Phosphoprotein Phosphatase Activity Associated with Estrogen-Induced Protein in Rat Uterus (cyclic AMP/cyclic GMP/hormone/steroids)

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ABSTRACT Estrogen-induced protein was purified from rat uterus and assayed for several enzymatic activities involved in the metabolism and action of cyclic nucleotides. No adenylate and guanylate cyclase (EC 4.6.1.1 and 4.6.1.2, respectively), protein kinase (EC 2.7.1.33), and cyclic nucleotide binding activities could be demonstrated in three independent preparations of the protein. However, all three preparations exhibited significant phosphoprotein phosphatase activity (EC 3.1.3.16) on phosphorylated protamine and histones F1. This activity is optimal at neutral pH, inhibited by Zn++, and unaffected by cyclic AMP or cyclic GMP.

One of the earliest effects of estrogen on rat uterus is the induction within 1 hr after hormone administration, of the synthesis of a specific protein (IP) (1). IP represents a very minor component of uterine soluble proteins that can only be demonstrated on the basis of increased incorporation of labeled amino acids in this protein after estrogen treatment. IP has been purified by ion exchange chromatography and preparative gel electrophoresis as a polypeptide of 45,000 molecular weight (2).

The biological function of this induced protein (IP) is still unknown, although it has been implicated by some authors to play a decisive role in the subsequent increase in RNA and protein synthesis involved in the trophic effects of the hormone on its target tissues (3).

Cyclic AMP and cyclic GMP control several processes related to cell growth in fibroblastic and lymphoid cell lines in vitro (4–7). It is, therefore, interesting to examine whether the same mediators might be implicated in the growth-promoting action of hormones such as steroids on epithelial tissues. Despite earlier results (8), estrogens do not seem to affect cyclic AMP levels in the uterus (9, 10), but treatment with cyclic AMP induces a response that can mimic estrogen effects (11–13). Indeed, the endometrium contains a cyclic AMP-dependent protein kinase (14). Although cyclic AMP does not influence the early synthesis of IP (1), cyclic nucleotides might play a role in subsequent effects of estrogens.

We have investigated whether IP has enzymatic activity related to the regulation of uterine cell functions by the intracellular mediators, cyclic AMP and cyclic GMP. In this study, we report that IP exhibits a significant phosphoprotein phosphatase (EC 3.1.3.16; phosphoprotein phosphohydrolase) activity strongly inhibited by Zn++ and unaffected by either cyclic AMP or cyclic GMP. This phosphatase activity shows great substrate specificity, being much more efficient on phosphorylated protamine and histone F1 than on phosphitin.

MATERIALS AND METHODS

Isolation of Purified IP. IP was prepared following the described general procedure (2) with a slight modification. Immature female rats, 21–24 days old (Wistar), were injected intraperitoneally with 5 µg of 17β-estradiol; 1 hr later, the animals were killed and the excised uteri were incubated for 1 hr with [14C]leucine (100 µCi/ml). Uteri from control rats were incubated with [3H]leucine (500 µCi/ml). At the termination of the incubation period, control and treated uteri were rinsed and combined with unincubated uteri from 40 to 50 mature virgin female rats. After homogenization in 1.5 mM EDTA (pH 7.6), the homogenates were centrifuged at 105,000 × g for 30 min, and the combined cytosol fractions were applied to a DEAE-cellulose column (1 × 20 cm). After the column was washed, fractionation was performed by means of a parabolic gradient to 1.0 M NaCl provided by a 9-chambered gradient device (15). Eluted fractions of a high 14C/3H ratio (indicating the estrogen-induced protein) were pooled and subjected to electrophoresis on cellulose acetate gel, exactly as described by Sømjen et al. (16).

Three independent preparations of IP were used in the present study.

Adenylate and Guanylate Cyclase Assay. Either [α-32P]ATP or [α-32P]GTP was used as substrate for the corresponding cyclase (EC 4.6.1.1 and 4.6.1.2, respectively) assay. The assay and the separation of the 32P-labeled cyclic nucleotides on columns of aluminium oxide were carried out according to White and Zenser (17).

Cyclic Nucleotide Binding Assay. Cyclic AMP and cyclic GMP binding activities of IP were assayed by the method of Gilman (18), except for the omission of the heat-stable inhibitor. The assays were carried out at pH 4.0 and pH 6.0, in a total volume of 100 µl. The incubation mixture contained 50 mM sodium acetate (pH 4.0 or pH 6.0), 0.1 mM of either [3H]cAMP or [3H]cGMP (60,000 cpm/assay), and 0.2–20 µg of IP.

Protein Kinase Assay. The protein kinase (EC 2.7.1.37) activity of IP was determined by measuring the incorporation of 32P into various substrates: lysine-rich histone F1, slightly lysine-rich histone F2a, arginine-rich histone F3, and protamine.

Abbreviations: IP, estrogen-induced protein; NaDodsO4, sodium dodecyl sulfate.
The reaction mixture contained, in a final volume of 120 μl: 50 mM glycerophosphate (pH 6.0), 5 mM 2-mercaptoethanol, 10 mM magnesium acetate, 0.3 mM EDTA, and 10 μM [γ-32P]ATP (1 x 10^6 cpm) with or without either 5 μM cAMP or 5 μM GMP. Substrate concentrations ranged from 30 μg for the histones to 100 μg for protamine. After the addition of IP (0.2-1 μg), incubations were carried out for 10 min at 30°. The reaction was stopped by addition of 2 ml of 10% trichloroacetic acid containing 10 mM sodium phosphate. The protein precipitates were collected on Whatman GFC filters and washed five times with 5% trichloroacetic acid containing 5 mM sodium phosphate.

**Phosphoprotein Phosphatase Assay.** An indirect assay measured the effect of added IP on the rate of phosphorylation of histones by the cAMP-dependent kinase of beef muscle. Direct assays of this enzymatic activity were based on the release of [32P]orthophosphate from phosphorylated substrates, according to Maeno and Greengard (19). A cAMP-dependent kinase from beef muscle was used to phosphorylate the various substrates: histones (F1, F2A, and F3) and protamine. Phosvitin was, on the other hand, phosphorylated by a cAMP-independent protein kinase extracted from *Xenopus laevis* oocytes (20).

The standard incubation medium contained, in a final volume of 0.5 ml: 20 mM glycerophosphate (pH 6.0); 20 mM sodium acetate buffer (pH 6.0); 1 mg of protein substrate (except for histone F1, 0.6 mg); 10 mM magnesium acetate; 0.3 mM EDTA; 5 mM 2-mercaptoethanol; 2 mM caffeine; 10 mM sodium fluoride; 5 μM cAMP when necessary; 80 μM [γ-32P]ATP (24 x 10^6 cpm); and either 50 μg of cAMP-dependent protein kinase (beef muscle) or 90 μg of cAMP-independent protein kinase (Xenopus oocyte).

The specific activities of the phosphorylation reactions were adjusted in order to recover suitable amounts of the various 32P-labeled substrates. The reactions were carried out at 30° for 1 hr and terminated with the addition of 0.5 ml of 50% trichloroacetic acid. The resulting precipitates were centrifuged, washed twice by redissolving in water and reprecipitating in 25% trichloroacetic acid, and finally dialyzed against distilled water for 48 hr.

Phosphorylated histone F1 was prepared by the same procedure and then separated from labeled precursor by extensive dialysis against distilled water at the end of the reaction.

The phosphoprotein phosphatase assays were performed at 30° for 30 min in a total volume of 100 μl containing 0.1 M Tris·HCl buffer at pH 7.2, 0.05-3 μM substrates, and 0.2-20 μg of IP. The reaction was terminated by the addition of 0.1 ml of 0.5% bovine serum albumin and of 0.4 ml of 25% trichloroacetic acid. Orthophosphate was extracted from the deproteinized supernatant by the method of Plaut (21) as modified by Maeno and Greengard (19). To 0.8 ml of isobutyl alcohol extract were added 10 ml of a toluene scintillation mixture containing 0.4% of Omnifluor and 15% of NCS tissue solubilizer.

All substrate concentrations are expressed as phosphate concentrations and were calculated from the specific activity of the precursor [γ-32P]ATP. One unit of phosphoprotein phosphatase activity is defined as 1 pmole of [32P]orthophosphate released under the above assay conditions.

**Materials.** Calf thymus histones: type II A, type V (F1), type VI (F2), type VIII (F3), salmon protamine sulfate, and egg vitellin phosvitin were obtained from Sigma. [γ-32P]ATP and [3H]cAMP were from New England Nuclear Corp; [3H]GMP, [α-32P]ATP, [α-32P]GTP, [3H]leucine, [14C]-leucine, and NCS tissue solubilizer were obtained from Amer sham Radiochemical Centre. DEAE-cellulose (DE 52, microgranular) was purchased from Whatman Biochemicals. Cellogel strips of cellulose acetate were obtained from Chemtron, Milan, Italy. Other reagents were analytical grade.

**RESULTS**

Sizable amounts of IP were obtained by copurifying it from 14C-labeled proteins from estrogen-treated immature animals, 3H-labeled proteins from untreated controls, and unlabeled uteri from mature virgin females. IP synthesis is indeed continuous during the estrous cycle in postpubertal animals (22). Fig. 1 illustrates the purity of IP used in this study. It migrates on NaDodSO4-polyacrylamide gel as an homogeneous polypeptide chain of approximately 45,000 molecular weight. This single band has electrophoretic mobility identical to that obtained with IP purified from estrogen-treated uteri of immature animals (2).

In order to evaluate the possible involvement of IP in cyclic nucleotide metabolism, three independent preparations of the protein were assayed for a number of enzymatic activities related to cAMP metabolism and action.
No adenylate or guanylate cyclase activity was detected. IP did not bind either of the cyclic nucleotides. IP did not catalyze the phosphorylation of either histones or protamine, in the absence or in the presence of either cyclic nucleotide at 5 μM. Furthermore, IP did not serve as a substrate for the cyclic AMP-dependent protein kinase from beef muscle.

Phosphoprotein Phosphatase Activity of IP. The addition of IP slowed the apparent rate of histone phosphorylation by the cAMP-dependent kinase by 10-20%. Direct evidence for the presence of a phosphoprotein phosphatase could be demonstrated by the release of [32P]orthophosphate from various phosphorylated substrates.

When the enzymatic activity is tested during the last steps of purification of IP, it appears that the phosphoprotein phosphatase activity is purified with the peak of IP characterized by a high 14C/3H ratio. Indeed, the material eluted from the DEAE-cellulose column at 0.2 M NaCl comprises four main bands upon analytical polyacrylamide gel electrophoresis. Only one of these bands contains estrogen-induced protein, as evidenced by the high 14C/3H ratio (2). This band, which is further purified as IP by electrophoresis on cellulose acetate strips, contains about 15% of the total proteins in the DEAE-cellulose eluate. Table 1 shows a 6- to 7-fold purification of the histone phosphatase activity, in agreement with the extent of purification of IP achieved during the last step of its preparation.

The DEAE-cellulose eluate also catalyzes, although at a significantly lower rate, the release of inorganic phosphate incorporated into phosvitin by a cAMP-independent kinase from Xenopus laevis oocytes. It is of interest that the phosphatase activity towards this substrate disappears upon further purification of IP.

Properties of the Phosphoprotein Phosphatase Activity of IP. Phosphorylated protamine was the most efficient substrate for the enzymatic activity of IP. Because of the limited amount of available purified protein, the properties of the phosphatase reaction were studied with protamine-phosphate as the substrate. The rate of dephosphorylation was linear with respect to increasing concentrations of IP, in the range of 0.2–8 μg per assay. Optimal reaction rates were observed at neutral pH (Fig. 2). The effect of various ions was not tested because of limitations in the availability of IP. However, as in the case of other phosphoprotein phosphatases (19, 23, 24), the activity was more than 90% inhibited in the presence of Zn++ at 10 mM. This effect of Zn++ was also observed with phosphorylated histones FIIA and phosvitin (Table 2) whether the enzyme preparation used was the DEAE-cellulose eluate or purified IP. The addition of cAMP or cGMP at 5 μM did not affect the rates of dephosphorylation (Table 2) of either phosphorylated histones FIIA or phosvitin by both enzyme preparations.

Table 1. Phosphoprotein phosphatase activity during purification of induced protein

<table>
<thead>
<tr>
<th>Enzyme fraction</th>
<th>Histone FIIA</th>
<th>Phosvitin</th>
</tr>
</thead>
<tbody>
<tr>
<td>Preparation A</td>
<td></td>
<td></td>
</tr>
<tr>
<td>DEAE-cellulose eluate</td>
<td>90</td>
<td>3.7</td>
</tr>
<tr>
<td>Purified IP</td>
<td>630</td>
<td>&lt;1</td>
</tr>
<tr>
<td>Preparation B</td>
<td></td>
<td></td>
</tr>
<tr>
<td>DEAE-cellulose eluate</td>
<td>150</td>
<td>9.4</td>
</tr>
<tr>
<td>Purified IP</td>
<td>990</td>
<td>&lt;1</td>
</tr>
</tbody>
</table>

The purification procedure is described in Materials and Methods. Substrate concentrations, expressed as the concentration of phosphate moiety, were 1 and 3.5 μM, respectively, for the assays carried out with the two independent preparations A and B. Histone FIIA was phosphorylated by the beef muscle cAMP-dependent protein kinase, whereas phosvitin was phosphorylated by the cAMP-independent protein kinase from Xenopus laevis oocytes.

![Fig. 2. pH dependence of protein phosphatase activity of purified IP. The incubation mixture contained 3P-labeled protamine. Substrate concentration as phosphate was 0.04 μM, and the amount of enzyme was 0.4 μg in an assay volume of 0.1 ml. Assays were performed as described in Materials and Methods, with 0.1 M acetate buffer for determinations at pH 5 and 6 and 0.1 M Tris-HCl buffer for assays at pH 7–9.](image-url)

Table 2. Effect of Zn++, cyclic AMP, and cyclic GMP on phosphatase activity of DEAE-cellulose fractions and purified IP

<table>
<thead>
<tr>
<th>Enzyme fraction</th>
<th>Histone FIIA</th>
<th>Phosvitin</th>
</tr>
</thead>
<tbody>
<tr>
<td>DEAE-cellulose eluate</td>
<td>90</td>
<td>3.7</td>
</tr>
<tr>
<td>+ cAMP</td>
<td>630</td>
<td>&lt;1</td>
</tr>
<tr>
<td>+ cAMP</td>
<td>602</td>
<td>&lt;1</td>
</tr>
<tr>
<td>+ cAMP</td>
<td>702</td>
<td>&lt;1</td>
</tr>
<tr>
<td>+ Zn++</td>
<td>24</td>
<td>&lt;1</td>
</tr>
</tbody>
</table>

Substrate concentration was 1 μM as phosphate for both histone and phosvitin. The 32P-labeled proteins were obtained as described in Table 1; cyclic AMP and cyclic GMP were added at a final concentration of 5 μM, Zn++ at a concentration of 10 mM.
Because phosphovitin was a poor substrate for the cAMP-dependent kinase of muscle, we used, for the phosphatase assays, phosphovitin that had been phosphorylated by a cAMP-independent kinase from *Xenopus laevis* ovaries. Table 3 illustrates the relative activities of IP on the various substrates, as compared to protamine phosphorylated by the cAMP-dependent kinase. Interestingly enough, histone F1 phosphorylated by the same kinase was far a much better substrate than histones FIIA and F3. The release of $[^{32}P]$-orthophosphate from phosphovitin was barely detectable, even at substrate concentrations one order of magnitude greater than the concentrations of other substrates assayed.

**DISCUSSION**

Since the first report in 1966 (25) of estrogen-stimulated synthesis of a specific rat uterine protein, numerous studies have been devoted to the mechanism of its induction and its relations to the binding of the hormone by specific receptors (22, 26). Although this induction represents one of the earliest effects of the hormone, both in *vivo* and in *vitro*, the functional significance of IP has attracted much less attention. Such studies have been made difficult by the fact that IP represents a very minor and transient component of uterine soluble proteins.

We decided to study activities implicated in the metabolism and action of cyclic nucleotides for two reasons: first, because of the role of these mediators in the regulation of cell growth in fibroblastic and lymphoid cell lines (4, 7); second, for studying the controversial question of the involvement of cyclic nucleotides in steroid hormone action in general (27) and in estrogen-induced responses in the uterus.

Among various functional activities tested, we reproducibly observed a phosphoprotein phosphatase activity that is enriched upon purification of IP. The observation is interesting because of the important role that this class of enzymes plays in the control of cell functions, for instance, the regulation of glycogen metabolism (23, 28).

A few recent reports have identified phosphoprotein phosphatases in several vertebrate tissues (19, 24, 29) that share some of the properties exhibited by IP. These features include optimal activity at neutral pH, inhibition by Zn$^{2+}$, and faster rates of reaction with protamine and histone F1 as substrates. These characteristics make it unlikely that the activity reported here for IP might reflect a contamination by a common "nonspecific" phosphatase rather than an authentic phosphoprotein phosphatase.

At variance with the enzyme present in the toad bladder (24), the phosphatase activity of IP was unaffected by either cAMP or cGMP. Meisler and Langan also failed to observe a dependence on cAMP of the rat liver histone phosphatase (29).

In the rat brain, cAMP can, however, under certain conditions, activate the release of phosphate from a membrane-bound substrate protein (24). It is not impossible that these effectors might regulate the activity of IP by a cyclic-nucleotide-dependent phosphorylation. However, as far as one can conclude from our experiments with the beef muscle cAMP-dependent kinase, IP does not appear to be a substrate for this class of phosphorylating enzymes. Alternatively, such a phosphorylation of IP might have occurred *in vivo* prior to its isolation.

Although the nature of the real substrate for IP in the uterus remains to be investigated, our studies indicate faster rates of reaction with phosphorylated protamine and histone F1 than with phosphovitin. Whether this difference reflects the nonmammalian origin of the ovarian kinase used to phosphorylate the latter substrate or a preference of IP for substrates phosphorylated by cAMP-dependent kinases, is an intriguing question to be answered. On the other hand, it is difficult to accept the observed rate of release of $[^{32}P]$-orthophosphate from phosphovitin as the true rate of reaction because IP probably also attacks unlabeled phosphate residues in this already extensively phosphorylated protein (30). Nevertheless, it is of interest that the incorporated $[^{32}P]$-residues in phosphovitin could serve as substrates for the DEAE-enzyme preparation and that this activity was lost upon further purification of IP. Whatever its significance, this fact strengthens our conclusion that IP represents an authentic and specific phosphoprotein phosphatase.

One must await the identification of the physiological substrate of IP in the uterus before speculating on the possible implications of our findings in the control by estrogens of cell division (31), gene expression (32, 33), and glycogen metabolism (34) in the uterus.


**TABLE 3. Phosphatase activity of induced protein towards various phosphorylated substrates**

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Concentration (μM)</th>
<th>Relative activity (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Protamine</td>
<td>0.2</td>
<td>100</td>
</tr>
<tr>
<td>Histone F1</td>
<td>0.2</td>
<td>28</td>
</tr>
<tr>
<td>Histone F II</td>
<td>0.2</td>
<td>11.6</td>
</tr>
<tr>
<td>Protamine</td>
<td>0.08</td>
<td>100</td>
</tr>
<tr>
<td>Histone F II</td>
<td>0.08</td>
<td>6.6</td>
</tr>
<tr>
<td>Histone F3</td>
<td>0.08</td>
<td>3.6</td>
</tr>
<tr>
<td>Phosvitin</td>
<td>1</td>
<td>0.2</td>
</tr>
<tr>
<td>Phosvitin</td>
<td>3.5</td>
<td>0.2</td>
</tr>
</tbody>
</table>

Experiments were performed with purified IP at a concentration ranging from 0.5 to 20 μg. $[^{32}P]$-Labeled substrates were all obtained with beef muscle protein kinase, except for phosvitin for which cAMP-dependent protein kinase of *Xenopus laevis* oocytes was used.

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