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PHOSPHORYLATION OF ZYMOGEN GRANULE MEMBRANE PROTEINS IN INTACT RAT PANCREATIC ACINAR CELLS

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SUMMARY: Phosphorylated substrates of molecular weights 130,000, 70,000, and 29,000, were identified by SDS-gel electrophoresis in zymogen granule membranes of rat pancreatic acinar cells incubated *in vitro* with protein kinase catalytic subunit. However, when intact cells were incubated with [^{32}P]-orthophosphate, only the 29,000 molecular weight protein was phosphorylated.

The existence of specific protein kinase substrates on zymogen granule membranes isolated from pancreatic acinar cells has previously been investigated (1-3). Incubating purified membranes with [γ-^{32}P]-ATP, MacDonald and Ronzio observed the phosphorylation of a single protein with a molecular weight of 130,000 (1) whereas Lambert *et al* (2) reported the presence of 8-10 phosphoproteins ranging in molecular weight from 12,000 to 70,000. In neither case was phosphorylation stimulated by cAMP. However, in the presence of a postmicrosomal supernatant, a known source of cAMP-dependent protein kinase, cAMP increased the incorporation of [^{32}P] into all the granule membrane phosphoproteins (2). Since the *in vitro* conditions used to study phosphoproteins on zymogen granule membrane are not representative of *in vivo* conditions, we have investigated the phosphorylated state of these membrane proteins after labelling intact, isolated pancreatic acinar cells with [^{32}P]-orthophosphate.

In the present communication, we report that under basal conditions, only one major phosphoprotein is labelled. This protein has a molecular weight of 29,000.

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METHODS

Preparation and subfractionation of dispersed acinar cells. Rat pancreatic acinar cells were isolated according to the method of Chauvelot et al (5). Cells were resuspended and homogenized in cold (4°C) 5.0 mM TES buffer (pH 6.5) containing 2.0 mM pyrophosphate (to inhibit phosphatase activity), and 0.31 M sucrose (buffer A). Zymogen granules, zymogen granule membranes and an intragranular fraction were obtained by the method of Heisler and Lambert (6). The zymogen granule membrane suspension was diluted 5-fold with 0.25 M NaBr solution in order to remove any non-specifically adsorbed proteins (7) and membranes pelleted at 180,000 x g (45 min).

A ribosomal fraction was prepared from homogenates of the dispersed acinar cells according to the technique of Tashiro and Siekevitz (8). Protein concentrations were determined by the method of Lowry et al (9).

Phosphorylation of zymogen granule membranes in vitro. Zymogen granule membranes were incubated (final volume 180 µl) at 31°C in 50 mM Hepes (pH 7.0), 10 mM $MgCl_2$, 115 µM EGTA, and 5.0 µg protein kinase catalytic subunit (Sigma Chem., St. Louis, MO; bovine heart). After a preincubation of 1 min at 31°C, 20 µl of 100 µM [γ-^{32}P]-ATP (0.35 µCi/tube) were added and samples incubated for 3 min. Phosphorylation was terminated by the addition of 100 µl of 0.1 M Tris-HCl buffer (pH 7.4), containing 5.0 mM β-mercaptoethanol, 10% (w/v) SDS, 10% (w/v) sucrose and 0.002% (w/v) bromophenol blue. Proteins were then resolved by SDS-polyacrylamide gel electrophoresis.

Incubation of intact acinar cells. Dispersed acinar cells were resuspended (0.5×10^8 cells/ml) in 5.0 mM Hepes buffer (pH 7.4), containing 0.1 mM $CaCl_2$, 6.0 mM glucose, 5.0 mM KCl, 0.15 M NaCl, 0.1% bovine serum albumin and 0.036% soyabean trypsin inhibitor (Sigma) (buffer B). [^{32}P]-Orthophosphate (0.38 mCi) was added and the cells were incubated for 30 min at 37°C in a shaker bath under 95% O_2-5% CO_2. Incubation was stopped by diluting the cell suspension 5-fold with buffer B and centrifuging the suspension at 120 x g (5 min). Pelleted cells were washed twice by centrifugation, resuspended in cold (4°C) buffer A and fractionated as above.

Incubation of subfractions with ribonuclease. Ribosomes and zymogen granule membranes were isolated from acinar cells incubated in the presence of [^{32}P]-orthophosphate as described above. These fractions were then incubated for 20 min (37°C) with or without ribonuclease A (Sigma; beef pancreas: 50 Kunitz units/ml), in 1.0 mM Tris-HCl buffer (pH 7.6) containing 1.0 mM $MgCl_2$, pelleted (180,000 x g; 60 min) and pellets were subsequently resolved by gel electrophoresis.

Gel electrophoresis. Proteins were separated on SDS-polyacrylamide slab gels (9% or 13% acrylamide) according to the method of Laemmli (10) as modified by Rudolph and Krueger (11) and protein bands were visualized by Coomassie Blue stain. Dried gels were exposed to Kodak-X-Omat film and an intensifying screen (Dupont Instruments, Inc., Wilmington, PA) for 3 days at -70°C. The resulting autoradiographs were analyzed using a Clifford densitometer.

RESULTS

Phosphorylation of zymogen granule membrane proteins by the protein kinase catalytic subunit. To establish a basic pattern of phosphorylation of our purified zymogen granule membrane, bovine protein kinase catalytic subunit and [γ-^{32}P]-ATP were added to the membrane preparation. Proteins of 130,000, 70,000, 42,000, and 29,000 molecular weight were phosphorylated (Fig. 1). All

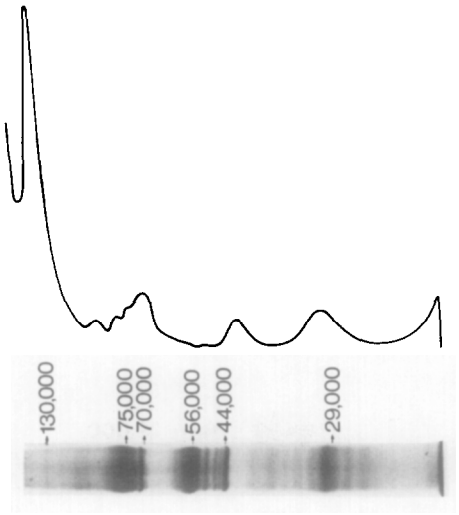

Figure 1 Phosphoproteins of zymogen granule membranes incubated in vitro. Purified zymogen granule membranes were incubated with bovine heart protein kinase catalytic subunit and [^{32}P]-ATP. Proteins (150 µg) were resolved by gel electrophoresis and a densitometric scan of an autoradiograph prepared. Numbers refer to molecular weights of protein standards run under identical conditions. This result is representative of two other experiments.

three substrates, with the exception of the 42,000 molecular weight protein, corresponded to bands on the electrophoretic pattern of zymogen granule membrane proteins stained with Coomassie blue. The 130,000 molecular weight phosphoprotein was by far the most heavily labelled membrane protein. The protein kinase catalytic subunit preparation incubated in the absence of zymogen granule membranes undergoes autophosphorylation (data not shown), and accounts for the peak of radioactivity corresponding to the 42,000 molecular weight substrate.

Zymogen granule protein phosphorylation in intact cells. Dispersed acinar cells were incubated with [^{32}P]-orthophosphate and subsequently zymogen granule membranes isolated. Only one major protein with a 29,000 molecular weight was phosphorylated in intact cells (Fig. 2). This protein corresponded to one of the phosphoproteins labelled by the protein kinase catalytic subunit in the study above. In contrast to granule membrane proteins, proteins contained within zymogen granules were not phosphorylated in intact cells (Fig. 2).

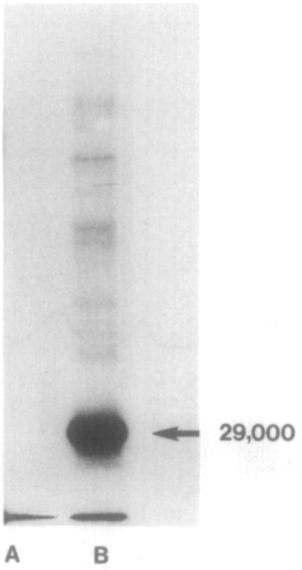

Figure 2 Phosphoproteins of zymogen granule membranes isolated from intact acinar cells. Zymogen granule subfractions were isolated from cells incubated with [^{32}P]-orthophosphate. Equal amounts (50 µg) of intragranular protein (A) and zymogen granule membrane protein (B) were resolved by gel electrophoresis. The position of the 29,000 molecular weight membrane phosphoprotein is indicated on the autoradiograph. This result is representative of two other experiments.

Incubation of ribosomes and zymogen granule membrane with ribonuclease. Freedman and Jamieson (12) have recently reported that the ribosomal protein S6 (29,000 molecular weight) is phosphorylated in rat pancreatic lobules. To determine whether the 29,000 molecular weight protein of zymogen granule membranes was a ribosomal contaminant, ribosomes and zymogen granule membranes were isolated from acinar cells labelled with [^{32}P]-orthophosphate. Both fractions were incubated with ribonuclease. Following this enzymatic treatment the undigested subcellular fractions were pelleted by centrifugation and analyzed by electrophoresis. Treatment with this enzyme should digest ribosomal RNA and would thus disintegrate any ribosomes present in a subcellular fraction.

As expected, ribonuclease treatment dissociated the ribosomes, thereby causing complete disappearance of ribosomal phosphoproteins (i.e. no [^{32}P]-labelled proteins pelleted at 180,000 x g for 60 min) (Fig. 3). On the other hand, autoradiographic analysis of ribonuclease-treated zymogen granule

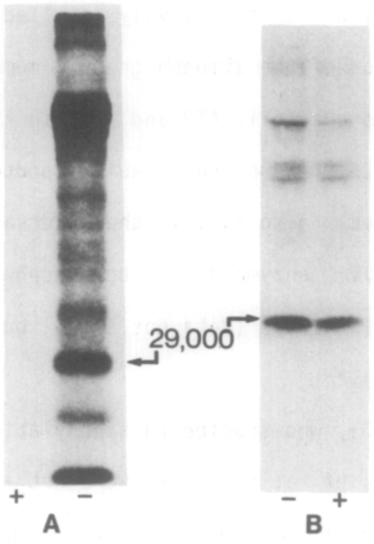

Figure 3 Effect of ribonuclease treatment on ribosomal and zymogen granule phosphoproteins. Ribosomes (A) and zymogen granule membranes (B) were incubated in the presence (+) or absence (-) of ribonuclease A as described in the text. The position of the 29,000 molecular weight phosphoprotein is indicated on the autoradiograph. This result is representative of two other experiments.

membranes showed that incubation with this enzyme had no effect on the presence of phosphoproteins found in the 180,000 x g pellet (Fig. 3).

DISCUSSION

Zymogen granule membranes from rat pancreatic acinar cells contain three proteins of 130,000, 70,000, and 29,000 molecular weight which can serve as *in vitro* substrates for a membrane bound protein kinase (1-3), a cytosolic cAMP-dependent protein kinase (2), or as shown in this study, a bovine heart protein kinase catalytic subunit. However, studies in whole cells under basal conditions indicates that of the three proteins, only the 29,000 molecular weight protein is a major substrate for an intracellular protein kinase. While the discrepancy between *in vitro* and intact cell findings is not known, it is possible that phosphorylation of the 130,000 and 70,000 molecular weight proteins is by a process which may be rapidly modified under different physiological conditions not studied here (e.g. stimulated secretion; granule repletion). Alternately, it is possible that the 130,000 and 70,000 molecular weight pro-

teins, the former of which is so heavily labelled in vitro, are oriented towards the inner surface of the zymogen granule membrane. As a result, they may not be accessible to cytosolic ATP and protein kinase(s) which catalyze phosphorylation of the 29,000 molecular weight protein in the intact acinar cell. This possibility may also explain the observation that several intragranular proteins (digestive enzymes) can be phosphorylated in vitro by the catalytic subunit of protein kinase (data not shown) but are not phosphorylated in the intact cell (Fig. 2).

Lewis and Ronzio (3), who studied phosphorylation of zymogen granule membrane proteins in slices of rat pancreas, were not able to detect the 29,000 molecular weight phosphoprotein, as currently demonstrated. Endogenous phosphatase activity may account for the differences in our findings since without inclusion of a phosphatase inhibitor (2.0 mM pyrophosphate) in our subfractionation medium, the 29,000 molecular weight phosphoprotein could not be visualized.

Since Freedman and Jamieson (12) reported the presence of a similar molecular weight phosphoprotein in ribosomes of pancreatic acinar cells, studies were conducted to show that the 29,000 molecular weight phosphoprotein on zymogen granule membranes was not a ribosomal contaminant. The lack of effect of ribonuclease treatment on the presence of zymogen granule membrane phosphoproteins precludes the possibility of ribosomal contamination in the granule membrane fraction.

Although we have shown that a 29,000 molecular weight zymogen granule membrane protein is phosphorylated in intact acinar cells, its biological function and regulation by cyclic nucleotides and/or calcium, at present, remain unknown.

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REFERENCES

1. MacDonald, R.J., and Ronzio, R.A. (1974) Febs Lett. 40, 203-206.
2. Lambert, M., Camus, J., and Christophe, J. (1974) Febs Lett. 49, 228-232.

3. Lewis, D.S., and Ronzio, R.A. (1979) Biochim. Biophys. Acta 583, 422-433.
4. Jensen, R.T., and Gardner, J.S. (1978) Gastroenterology 75, 806-817.
5. Chauvelot, L., Heisler, S., Huot, J., and Gagnon, D. (1979) Life Sci. 25, 913-920.
6. Heisler, S., and Lambert, H. (1982) Biochemistry of S-Adenosyl Methionine and Related Compounds, pp 71-74, McMillan Press, New York.
7. Ronzio, R.A., Kronquist, K.E., Lewis, D.S., MacDonald, R.J., Mohrlok, S.H., and O'Donnell, J.J., Jr. (1978) Biochim. Biophys. Acta 508, 65-84.
8. Tashiro, Y., and Siekevitz, P. (1965) J. Molec. Biol. 11, 149-165.
9. Lowry, O.H., Rosebrough, N.I., Farr, A.L., and Randall, R.J. (1951) J. Biol. Chem. 193, 265-275.
10. Laemmli, U.K. (1970) Nature (London) 227, 680-685.
11. Rudolph, S.A., and Krueger, B.K. (1979) Cyclic AMP Research, pp. 107-133, Raven Press, New York.
12. Freedman, S.D., and Jamieson, J.D. (1982) J. Cell. Biol. 95, 909-917.