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The Absence from the Oocyte Secretory Apparatus of a Protein Kinase Capable of Phosphorylating Sequestered Caseins[1]

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The lactating guinea-pig mammary gland synthesizes and secretes four major milk proteins, i.e., three caseins and α-lactalbumin. Of these, the caseins are highly phosphorylated, a post-translational event which in the mammary gland involves a specific casein kinase, which is an integral membrane protein probably of Golgi origin. The microinjection of milk protein mRNA into *Xenopus* oocytes in the presence of [^{35}S]methionine leads to the synthesis, sequestration, and secretion of proteins which coelectrophorese with α-lactalbumin and with partially processed caseins. That the secreted caseins were not phosphorylated was shown by the use of ^{32}P. Either the oocytes were injected with mammary gland mRNA followed by incubation with [^{32}P]phosphate containing media or the mRNA was co-injected with [γ-^{32}P]ATP and the oocytes were then incubated. In neither case were ^{32}P-labeled caseins secreted. Golgi-rich fractions, identified by the marker enzyme galactosyltransferase, were isolated from the postnuclear supernatant of both oocytes and lactating mammary gland by sucrose density gradient fractionation. In contrast to the mammary gland fractions those derived from the oocytes contained no detectable casein kinase activity. Homogenates of oocytes do effect the phosphorylation of casein but the enzyme activity appears to be present in the soluble fraction and is not membrane bound. It is concluded that the *Xenopus* oocyte lacks the specific kinase that in the mammary gland phosphorylates sequestered caseins and that the phosphorylation of the caseins is not a prerequisite for their secretion by the oocyte.

The *Xenopus laevis* oocyte has proved to be a useful surrogate system in which to study gene expression. The synthesis from injected DNA or RNA of processed and secreted foreign proteins occurs with reasonable fidelity and thus by perturbing specific steps the sequence and interdependence of events can be studied in a living cell [see (1, 2)]. It is important, therefore, to define the limits of the fidelity of the oocyte system, including its processing ability which is known to encompass signal peptide removal, glycosylation, and many other secondary modifications of proteins [see (3)]. Moreover it is of interest to consider whether failure of the oocyte to modify secretory proteins will affect its ability to secrete proteins.

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There have been several studies (4–6) which describe the synthesis and secretion of guinea-pig milk proteins from oocytes. These have shown that α-lactalbumin exported by the oocyte has an electrophoretic mobility on SDS[4]–polyacrylamide gels identical to that of α-lactalbumin isolated from guinea-pig milk. In contrast, the caseins made in the oocyte migrate faster than the caseins exported by guinea-pig mammary gland cells or isolated from milk, a result we have attributed to the inability of the oocyte or heterologous cell-free systems to phosphorylate caseins (4, 7). In the present paper we provide evidence that the *Xenopus* oocyte lacks the enzymes (8) required to phosphorylate sequestered guinea-pig caseins and that addition of phosphate residues is not required for the export of milk proteins.

MATERIALS AND METHODS

All materials were obtained from sources previously described (5, 7, 9). Radiochemicals, [^{32}P]orthophosphate (carrier free), [γ-^{32}P]ATP (3000 Ci/mol), and [^{35}S]methionine (800–1300 Ci/mol), were purchased from Amersham International, Amersham, Bucks, UK, and calf thymus histone was from Sigma London Chemical Company Ltd., Fancy Road, Poole, Dorset, UK.

Poly(A)-containing RNA from the lactating guinea-pig mammary gland was isolated and translated in the wheat-germ system with or without microsomal membranes, as previously described (7, 9). Oocytes were injected and incubated in Barth X solution and then fractionated as described elsewhere (5), while explants were incubated in Eagle's minimal essential medium containing 20 mCi ml^{-1} sodium [^{32}P]orthophosphate (10), except that the preincubation step was omitted.

Oocytes microinjected with [γ-^{32}P]ATP (5 mCi ml^{-1}) were incubated in Barth X solution, and other batches of oocytes were incubated as appropriate in Barth X solution containing L-[^{35}S]methionine (1.5 mCi ml^{-1}) or sodium [^{32}P]orthophosphate (10 mCi ml^{-1}). Antibody precipitation and polyacrylamide gel electrophoresis were carried out as previously described (7).

Postnuclear supernatants were prepared from oocytes and lactating guinea-pig mammary gland and centrifuged on linear 20–50% (w/v) sucrose gradients [see (8)], and casein kinase and galactosyltransferase activities were measured as previously described (8).

RESULTS AND DISCUSSION

Translation of postnuclear poly(A)-containing RNA from the lactating guinea-pig mammary gland in the wheat-germ cell-free system leads to the synthesis of four major polypeptides which are antibody precipitable with antisera raised against α-lactalbumin, caseins A, B, and C isolated from guinea-pig milk (see Fig. 1, lane 1). These polypeptides are the primary translation products of the mRNA from guinea-pig lactating mammary gland (11, 12). The addition of dog pancreas microsomes (Fig. 1, lane 2) to the wheat-germ system leads to cotranslational processing and the appearance of four additional peptides sequestered within membrane vesicles, namely the partially processed caseins A′, B′, and C′ and α-lactalbumin [see (4, 12)]. Of these the caseins, although antibody precipitable, do not comigrate with authentic guinea-pig milk proteins isolated from milk. The microinjection of the same poly(A)-

[4] Abbreviation used: SDS, sodium dodecyl sulfate.

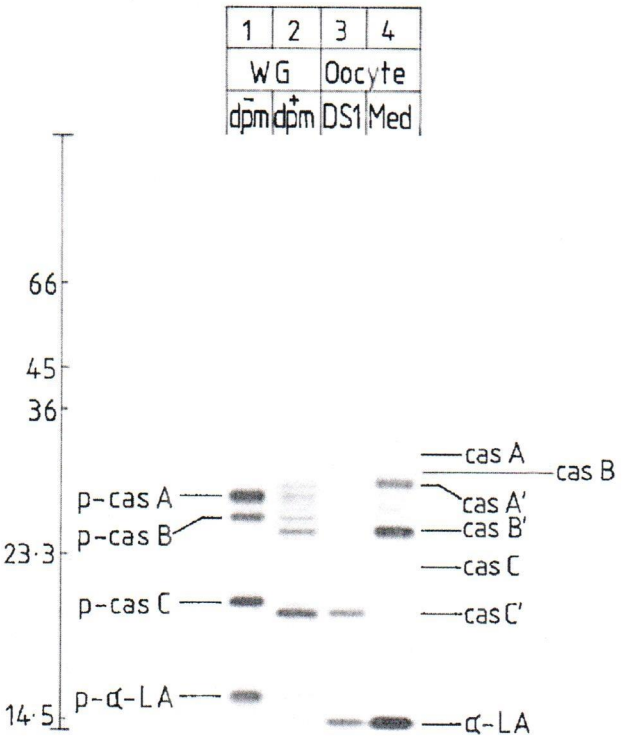

FIG. 1. Comparison by SDS–polyacrylamide gel electrophoresis of the relative electrophoretic mobility of milk proteins synthesized and sequestered in the wheat-germ cell-free system or sequestered and secreted by the oocyte. Procedures were as described under Materials and Methods. [^{35}S]Methionine-labeled antibody-precipitable milk proteins synthesized under the direction of guinea-pig mammary gland postnuclear poly(A)-containing RNA were separated by SDS–polyacrylamide gel electrophoresis and visualized by fluorography. The following heterologous systems were used: Lanes 1 and 2, wheat-germ cell-free system in the absence and presence of dog pancreas microsomes (dpm), respectively, Lanes 3 and 4, the *Xenopus* oocyte showing the milk proteins present in the vesicle fraction (DS1, lane 3) and those secreted into the incubation medium (Med, lane 4). The positions of the primary translation products, the precaseins (p-cas A, p-cas B, and p-cas C) and pre-α-lactabulmin (p-LA), the partially processed caseins (cas A', cas B', and cas C'), and the authentic milk proteins (cas A, cas B, cas C, and α-LA) electrophoresed in parallel and stained with Coomassie blue are shown. The molecular weight markers were bovine serum albumin (Mr 66 × 10^3), ovalbumin (M_r 45 × 10^3), lactate dehydrogenase (M_r 36 × 10^3), trypsin (M_r 23.3 × 10^3), and α-lactalbumin (M_r 14.5 × 10^3).

containing RNA into *Xenopus* oocytes leads to the synthesis and secretion of processed milk proteins with electrophoretic mobilities identical to those synthesized in the microsomal membrane supplemented wheat-germ system (Fig. 1, lanes 2–4). In the oocyte no primary translation products are observed, only caseins A', B', C', and α-lactalbumin. Thus the caseins synthesized and secreted by the oocyte do not comigrate with their processed phosphorylated counterparts isolated from milk which were electrophoresed in parallel and stained with Coomassie blue (see Fig. 1).

Guinea-pig milk caseins are phosphorylated, but α-lactalbumin is not phosphorylated (13). The possibility arises therefore that the oocytes lack the specific casein kinase that is responsible for the phosphorylation of the caseins and that this accounts for the difference between the electrophoretic mobility of the "caseins" secreted from the oocytes and the milk proteins.

To examine this possibility, oocytes were injected with poly(A)-containing RNA

from the lactating mammary gland, or, as a control, with water, before incubation in [^{32}P]orthophosphate-containing medium. The oocytes were disintegrated and subfractionated and the resulting vesicle fraction (DS1) was analyzed by SDS–polyacrylamide gel electrophoresis. The results (Fig. 2A) reveal strikingly similar patterns of phosphoproteins whether mRNA or water had been injected (lanes 2 and 4). Few radiolabeled proteins were found in the incubation medium (Med), and antibody precipitation failed to reveal ^{32}P-labeled proteins having the mobility of phosphorylated caseins, such as those secreted by mammary explants (Fig. 2A, lane 1). As a positive control, oocytes injected with the same batch of poly(A)-containing RNA were incubated in the presence of [^{35}S]methionine and were then processed in parallel. Radiolabeled immunoprecipitable milk proteins of the expected electrophoretic mobility were synthesized (the results are not shown—but Fig. 1 shows a typical electrophoretic pattern).

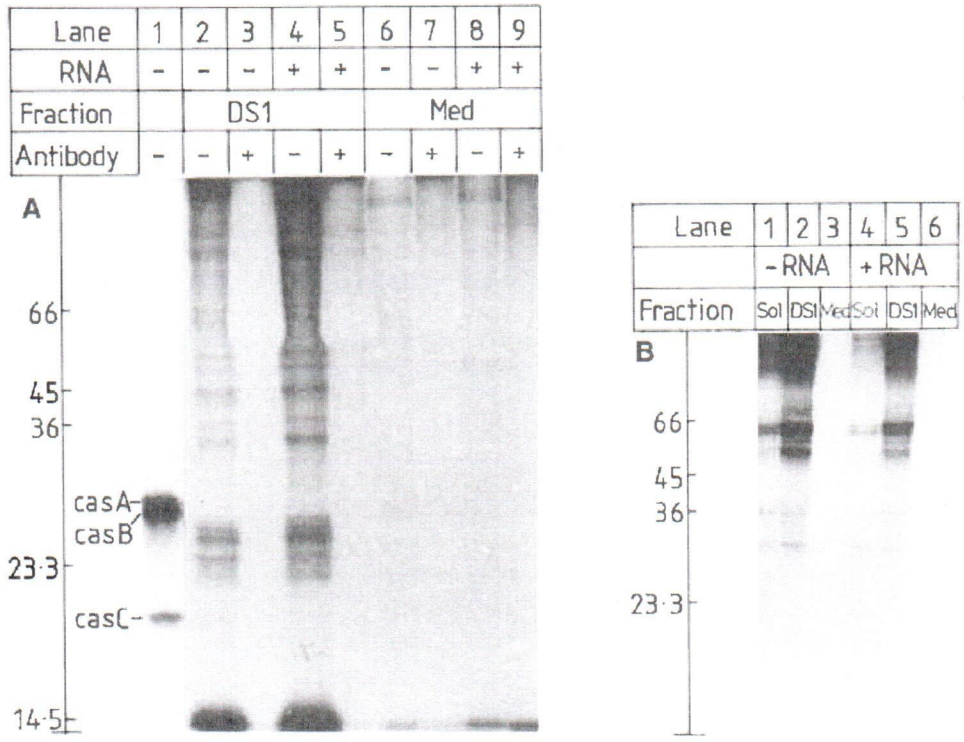

FIG. 2. Synthesis of ^{32}P-labeled proteins in oocytes microinjected with postnuclear poly(A)-containing RNA from the lactating guinea-pig mammary gland. Oocytes were injected with postnuclear poly(A)-containing RNA from the lactating guinea-pig mammary gland as described under Materials and Methods and either (A) incubated in medium containing [^{32}P]orthophosphate or (B) co-injected with [γ-^{32}P]ATP prior to incubation in normal medium. After incubation the cells were separated from the medium (Med) and then disintegrated, and vesicle (DS1) and soluble (Sol) fractions were obtained. Where indicated these were treated with a mixture of antisera raised against guinea-pig caseins A, B, and C, and analyzed by SDS–polyacrylamide gel electrophoresis, and finally visualised by fluorography. In both cases 2.5% of the total protein extract was loaded onto gels, 50% of the total protein extract was treated with antibodies, and half of the resulting precipitate was loaded in parallel. Autoradiographs were exposed for 6–8 weeks. The positions of the mature ^{32}P-labeled caseins secreted by explants from the lactating guinea-pig mammary gland incubated in the presence of [^{32}P]orthophosphate are shown (A, lane 1); the molecular weight markers are as described in Fig. 1.

Our results suggest that caseins synthesized and secreted by the oocyte are not phosphorylated. To investigate the possibility that the apparent lack of phosphorylation might simply reflect slow incorporation of [^{32}P]orthophosphate into ATP pools, oocytes were injected either simultaneously with mammary gland poly(A)-containing RNA and [γ-^{32}P]ATP or with [γ-^{32}P]ATP alone. The cells were then incubated and disintegrated, and the distribution of phosphoproteins in the soluble (Sol) vesicle (DS1) fractions and medium (Med) was examined. Although the distribution of phosphoproteins differed from that observed in the previous experiment (compare Figs. 2A and B), there was no evidence for the synthesis of ^{32}P-labeled caseins.

Although the oocyte is deficient in the phosphorylation of the primary translation product of casein mRNA, oocyte extracts do possess protein kinase activity. In the guinea-pig mammary gland the specific casein kinase is an integral membrane protein probably of Golgi origin which sediments into sucrose gradients on centrifugation of mammary gland postnuclear supernatants (8). We have therefore examined the subcellular distribution of this enzyme as revealed by sedimentation analysis on 20–50% (w/v) sucrose gradients. Assay of each fraction for protein kinase activity and galactosyltransferase activity [a Golgi marker enzyme (14)] revealed that most of the galactosyltransferase activity was well within the gradient (Fig. 3A). In contrast most of the protein kinase activity remained in the soluble fraction, although some of the enzyme did sediment into the gradient. The soluble enzyme activity was sensitive to trypsin digestion, while the membrane-associated enzyme was insensitive (see Fig. 3A). Analysis of an oocyte postnuclear supernatant sedimented on a parallel sucrose gradient showed that the majority ($\geqslant$90%) of the protein kinase activity remained at the top (Fig. 3B). Consistent with its expected intracellular location, the bulk of the oocyte galactosyltransferase activity sedimented well into the gradient, although a small proportion appeared to be soluble. Unfortunately, the presence in the oocyte soluble fraction of endogenous inhibitors prevented testing for membrane-bound kinases by the addition of proteolytic enzymes (8).

The protein kinase assay we have used throughout these experiments has employed casein C as a substrate. A commonly used substrate is histone, so we have compared the specificty of the kinases present in the mammary gland, oocytes, and liver (Table I). The protein kinases present in a postnuclear supernatant from oocytes phosphorylate dephosphorylated guinea-pig casein C *in vitro,* with kinetics similar to those obtained using a histone substrate. A similar result was observed when a guinea-pig liver postnuclear supernatant was used as a source of enzyme. In contrast, the enzyme present in a postnuclear supernatant from lactating mammary gland showed a 50-fold preference for casein C as a substrate as compared to calf thymus histone. These results are consistent with the view that the oocyte lacks an enzyme equivalent to the mammary gland casein kinase.

CONCLUSION

Although the oocyte contains soluble protein kinase activity and a functional secretory pathway, capable of signal peptide removal and glycosylation (3), necessary events in the synthesis of mature caseins, it does not, in contrast to the mammary gland, contain within its secretory apparatus a protein kinase capable of phosphorylating detectable amounts of sequestered caseins. Casein kinase therefore represents

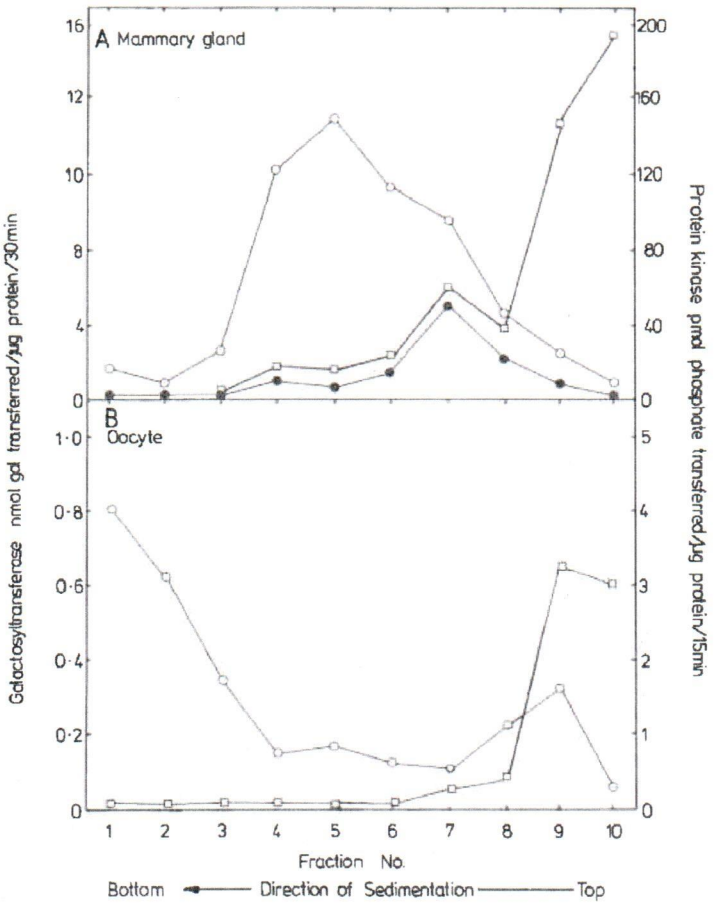

FIG. 3. Distribution of protein kinase and galactosyltransferase activities in postnuclear supernatant fractions from the lactating guinea-pig mammary gland and oocytes as determined by sucrose gradient centrifugation. Postnuclear supernatant fractions were prepared from (A) the lactating mammary gland or (B) oocytes, fractionated on linear 20–50% (w/v) sucrose gradients, and assayed for galactosyltransferase activity (○) or protein kinase (□) activities using guinea-pig casein C as substrate. In the case of the mammary gland (A) additional aliquots (10 μl) were incubated at 0°C for 60 min in the presence of 50 μg ml^{-1} trypsin and assayed for protein kinase (●) following inhibition of trypsin activity by the addition of 150 units of trasylol.

an enzyme, involved in the post-translational modification of secretory proteins, which is not an obligatory component of the secretory pathway. Such an enzyme may be unique to mammary epithelial cells which synthesize caseins. In comparison authentic α-lactalbumin appears in the oocyte, as the only processing event required in its synthesis is signal peptide removal (7).

Our experiments are analogous to those of Rapaport (15), who demonstrated the secretion of carp proinsulin rather than insulin by the oocyte. This inability to process the precursor presumably reflects the absence from the oocyte secretory apparatus of the appropriate proteolytic enzymes present in the pancreatic β-cells (16); the failure to process promelitin provides another example (17). Thus the use of the oocyte as a surrogate secretory system reveals that a number of enzymes within the secretory pathway required for the post-translational modification of

TABLE I

Substrate Specificity of Protein Kinase Activity in Guinea-Pig Tissues and the *Xenopus* Oocyte

	Substrate	
Source of kinase	*Casein C*	*Histone*
Mammary gland	51.5	1.1
Liver	1.2	0.5
Oocytes	4.1	4.0

Note. Protein kinase activity was assayed in postnuclear supernatants from lactating guinea-pig mammary gland, the liver of the lactating guinea-pig, and *Xenopus* oocytes using either guinea-pig casein C or calf thymus histone as the phosphate acceptor. Results are expressed as pmol phosphate transferred/ μg protein/20 min [see (8)].

proteins prior to their secretion may be expressed in a cell-type-specific manner and are not obligatory components of the secretory pathway.

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