ACTIN IN XENOPUS YOLK PLATELETS: A PECULIAR AND DEBATED PRESENCE

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SUMMARY

Our previous work showed that Xenopus yolk platelets exhibited, when reacted with actin antibodies or fluorescent DNase 1, a shiny halo of fluorescence, which revealed an external actin cover around the platelets themselves. This structure was named the 'actin-shell'. Now we have demonstrated the actin-containing coat of Xenopus yolk platelets by agglutination tests, using anti-Xenopus-muscle-actin rabbit serum, and by indirect immunofluorescence microscopy, when the platelets reacted with antibodies directed against DNase 1. We chose these procedures to minimize the difficulties of the techniques used previously and to confirm the data obtained.

INTRODUCTION

We recently described, by means of indirect immunofluorescence tests, the presence of an actin coat (actin-shell) around Xenopus laevis yolk platelets (Colombo, Benedusi & Valle, 1981), in paraffin-embedded egg sections (method of Sainte-Marie, 1962). In fact, the main result of these experiences was the appearance, using a rabbit serum against Xenopus muscle actin, of a shiny halo of fluorescence around the yolk platelets, when paraffin sections of Xenopus eggs had been exposed to the fluorescent antibodies. To emphasize the presence of actin in the yolk platelet structure, during a second series of experiments (Colombo, 1982), we isolated the platelets and then exposed them to the DNase–FITC (fluorescein isothiocyanate) complex, according to Wang & Goldberg (1978). Ultraviolet light micrographs showed perfect superimposition of the results with those obtained in the first experiments. We related this peculiar location of the contractile protein to the formation mechanism of the yolk platelet itself.

Yolk, in Amphibia and in all so-called etherosynthetic animals, is not a synthetic product of the growing oocyte, but is synthesized by the maternal liver (Wallace & Dumont, 1968; Wallace & Jared, 1969) and is then haematically transferred to the ovary. The process by which the oocyte takes up the yolk material from the external medium is an active mechanism (Roth & Porter, 1964), which is called 'receptor-mediated endocytosis' (Goldstein, Anderson & Brown, 1979). During the development of this process, the clustering of the membrane yolk receptors is probably mediated by the membrane-linked cytoskeletal actin filaments. The data obtained by Salisbury, Condeelis & Satir (1980) on cultured B-lymphoblastoid cells support this idea.
We interpreted the actin shell as a vestigial structure, i.e. as evidence of the oocyte yolk uptake mechanism.

Many colleagues, during several discussions, have emphasized the possibility that there is an aspecific adsorption of proteins in the platelet coat, when the platelets are submitted to dehydration. To estimate the influence of the preceding techniques on the credibility of the results, we prepared another series of tests.

**MATERIALS AND METHODS**

*Isolation of Xenopus yolk platelets*

We used the polyvinyl pyrrolidone-sucrose technique (PVP–sucrose discontinuous gradient) first used by Wallace & Karasaki (1963), with slight modifications. In fact, when dejelled eggs were homogenized with a Teflon pestle and the platelets were isolated on the discontinuous gradient of PVP–sucrose, the external membrane of the studied organelles had often disappeared or was damaged (Fig. 1A) (many of these platelets, when submitted to immunofluorescence tests, in order to visualize the actin-shell, show none or a discontinuous fluorescent halo; Colombo, unpublished).

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Fig. 1. A. Electron micrograph of a detail of *Xenopus* yolk platelet, isolated by PVP–sucrose discontinuous gradient, after Potter homogenization (according to Wallace & Karasaki, 1963). *y*, yolk crystal. The external membrane has disappeared. ×159000.

B. Electron micrograph of a detail of *Xenopus* yolk platelet isolated by PVP–sucrose discontinuous gradient, without previous homogenization. *y*, yolk crystal; *m*, membrane. The external membrane is visible. ×219000. Yolk platelets were prepared for electron microscopy by the usual techniques.
data). In our experiments, dejelled eggs were not homogenized, but were broken with forceps and the external membranes were removed. The suspension obtained was then centrifuged on a discontinuous gradient of PVP-sucrose. Most of the yolk platelets obtained in this way exhibited an external membrane (Fig. 1a).

**Anti-Xenopus-muscle-actin antibodies**

The rabbit $\gamma$-globulin fraction, which contained antibodies against *Xenopus* muscle actin, was the same as the one we obtained and used previously (Colombo et al. 1981).

**Anti-DNase 1 antibodies**

Anti-DNase antibodies were obtained by the method of Wang & Goldberg (1978). Rabbits were immunized by intramuscular injection with 500 $\mu$g of DNase 1 obtained from Boehringer (DNase 1, grade I, RNase-free, Boehringer-Mannheims). The lyophilized enzyme had been emulsified with Freund's complete adjuvant. One week later, the animals were again intramuscularly injected with another 500 $\mu$g of the antigen, which had been emulsified with Freund's incomplete adjuvant. Two weeks later a third injection containing 200 $\mu$g of DNase was administered intravenously. Ten days after the last injection, the rabbit blood was collected and the serum was prepared. The $\gamma$-globulin fraction was partially purified by ammonium sulphate precipitation. The antiserum was specific for DNase 1, as shown by the immunodiffusion test (Fig. 2). When non-immune rabbit serum was tested against the antigen, no precipitin band appeared.

**Agglutination tests**

Yolk platelets, isolated as previously described, were resuspended in 0.1 M buffered phosphate (pH 6.5–6.7). One millilitre of this suspension contained the platelets, which were extracted from about 15–20 eggs. In the first test, 1 vol. of this suspension was added to 0.5 vol. of anti-Xenopus-muscle-actin rabbit serum (using a smaller quantity of serum, the agglutination is not dramatic). A

Fig. 2. Immunodiffusion prepared by the Ouchterlony technique (1958); 1 % agarose in 0.05 M-phosphate buffer (pH 7.2)+0.02 M-NaCl. In the central well: anti-DNase rabbit serum ($\gamma$-globulin fraction). Well a: DNase 1, 62.5 $\mu$g/ml in phosphate buffer containing 1 mM-PMSF. Well b: DNase 1, 31.2 $\mu$g/ml. Well c: DNase 1, 15.6 $\mu$g/ml. Well d: DNase 1, 7.8 $\mu$g/ml.
control experiment in which an anti-boar-acrosin rabbit serum took the place of anti-actin was performed in parallel. Other tests were prepared using anti-Xenopus-muscle-actin rabbit serum adsorbed against paraformaldehyde-insolubilized Xenopus muscle actin. Finally, part of the yolk platelet suspension was treated with trypsin (0.1% at 37°C) for 1 min. The reaction was stopped by addition of an excess of calf serum. The trypsin-treated yolk platelets were collected by low-speed centrifugation, resuspended in phosphate buffer, and then mixed with anti-Xenopus-muscle-actin rabbit serum. A negative control, consisting of only yolk platelet suspension in phosphate buffer, was also performed.

**Immunofluorescence experiments**

Some drops of the yolk platelet suspension were layered on a glass slide previously coated with a thin layer of BSA solution (bovine serum albumin, 10% in phosphate buffer). The slides were air-dried, and the platelets were then fixed in absolute methanol. Some samples of fixed yolk platelets were soaked in a buffered solution of BSA (1 mg/ml in phosphate buffer) to eliminate all unspecific adsorption phenomena, and then the platelets were prepared for immunofluorescence tests according to Wang & Goldberg (1978). Each slide was reacted with a solution of DNase (1 mg/ml) in the presence of 1 mM-phenylmethylsulphonyl fluoride (PMSF) and incubated for 10 min at 37°C. Unbound DNase was washed off with phosphate buffer. Slides were then reacted with anti-DNase rabbit serum (diluted 1:20) and incubated for 30 min at 37°C in a water-saturated atmosphere; they were then washed in phosphate buffer and treated for staining with fluorescein-conjugated goat anti-rabbit immunoglobulin G (IgG) (diluted 1:30). After the incubation period, the slides were washed again with phosphate buffer and mounted in buffered glycerol. A second group of samples was treated according to Wang & Goldberg, but the immersion in BSA was omitted. Finally, a third stock of fixed platelets was treated as previously described, omitting, however, both immersion in BSA and DNase incubation steps.

**RESULTS**

We thought that, if the Xenopus yolk platelets have actin microfilaments bound to the external surface of their peripheral membrane, they should agglutinate in the presence of actin antibodies. In fact, Fig. 3A clearly shows the dramatic clustering of the platelets, when the anti-Xenopus-muscle-actin rabbit serum was added to the yolk platelet suspension. On the contrary, when the serum used had been previously adsorbed with specific antigen, the agglutination was not present (Fig. 3B).

To recognize the position of the actin in relation to the external membrane of the yolk platelet, we treated the organelles with trypsin. Trypsinized platelets showed no agglutination when they were mixed with actin antibodies (Fig. 3C). This fact suggests that the antigen (or its connection to the platelet membrane) is affected by the enzyme, so it is reasonable to think that actin has an external position.

Non-immune rabbit serum did not excite yolk platelet agglutination (Fig. 3D). Fig. 3E shows that yolk platelets aggregate naturally in small clusters, which are clearly smaller than the clods that were visible after treatment with the actin antibodies.

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Fig. 3. A. Appearance of yolk platelet suspension after addition of anti-Xenopus-muscle-actin rabbit serum (γ-globulin fraction). B. Appearance of yolk platelet suspension after addition of anti-Xenopus-muscle-actin rabbit serum (γ-globulin fraction) adsorbed with glutaraldehyde-insolubilized Xenopus muscle actin. C. Appearance of trypsinized yolk platelet suspension after addition of anti-Xenopus-muscle-actin rabbit serum (γ-globulin fraction). D. Appearance of yolk platelet suspension after addition of anti-boar-acrosin rabbit serum (γ-globulin fraction). E. Appearance of yolk platelet suspension without any addition. A–E ×1200.
Actin in Xenopus yolk platelets

Fig. 3
We also performed immunofluorescence tests in which isolated yolk platelets layered on glass slides were differently treated. It is well known that DNase 1 specifically interacts with actin (Lazarides & Lindberg, 1974). The result of this interaction is a well-bound complex, DNase–actin. When we supplied exogenous DNase 1 to the yolk platelets and then checked the localization of the DNase–actin complex by means of indirect immunofluorescence using an anti-DNase rabbit serum, we could see the actin-shell around the platelets (Fig. 4A). When the yolk platelets were soaked in BSA solution before treatment with exogenous DNase and then prepared for immunofluorescence experiments, the actin-shell was clearly visible (Fig. 4B). This fact suggests that DNase is specifically bound to the yolk platelet membrane and not attached by means of a simple physical phenomenon.

When exogenous DNase 1 was not supplied, immunofluorescence tests showed no shiny structure (not shown).

**DISCUSSION**

Actin around yolk platelets of *X. laevis* was detected in our previous studies (Colombo et al. 1981; Colombo, 1982). This presence was interpreted as a remnant of the oocyte yolk uptake mechanism, and it is likely that the contractile protein is also involved in platelet migration through the egg and blastomere cytoplasm. These data are very interesting because it is possible to use the actin-shell as a marker of membranes enclosing the nutritive material captured from the extracellular environment. In fact, preliminary experiments performed on the autosynthetic eggs of *Ciona intestinalis* showed the absence of actin around the yolk platelets (Colombo, 1982).

We recently discussed with many colleagues the suitability of paraffin-embedded sections for immunohistochemical tests. Using this method, yolk platelet external coat, submitted to dramatic dehydration, probably behaves like a sponge and adsorbs the supplied proteins aspecifically. To eliminate this eventuality, we performed
agglutination tests in which the platelets were not submitted to any dehydration. When actin antibodies were added to a suspension of isolated yolk platelets, these organelles agglutinated dramatically in large clods that are easily recognized microscopically (Fig. 3A). If the antiserum added to the platelet suspension had been adsorbed against glutaraldehyde-insolubilized actin, yolk platelet agglutination was not visible (Fig. 3B). The platelets also showed no agglutination when they were mixed with a rabbit serum against boar acrosin (a protein that, certainly, is not present in the egg) (Fig. 3D). These facts suggest that platelet agglutination is only related to the presence, in the serum used, of specific γ-globulins directed against the antigenic sites of actin.

When yolk platelets are first treated with trypsin and then mixed with the anti-Xenopus-muscle-actin rabbit serum, the platelets themselves do not agglutinate (Fig. 3C). Since it is well known that trypsin does not affect the actin molecule (Laki, 1971), it is likely that the proteolytic enzyme interacts with a protein that binds actin to the yolk platelet membrane, liberating actin microfilaments from the membrane itself (trypsinized yolk platelets do not show actin-shell when submitted to immuno-fluorescence tests; Colombo, unpublished data).

Furthermore, we performed immunochemical experiments in which DNase and anti-DNase rabbit serum were used. Because of the dehydration process to which yolk platelets were submitted during the preparation, we first soaked the samples in BSA solution to prevent all unspecific adsorption phenomena of proteins by the platelet’s external coat. BSA-soaked yolk platelets exhibited, when submitted to DNase–anti-DNase treatment, the same shiny halo of fluorescence clearly shown by untreated organelles (Fig. 4A, B). When exogenous DNase was not added to the sample and the fixed yolk platelets were then treated with anti-DNase rabbit antibodies, the addition of fluorescent goat anti-rabbit IgG did not reveal any fluorescent structure (not shown). It is easy to conclude, at this point, that DNase 1 specifically binds one of the molecular components of the yolk platelet membrane and that this component is very likely to be the natural inhibitor of the enzyme: i.e., the actin (Lazarides & Lindberg, 1974).

The problem of actin localization in Xenopus eggs is not yet completely solved; it requires proof, which could emerge from immunoelectron microscopic studies. This approach is not easy because the Xenopus egg is a ‘dirty’ system. There is, in this cell, a large amount of actin in the cytoplasmic protein pool (Clark & Merriam, 1978; Merriam & Clark, 1978), which could mask or mime the contractile protein molecules with a specific localization.

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REFERENCES


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