SECRETED PROTEINS IN THE MEDIUM OF MICROINJECTED *XENOPUS* OOCYTES ARE DEGRADED BY OOCYTE PROTEASES

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1. Introduction

Oogenesis in *Xenopus laevis*, the South African clawed frog, proceeds as a continuous asynchronous process; therefore oocytes in all stages of development are simultaneously present in the ovary of the adult frog [1]. Mature *Xenopus* oocytes provide a convenient heterologous system for the translation of a variety of microinjected mRNAs [2–4]; when mRNAs for secretory proteins are employed, compartmentalization and secretion of these proteins also occur [5]. *Xenopus* oocytes therefore serve as an experimental system for the investigation of secretory processes [6], and the cumulative yield of secreted proteins directed by microinjected mRNAs provides a measure to estimate the half-life of their corresponding mRNAs [5,7,8]. Proteolytic activities of the oocyte, if secreted, would be expected to reduce the apparent yield of exogenous secretory products and thereby also affect the calculated half-life of their coding mRNAs. We therefore examined the presence of proteolytic activities secreted by *Xenopus* oocytes.

To further estimate the effect of oocyte proteases on proteins secreted into the incubation medium, we measured the stability of proteins excreted from, or coincubated with *Xenopus* oocytes, both in the absence and presence of protease inhibitors.

2. Materials and methods

2.1. Xenopus *oocytes*

Adult *Xenopus laevis* females were obtained from the South African Snake Farm (Fish Hoek, South Africa). The toads were anaesthesized by cooling in ice, ovarian lobes were removed and individual oocytes were dissected and staged [1]. The oocytes were incubated at 21°C in modified Barth medium [2] or under casein containing agar overlays [9]. Samples of incubation medium were removed and stored at −70°C until use.

2.2. *Gel analysis of proteolytic activities*

Samples of oocyte incubation medium were electrophoretically analyzed on 0.5 mm thick SDS–polyacrylamide gels containing gelatin [10]. Following electrophoresis, gels were washed in 2.5% Triton X-100 to remove SDS and incubated at 37°C. Gels were stained with Coomassie brilliant blue, and areas where gelatin was digested appeared as white transparent zones.

2.3. *Overlay analysis of individual oocytes*

Oocytes were laid in a 30 mm dish and covered with 0.8 ml agar–casein overlay [9], which contained 3 parts of Barth medium, 2 parts of 2.5% purified agar [11] in Barth medium, 1 part of a 8% solution of commercial non-fat milk powder (Carnation) and, where indicated, also 20 μg/ml of human plasminogen [12]. Following 15 h incubation at 21°C, oocytes were fixed with Dulbecco medium containing 10% pyridine and 2.5% glutaraldehyde and photographed.

2.4. *Determination of the protective effect of protease inhibitors*

A mixture of commercially purified proteins (Sigma) was iodinated in the presence of chlormaine T as in [13]. The mixture contained the following proteins (M_r): bovine serum albumin (67 000); ovalbumin (45 000); chemotrypsinogen (24 300); β-lacto-

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globulin (18 400); and lysosyme (14 300). Aliquots of the mixture, containing 2×10^6 cpm of ^{125}I-labeled proteins were added to 100 μl incubation media of groups of 10 oocytes, and coincubated with the oocytes in the presence and absence of protease inhibitors. At 0, 20 and 40 h incubation, 10 μl samples were removed and the different proteins were electrophoretically separated on a SDS–polyacrylamide gel together with non-labeled M_r markers [14]. Gels were vacuum-dried and autoradiographed, and radiolabeled bands were cut out from the gel and counted in the γ-counter. The inhibitors included were: trasylol (Sigma, aprotinin no. A-6012, 0.23 TIU/ml final conc.); soybean trypsin inhibitor (Worthington, 10 μg/ml); 6-aminocaproic acid (Aldrich Chemicals (Milwaukee WI) 2×10^{-3} M); leupeptin (5×10^{-4} M); and antipain (5×10^{-4} M).

2.5. *Determination of protein synthesis activity in* Xenopus *oocytes*

Oocytes were incubated in groups of 10 in 100 μl Barth medium containing 15 μCi [^{35}S]methionine (730 Ci/mmol, Amersham) and when indicated, also the protease inhibitors specified section in 2.4. Incorporation of [^{35}S]methionine into trichloroacetic acid-insoluble protein was determined in [8], in 5 μl aliquots of oocyte extracts after 0, 20 and 40 h incubation at 21°C. No difference could be observed in the electrophoretic migration patterns of labeled proteins synthesized in oocytes incubated with or without protease inhibitors, as analyzed by autoradiography of polyacrylamide gels of these proteins.

2.6. *Analysis of interferon in medium of microinjected oocytes*

Human fibroblast mRNA [15] was injected into single stage 6 selected *Xenopus* oocytes 50 ng/oocyte) and oocytes were incubated in groups of 10 in 100 μl Barth medium, with or without 10^{-4} M phenylmethylsulfonylfluoride. Interferon titers were bioassayed as in [15] following 20 h and 48 h incubation at 21°C.

3. Results

3.1. *Heterogeneity of proteolytic activities in oocytes from different frogs*

As substrates to assay proteases of the oocytes we used fibrin [16], casein [9] and gelatin [10]. Assays were also performed in the presence of added plasminogen, in order to detect plasminogen-activator-like proteases. The latter are highly specific proteases which convert the inactive zymogen plasminogen into the active non-specific endopeptidase plasmin, which can digest the 3 protein substrates employed [17].

A survey of oocytes from 15 frogs revealed a prominent qualitative and quantitative heterogeneity of proteolytic activities, as demonstrated for 6 frogs in fig.1. The pattern of the electrophoretic mobility of active proteases secreted by oocytes obtained from different frogs was determined by analysis on gelatin-containing polyacrylamide gels [10]. For this analysis, groups of separated oocytes were incubated for 20 h in Barth medium [2], and 5% of the incubation medium containing the secreted products were analyzed. A representative experiment of this type is shown in fig.1. Out of 6 different frogs, oocytes from 1 frog did not show any activity, while oocytes from 5 frogs expressed activities which migrated in the range corresponding to 20 000–>80 000 M_r. In 4 cases >1 active species was seen. It is not known whether all of the different bands represent distinct molecular species, or are, at least in part, active degradation products derived from higher M_r forms. These findings clearly demonstrate the variability between oocytes from different frogs in the amount of the total apparent proteolytic activities and in the number and the electrophoretic migration patterns of the active bands. Identical results were obtained with gels containing plasminogen, indicating that the ability to digest gelatin was in these cases plasminogen independent. Variability of a similar nature was

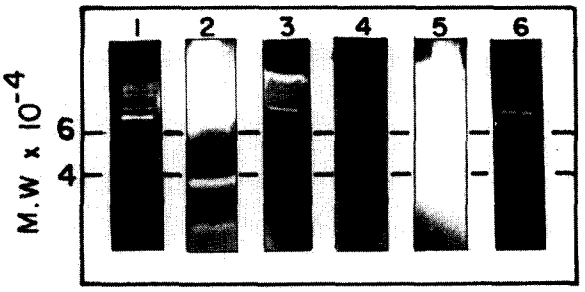

Fig.1. Electrophoretic analysis of proteolytic activities secreted by *Xenopus* oocytes from different frogs. Groups of 10 oocytes taken from 6 different frogs were incubated in 100 μl Barth medium for 15 h at 21°C. Medium samples (6 μl) of similar protein content were then electrophoretically analyzed as in section 2.

obtained also with intracellular proteases tested in oocyte extract. In quantitative assays in which fibrin was utilized as a substrate [13] we observed differences in the ratio between intracellular and secreted proteolytic activities of oocytes from different frogs (not shown).

It should be noted that a lower variability and also a lower level of proteolytic activity was observed in oocytes obtained from frogs grown by the Nasco Co., as compared with the wild-caught South African frogs. However, the wild-caught frogs also displayed significantly higher levels of endogenous protein synthesis and of specific translation of exogenous microinjected mRNA (submitted; unpublished).

The activity of individual stage 6 oocytes [1] of the same frog was assayed in a casein overlay [9] with and without plasminogen (fig.2). The clear proteolytic zones surrounding the oocytes were absolutely plasminogen-dependent and exhibited a wide range of sizes, indicative of quantitative differences in the level of activities which are membrane bound and/or secreted by single oocytes. The dependence of the proteolysis on the addition of plasminogen to the overlay assay indicated that, in this experiment, it was due to a plasminogen-activator-like protease. Plasminogen-dependent fibrinolysis was also observed with oocytes from several frogs, and it was much higher with human plasminogen as a substrate as compared with chicken plasminogen (not shown).

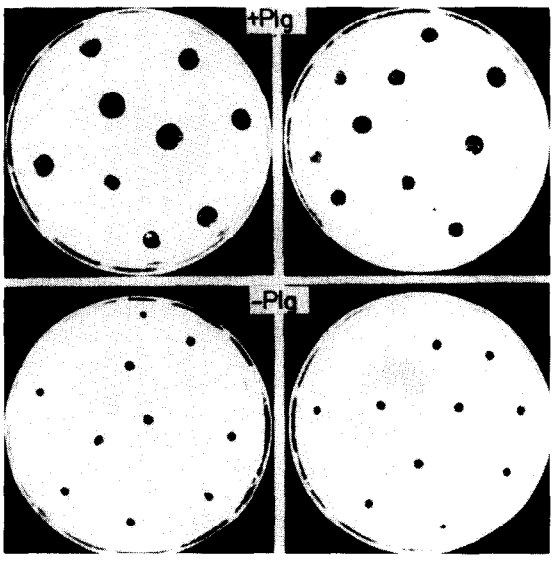

Fig.2. Detection of plasminogen-dependent proteolytic activity of single oocytes. Oocytes from a single frog were incubated under an agar–casein overlay [9] as in section 2, with and without human plasminogen [12] (20 μg/ml). Dark field photographs are presented.

3.2. *Extracellular proteolytic activities of staged oocytes*

Xenopus oocytes serve as a unique experimental model for developmental studies related to gene expression [1,18]. Several developmental processes have been shown to be modified under precisely controlled mechanisms during the maturation of *Xenopus* oocytes [19–22]. To test whether or not the proteolytic activities of oocytes may be related to their developmental stage, oocytes from a single frog were separated into 3 groups containing oocytes in stages 1–2, 3–4, and 5–6 [1], and then were assayed for extracellular fibrinolytic activity [13]. Plasminogen dependent fibrinolysis was found in all 3 stages, but with quantitative differences; whereas the amount of activity secreted/oocyte was 2-fold higher in stage 5–6 as compared with stage 1–2, the specific activity, e.g., activity secreted/mg oocyte protein content, was 3-fold higher than in stage 1–2, and stage 3–4 oocytes gave intermediate values. Plasminogen-independent fibrinolysis was also found in all 3 groups, and the amount secreted/oocyte was highest in stage 1–2. Microinjection experiments are performed with either mature selected oocytes at stage 6 of their development [1] or with ovarian fragments, which contain connective tissue and immature oocytes in addition to mature oocytes [2,19]. Therefore, we suggest the use of selected oocytes rather than whole ovarian fragments for injection of mRNAs of secretory proteins, to achieve a fairly homogeneous system.

Around the oocytes a layer of follicle cells appears during oocyte development [1] and is generally not removed before microinjection of mRNA [19]. In the rat it has been shown that granulosa cells, which encapsulate the ovarian follicles and surround the rat oocytes, synthesize and secrete plasminogen activitor just prior to ovulation [23]. To determine whether follicle cells are the major contributors of proteolytic activities secreted by *Xenopus* oocytes, mature selected oocytes were manually defolliculated [20]. Intact and defolliculated oocytes, as well as the removed follicle cells, were incubated in Barth medium. Cell extracts and incubation medium were then analyzed for active proteolytic bands (fig.3). Activities of apparently similar M_r were clearly detected in medium and extracts of both cell types,

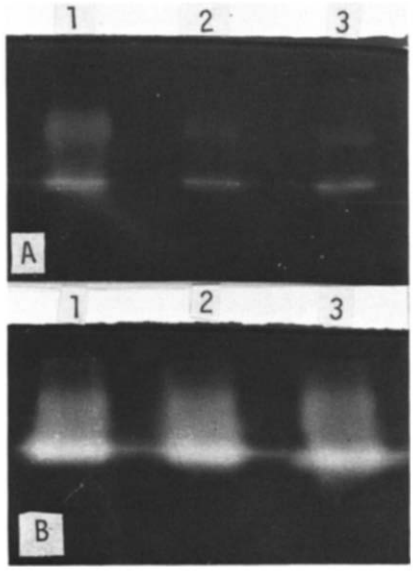

Fig.3. Electrophoretic analysis of intracellular and secreted proteolytic activities of oocytes and follicle cells. Oocytes from a single frog were manually defolliculated [20]. Intact oocytes [1], defolliculated oocytes [2] and separated follicle cells [3] were washed twice in Barth medium and incubated for 15 h at 21°C. Extract (A) and medium (B) samples representing activity of 0.5 oocyte were then electrophoretically analyzed as in section 2. Similar patterns were observed when plasminogen was included in the gel [10].

indicating that defolliculation procedures would not have prevented proteolysis in the incubation medium of *Xenopus* oocytes.

3.3. *Effect of protease inhibitors on proteins in oocyte medium*

It is suggested by our findings, that secretory proteins accumulated in oocyte incubation medium would be exposed to proteolytic degradation by the proteases secreted into the medium, especially the plasminogen-independent proteases in fig.1. Partially purified human interferon incubated with viable *Xenopus* oocytes undergoes appreciable loss of activity [8]. To minimize such proteolytic degradation we employed the mixture of protease inhibitors specified in fig.4, which has a wide range of action. The presence of the tested inhibitors in the medium of viable oocytes did not affect protein synthesis in the oocytes, as shown in table 1. This mixture can thus be safely added to the incubation medium immediately following microinjection of exogenous mRNA, and be kept there for ⩾40 h. Several iodinated proteins of different M_r-values were coincubated with oocytes for 20 and 40 h, in the presence and absence of this mixture. A prominent decrease in the amounts of the different proteins occurred in the absence of the inhibitors and was partially prevented in their presence (fig.4). This experiment therefore indicated that protease inhibitors may increase the apparent yield of secretory translational products.

The mixture of inhibitors utilized here was found to be toxic when tested in the bioassay for human

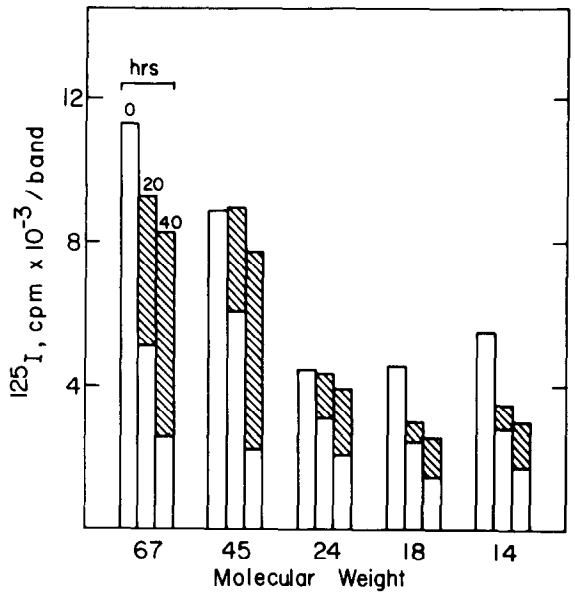

Fig.4. Protection by protease inhibitors of labeled proteins coincubated with oocytes. A mixture of commercially purified proteins (Sigma) was iodinated in the presence of chloramine T as in [13]. The mixture contained the following proteins (M_r): bovine serum albumin (67 000); ovalbumin (45 000); chemotrypsinogen (24 300); β-lactoglobulin (18 400); and lysosyme (14 300). Aliquots of the mixture, containing 2 × 10^6 cpm of ^{125}I-labeled proteins were added to 100 μl incubation media of groups of 10 oocytes, and coincubated with the oocytes in the presence and absence of protease inhibitors. At 0, 20 and 40 h incubation, 10 μl samples were removed and the different proteins were electrophoretically separated on a SDS–polyacrylamide gel together with non-labeled M_r markers [14]. Gels were vacuum-dried and autoradiographed, and radiolabeled bands were cut out from the gel and counted in the γ-counter. Empty bars represent values obtained without inhibitors. Hatched bars represent values obtained in the presence of inhibitors. The inhibitors included were: trasylol (Sigma, aprotinin no. A-6012, 0.23 TIU/ml final conc.); soybean trypsin inhibitor (Worthington, 10 μg/ml); 6-aminocaproic acid (Aldrich chemical (Milwaukee WI 2 × 10^{-3} M); leupeptin (5 × 10^{-4} M); and antipain (5 × 10^{-4} M).

Table 1
Protein synthesis in oocytes incubated with protease inhibitors (PI)

Incubation time (h)	[^{35}S]Methionine incorp. (cpm/oocyte)	
	−PI	+PI
20	8.9×10^4	8.3×10^4
40	3.1×10^5	3.1×10^5

Oocytes at stage 6 [1] were incubated in groups of 10 in the presence or absence of the mixture of protease inhibitors specified in section 2 and incorporation of [^{35}S]methionine into protein was determined on 5 μl samples of oocyte homogenates

interferon, which employs human cells in culture [15]. It was therefore replaced by PMSF in experiments which involved the microinjection of interferon mRNA. PMSF increased the yield of biologically active secreted interferon. Its effect was most prominent at 48 h post-injection, when a five-fold increase was obtained as compared with oocytes incubated without PMSF (table 2).

Discussion

Xenopus oocytes secrete variable levels of proteolytic activities of heterogeneous electrophoretic migration, which differ between oocytes from different frogs. Proteolytic activities are expressed by oocytes at all developmental stages, both by defolliculated oocytes and by follicle cells. Exogenous proteins coincubated with viable oocytes undergo proteolytic degradation, which is reduced by the addition of protease inhibitors to the medium. Similarly, the yield of active human interferon secreted from oocytes microinjected with fibroblast mRNA has also been improved when a protease inhibitor was added to the oocyte medium.

We suggest the use of stage 6 selected single oocytes for microinjection experiments, and routinely to add protease inhibitors to the incubation medium of *Xenopus* oocytes following microinjection of mRNAs coding for secretory proteins. Because of the heterogenous patterns of the proteolytic activities found in oocytes from different frogs, it is preferable to use a mixture of different inhibitors, which react with a wide range of proteases but do not affect protein synthesis of the oocyte. When a protein product with a biological activity is pursued, inhibitors which do not affect the bioassay of the product should be selected.

Table 2
Protection of PMSF of active human interferon secreted by microinjected oocytes

Medium	Hours	Human interferon (CPE unit/ml)	
		Poly(A)$^+$ RNA	Control
Barth	20	120	<10
Barth	48	20	<10
Barth + PMSF	20	160	<10
Barth + PMSF	48	100	20

Oocytes were microinjected with human fibroblast mRNA [15]. Control oocytes were injected with Barth medium [2]. Interferon activity, as measured by the interference with cytopathic effect (CPE units), was assayed by inhibition of growth of vesicular stomatitis virus (VSV) on FS11 human fibroblasts [15]

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