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Phosphoprotein Phosphatase Activity in the Thyroid[1] (39080)

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(Introduced by P. J. Mulrow)

Cyclic-AMP activates cyclic-AMP-dependent protein kinases, which in turn are thought to mediate hormone action by phosphorylating serine and threonine groups of certain proteins with a concomitant change of the activity of these substrates. If the hypothesis is correct, the dephosphorylation of these substrates would serve to modulate the strength and duration of the cyclic-AMP response. This study demonstrates the presence of phosphoprotein phosphatase activity in the thyroid with a preliminary characterization of the enzyme including its activity in several subcellular fractions. The recent demonstration of an apparent hormonal regulation of phosphoprotein phosphatase has provided further interest in this enzyme as a control mechanism (1, 2).

Methods. Thyroid slices were homogenized in four volumes of 1 m*M* DTT (dithiothreitol), 5 m*M* HEPES (*N*-2-hydroxyethyl piperazine, *N*-2 ethane sulfonic acid), and 0.33 *M* sucrose, pH 7.2. After filtering through two layers of cheesecloth, the homogenate was centrifuged at 500*g*, and the supernate successively spun at 5000, 20,000 and 105,000*g*. The 20,000*g* fractionation was performed to better separate the 5000*g* pellet from the 105,000*g* pellet. Each fraction was washed and then resuspended in the initial buffer.

For the chromatographic study, approximately 500 mg of the 105,000*g* supernatant protein was applied to a 2 × 5 cm DEAE-Sephadex column previously equilibrated with 1 m*M* DTT, 5 m*M* HEPES, pH 7.0. Any nonabsorbed protein was removed by washing the column with 100 ml of the same buffer. The column was then eluted with a linear gradient of 250 ml of 5 m*M* HEPES, pH 7.0, 1 m*M* DTT, and 250 ml of the same buffer containing 500 m*M* NaCl.

Substrates for the phosphoprotein phosphatase assay were prepared by adding 1 mg/ml mixed calf thymus histone or 1 mg/ml protamine sulfate to 100 μg of skeletal muscle protein kinase (Peak A from the DEAE step (3) in the presence of 5 μ*M* cyclic-AMP, 10 m*M* NaF, 2 m*M* theophylline, 0.3 m*M* EDTA, and 50 m*M* HEPES, pH 7.0 [γ-^{32}P]ATP was then added (final concentration 8 μ*M*, specific activity about 1 C_i/μmole) and the tubes were incubated at 30°C for 45 min. Each 1-ml reaction was stopped with 0.25 ml of 100% trichloroacetic acid (TCA). The precipitate was recovered by centrifugation, rinsed with water, resuspended and carefully neutralized to redissolve it. The solutions were then reprecipitated and washed twice with 0.25 ml of 25% TCA, dialyzed against distilled water overnight and the 20,000*g* supernatant kept at 4°C (4).

The phosphatase assay was performed in a final volume of 100 μl containing 10–50 μg enzyme protein, 20 to 100 μg of [^{32}P]histone or [^{32}P]protamine (5–10 × 10^4 cpm), 10 m*M* HEPES, 0.1 m*M* DTT, pH 7.0, and incubated at 30°C for 10 min. The reaction was stopped with 400 μl of 25% TCA, then 100 μl of (625 mg%) bovine serum albumin was added and the precipitate removed by centrifugation. To 400 μl of the supernatant, 50 μl of 10 m*M* KH_2PO_4 and then 150 μl of ammonium molybdate (5 gm/100 ml) were added, and the phosphomolybdate was then extracted with 1 ml isobutyl alcohol and counted. Boiled enzyme blanks and zero time incubations revealed no spontaneous dephosphorylation of the substrates. Over a range of enzyme protein concentrations from 100 to 500 μg/ml, phosphatase activity was linear in all fractions for up to 20 minutes at the substrate concentrations employed. Enzyme activity was rather labile, with losses up to 50% in 48 hours when stored at 0°C. Storage at −78°C did not significantly increase enzyme stability.

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TABLE I. PHOSPHOPROTEIN PHOSPHATASE ACTIVITY OF SUBCELLULAR FRACTIONS PREPARED FROM A SINGLE HOMOGENATE

Fraction	Mg protein per gram thyroid	[^{32}P]protamine		[^{32}P]histone	
		Activity[a]	% change with Triton	Activity[a]	% change with Triton
500*g* pellet	5.2 + 0.2	46.6 ± 1.1[b]	130 ± 12[c]	18.7 ± 5.0[b]	95 ± 16[c]
5000*g* pellet	2.2 ± 0.4	39.0 ± 7.4	115 ± 12	24.3 ± 8.9	85 ± 18
105,000*g* pellet	2.0 ± 0.2	13.2 ± 2.8	126 ± 32	6.3 ± 1.3	171 ± 45
105,000*g* supernatant	62.0 ± 12.0	12.6 ± 1.5	101 ± 12	1.9 ± 0.4	117 ± 10
Starting material homogenate	127.0 ± 17.0	15.0 ± 2.7	95 ± 10	4.0 ± 0.8	114 ± 7

[a] Sp act in pmoles P_i released per minute per mg protein.
[b] Mean ± SEM, $n = 4$.
[c] Mean ± SEM, $n = 3$. Percent of basal activity in the presence of 0.1% Triton.

Results. The subcellular distribution of phosphoprotein phosphatase activity with [^{32}P]protamine or [^{32}P]histone as substrate is given in Table I. With [^{32}P]protamine, the highest specific activity was in the 500*g* pellet (46.6 pmoles P_i/mg/min). A slightly different distribution of phosphoprotein phosphatase activity was found in the subcellular fractions when [^{32}P]histone was used as the substrate; the highest specific activity occurred in the 5000*g* fraction (24.3 pmoles P_i/mg/min), although in general, the distribution of phosphatase activity was similar for the two substrates.

To determine whether phosphoprotein phosphatase was membrane bound, subcellular fractions were treated with Triton X-100. Phosphatase activity was increased by the addition of Triton X-100 (0.1% w/v). Although the detergent increased the variability of the phosphatase assay, activity of the 105,000*g* pellet increased by about 70 percent when the substrate was [^{32}P]histone but only by 25 percent with [^{32}P]protamine.

A representative chromatographic pattern of phosphoprotein phosphatase activity in the 105,000*g* supernatant as eluted from DEAE-Sephadex is shown in Fig. 1. There are at least three peaks, the first two having greater activity with [^{32}P]protamine, and the last having relatively more activity with [^{32}P]histone. Phosphoprotein phosphatase activities were assayed with several different divalent cations. Peak III was stimulated only by 2 m*M* manganese, while Peaks I and II were also stimulated by 2 m*M* magnesium.

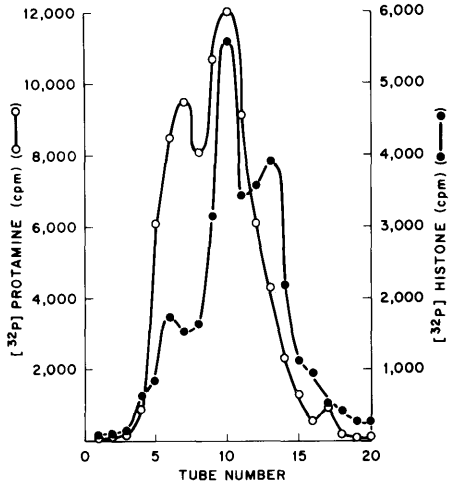

FIG. 1. Chromatographic pattern of phosphoprotein phosphatase activity in the 105,000*g* supernatant from DEAE-Sephadex: cpm ^{32}P released from ^{32}P-labeled protamine are shown as open circles, and ^{32}P released from ^{32}P-labeled histone are shown as closed circles.

Two m*M* cobalt was inhibitory to the activity of all three peaks, as was 15 m*M* NaF.

Discussion. The presence of phosphoprotein phosphatases in the thyroid may provide the means for terminating the action of TSH by removing phosphate from proteins previously phosphorylated by cyclic-AMP-dependent protein kinase. TSH-dependent phosphorylation of both endogenous and exogenous histone has been shown in the thyroid (5, 6).

Although lability of the phosphoprotein phosphatase activity in the thyroid made ex-

tensive characterization difficult, the specific enzyme activities were about one-tenth to one-fiftieth of those found in the brain (4). The addition of 0.1% Triton X-100 resulted in a 71% increase in phosphatase activity with the 105,000*g* pellet, suggesting that membrane-bound enzyme was present.

DEAE-Sephadex chromatography resolved at least three peaks of phosphatase activity in the 105,000*g* supernatant. The first two behaved very similarly, both with respect to substrate specificity and divalent metal activity. The third, however, was clearly different in both respects. All three phosphatases were less active in the presence of 2 m*M* cobalt. This finding of an inhibition of phosphatase activity may be useful when kinase activity is measured concomitantly, since protein kinase activity is stimulated by cobalt (7). The presence of phosphoprotein phosphatase activity with different substrate specificities in different subcellular fractions provides a possible explanation for the different effects which cyclic-AMP mediates within the cell. However, the significance of these findings must await the identification of functionally important endogenous substrates for the cyclic-AMP-dependent protein kinase.

Summary. Phosphoprotein phosphatase activity in the calf thyroid was found in various subcellular fractions. The relative amount in each fraction varied according to the substrate used: The 500*g* fraction had the highest specific activity when protamine was used, while the 5000*g* fraction was highest when histone was used. Triton X-100 tended to increase activity in all the particulate fractions, the greatest change being found in the 105,000*g* pellet. DEAE chromatography of the 105,000*g* supernatant resolved at least three peaks of phosphoprotein phosphatase activity.

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