Hormone-dependent Phosphorylation of Ribosomal and Plasma Membrane Proteins in Mouse Mammary Gland in Vitro*

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SUMMARY

Mouse mammary cells in organ culture incorporate $^{32}$P into the serine and threonine residues of 19 specific plasma membrane proteins and 8 specific ribosomal proteins as resolved by polyacrylamide electrophoresis. These proteins are also phosphorylated in cell-free reactions by tightly bound protein kinases which are endogenous to these organelles and not activated by cyclic adenosine 3',5'-monophosphate (cyclic AMP), and by a purified cytosol protein kinase which is activated by cyclic AMP. The radioactivity patterns of phosphorylated plasma membrane proteins are nearly identical after reactions with the endogenous or cytosol protein kinases or after phosphorylation in intact cultured cells. Certain differences in the radioactivity profiles of $^{32}$P-labeled ribosomal proteins are observed after phosphorylation by the ribosomal or cytosol protein kinases as compared to the $^{32}$P proteins of ribosomes labeled in cultured cells.

Insulin stimulates the rate of phosphorylation of specific plasma membrane and ribosomal proteins in undifferentiated mammary stem cells with maximal stimulation observed after 16 hours of culture. Prolactin synergizes with insulin to stimulate the rate of phosphorylation of all 19 membrane proteins and four ribosomal proteins in cells which differentiate in vitro after treatment with insulin and hydrocortisone. Stimulation of phosphorylation of these specific proteins occurs subsequent to the rapid induction by prolactin of the catalytic and regulatory subunits of the cyclic AMP-dependent protein kinase of the cytosol, as previously described. The results suggest that the phosphorylation of multiple specific proteins in these organelles represents the propagation of the initial prolactin stimulus at the cell surface to various functionally distinct compartments of the mammary epithelial cell.

EXPERIMENTAL PROCEDURE

Chemicals—Adenosine 3',5'-monophosphate, pancreatic DNase, colchicine, O-phosphoserine, and O-phosphothreonine were obtained from Calbiochem. Calf thymus histones, pancreatic RNase (five times crystallized), and crude collagenase were purchased from Worthington. cGMP, cUMP, and cCMP were products of Boehringer Mannheim, and [Y-$^{32}$P]-ATP (31 to 34 Ci per mmole) and inorganic [3'P]phosphate (carrier-free) were from International Chemical and Nuclear Corporation. [8-$^3$H]Adenosine 3',5'-monophosphate (163 Ci per mmole) was obtained from Schwarz BioResearch. Cycloheximide was a product of Nutritional Biochemicals, density gradient grade sucrose (ribonuclease-free) was from Mann, and actinomycin D was obtained from Merck. DEAE-cellulose and calcium phosphate gel, ATP, and bovine serum albumin were purchased from Sigma. Hydroxylapatite (Bio-Gel HT).

Previous studies from our laboratory have characterized the enzymatic properties of two forms of protein kinase (I and II)

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The abbreviations used are: cAMP, adenosine 3',5'-monophosphate; eGMP, guanosine 3',5'-monophosphate; eUMP, uridine 3',5'-monophosphate; eCMP, cytidine 3',5'-monophosphate.
was from Bio-Rad and ultrapure guanidine HCl was a product of Heico.

Isolation of Mammary cAMP-binding Protein—Lactating mouse mammary gland was homogenized (1:8, w/v) in 5 mm sodium glycophosphate-HCl buffer (pH 6.5) containing 0.15 M KCl and 0.2 mM dithiothreitol. The homogenate was centrifuged at 110,000 × g for 60 min, and the sediment was discarded (Step 1). Solid ammonium sulfate was added to the above supernate (31.5 g per 100 ml) with stirring. After 30 min the precipitate was collected by centrifugation at 16,000 × g for 10 min and the supernatant fluid was discarded. The residue was dissolved in 10 mM Tris-HCl buffer (pH 7.5) containing 10% glycerol and 6 mM 2-mercaptoethanol (Buffer A) and dialyzed against 100 volumes of the same buffer with two changes of the buffer. Calcium phosphate gel suspension in Buffer A was added to the above dialyzed protein preparation (50 mg per ml) with stirring. After 15 min the gel was sedimented by centrifugation at 3,000 × g for 5 min and the supernate was discarded. The sedimented gel was washed three times with 50 ml of Buffer A. Protein was finally eluted from the gel by extracting four times with 50 ml of 0.2 M potassium phosphate buffer (pH 8.1) containing 10% glycerol and 6 mM 2-mercaptoethanol. To the combined extracts of the calcium phosphate gel, solid ammonium sulfate was added (31.5 g per 100 ml). After 30 min the suspension was centrifuged at 16,000 × g for 10 min and the supernate was discarded. The pellet was dissolved in Buffer A and dialyzed extensively against the same buffer (Step 2). Solid ammonium HCl was added to the above solution with stirring to make the final concentration of ammonium HCl 6 M. After 72 hours the solution was dialyzed against 30 volumes of the same buffer with six changes of the buffer. The dialyzed turbid solution was centrifuged at 100,000 × g for 20 min and the pellet was discarded (Step 3). The resulting supernate was applied to an hydroxylapatite column (1.5 × 3.5 cm) previously equilibrated with Buffer A. The column was washed with 5 ml of the equilibrating buffer prior to further elution with a linear gradient of potassium phosphate (0 to 400 mM) in a total volume of 250 ml of the buffer. The flow rate was 1.3 ml per hour and the volume in each fraction was 1.5 ml (Step 4). All steps were carried out at 0-4°C.

Isolation of Mammary Cell Membranes—Minced tissue, explants from organ culture, or isolated epithelial cells were stirred in deionized water, the pH was adjusted to 7.0 with sodium bicarbonate (1:8, w/v) for 10 min, and the suspension was centrifuged at 1,000 × g for 10 min. The pellet was homogenized in 0.25 M sucrose containing 0.1 M KCl, 0.01 M Tris-HCl buffer (pH 7.4), and 0.003 M MgCl₂ in a glass homogenizer with eight passes of a loosely fitting glass pestle driven by motor at 60 rpm. The homogenate was filtered through four layers of cheesecloth and then centrifuged at 3,000 × g for 10 min, and the pellet was washed with the same buffer. Cell membranes were isolated from the pellet by discontinuous sucrose density centrifugation (2). The membrane preparation recovered at the interface between layers of 1.08 M and 1.50 M sucrose was diluted with 3 volumes of water and the membranes were finally resedimented by centrifugation at 27,000 × g for 30 min. For the assay of protein kinase activity in membranes, the sediment was suspended in 0.35 M sucrose containing 10 mM sodium glycerophosphate-HCl buffer (pH 6.5) and the suspension was dialyzed against the same buffer.

Isolation of Mammary Ribosomes—Tissues or isolated epithelial cells were homogenized in 0.25 M sucrose containing 0.1 M KCl, 0.01 M Tris-HCl (pH 7.4), and 0.003 M MgCl₂ in an all glass homogenizer, and the homogenate was centrifuged at 17,000 × g for 10 min. The resulting supernate was centrifuged at 110,000 × g for 60 min to sediment the microsomes, and the crude ribosomes were isolated from the microsomal pellet by extraction with sodium deoxycholate (3). For studies involving the assay of endogenous protein kinase activity, the crude ribosomes were further purified by treatments with 0.05 M MgCl₂ and 0.01 M MgCl₂ and by repeated extractions with 0.5 M NH₄Cl (3); they were finally dialyzed against 0.35 M sucrose containing 10 mM sodium glycophosphate-HCl buffer (pH 6.5).

Organ Culture Methods—The abdominal, thoracic, and inguinal mammary glands were removed with aseptic technique from C3H/HeJ mice at the midpoint of their first pregnancy, and explants were prepared and cultured in Medium 199 (Microbiological Associates) as previously described (4, 5). Crystalline bovine insulin (Lilly), hydrocortisone, and ovine prolactin (Endocrinology Study Section, National Institutes of Health) were each present in the medium at a concentration of 5 μg per ml. The culture medium was replenished after each 48-hour period.

Culture of Isolated Mammary Epithelial Cells—Explants prepared fresh or removed from organ cultures were incubated in plastic vials at 37°C for 60 min with collagenase (0.8 mg per ml) prepared in Medium 199 containing 0.1% serum albumin and penicillin and streptomycin (50 units each per ml) (6). The epithelial cells were separated from fat cells by centrifugation at 1000 × g for 10 min at 0-4°C and washed four times with Medium 199. The suspension of the cells in Medium 199 was finally cultured in plastic Petri dishes for specified periods at 37°C.

Assay of cAMP-binding Protein—The activity of cAMP-binding protein was measured by a slight modification of the method described earlier (2). The standard assay medium contained 5 μmoles of Tris-HCl (pH 7.5), 2 μmoles of magnesium acetate, 0.4 μmoles of theophylline, 15 μmoles of [8-3H]cAMP containing 1.6 × 10⁶ cpm, and cAMP-binding protein in a total volume of 0.1 ml. The incubation was carried out at 0°C for 60 min and [PH]cAMP-protein complex was selectively adsorbed on 24-mm cellulose ester (Millipore) filters (pore size, 0.45 μm) (2). The filter was finally counted in a liquid scintillation spectrometer in a toluene-2,5-diphenyloxazole (PPO) (0.4%)-1,4-bis[2-(5-phenyloxazolyl)]benzene (POPOP) (0.005%) scintillation fluid. One unit of the cAMP-binding protein activity was defined as the amount of protein which bound 1 pmole of [8-3H]cAMP during 60 min under the standard assay conditions.

Assay of Protein Kinase—The activity of protein kinase in the purified cell membranes and ribosomes was measured by a slight modification of the method described earlier (2). The endogenous protein of these preparations served as the substrate for the endogenous protein kinase. The standard assay medium contained 10 μmoles of sodium glycophosphate-HCl (pH 6.5), 1 nmole of [γ-32P]ATP containing 2 to 5 × 10⁶ cpm, 2 μmoles of sodium fluoride, 0.4 μmoles of theophylline, 0.06 μmole of ethylene glycol bis(β-aminoethyl ether)-N,N',N'-tetraacetate acid, 2 μmoles of cobalt chloride, and cell membranes or ribosomes with or without 200 pmoles of cAMP in a total volume of 0.2 ml. The incubation was carried out at 30°C for 30 min and the reaction was stopped by the addition of 5 ml of cold 6% trichloroacetic acid. Bovine casein (2.5 mg), nonspecific ATP (1.25 μmoles), and disodium phosphate (10 μmoles)
were then added. After 30 min the suspension was heated at 90° for 20 min to hydrolyze nucleic acids. The precipitate was collected on Whatman GF/C glass fiber discs and washed with 25 ml of 6% trichloroacetic acid. To remove lipid, the discs were washed successively with 25 ml each of ethanol, ethanolether (3:1, v/v), and ether. The filter discs were finally counted in toluene scintillation liquid as described above. A unit of enzyme activity was defined as the amount of enzyme which catalyzed the transfer of 1 pmole of 32P from [γ-32P]ATP to the recovered protein during 30 min under the standard assay conditions. cAMP-independent protein kinase (I) and cAMP-dependent protein kinase (II) were isolated from lactating mouse mammary gland (1). The protein kinase activities of these enzymes and of CAMP-binding protein preparations were evaluated using calf thymus histones (1 mg) as the substrate in the above standard assay medium. After termination of the reaction by the addition of 10% trichloroacetic acid, the precipitate was assayed for radioactivity (2).

**DEAE-Cellulose Chromatography of Protein Kinases—**Isolated mammary protein kinases (1 and II), fresh or pretreated with cAMP, were chromatographed on a column of DEAE-cellulose by a slight modification of the procedure reported earlier (2). Fresh preparations of these enzymes were applied to a DEAE-cellulose column (0.9 × 24 cm) previously equilibrated with 5 mM potassium phosphate buffer (pH 7.0) containing 2 mM EDTA. The column was washed with 18 ml of the equilibrating buffer prior to elution with a linear gradient of potassium phosphate (3 to 400 mM) in a total volume of 300 ml of the buffer. The flow rate was 8 ml per hour and the volume of each fraction was 3 ml. Enzyme preparations, preincubated with 0.5 μM cAMP, were also chromatographed by the above procedure except that all of the buffer solutions also contained 0.5 μM cAMP.

**Sucrose Density Gradient Sedimentation—**Isokinetic sucrose density gradients (ρ = 23.7%) were prepared according to McCarty et al. (7). The sucrose solutions contained 5 mM sodium glycerophosphate-HCl (pH 6.5) and 0.2 mM dithiothreitol. Protein kinase and CAMP-binding protein preparations (0.2 ml), dialyzed previously against the above buffer, were layered over 4.5 ml of the sucrose gradient. After centrifugation for 60 min. The phosphorylation of membrane protein was arrested by the addition of 6 ml of cold 0.35 M sucrose containing 0.035 M K2HPO4, 0.025 M KCl, 0.004 M MgCl2, and 0.02 M K3HPO4 (pH 7.4) (3) and the labeled ribosomes were isolated by centrifugation at 110,000 × g for 60 min.

Ribosomal protein was extracted from the labeled ribosomes with 67% acetic acid in the presence of 0.1 mg magnesium chloride (5), the acetic acid extract of the protein was dialyzed against 50 volumes of 7% acetic acid with two changes of the acid, and the dialyzed was finally lyophilized (9). For the isolation of membrane protein, 32P-labeled membranes were digested at 37° for 60 min with RNase (75 μg) and DNase (70 μg) in 0.1 mg sodium acetate buffer (pH 6.0) containing 0.05 mM magnesium chloride in a total volume of 1.0 ml. The reaction was stopped by the addition of 7 ml of 0.1 mg sodium acetate buffer (pH 6.0) and the membranes were recovered by centrifugation at 27,000 × g for 30 min. The phosphorylation of ribosomal protein was arrested by the addition of 6 ml of cold 0.35 M sucrose containing 0.035 M K2HPO4, 0.025 M KCl, 0.004 M MgCl2, and 0.02 M K3HPO4 (pH 7.4) (3) and the labeled ribosomes were isolated by centrifugation at 110,000 × g for 60 min.

Ribosomal protein was extracted from the labeled ribosomes with 67% acetic acid in the presence of 0.1 mg magnesium chloride (5), the acetic acid extract of the protein was dialyzed against 50 volumes of 7% acetic acid with two changes of the acid, and the dialyzed was finally lyophilized (9). For the isolation of membrane protein, 32P-labeled membranes were digested at 37° for 60 min with RNase (75 μg) and DNase (70 μg) in 0.1 mg sodium acetate buffer (pH 6.0) containing 0.05 mM magnesium chloride in a total volume of 1.0 ml. The reaction was stopped by the addition of 7 ml of 0.1 mg sodium acetate buffer (pH 6.0) and the membranes were recovered by centrifugation at 27,000 × g for 30 min. To the membrane pellet 8 ml of 5% trichloroacetic acid were added, and the suspension was centrifuged at 1,000 × g for 10 min. For the removal of lipid, the precipitate was extracted with ethanol, ethanol-ether (3:1, v/v), and finally with ether.

**Polyacrylamide Gel Electrophoresis of Ribosomal Protein—**32P-Labeled ribosomal proteins prepared by the above procedure were subjected to polyacrylamide gel electrophoresis by a slight modification of the method of Leboy et al. (10). The lower gel (60 × 6 mm) contained 7.5% acrylamide (Eastman) and 0.15% methylenebisacrylamide (Eastman) and the upper gel
(10 × 6 mm) contained 2.5% acrylamide and 0.075% methylenebisacrylamide. The lyophilized 32P-labeled ribosomal protein was suspended in 7 M urea containing 0.1 M 2-mercaptoethanol and the mixture was incubated at 37° for 2 hours (9). The protein in suspension was solubilized by the addition of glacial acetic acid (final concentration, 10%). Both the polyacrylamide gels and protein samples were 6 m in ures, and 150 to 200 μg of protein were applied to each gel for electrophoretic analysis. Electrophoresis was carried out at 4° with a current of 3 ma per gel, and the time of electrophoresis, approximately 3½ hours, was judged by the migration of Pyrnonine Y to the tip of the gel cathode from the upper reservoir. After the electrophoresis, the gels were stained in 1% Amido black in 7% acetic acid for 30 min, destained in 7% acetic acid, and scanned for absorbance at 600 nm in a Gilford model 2400-S spectrophotometer equipped with a Gilford linear transport apparatus. The gels were then sectioned with a manual slicing device (11), and the thickness of each gel section was 1 mm. The gel sections were incubated in 1 ml of Soluteon 100 (Packard) at 60° for 3 hours prior to incubation at room temperature overnight, and the samples were counted in toluene scintillation liquid as described above. The relative electroforetic mobility (Rf) of the protein bands was calculated from the ratio of the distance migrated by the protein band to the distance migrated by the tracking dye.

Polyacrylamide Gel Electrophoresis of Membrane Protein—32P-Labeled membrane protein prepared by the above procedure was subjected to polyacrylamide gel electrophoresis by a modification of the method of Fairbanks et al. (12). The gel (60 × 6 mm) contained 5.6% acrylamide, 0.21% methylenebisacrylamide, 0.035 M Tris-0.017 M sodium acetate-0.0017 M EDTA-acetic acid buffer (pH 7.4), 0.13% potassium persulfate, 6 M urea, 0.5% sodium dodecyl sulfate (Fisher), 0.025% Triton X-100, 0.05% sodium deoxycholate and a minimal amount of N,N',N'-tetrakis(dimethylamino)ethylenediamine. 32P-Labeled membrane protein was suspended in 0.01 M Tris-HCl (pH 8.0) containing 0.04 M diithiothreitol, 0.002 M EDTA, 6 M urea, 0.4% sodium deoxycholate, 0.1% Triton X-100, and 0.2% sodium dodecyl sulfate, and the mixture was incubated at 37° for 2 hours. The suspension was centrifuged at 500 × g for 5 min to sediment the small amount of insoluble material, and the supernate (30 to 40 μg of protein) containing 10% sucrose was used for the electrophoretic separation of proteins. The buffer for both of the reservoirs was 0.04 M Tris-0.02 M sodium acetate-0.002 M EDTA-acetic acid buffer (pH 7.4) containing 1% sodium dodecyl sulfate. Electrophoresis toward the anode was carried out at 30° for approximately 75 min with a current of 8 ma per gel using Pyrnonine Y as the tracking dye. After the electrophoresis, the gels were stained overnight in 0.05% Coomassie blue in 25% isopropyl alcohol containing 10% acetic acid (12). The gels were destained first in 0.0025% Coomassie blue in 10% isopropyl alcohol containing 10% acetic acid for 4 to 5 hours and finally in 10% acetic acid (12). The gels were scanned for absorbance at 550 nm and then sectioned with a manual slicing device for the assay of radioactivity in the gel sections by the procedure described above.

Other Analytical Procedures—32P-Labeled membrane and ribosomal proteins were hydrolyzed in 2 × HCl in a boiling water bath for 16 hours (13). HCl was removed under vacuum, and the hydrolysate was subjected to paper electrophoresis on Whatman No. 1 paper strips (5 × 26 cm) using 8% formic acid as the electrophoresis buffer. Electrophoresis was conducted at 4° for 10 hours at a constant voltage of 400 volts, the current flow being 2.8 to 3.0 ma per filter paper strip. Ninhydrin-staining spots were cut out and counted in toluene scintillation liquid.

The protein concentration of membrane and ribosome preparations and homogenates of the isolated mammary epithelial cells was estimated by the method of Lowry et al. (14). The protein content of the other samples was measured by the micro-biuret method (15). In both methods bovine serum albumin was used as the standard protein.

RESULTS

Properties of cAMP-binding Protein from Mouse Mammary Gland—Mammary cAMP-binding protein was separated partially from the protein kinase activity by hydroxylapatite chromatography. Fractions containing cAMP-binding protein (eluted at 15 to 35 mM potassium phosphate) were pooled together and dialyzed against 5 mM sodium glycerophosphate-HCl buffer (pH 6.5) containing 0.2 mM dithiothreitol. The dialyzed preparation of the cAMP-binding protein was used for studies reported here. Results of a typical purification procedure are shown in Table I. Treatment with 0 μg guanidine HCl selectively removed approximately 94% of the protein kinase activity. The cAMP-binding protein was purified to approximately 200-fold as compared to the whole tissue homogenate and the preparation was approximately 99% free of the initial protein kinase activity per unit of activity of the cAMP-binding protein. The binding protein showed a higher degree of specificity for the binding of cAMP as compared to the binding of other cyclic nucleotides (cGMP, cUMP, and cCMP). Isokinetic sucrose gradient centrifugation showed that cAMP-binding protein sediments as a single peak (2.4 S). cAMP-binding protein markedly inhibited the activity of protein kinase I, and the addition of cAMP completely restored the original activity of the protein kinase. However, the cAMP-binding protein had no significant effect on the activity of

| Fraction | Activity | Specific activity | Purification | Recovery | Activity of cAMP-binding protein/activity of protein kinase × 10⁴ |
|----------|----------|------------------|--------------|----------|-------------------------------------------------------------|
|          | Protein kinase | cAMP-binding protein | Protein kinase | cAMP-binding protein | Protein kinase | cAMP-binding protein | Protein kinase | cAMP-binding protein | Protein kinase | cAMP-binding protein |
| Step 1   | 60,000 | 780 | 430 | 0.55 | 1.0 | 1.0 | 100 | 100 | 1.3 |
| Step 2   | 37,500 | 390 | 127.5 | 1.33 | 2.9 | 2.4 | 62 | 50 | 1.0 |
| Step 3   | 880 | 190 | 85.0 | 19.40 | 1.9 | 35.3 | 1.4 | 25 | 23.0 |
| Step 4   | 127 | 160 | 34.3 | 0.2 | 0.7 | 78.5 | 0.2 | 21 | 120.0 |

Table I

Summary of purification of cAMP-binding protein from lactating mouse mammary gland
cAMP-binding protein was isolated from 20 g of mammary gland. The crude homogenate of the tissue contained 3 g of protein.
cAMP-activated protein kinase II. The other characteristics of CAMP-binding protein were almost identical with those reported earlier (2) using a less purified preparation of CAMP-binding protein.

Fig. 1 shows the DEAE-cellulose chromatography profiles of protein kinase II before and after treatment with 0.5 μM CAMP. Untreated protein kinase II showed the presence of a small amount of protein kinase I as a contaminant. Pretreatment of kinase II with CAMP caused it to be eluted in the form of protein kinase I. Analysis of the dialyzed fractions showed that the protein kinase activity recovered in the peak corresponding to kinase I was not activated by CAMP, and there was no detectable protein kinase peak corresponding to kinase II. There was, however, no change in the DEAE-cellulose chromatography pattern of protein kinase I after preincubation with CAMP.

The product of the interaction of protein kinase I with CAMP-binding protein was analyzed by isokinetic sucrose density gradient centrifugation (Fig. 2). Purified protein kinases I and II sedimented in the sucrose gradient as 2.6 S and 3.9 S peaks. Preincubation of protein kinase I with CAMP-binding protein caused the enzymatic activity to have a sedimentation pattern which was nearly identical with that of native protein kinase II and caused the enzymatic activity to be dependent upon CAMP. Such interaction with protein kinase I caused the 2.4 S CAMP-binding protein to sediment as two peaks (2.4 S and 3.9 S). Treatment of protein kinase II with CAMP-binding protein did not change its sedimentation pattern on the sucrose gradient.

Phosphorylation of Mammary Membrane and Ribosomal Proteins by Protein Kinase in Vitro—Endogenous protein kinase activity of purified mouse mammary cell membranes and ribosomes was measured using the endogenous proteins in each particulate preparation as the substrate for phosphorylation. Both the cell membranes and the ribosomes contained protein kinase activity which was not stimulated significantly by CAMP, and this protein kinase activity was lost completely when the membranes and ribosomes were heated at 100° for 3 min. Treatment of the membranes with cetyltrimethylammonium bromide enhanced the membrane-associated protein kinase activity by approximately 5-fold, whereas no such latent protein kinase activity was detected in the ribosomes. There was no appreciable extraction of the membrane- and ribosome-bound protein kinase activity by treatments with 0.2% Triton X-100, 0.2% sodium deoxycholate, 0.2% cetyltrimethylammonium bromide, 0.1% phosphatidylcholine, 0.9 M potassium chloride, 6 M guanidine HCl, 1.5 M 2-mercaptoethanol, or 5 M urea. Isolated cell membranes (cetyltrimethylammonium bromide treated) and ribosomes contained approximately 1% and 0.4%, respectively, of the total protein kinase activity of lactating mammary tissue when calf thymus histones were used as the substrate for phosphorylation.

Fig. 3 shows the rate of phosphorylation at varying concentrations of membrane and ribosomal protein substrates by exogenous cytosol protein kinase II. Protein kinase II phosphorylated ribosomal proteins at a faster rate (approximately 2-fold greater) than membrane proteins, and the rate of phosphorylation of mammary histones by protein kinase II was approximately six times greater than that of ribosomal proteins. The apparent Kₘ value for membrane protein (1.05 mg per ml) was slightly greater than that of ribosomal protein (0.8 mg per ml) for protein kinase II.

Plasma membrane proteins were phosphorylated with 32P in cell-free reactions with endogenous protein kinase of membrane or with cytosol protein kinase II or by incubating lactating
mammary gland explants on medium containing $^{32}$P orthophosphate. The purified membrane proteins were then fractionated by polyacrylamide gel electrophoresis (Fig. 4). Thirty protein bands were detected in the electrophoretogram of the membrane proteins, and 19 of these bands were labeled with $^{32}$P. Four proteins with $R_F$ values of 0.58, 0.61, 0.65, and 0.68 represent the major $^{32}$P-labeled phosphoproteins. The pattern of phosphorylation of membrane proteins by membrane-bound protein kinase and by cytosol protein kinase II was nearly identical with that obtained with $^{32}$P-proteins derived from plasma membranes of intact mammary cells incubated with $^{32}$Pi.

Fig. 5 shows the electrophoretogram radioactivity profiles of $^{32}$P-labeled ribosomal proteins which were phosphorylated by endogenous protein kinase of purified ribosomes or by reacting ribosomes with cytosol protein kinase II, or by incubating lactational mammary gland with $^{32}$P orthophosphate in organ culture. Eighteen protein bands were detected in the electrophoretogram of ribosomal proteins and at least eight of these proteins are phosphorylated in intact mammary tissue in vitro. Addition of cytosol protein kinase II to suspensions of purified ribosomes augmented the phosphorylation of four ribosomal proteins ($R_F = 0.28, 0.40, 0.45$, and $0.73$) which were also phosphorylated by the endogenous protein kinase activity of the ribosomes. Cytosol protein kinase II also phosphorylated three other ribosomal proteins ($R_F = 0.50, 0.60$, and $0.67$) which were either not phosphorylated or phosphorylated to a small extent by the endogenous protein kinase of ribosomes. The patterns of ribosomal $^{32}$P-protein formed by reacting the endogenous protein kinase or the cytosol protein kinase II with ribosomes were quite different from that obtained from intact lactating mammary tissue cultured in vitro with $^{32}$Pi. Two ribosomal proteins ($R_F = 0.33$ and $0.37$) which were most heavily phosphorylated in the whole lactating tissue were not phosphorylated significantly by the endogenous protein kinase of isolated ribosomes or by the cytosol protein kinase II in cell-free systems. The ribosomal proteins having $R_F$ values of 0.60 and 0.67 were phosphorylated to a small extent in the lactating mammary gland, whereas these proteins were phosphorylated to the greatest degree in purified ribosomes incubated with protein kinase II.

Effect of Hormones on Phosphorylation of Membrane and Ribosomal Proteins in Vitro—The effects of hormones on the rate of phosphorylation of membrane and ribosomal proteins were evaluated by culturing intact midpregnancy mammary explants or isolated mammary epithelial cells in medium containing various hormonal additions. It has been previously shown that the development of mammary gland depends upon the actions of insulin, hydrocortisone, and prolactin (16). Fig. 6 shows the time course of the action of insulin on the incor.
Fig. 6. Time course of the action of insulin on the incorporation of $^{32}$P into membrane protein (circles) and ribosomal protein (triangles) of mammary epithelial cells. Explants were incubated in Medium 199 in the absence of any hormone for 48 hours prior to the transfer to the indicated media for the specified periods. The explants after exposure to $^{32}$P (50 μCi per ml) during the final 4 hours of incubation were digested with collagenase to separate the epithelial cells from the fat cells, and $^{32}$P-labeled membrane and ribosomal proteins were isolated from the epithelial cells. Each point represents the amount of isotopic precursor incorporated during the preceding 4-hour period. O-O, membrane $^{32}$P-protein, control; A-A, ribosomal $^{32}$P-protein, control; Q-Q, membrane $^{32}$P-protein, insulin and hydrocortisone; Δ-Δ, ribosomal $^{32}$P-protein, insulin and hydrocortisone. I, insulin; F, hydrocortisone; NH, nonhormonal control.

Fig. 7. Time course of the action of prolactin on the incorporation of $^{32}$P into membrane protein (O-O) and ribosomal protein (Δ-Δ) of mammary epithelial cells of cultured explants. Tissues previously incubated on medium containing insulin and hydrocortisone for 72 hours were then exposed to the same medium or to the medium containing insulin, hydrocortisone, and prolactin for the specified periods. The explants after exposure to $^{32}$P (45 μCi per ml) were digested with collagenase, and $^{32}$P-labeled membrane and ribosomal proteins were obtained from the isolated epithelial cells. Each point represents the amount of isotopic precursor incorporated during the preceding 4-hour period. O-O, membrane $^{32}$P-protein, insulin and hydrocortisone; Δ-Δ, ribosomal $^{32}$P-protein, insulin and hydrocortisone.

It has been previously shown that prolactin induces specific milk proteins only in alveolar cells formed in vitro and previously treated with insulin and hydrocortisone (16). The period of cell division required to form these cells is essentially complete after 72 hours of incubation (17). The maximal effect of insulin on the phosphorylation of membrane and ribosomal proteins was observed after 18 hours of incubation. The degree of stimulation of membrane and ribosomal protein phosphorylation was slightly lower in explants incubated with insulin and hydrocortisone as compared with those incubated with insulin alone. Results similar to those shown in Fig. 6 were also observed with epithelial cells isolated from the cultured explants.

It has been previously shown that prolactin induces specific milk proteins only in alveolar cells formed in vitro and previously treated with insulin and hydrocortisone (16). The period of cell division required to form these cells is essentially complete after 72 hours of incubation (17). Fig. 7 shows the time course of the action of prolactin on the incorporation of $^{32}$P into membrane and ribosomal proteins of the mammary epithelial cells of cultured explants. The explants were incubated on medium containing insulin and hydrocortisone for 72 hours prior to the addition of prolactin. Prolactin stimulated the rate of incorporation of $^{32}$P into mammary cell membrane and ribosomal proteins by approximately 90%. The kinetics of the prolactin-mediated phosphorylation of membrane protein was faster than that of the ribosomal protein. The stimulatory action of prolactin for the phosphorylation of membrane protein was maximal after 8 hours, and the half-maximal effect was observed after approximately 4 hours. The maximal effect of prolactin on the phosphorylation of ribosomal protein was observed after 16 hours, and the half-maximal effect was noted after approximately 8 hours. Similar results were obtained when epithelial cells, isolated from explants previously incubated first with insulin and hydrocortisone for 72 hours and then with prolactin for the specified periods, were exposed to medium containing $^{32}$P.

Table II shows the effect of insulin and hydrocortisone for the prolactin-mediated stimulation of the phosphorylation of mammary membrane and ribosomal proteins. Prolactin stimulated the rate of incorporation of $^{32}$P into these proteins in cells which were previously treated with hydrocortisone, and prolactin required the continued presence of insulin for its stimulatory action on the phosphorylation of membrane and ribosomal proteins. Results similar to those in Table II were also observed with isolated epithelial cells derived from the $^{32}$P-labeled mammary explants.

In order to distinguish the responses of the daughter cells formed in vitro from those of the epithelial cells which did not divide, colchicine was added to the culture to arrest dividing cells in mitosis. As shown by the results listed in Table III, prolactin did not stimulate the incorporation of $^{32}$P into membrane and ribosomal proteins after colchicine treatment, in-
indicating that prolactin stimulates the phosphorylation of these proteins in cells formed in vitro. Addition of actinomycin D or cycloheximide to the culture along with prolactin prevented the stimulation of phosphorylation of membrane and ribosomal proteins, a result consistent with the conclusion that these prolactin-mediated increases in protein phosphorylation require concomitant synthesis of RNA and protein.

The $^{32}$P-labeled membrane and ribosomal proteins derived from each hormone system were subjected to partial acid hydrolysis, and the phosphorylated amino acids were separated by paper electrophoresis. As shown in Table IV prolactin stimulated the phosphorylation of serine and threonine residues of membrane and ribosomal proteins and the degrees of stimulation were similar to those observed with whole membrane and ribosomal proteins. This result indicates that the hormonal effects relate to the phosphorylation of the polypeptide chains and not to the phosphorylation of some protein-associated molecules. Serine was phosphorylated at more than twice the rate of the threonine residues in both the membrane and ribosomal proteins.

Mammary explants previously incubated first with insulin and hydrocortisone for 0 to 72 hours and then with or without prolactin for 72 to 85 hours were labeled with $^{32}$P in vitro, and the purified cell membrane and ribosomal proteins were subjected to polyacrylamide gel electrophoresis. It is clear from the electrophoretogram radioactivity profiles of $^{32}$P-labeled membrane protein (Fig. 8) that at least 19 proteins of the membrane are phosphorylated when the mammary explants are incubated in insulin and hydrocortisone, and the addition of prolactin caused a general stimulation of the phosphorylation of these proteins of the membrane. Fig. 9 shows the electrophoretogram radioactivity profiles of ribosomal protein labeled

![Fig. 8. Effect of prolactin on the rate of phosphorylation of specific mammary membrane proteins.](#)

**Fig. 8.** Effect of prolactin on the rate of phosphorylation of specific mammary membrane proteins. Explants were incubated on medium containing insulin and hydrocortisone for 72 hours. Prolactin was then added to some dishes and the incubation was continued for 9 hours prior to labeling with $^{32}$P (75 μCi per ml) for 4 hours. $^{32}$P-Labeled membrane protein was isolated from the epithelial cells obtained from $^{32}$P-labeled explants after digestion with collagenase. ○ ○, insulin and hydrocortisone; ●, insulin, hydrocortisone and prolactin.

### Table II

**Effect of various hormones on phosphorylation of mammary membrane and ribosomal proteins**

Midpregnancy mammary explants were exposed to the indicated medium for the specified times. At the end of each incubation period the explants were rinsed and transferred to the new medium. $^{32}$P was present during the period from 81 to 85 hours, and all explants were assayed at 85 hours. $^{32}$P-Labeled membrane and ribosomal proteins were isolated from mammary epithelial cells derived from $^{32}$P-labeled explants by digestion with collagenase. I, insulin; F, hydrocortisone; P, prolactin.

| Hormone system | Membrane $^{32}$P protein | Ribosomal $^{32}$P protein |
|----------------|---------------------------|---------------------------|
| 0 to 72 Hours  | 72 to 85 Hours             | 72 to 85 Hours             |
| IF            | IF                        | IF                        |
| IF            | IFFP                      | IFFP                      |
| IF            | IF                        | IFFP + cycloheximide      |
| IF + cycloheximide | IFFP + actinomycin D |
| IF            | IFFP                      | IFFP + cycloheximide      |

### Table III

**Effect of cycloheximide, actinomycin D, and colchicine on stimulation of phosphorylation of mammary membrane and ribosomal proteins by prolactin**

Midpregnancy mammary explants were exposed to the indicated medium for the specified periods. At the end of each incubation period the explants were rinsed and transferred to the new medium. The explants were exposed to $^{32}$P (40 μCi per ml) during the period 81 to 85 hours and all of the explants were assayed at 85 hours. $^{32}$P-Labeled membrane and ribosomal proteins were isolated from mammary epithelial cells obtained from $^{32}$P-labeled explants by digestion with collagenase. The concentrations of actinomycin D, cycloheximide, and colchicine were 1 μg, 80 μg, and 0.1 μg per ml respectively. I, insulin; F, hydrocortisone; P, prolactin.

| Hormone system | Membrane $^{32}$P protein | Ribosomal $^{32}$P protein |
|----------------|---------------------------|---------------------------|
| 0 to 72 Hours  | 72 to 85 Hours             | 72 to 85 Hours             |
| IF            | IF                        | IF                        |
| IF            | IFFP                      | IFFP                      |
| IF            | IFFP + cycloheximide      | IFFP + actinomycin D      |
| IF + colchicine | IFFP + cycloheximide  | IFFP + actinomycin D      |

### Table IV

**Effect of prolactin on phosphorylation of serine and threonine residues of mammary membrane and ribosomal proteins**

Midpregnancy mammary explants were cultured on the indicated media for the specified periods. During the period 81 to 85 hours, the media contained $^{32}$P (50 μCi per ml). After 85 hours the explants were digested with collagenase to isolate the epithelial cells, and $^{32}$P-labeled membrane and ribosomal proteins were extracted from the isolated epithelial cells and subjected to partial hydrolysis in 2 N HCl. At least 85% of the counts subjected to paper electrophoresis were recovered in phosphoserine and phosphothreonine for each protein fraction derived from each hormone system. I, insulin; F, hydrocortisone; P, prolactin.

| Hormone system | Membrane $^{32}$P protein | Ribosomal $^{32}$P protein |
|----------------|---------------------------|---------------------------|
| 0 to 72 Hours  | 72 to 85 Hours             | 72 to 85 Hours             |
| IF            | IF                        | IFFP                      |
| IF            | IFFP                      | IFFP + cycloheximide      |
| IF            | IFFP + cycloheximide      | IFFP + actinomycin D      |
| IF + colchicine | IFFP + cycloheximide  | IFFP + actinomycin D      |

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with \(^{32}\)P, by mammary explants in vitro. Ribosomal proteins having \(R_F\) values of 0.28, 0.33, 0.37, 0.40, 0.45, 0.50, 0.60, and 0.67 were phosphorylated significantly when mammary epithelial cells in intact explants were cultured with insulin and hydrocortisone. Addition of prolactin stimulated selectively the phosphorylation of ribosomal proteins having \(R_F\) values of 0.28, 0.33, 0.37, and 0.40 without changing appreciably the rate of phosphorylation of the other phosphoproteins of the ribosomes.

To evaluate the possibility of contamination of \(^{32}\)P-labeled ribosomal protein with \(^{32}\)P-casein, which is the major phosphoprotein of the differentiated mammary epithelial cells, \(^{32}\)P-labeled mouse casein was subjected to co-electrophoresis with the nonradioactive mammary ribosomal protein. \(^{32}\)P-Casein resolved into three radioactive peaks (results not shown) which had low electrophoretic mobilities (\(R_F = 0.06, 0.12, 0.18\)), indicating that all of the major \(^{32}\)P-labeled ribosomal phosphoproteins as detected in the electrophoretogram (Figs. 3, 5, 9) are free from any possible casein contamination. \(^{32}\)P-Labelled mouse casein when subjected to co-electrophoresis with nonradioactive membrane protein in the polyacrylamide gel (5.6%), resolved into several radioactive peaks (results not shown) with \(R_F\) values of 0.20, 0.40, 0.48, 0.52, 0.62, and 0.65. To evaluate the degree of possible contamination of \(^{32}\)P-labeled membrane protein with \(^{32}\)P-casein, \(^{32}\)P-labeled mouse casein was added to the midpregnancy mammary explants prior to homogenization of the tissue, and cell membranes were isolated and assayed for \(^{32}\)P-protein. The recovery of \(^{32}\)P-casein in the isolated membrane protein was 0.2%. Estimation of the phosphorylation of casein and membrane protein in mammary explants incubated in different hormone systems disclosed that less than 1% of the observed radioactivity of the membrane protein may be due to contamination with \(^{32}\)P-casein. This conclusion is further strengthened by the marked differences in the kinetics of prolactin-induced phosphorylation of cell membrane protein (Fig. 7) and of casein (18).

**DISCUSSION**

Our previous studies demonstrated the presence of a specific protein in mammary gland cytosol which has high affinity and a high degree of specificity for the binding of cyclic AMP (2). This protein was rapidly and coordinately induced with cyclic AMP-activated protein kinase in cultured mammary epithelial cells stimulated with prolactin. In the present studies the cyclic AMP-binding protein has been partially purified from mammary gland and its functional relationship to the cyclic AMP-activated protein kinase has been further studied. These studies demonstrate that the prolactin-induced, cyclic AMP binding protein is identical with the regulatory subunit of protein kinase demonstrated in a number of other tissues by other investigators (19-22). Although each of these enzyme subunits has different hormonal requirements for its induction (2), a cyclic AMP-binding subunit combines with protein kinase I to form an inactivated complex, and interaction with cyclic AMP releases kinase II, the active catalytic form of the enzyme, from this complex.

It was of interest to observe in these studies that the catalytic subunit of cytosol protein kinase is highly unstable after dissociation from the cyclic AMP-binding regulatory subunit. The catalytic subunit lost 80% of its enzymatic activity during storage at 4°C for 20 hours. After reaction with the partially purified cyclic AMP-binding subunit and storage under identical conditions, no significant loss of activity of the catalytic subunit of protein kinase I could be detected. It is possible that another physiological function of the cyclic AMP-binding protein is to stabilize and protect the catalytic subunit of protein kinase, which may have a high turnover rate in the cell. Purified mammary plasma membranes were observed to selectively and rapidly inactivate the catalytic subunit of protein kinase, although such preparations did not influence the activity of protein kinase II complex. Thus it may be of advantage to the cell to induce coordinately both the catalytic and regulatory subunits of the enzyme, as is observed after hormonal stimulation in mammary cells (2). The mammary protein kinase thus exhibits properties expected of a regulatory protein which mediates acute adaptations to environmental stimuli; it is rapidly induced, is activated by cyclic AMP, and the activated form can be rapidly inactivated.

Purified cell membranes and ribosomes of mammary cells also contain tightly bound protein kinase enzymes which are not activated by cyclic AMP. Latent protein kinase activity associated with the membrane preparations was labilized and activated following treatment of the membranes with cetyltrimethylammonium bromide. The substrate specificities of the cytosol protein kinase and of the latent, organelle-associated protein kinase were compared by the analysis of the phosphorylated amino acid residues and by polyacrylamide gel electrophoresis of the phosphorylated products. All of the enzyme activities showed a high degree of specificity for the phosphorylation of serine and threonine residues, which represented 97% and 3%, respectively, of the phosphorylated amino acids in proteins derived both from the plasma membrane and from ribosomes. The radioactivity profiles of the phosphorylated proteins of the plasma membrane were similar after phosphorylation by either the endogenous protein kinase enzymes or by cytosol protein kinase in cell-free reactions or by intact cellular mechanisms in organ culture (Fig. 4). This result suggests that the membrane-associated protein kinase is similar or identical with the cytosol protein kinase, and is consistent with the concept that the hormonally induced phosphorylation of specific proteins in the plasma membrane may be catalyzed by the cytosol kinase. Slight differences were observed in the phos-
phorylation by the endogenous protein kinase or by the cytosol kinase as compared to the pattern of phosphorylation by intact mammary cells in organ culture (Fig. 5). These differences may relate to differences in relative substrate availability in the intact cells as compared to the broken cell preparations. The phosphorylation of membrane and ribosomal proteins by endogenous protein kinase enzymes and by exogenous cytosol protein kinases has been previously reported (9, 23–26). In the present studies, it has been possible to compare the patterns of protein phosphorylation of membrane and ribosomal proteins by purified enzyme preparations with the patterns of phosphorylation observed in cultured cells responding to specific hormonal signals.

Previous studies demonstrated that prolactin and insulin act synergistically to induce rapidly the cytosol protein kinase in mammary epithelial cells in organ culture (2). The present studies demonstrate that this induction is associated with a marked increase in the rates of phosphorylation of specific membrane and ribosomal proteins in the cultured mammary cells formed in vitro (Fig. 7). Both enzyme induction and the phosphorylation of specific plasma membrane and ribosomal proteins occur selectively in the mammary epithelial cells formed during the period of incubation in vitro as shown by the experiments with colchicine (Table III). The induction of protein kinase required only the actions of insulin and prolactin on the cultured cells (2). A stimulation of the rate of phosphorylation of the membrane and ribosomal proteins required in addition a preliminary period of treatment of the cells with hydrocortisone (Table II). Experiments with inhibitors of RNA and protein synthesis indicated that the prolactin-mediated stimulation of phosphorylation of specific membrane and ribosomal proteins is dependent upon the concomitant synthesis of RNA and protein. It has been previously shown that the specific activity of the ATP pool of these cells does not change appreciably after stimulation with these hormones (13), indicating that the increased rate of incorporation of 32P into specific proteins represents an increase in the net rate of phosphorylation of these proteins rather than a change in the availability of isotopically labeled precursor. Since no turnover of the 32P-labeled protein was detected during the 8-hour chase periods, the net rate of incorporation of isotope into the phosphorylated proteins may be taken to represent the rate of phosphorylation of these substrates.

Studies on the time course of the hormonally induced phosphorylation of membrane and ribosomal proteins (Fig. 7) demonstrated that the maximal rates of phosphorylation were obtained following the induction of maximal levels of cytosol protein kinases (2). This result is consistent with the concept that the protein kinase may be rate-limiting for protein phosphorylation, and that the phosphorylation of these proteins occurs as a consequence of the induction of protein kinase by prolactin. Characterization of the 32P-labeled proteins derived from plasma membrane demonstrated that prolactin causes a general increase in the rate of phosphorylation of all 19 phosphoproteins identified by this technique in the plasma membrane preparations. Previous studies have demonstrated that prolactin causes the formation of new ribosomes and polysomal aggregates, and the kinetics of the prolactin-mediated formation of new polysomes (27) is similar to that of the prolactin-induced phosphorylation of ribosomal proteins. The selective stimulation of the phosphorylation of four ribosomal proteins in response to prolactin may reflect factors relating to substrate availability as well as the concentration of induced protein kinase.

Insulin stimulated the rate of phosphorylation of specific membrane and ribosomal proteins in the undifferentiated mammary stem cells, and the maximal stimulation was observed after 16 hours of culture (Fig. 6). This insulin-mediated phosphorylation of membrane and ribosomal proteins occurred concomitantly with the induction by insulin of the cytosol protein kinase (2) and with the phosphorylation of histones and nuclear acidic proteins (13). These results support the concept that the insulin-stimulated phosphorylation of nuclear, ribosomal, and membrane proteins may be mediated by the induced protein kinase.

The previous studies on the action of prolactin on mammary epithelial cells have indicated that prolactin initially interacts with specific hormone receptors which are part of the superficial cell surface (28, 29). Subsequently, both subunits of the cytosol protein kinase are induced, and this induction represents one of the earliest intracellular actions of prolactin (2). The present studies, together with those previously reported (13), indicate that stimulation by prolactin is associated with the phosphorylation of specific proteins of the nucleus, plasma membrane, and ribosomes of mammary cells. The phosphorylation of a variety of proteins in a number of functionally distinct organelles in various compartments of the cell may thus serve to propagate and amplify the initial stimulus of prolactin at the cell surface. Elucidation of the physiological significance of these phosphorylation reactions in the functional activation of the mammary secretory cell is currently under investigation.

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