and anti-follistatin peptide antibody. Gold particles representing activins A and B and follistatin proteins were observed specifically over yolk platelets (Fig. 2), whereas no significant localization of the particles was seen over other cytoplasmic organelles. The staining was judged to be specific because of the lack of staining with preabsorbed antibodies. Incomplete absorption of anti-follistatin antibody with the synthetic peptide may have been due to its insolubility in the buffer (Fig. 2f). Staining spots of both activin and follistatin proteins lay scattered uniformly over the yolk platelets.

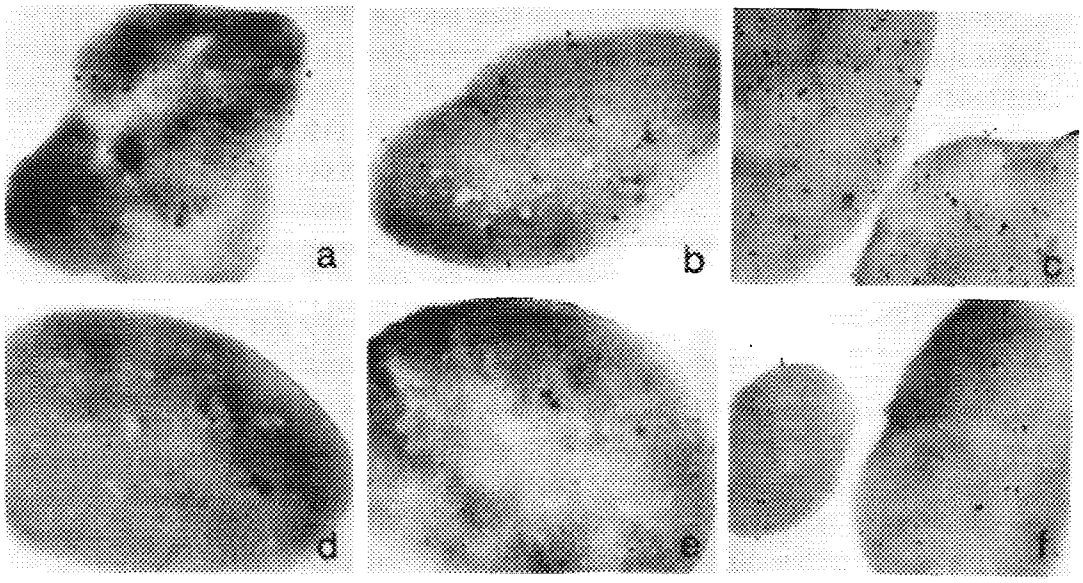

Fig. 2. Subcellular localization of activins and follistatin in *Xenopus laevis* eggs. Sections of *X. laevis* eggs were incubated with anti-*X.* activin β_A subunit peptide antiserum (a), with anti-*X.* activin β_B subunit peptide antiserum (b), with anti-*X.* follistatin peptide antiserum (c), and with immune serum pretreated with the synthetic peptides, *X.* activin β_A subunit peptide (d), *X.* activin β_B subunit peptide (e) and *X.* follistatin peptide (f), respectively. x12,600.

Discussion

We found that activin and follistatin bound preferentially to vitellogenin in female *Xenopus* serum. In ligand blot analysis, a faint α_2M band was visible after long exposure, but no significant band other than that of vitellogenin was detected (data not shown). Only weak affinity of activin-related factors, inhibin and TGF-ß, for vitellogenin was evident (data not shown). On the basis of these results, the binding of activin and follistatin to vitellogenin seems to be specific.

Recently, we attempted to purify activin and follistatin proteins from 10,000 *Xenopus* embryos and found that the yields of the three isoforms of activins A, AB and B were 1.4 ng, 1.3 ng and 6.4 ng, respectively, whereas that of follistatin was 402 ng (7). In the immunogold labeling experiments, the observed numbers of particles for activin B was obviously higher than that for activin A, and furthermore, the density of particles for follistatin was much greater than those for both activins, indicating that a marked excess of follistatin is present in *Xenopus* eggs. This may reflect the content of each protein molecule in yolk platelets, and agrees well with the purification yields of the proteins from *Xenopus* egg. Although we were able to confirm the affinity of activin and follistatin for vitellogenin, the photographs did not reveal whether activin and follistatin are present as a complex in the yolk platelet.

Vitellogenin is produced by the liver, released into the circulation and selectively sequestered in developing oocytes. After internalization by the oocyte, the vitellogenin molecule is cleaved into several smaller molecules, lipovitellins, phosvitin and phosvettes (15, 16). These molecules are thought to be stored as nutritive materials within yolk platelets. Taken together, it is conceivable that activin and follistatin proteins are synthesized in somatic cells including follicular cells, carried on a vitellogenin molecule and transported into the oocytes to be stored in yolk platelets. Although yolk platelets must play an important role in embryogenesis, we still do not know in detail how they are utilized during mesoderm induction in *Xenopus* embryos. The present findings suggest a novel function of yolk platelets as a reservoir for inductive signal molecules such as activin and follistatin in the cytodifferentiation and patterning of cells in *Xenopus* embryos. However, several intriguing questions still remain unanswered. These include how the maternal proteins are stored in *Xenopus* yolk platelets, and how they can switch on the mesoderm-inducing reaction at the critical time.

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