Identification and subsequent phosphorylation of sequestered partially processed caseins in the lactating guinea-pig mammary gland

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1. Golgi and endoplasmic-reticulum fractions were prepared from the lactating guinea-pig mammary gland. The endoplasmic-reticulum fraction was highly active in the processing and sequestration of milk-protein primary translation products. 2. Explants from the lactating gland in organ culture were used to identify milk-protein intermediates present in the secretory pathway, and the timing of the events leading to their post-translational modification. With [^{35}S]methionine, the milk proteins labelled after a short pulse (3 min) were represented by the partially processed (but not phosphorylated) caseins and α -lactalbumin sequestered within membrane-bound vesicles. After a 30 min labelling period, higher- M_r caseins with electrophoretic mobilities identical with those of the phosphorylated caseins isolated from milk were identified in the incubation medium, and sequestered within membrane-bound vesicles. Pulse-chase experiments established a precursor-product relationship between these forms. Secretion is apparent approx. 30 min after sequestration. 3. Caseins are highly phosphorylated; removal of the phosphate residues with acid phosphatase results in proteins with increased electrophoretic mobility, similar to those of the partially processed early casein intermediates found sequestered in explants after a 3 min pulse with [^{35}S]methionine, and those sequestered within microsomal membranes after mRNA-directed cell-free protein synthesis. 4. A comparison of the proteins labelled during both short (5 min) and long (30 min) pulses with [^{35}S]methionine and [^{32}P]P_i shows that, in contrast with the ^{35}S -labelled caseins, those labelled with [^{32}P]P_i exhibit only electrophoretic mobilities identical with those of the mature caseins isolated from milk and those identified after long labelling periods with [^{35}S]methionine. No phosphorylated early intermediate forms of caseins were identified. 5. We conclude that the synthesis and post-translational modification of guinea-pig caseins occurs in two stages, (i) an early event involving synthesis and sequestration within the endoplasmic reticulum, an event that involves signal-peptide removal, followed (ii) 10–20 min later by phosphorylation at a different point in the secretory pathway, probably in the Golgi complex. Secretion of the phosphorylated caseins occurs 10–20 min later.

The lactating guinea-pig mammary gland synthesizes and secretes four predominant milk proteins, three caseins (A, B and C) and the whey

Abbreviations used: SDS, sodium dodecyl sulphate; Tes, 2-[[2-hydroxy-1,1-bis(hydroxymethyl)ethyl]amino]-ethanesulphonic acid; Hepes, 4-(2-hydroxyethyl)-1-piperazine-ethanesulphonic acid.

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protein α -lactalbumin (Craig *et al.*, 1976). The caseins are heavily phosphorylated and to a lesser extent glycosylated (Craig *et al.*, 1978), whereas α -lactalbumin remains unmodified (Brew, 1972). The translation of milk-protein mRNA isolated from the lactating guinea-pig mammary gland in heterologous cell-free protein-synthesizing systems (Craig *et al.*, 1976, 1979a,b), or after micro-injection into the *Xenopus* oocyte (Lane *et al.*, 1980), supported by the nucleotide-sequence analysis of cloned milk-protein complementary-DNA

sequences (Hall *et al.*, 1982, 1984), has provided insight into aspects of cellular secretory mechanisms shared by diverse cell types, but has yielded no information which defines the time-course of events, or localization of intracellular post-translational mechanisms within the lactating guinea-pig mammary gland.

One end-point of the studies mentioned above showed that pre- α -lactalbumin was appropriately processed to α -lactalbumin, whether sequestered within microsomal membranes, or sequestered and ultimately secreted by the secretory apparatus of the *Xenopus* oocyte. In contrast, the guinea-pig caseins, whether sequestered within a membrane-bound environment or secreted by oocytes, although processed when compared with the primary translation products, differed significantly in electrophoretic mobility from caseins secreted by the lactating mammary gland (see Zehavi-Willner & Lane, 1977; Colman & Morser, 1979; Lane *et al.*, 1979; Colman *et al.*, 1981; Pascall *et al.*, 1981a).

We have argued that this disparity reflects the inability of heterologous cell-free systems, or *Xenopus* oocytes, to phosphorylate caseins correctly, owing to the absence of a specialized casein kinase. This enzyme we have shown to be an integral component of a Golgi-derived membrane fraction of the lactating guinea-pig mammary gland (Pascall *et al.*, 1981b).

Here we demonstrate, by a combination of mRNA-directed cell-free protein synthesis in the presence of purified mammary-gland microsomal membranes, and pulse-chase experiments using lactating mammary-gland explants labelled in culture with [35 S]methionine or [32 P]P_i, that the partially processed sequestered caseins identified in previous experiments described above represent early sequestered intermediates of the mammary-gland secretory pathway. These are then phosphorylated before secretion. In explants, the phosphorylation process is initiated approx. 10 min after sequestration of the early intermediate forms within the endoplasmic reticulum. This takes place probably in the Golgi and continues over a further 15–20 min period before phosphorylated caseins are found secreted in the medium.

Materials and methods

Materials

All tissue-culture media were obtained from Gibco Biocult. Triton X-100 was from BDH Chemicals, Poole, Dorset, U.K.; dextran (average M_r 250 000) and phenylmethanesulphonyl fluoride were from Sigma (London) Chemical Co., Poole, Dorset, U.K.; proteinase K and potato acid phosphatase were from Boehringer (London), Lewes, E. Sussex, U.K.; L-[35 S]methionine (690–

1300 Ci/mmol) and [32 P]P_i (carrier-free) were from Amersham International, Amersham, Bucks., U.K. All other chemicals and solvents were obtained from sources described by Craig *et al.* (1976). Animals were Duncan Hartley guinea pigs either bred in our Animal House or obtained from A. Tuck and Son, Battlesbridge, Essex, U.K.

Preparation of solutions

Preparation of buffers, scintillation fluid and all recrystallization and distillation procedures were as described previously (Craig *et al.*, 1976; Pascall *et al.*, 1981b). Buffer A was 50 mM-triethanolamine/HCl (pH 7.5 at 4°C) containing 250 mM-KCl and 5 mM-magnesium acetate.

Methods

Preparation of EDTA-stripped lactating guinea-pig mammary-gland endoplasmic reticulum. Lactating guinea pigs 3–6 days *post partum* were killed by cervical dislocation, and the mammary glands were rapidly dissected into cold Buffer A containing 0.15 M-sucrose. All subsequent procedures were carried out at 4°C. The glands were blotted dry, connective and fatty tissue was removed, and the gland was then forced through a 1 mm steel mesh. The minced tissue was then weighed, and homogenized at a concentration of 1 g/2 ml of Buffer A containing 0.15 M-sucrose by four strokes of a glass/Teflon homogenizer (0.38 mm clearance) rotating at 1500 rev./min.

A post-nuclear supernatant was prepared by centrifugation at 850 g_{av} for 10 min in an MSE Minor centrifuge. The supernatant was centrifuged at 20 000 g_{av} for 20 min in the 8 × 50 ml rotor of an MSE 21 centrifuge, and the sedimented material was resuspended in Buffer A containing 2.1 M-sucrose, to a final concentration of 6 ml/g of initial tissue. Portions (10 ml) were placed in the bottom of Beckman SW27 tubes and overlaid successively with 10 ml of 1.9 M-sucrose in Buffer A, 10 ml of 1.3 M-sucrose in Buffer A and 5 ml of 0.5 M-sucrose in Buffer A, and the tubes were centrifuged at 100 000 g_{av} for 5 h in the SW27 rotor of a Beckman L5-65B ultracentrifuge. The material collecting at the 1.3 M-/1.9 M- and 0.5 M-/1.3 M-sucrose interfaces was removed and diluted 1:1 with 0.25 M-sucrose in Buffer A. That from the 1.3 M-/1.9 M-sucrose interface was overlaid on to 5 ml of 1.3 M-sucrose in Buffer A in a Beckman SW40 tube and centrifuged at 190 000 g_{av} for 1 h. The sedimented material was resuspended in 50 mM-triethanolamine/HCl, pH 7.5 at 4°C, and the polyribosomes were removed with EDTA as described by Jackson & Blobel (1977).

Incubation and fractionation of mammary-gland explants. Explants were removed from the lactating mammary gland as described by Burditt *et al.*

(1981) and either incubated directly in Hepes-buffered Eagle's minimal essential medium (MEM) containing [32 P]P_i (20 mCi/ml) or preincubated in methionine-free medium for 45 min before transfer to Hepes-buffered Eagle's MEM containing [35 S]methionine (1 mCi/ml). Explants were then rinsed briefly in Hepes-buffered Eagle's MEM at 0°C, and homogenized in 100 μ l of 50 mM-Tris [titrated to pH 6.75 at 4°C with maleic acid and containing 1 mM-magnesium acetate, 1% (w/v) dextran, 5 mM-2-mercaptoethanol and 0.5 M-sucrose] and centrifuged in 1.5 ml tubes at 850 g_{av} for 10 min in an MSE Minor centrifuge at 4°C. The sedimented material was discarded, and the post-nuclear supernatant retained as a source of membranous material.

Cell-free translations. Poly(A)-containing RNA was isolated from the lactating guinea-pig mammary gland as described by Craig *et al.* (1976, 1979a) and used to direct the synthesis of milk proteins in the wheat-germ system (Craig *et al.*, 1976) or reticulocyte-lysate system (Pelham & Jackson, 1976). When required, dog pancreas microsomal membranes were prepared as described by Katz *et al.* (1977).

Enzyme assays. Casein kinase, galactosyltransferase and glucose-6-phosphatase were assayed as described by Pascall *et al.* (1981b).

Dephosphorylation of caseins. Guinea-pig caseins isolated from guinea-pig milk as described by Craig *et al.* (1978) were dephosphorylated with potato acid phosphatase essentially as described by Bingham *et al.* (1976). Purified total casein (100 μ g) was incubated with 1 unit of the enzyme in a final volume of 0.5 ml in the presence of 1 mM-phenylmethanesulphonyl fluoride and 0.1 M-Tes, pH 7.0, at 37°C for 2 h. The volume was increased to 1.0 ml by the addition of water before dialysis against 1 litre of double-distilled water at 4°C for 16 h. The dephosphorylated caseins were recovered by precipitation with trichloroacetic acid, redissolved in 1.0 M-Tris/HCl, pH 8.0, and dialysed against water as described by Mackinlay *et al.* (1977). With this method the recovery of dephosphorylated caseins was approx. 80%.

Product analysis. SDS/polyacrylamide-gel electrophoresis was performed on slab gels (Craig *et al.*, 1979a) in a Tris/glycine buffer system as described by Maizel (1972). Antibody precipitation procedures were as described by Craig *et al.* (1976), and fluorography followed the procedure of Bonner & Laskey (1974), except that dimethyl sulphoxide was replaced by acetic acid (Burckhardt *et al.*, 1979). Protein M_r markers were bovine serum albumin (M_r 68000), ovalbumin (M_r 43000), lactate dehydrogenase (subunit M_r 36000), trypsin (M_r 23300) and guinea-pig α -lactalbumin (M_r 14500).

Results

Processing of milk-protein primary translation products by lactating-mammary-gland microsomal membranes

A microsomal membrane preparation essentially free of contaminating Golgi membranes was isolated from the post-nuclear supernatant of the lactating guinea-pig mammary gland by equilibrium centrifugation through a discontinuous sucrose gradient. As an estimate of cross-contamination of the organelle preparations, gradient fractions were assayed for glucose-6-phosphatase, galactosyltransferase and casein kinase activities. The results (Fig. 1a) show that 90% of the microsomal membrane preparation, as judged by glucose-6-phosphatase activity, accumulated at the 1.3 M-/1.9 M-sucrose interface, whereas 90% of the Golgi membranes as judged by casein kinase and galactosyltransferase activities accumulated at the 0.5 M-/1.3 M-sucrose interface. Membranous material recovered from each interface was then treated with EDTA to remove associated polyribosomes, and the ability of the resultant membrane preparations to process the primary translation products of guinea-pig casein in a mRNA-directed reticulocyte-lysate cell-free system was then determined, by using the criteria that the electrophoretic mobility of the processed [35 S]methionine-labelled protein translation products differed from those of the primary translation products synthesized in the absence of membranes (see Pascall *et al.*, 1981a).

Although processing activity was absent from the Golgi membrane preparation (J. C. Pascall, unpublished work), the mammary-gland microsomal-membrane preparation proved extremely active (Fig. 1b), as judged by the virtual absence of the primary casein translation products (pre-caseins A, B and C), the presence of the partially processed casein A', casein B' and casein C', and notably the high- M_r variant of casein A', casein hA' (see Pascall *et al.*, 1981a). As a control, a parallel experiment was performed with a microsomal preparation isolated from dog pancreas. Although in this instance processing did not go to completion, it was apparent that the processed caseins were identical as judged by their electrophoretic mobility when compared with those processed by the mammary-gland microsomal preparation. Thus, in common with caseins synthesized and sequestered in a wheat-germ cell-free system containing dog pancreas microsomal membranes (Craig *et al.*, 1979b) or caseins synthesized, sequestered and secreted as a result of microinjection of milk-protein mRNA into *Xenopus* oocytes (see Lane *et al.*, 1980), in the mammary gland, additional post-translational modification

Fig. 1. Processing of the mRNA-directed primary translation products of guinea-pig milk proteins synthesized in the presence or absence of microsomal membrane fractions

(a) A total microsomal fraction from the lactating guinea-pig mammary gland was prepared, and endoplasmic-reticulum (ER) and Golgi-enriched fractions were isolated by flotation on discontinuous sucrose gradients as described in the Materials and methods section. The gradients were fractionated from the bottom via a glass capillary inserted from above, and each fraction was assayed for glucose-6-phosphatase (□), casein kinase (○) and galactosyltransferase (■) activities. The distributions of the membrane fractions (A) and associated enzyme activities (B) on the sucrose gradients (concentrations indicated in both parts) are shown. (b) EDTA-stripped endoplasmic-reticulum preparations were incubated in a reticulocyte lysate in the presence or absence of post-nuclear poly(A)-containing RNA isolated from the lactating guinea-pig mammary gland: (lane 1) poly(A)-containing RNA alone; mammary-gland EDTA-stripped endoplasmic reticulum alone at concentrations of $0.25A_{280}$ unit/ml (lane 2), $0.5A_{280}$ unit/ml (lane 3), $1.0A_{280}$ unit/ml (lane 4); lanes 5, 6 and 7, as lanes 2, 3 and 4, except that poly(A)-containing RNA was present; lane 8, EDTA-stripped dog pancreas microsomal fraction at $1A_{280}$ unit/ml in the presence of poly(A)-containing RNA. In each case samples (5μ l) were analysed by SDS/polyacrylamide-gel electrophoresis as described in the Materials and methods section. The relative positions of the primary translation products (pre-caseins A, B and C), the partially processed caseins (A', B' and C'), the high- M_r variant casein hA' and the M_r -33000 protein ('33K'; see Pascall *et al.*, 1981a), assigned by comparison with M_r markers and authentic milk proteins electrophoresed in parallel and stained with Coomassie Blue, are shown.

events subsequent to sequestration and processing at the level of the endoplasmic reticulum are also required to ensure complete modification of caseins.

Identification of sequestered casein intermediates and fully processed caseins within the secretory pathway of the lactating mammary gland

Explants of lactating guinea-pig mammary gland were incubated for 3 or 30 min in medium containing [³⁵S]methionine. The explants were then homogenized, and nuclei and cell debris were removed by centrifugation. To determine whether newly synthesized caseins and α-lactalbumin were present, and, if so, whether they were sequestered

within a membranous environment, the distribution of radiolabelled proteins in the post-nuclear supernatant was determined by SDS/polyacrylamide-gel electrophoresis either directly, after digestion with proteinase K, or after digestion with proteinase K in the presence of detergent. The results (Fig. 2) revealed after 3 min incubation, a distribution of partially processed caseins A', B' and C' indistinguishable from those sequestered by microsomal membrane preparations. In contrast, and consistent with the observations from the analysis *in vitro* described above, α-lactalbumin appeared to be correctly processed, as judged by comparative electrophoretic mobility with α-lactalbumin isolated from milk. Digestion with

Fig. 2. Sequestration of processed and partially processed guinea-pig milk proteins within a membrane-bound environment in the lactating mammary gland

Explants were incubated in medium containing [³⁵S]methionine for either 3 or 30 min, and a post-nuclear supernatant was prepared from each as described in the Materials and methods section. Samples were incubated at 0°C for 2 h in the presence or absence of proteinase K with or without Triton X-100 before analysis by SDS/polyacrylamide-gel electrophoresis and fluorography. No additions (lanes 1 and 4): incubation with 200 μg of proteinase K/ml (lanes 2 and 5) or 200 μg of proteinase K/ml and 1% (v/v) Triton X-100 (lanes 3 and 6). The electrophoretic mobilities of the radiolabelled milk proteins are compared with those secreted by explants into the medium after a 30 min incubation in the presence of [³⁵S]methionine (lane 7). The identities of the mature milk proteins (caseins A, B, C and hA, and α-lactalbumin) secreted into the medium by explants and the relative positions of the partially processed caseins (A', B' and C'), assigned as described for Fig. 1, are shown.

proteinase K before electrophoresis resulted in no change in electrophoretic profile, whereas digestion with proteinase K in the presence of Triton X-100 resulted in proteolytic degradation of newly synthesized proteins, results consistent with the rapid sequestration of all four milk proteins within a membranous environment.

Analysis of sequestered milk proteins after a 30min incubation revealed a number of differences. Although partially processed caseins A', B' and C' were still present, over 50% of the radiolabelled casein now co-electrophoresed with caseins isolated from milk, indicating that additional modification had occurred at a later point in the secretory pathway. Electrophoretic analysis of [³⁵S]methionine-labelled proteins present in the medium revealed the almost exclusive presence of fully modified caseins which co-electrophoresed with their counterparts isolated from milk. Proteolytic digestion in the presence or absence of Triton X-100 again demonstrated that the processed or partially processed caseins existed in the mammary gland and were sequestered within a membranous environment. However, in contrast, sequestered α -lactalbumin remained resistant to proteolytic digestion even in the presence of deter-

gent, in agreement with results obtained in similar experiments using *Xenopus* oocytes (Zehavi-Willmer & Lane, 1977). Digestion of secreted milk proteins with proteolytic enzymes in the absence of detergent resulted in total degradation of all sequestered proteins, demonstrating their release from the membranous environment on secretion from the mammary gland.

To investigate the relative timing of the later post-translational modification events, explants were incubated for 3min in the presence of [³⁵S]methionine, then transferred to medium containing unlabelled methionine, and the electrophoretic mobilities of the radiolabelled caseins and α -lactalbumin were determined 5, 10, 20 and 30min after the initiation of the pulse. Consistent with our observations reported above, examination of the electrophoretic profile after the initial 3min incubation revealed the expected distribution of partially processed caseins and a trace only of α -lactalbumin (Fig. 3). This profile remained essentially unchanged except for the appearance of the M_r -33000 protein (Pascall *et al.*, 1981a) after a 5min chase period. By 10min some evidence for additional post-translational modification was apparent, with the appearance of

Fig. 3. Kinetics of synthesis and processing of milk proteins by explants from the lactating guinea-pig mammary gland. Explants from the lactating guinea-pig mammary gland were pulsed for 3min in the presence of [³⁵S]methionine, then chased for up to 30min in medium containing 20mM-methionine. Explants were extracted and the relative mobilities of the radiolabelled milk proteins after antibody precipitation were determined by SDS/polyacrylamide-gel electrophoresis as described in the Materials and methods section. The identities of the partially processed caseins (A', B' and C') and the relative positions of the mature milk proteins (caseins A, B and C and α -lactalbumin), assigned as described for Fig. 1, are shown.

[^{35}S]methionine-labelled material with electrophoretic mobilities similar to those of the phosphorylated caseins isolated from milk. After a 20 min chase, the shift in electrophoretic mobility was more marked, especially in the casein-C region, where multiple bands were apparent. After 30 min, except for a small amount of material with the electrophoretic mobility of partially processed casein B', essentially all the [^{35}S]methionine-labelled caseins electrophoresed with the same mobility as caseins A, B and C isolated from milk, an indication that post-translational processing had reached completion.

Phosphorylation of caseins in mammary explants

The experiments described above, though demonstrating the existence of at least two phases separated by time in the post-translational modification of caseins, failed to identify at which point within the secretory pathway phosphorylation occurred, and, moreover, whether the shift in electrophoretic mobility observed indeed reflected phosphorylation of guinea-pig caseins. To examine these questions the following experiments were performed.

Firstly, to determine whether phosphate groups affected the relative electrophoretic mobility of caseins, a total guinea-pig casein preparation was digested with potato acid phosphatase, and the relative distribution of the dephosphorylated caseins was compared with that of (i) the phosphorylated caseins isolated from milk (as judged by staining with Coomassie Blue) and (ii) the newly synthesized [^{35}S]methionine-labelled sequestered casein early intermediates extracted from mammary explants after a 3 min incubation period. This demonstrated (Fig. 4) that the dephosphorylation of caseins increased their electrophoretic mobility, such that the dephosphorylated proteins now co-electrophoresed with the newly synthesized partially processed caseins identified in explants, a result consistent with the view that the sequestered early intermediates of caseins A', B' and C' were not phosphorylated, and that subsequent post-translational events reflected predominantly the phosphorylation of the caseins. In this experiment the effect of dephosphorylation on the electrophoretic mobility of heavy casein A was not determined, since it co-electrophoresed with the bovine serum albumin present in the phosphatase preparation (lane 2). To establish whether in the mammary gland the partially processed caseins A', B' and C' were phosphorylated to any degree, and also if only those caseins with lower electrophoretic mobility owing to the later modification events were phosphorylated, explants were incubated for 5 min or 30 min in medium containing [^{32}P]P_i and then homogenized and fractionated as described in

Fig. 4. Effect of phosphorylation on the relative electrophoretic mobility of guinea-pig caseins

Guinea-pig caseins (100 μg) isolated as described by Craig *et al.* (1978) were taken up in 0.5 ml of 0.1 M-Tes containing 1 mM-phenylmethanesulphonyl fluoride, then dephosphorylated by incubation with 1 unit of potato acid phosphatase at 37° for 2 h. The volume was increased to 1 ml before dialysis for 16 h at 4°C against 1 litre of double-distilled water. The dephosphorylated caseins were recovered by trichloroacetic acid precipitation, redissolved in 1.0 M-Tris/HCl, pH 8.0, and dialysed overnight at 4°C against water. Samples (15 μg) of total guinea-pig caseins were subjected to SDS/polyacrylamide-gel electrophoresis both before (lane 1) and after (lane 2) dephosphorylation, and located by staining with Coomassie Blue. Their mobilities are compared with those (lane 3) of the radiolabelled milk proteins (caseins A', B' and C' and α -lactalbumin) synthesized in explants during a 3 min pulse with [^{35}S]methionine, separated as above and located by fluorography as described in the Materials and methods section. The position of the bovine serum albumin (BSA) present in the phosphatase preparation is also shown.

the Materials and methods section. The distribution of sequestered phosphorylated proteins was then determined by antibody precipitation and SDS/polyacrylamide-gel electrophoresis, and compared directly with caseins synthesized over the same time course in a parallel experiment in the presence of [^{35}S]methionine. The results (Fig. 5) showed, as expected, after a 5 min labelling period

Fig. 5. Comparison by SDS/polyacrylamide-gel electrophoresis of guinea-pig caseins synthesized in explants and radiolabelled with either $[^{32}\text{P}]\text{P}_i$ or $[^{35}\text{S}]\text{methionine}$ during short (5 min) or long (30 min) pulses

Explants from the lactating guinea-pig mammary gland were incubated in Eagle's MEM containing either $[^{32}\text{P}]\text{P}_i$ (20 mCi/ml; tracks 1–4) or $[^{35}\text{S}]\text{methionine}$ (1 mCi/ml; tracks 5–8), and post-nuclear supernatants were prepared as described in the Materials and methods section. Samples from each were incubated at 0°C for 2 h in the presence of 200 μg of proteinase K/ml, with (tracks 2, 4, 6 and 8) or without 1% Triton X-100 as indicated. Phenylmethane-sulphonyl fluoride was added to a final concentration of 1 mM, and the radiolabelled milk proteins were immunoprecipitated as described in the Materials and methods section and analysed by SDS/polyacrylamide-gel electrophoresis. The positions of the mature caseins (A, B and C), the high- M_r form of casein A (hA) and the partially processed form (hA') and the M_r -33000 protein (33 K; see Pascall *et al.*, 1981a), assigned by comparison with markers, are shown. Bands were detected by fluorography with exposure times of 21 days (tracks 1 and 2) and 7 days (tracks 3–8).

with $[^{35}\text{S}]\text{methionine}$, the presence of the three early intermediate major caseins sequestered within a membrane environment, as judged by proteolytic digestion in the presence and absence of detergent. After a 30 min labelling period with $[^{35}\text{S}]\text{methionine}$, the majority of the antibody-precipitable sequestered caseins co-electrophoresed with mature caseins isolated from milk (see also Figs. 2, 3 and 4). In sharp contrast, the electrophoretic mobility of sequestered antibody-precipitable caseins radiolabelled with $[^{32}\text{P}]\text{P}_i$, whether isolated after a 5 min or a 30 min labelling period, was identical with that of phosphorylated caseins A, B and C isolated from milk, or those sequestered within a membrane's environment or

secreted by explants after a 30 min incubation in the presence of $[^{35}\text{S}]\text{methionine}$. Thus, strikingly, no ^{32}P -labelled caseins co-electrophoresed with the $[^{35}\text{S}]\text{methionine}$ -labelled early intermediate forms of caseins A' and C' (electrophoretic resolution of casein A' from mature casein B prevents a similar definitive statement). Since we have previously demonstrated a precursor-product relationship between caseins A', B' and C' and the mature caseins A, B and C isolated from milk, as judged by pulse-chase experiments after radiolabelling with $[^{35}\text{S}]\text{methionine}$ (see Fig. 3), and that dephosphorylation resulted in an increased electrophoretic mobility (see Fig. 4), we must conclude that phosphorylation of guinea-pig caseins occurs

at some point after synthesis and sequestration. Furthermore, on the basis of the [^{35}S]methionine pulse-chase experiment (Fig. 3), it seems likely that no significant phosphorylation occurs until 10–15 min after the synthesis and sequestration of the newly synthesized caseins.

Discussion

Several earlier studies have provided evidence that casein phosphorylation occurs after, not during, casein peptide synthesis, and therefore implied that phosphorylation represented a post-translational modification of a casein precursor protein (Turkington & Topper, 1966; Singh *et al.*, 1967). More recently casein kinase activity has been reported to be associated with Golgi-enriched membrane fractions (Bingham *et al.*, 1972; Bingham & Farrell, 1974), and more specifically, a serine-specific casein kinase has been purified and shown to represent an integral membrane component of a Golgi-enriched membrane fraction isolated from the lactating guinea-pig mammary gland (Pascall *et al.*, 1981*b*). However, such studies did not identify sequestered unphosphorylated casein intermediates *in vivo*, and moreover did not establish the time scale involved between sequestration of the newly synthesized caseins within the lumen of the endoplasmic reticulum and their subsequent phosphorylation.

Our previous studies established that guinea-pig milk proteins, like most secretory proteins, are synthesized with nascent *N*-terminal signal-peptide extensions, which are removed by proteolytic processing during sequestration of the nascent proteins within the lumen of the endoplasmic reticulum. However, whether the individual sequestered processed caseins were synthesized by mRNA-directed heterologous cell-free systems in the presence of microsomal membranes, or sequestered and secreted after micro-injection of mRNA into *Xenopus* oocytes, the electrophoretic mobilities of the sequestered or secreted caseins differed significantly from those of the phosphorylated caseins present in guinea-pig milk, or secreted by guinea-pig explants in culture (see Burditt *et al.*, 1981). The experiments described above demonstrate that this disparity in electrophoretic mobility must reflect the inability of *Xenopus* oocytes or the various heterologous protein-synthesizing systems used to effect post-translational phosphorylation events. More important, the results demonstrate that these partially processed but unphosphorylated caseins represent biosynthetic intermediates sequestered within a membrane-bound environment in the lactating guinea-pig mammary gland.

A combination of experiments involving mRNA-directed cell-free protein synthesis in the presence of purified mammary-gland endoplasmic reticulum essentially free of casein kinase activity, and pulse-chase experiments using explants in culture, have also demonstrated that phosphorylation does not occur at the level of the endoplasmic reticulum, but is a relatively late event within the mammary-gland secretory pathway. It is apparent that phosphorylation of the newly synthesized caseins occurs 10–20 min after sequestration within a membranous environment, and that phosphorylation is rapidly followed by secretion, as judged by the appearance of fully modified caseins in the incubation medium within 30 min of sequestration. Moreover, phosphorylation requires an enzyme seemingly peculiar to the mammary gland, since the guinea-pig caseins when sequestered and secreted as a result of milk-protein mRNA micro-injected into *Xenopus* oocytes remain unphosphorylated, as judged by their electrophoretic mobility. Thus, although many facets of the secretory pathway in higher organisms lack cell type- and species-specificity, as judged by the synthesis and secretion of a wide range of secretory proteins by *Xenopus* oocytes (Lane *et al.*, 1980), it is apparent that, in common with the specialized enzymes required for the processing of proinsulin to insulin (Rapoport, 1981), the mammary-gland casein kinase(s) are not a feature common to the secretory pathway of oocytes, or presumably other tissues and cells, but represent a specialized mammary-specific enzyme(s).

The precise intracellular location within the mammary gland of this phosphorylation event remains to be established, but it is clearly not a post-translational event associated with the endoplasmic reticulum. This conclusion is based on (i) the absence of the casein kinase activity from a mammary-gland endoplasmic-reticulum preparation shown to process and sequester, but not to phosphorylate, caseins when added to a milk-protein-mRNA-directed cell-free system, and (ii) the inability of a Golgi-enriched membrane fraction containing the casein kinase activity to sequester and process the primary casein translation products when incubated under similar conditions. In addition, we have previously demonstrated the ability of the Golgi-associated casein kinase from the lactating guinea-pig mammary gland to phosphorylate caseins sequestered within isolated Golgi-enriched membrane fractions, an observation consistent with the view that the Golgi apparatus, not the endoplasmic reticulum, is the site of phosphorylation of the caseins *in vivo* (Pascall *et al.*, 1981*b*).

Additional evidence that casein phosphorylation does not occur at the level of the endoplasmic

reticulum has also been provided by others using a different approach. This utilized the mRNA-directed synthesis and sequestration within mammary-gland microsomal membranes of sheep β -casein (see Mercier & Gaye, 1980). In this instance the absence of phosphoserine residues from the sequestered β -casein provided the criteria that phosphorylation was not a function of the endoplasmic reticulum and must therefore occur at a later period in the secretory pathway.

In conclusion, we have established: (i) a precursor-product relationship between the casein polypeptides sequestered within different microsomal membrane preparations of guinea-pig endoplasmic reticulum and the phosphorylated caseins secreted by mammary-gland explants and isolated from milk; (ii) that the early casein intermediates are not phosphorylated, and that phosphorylation occurs 10–20 min after sequestration; and (iii) that two separate events, which occur in different organelles, are required for the post-translational modification of caseins, initially proteolytic processing/sequestration at the level of the endoplasmic reticulum, then phosphorylation, probably at the level of the Golgi apparatus, using an apparently mammary-specific casein kinase(s).

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