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A membrane-bound human placental protein kinase activated by endogenous polypeptides

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A protein kinase (PPdPK) was purified from plasma membranes of human placenta. Phosphorylation of casein, but not of phosvitin or lactalbumin, by [γ-^{32}P]ATP in the presence of PPdPK was stimulated about 10-fold by naturally occurring polypeptides prepared from a variety of sources similar to the procedure of Roberts et al. (Proc. Natl. Acad. Sci. U.S.A. **77**, 3494-3498, 1980). The amino acid phosphorylated on casein was serine. According to gel exclusion chromatography the mol.wt. of PPdPK was 95 000. In autoradiograms, following polyacrylamide-gel electrophoresis, the autophosphorylation of PPdPK was greatly enhanced by the polypeptide activators.

Several membranous protein kinases have recently been described that are associated with receptors and that are activated by specific polypeptide hormones or growth factors. Epidermal growth factor (EGF) (1), transforming growth factor (2), insulin (3), and platelet-derived growth factor (4,5) stimulate phosphorylation of membranous proteins. In all these examples a tyrosine residue of a protein becomes phosphorylated.

We have observed that addition of a crude preparation of polypeptides (6) to an extract of plasma membranes of human placenta stimulated the phosphorylation of [γ-^{32}P]ATP of several protein bands seen in autoradiograms. In this paper we describe a placental protein kinase (PPdPK) that was extracted from plasma membranes of human placenta with low concentrations (0.3%) of Triton X-100 and purified by hydroxylapatite and gel exclusion chromatography. The polypeptide which stimulated the phosphorylation of this protein kinase could not be substituted by cAMP, insulin, EGF, and other activators of protein kinase activity.

Materials and Methods

Materials

Hepes, Triton X-100, cAMP, phosphoserine, phosphothreonine, DPCC-treated trypsin, soybean trypsin inhibitor, insulin, lysozyme, transferrin, phosphorylase *b*, and crude casein were obtained from Sigma; phosphotyrosine was prepared by Mark Spector; carbonic anhydrase from Worthington; fibroblast growth factor from Collaborative Research; monothioglycerol from Evans Cosmetics, Inc.; and [γ-^{32}P]ATP from Amersham. Samples of purified caseins were generous gifts from Dr. E. W. Bingham and Dr. M. L. Groves. Spherogel-TSK 3000 columns (7.5 x 300 mm) and an Altex C-18 h.p.l.c. column (10 x 250 mm) were obtained from Beckman. EGF was prepared as described elsewhere (7).

Partial purification of polypeptide activators from placenta and hypothalamus

Term human placenta or bovine hypothalamus (Pel Freeze) was extracted by using an acid-ethanol procedure as described by Roberts et al. (6). Briefly, placenta or hypothalamus (200-300 g) was thawed in 4 ml per g of tissue of a solution made of 375 ml of 95% ethanol, 7.5 ml of concentrated HCl, 33 mg of phenylmethylsulfonyl fluoride, and 1.9 mg of pepstatin. The volume was adjusted with glass-distilled water to give 6 ml per g of tissue and the mixture was homogenized in a Waring blender for 30 s. After 18 h extraction at 4°C in a gyrotory shaker and centrifugation at 10 800 g for 1 h, the supernatant was adjusted to pH 5.2 with concentrated ammonium hydroxide. One ml of 2 M ammonium acetate buffer (pH 5.3) was added per 85 ml of extract (final pH of about 6.1). Cold absolute ethanol (1.7 vol.) and cold anhydrous ether (3.3 vol.) were added to the mixture, which was allowed to stand at -20°C for 48 h. The precipitate was collected by filtration through fluted filter paper (Whatman 2^V) and dissolved in 1 M acetic acid (2 ml per g of placenta). After centrifugation at 100 000 g for 30 min the acetic-acid-insoluble precipitate was discarded and the supernatant was lyophilized and stored in liquid nitrogen. The powder was dissolved in 20 mM Hepes buffer (pH 7.4) at 10 mg of protein per ml and centrifuged at 100 000 g for 20 min. The supernatant contained 5-7 mg of protein per ml and was further purified by reverse-phase high-performance liquid chromatography (h.p.l.c.). An aliquot (0.4 ml) was injected into an Altex C-18 column connected to a Beckman series 341 P liquid chromatograph and a 160 absorbance detector (214 nm). Fractions were eluted at 3 ml per min with 30% acetonitrile containing 0.1% trifluoroacetic acid for 10 min, followed by a linear gradient reaching 70% acetonitrile containing 0.1% trifluoroacetic acid in 30 min. Each fraction was evaporated in a Savant Speedvac Concentrator, washed twice with 0.2 ml of H_2O, and dissolved in 0.1 ml of glass-distilled water. The activity was measured in the protein kinase assay described below in the presence of excess PPdPK (1 µg from the peak of the TSK column).

Partial purification of PPdPK from human placental membranes

Frozen, normal-term human placenta was obtained from the local hospital and kept at -70°C until used for the preparation of either plasma membranes or placental polypeptide activator (PA). Plasma membranes were prepared as described by Hock and Hollenberg (8), resuspended in 20 mM Hepes buffer (pH 7.4) at 10-15 mg per ml, and immediately frozen at -70°C. For solubilization, the plasma membranes (625 mg) were resuspended in 20 mM Hepes buffer (pH 7.5) containing 0.3% Triton X-100 and 10% glycerol at a final protein concentration of 8 mg per ml. After 20 min at 0° the mixture was centrifuged for 1 h at 100 000 g at 4°C. The supernatant contained protein kinase activity which showed only slight stimulation on addition of PA but in autoradiograms a marked stimulation of the autophosphorylation of PPdPK by PA was seen similar to that shown in Fig. 2. The supernatant (250 mg) was loaded on a hydroxylapatite column (2.5 cm x 11 cm) previously equilibrated at 4°C with 20 mM Hepes buffer (pH 7.4) containing 10% glycerol. Fractions were eluted with a linear gradient (0-0.2 M) of potassium phosphate in the presence of the Hepes-glycerol buffer. An aliquot of each fraction was assayed for protein kinase with casein in the presence and absence of PA. Fractions containing PA-dependent protein kinase activity were combined. Aliquots of about 1 mg of protein were injected into two Spherogel-TSK columns linked in series that were equilibrated at room temperature with a buffer containing 50 mM KP_i (pH 7.1), 100 mM NaCl, and 0.05% sodium azide. The flow rate was 0.75 ml per min at an output pressure of the pump of 400-450 p.s.i. The fractions (0.75 ml) were collected into tubes containing glycerol (10% final concentration), and aliquots (10 µl) of each fraction were assayed with and without casein in the absence and presence of HA (the polypeptide activator prepared from bovine hypothalamus) as shown in Fig. 1. Estimations of molecular weights were based on calibration with the following standards: lysozyme (14 500), carbonix anhydrase (29 000), transferrin (72 000), and phosphorylase *b* (195 000).

Protein kinase assay

The reaction mixture contained 20 mM Hepes (pH 7.4), 35 mM $MgCl_2$, 10 mM monothioglycerol, 10 µM [γ-^{32}P]ATP (2 x 10^6 c.p.m. per incubation), enzyme (concentration as indicated in table and figure legends), in a final vol. of 50 µl. Crude PA (5-10 µg) and casein (50 µg) were added where indicated unless stated otherwise in the legends. The reaction was initiated by addition of [γ-^{32}P]ATP and incubated at room temperature for 1 h. The reaction was terminated by pipetting 10- or 20-µl aliquots of the reaction mixture onto 1.8- x 1.8-cm Whatman 3 MM filter papers which were immediately dropped into ice-cold trichloroacetic acid containing 10 mM pyrophosphate and washed as described elsewhere (9).

Other assays

Proteins were determined according to Lowry et al. (10) or Bradford (11). Sodium-dodecyl-sulfate/polyacrylamide-gel electrophoresis (SDS-PAGE) and Coomassie blue staining (12), autoradiography (9), and phosphoamino analysis (13) were performed as described in the references.

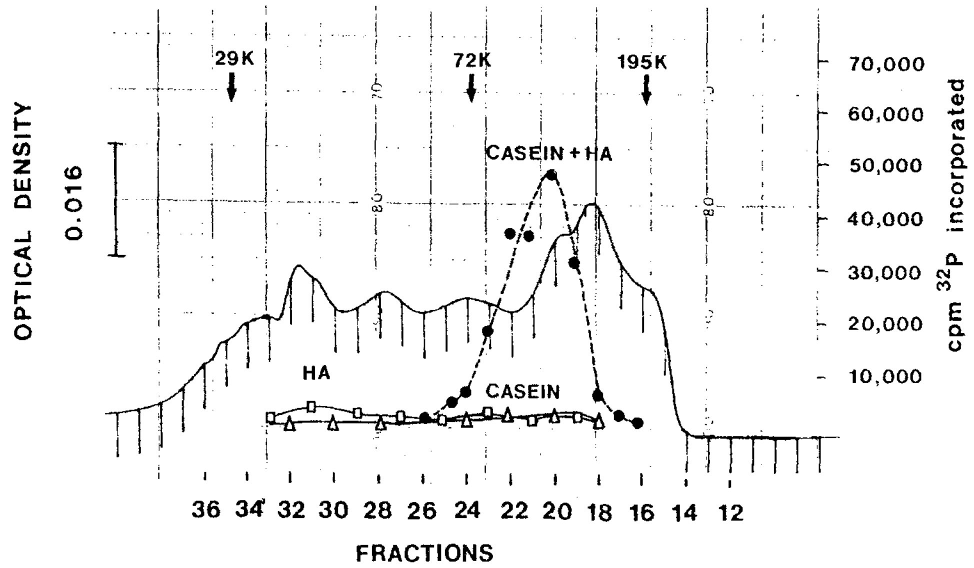

Fig. 1. Fractionation of PPdPK by gel exclusion chromatography. PPdPK obtained after hydroxylapatite chromatography was passed through TSK 3000 columns, and fractions (0.75 ml) were collected as described under 'Materials and Methods'. Optical density (0.16 full scale) was recorded at 280 nm. The positions of protein standards of mol.wt. of 195 000, 72 000, and 29 000 are indicated by arrows. Protein kinase activity in the presence of casein and HA (●) peaked at 95 K with a specific activity of 1300 pmol of ^{32}P incorporated into casein x min^{-1} x mg $protein^{-1}$. Activity with HA alone was negligible.

Results and Discussion

Purification of PPdPK

The enzyme was extracted from plasma membranes of human placenta with 0.3% Triton X-100. Fractionation on hydroxylapatite and gel exclusion columns as described under 'Materials and Methods' yielded an active preparation corresponding to a mol.wt. of about 95 000 (see Fig. 1). Since the assay of crude extracts was not quantitative because of the presence of alkaline phosphatase which hydrolyzed ATP as well as phosphoproteins, an exact estimation of the fold purification cannot be given. When the most active fraction obtained from the TSK column was analyzed by SDS-PAGE autoradiography (Fig. 2), a phosphorylated band at 95 000 dalton was barely detectable but was greatly enhanced on addition of PA or HA during assay. Neither PA nor HA alone yielded a phosphorylated band. Casein (see lower band) at low concentration (0.2 µg) was not visibly phosphorylated by PPdPK unless activator was added.

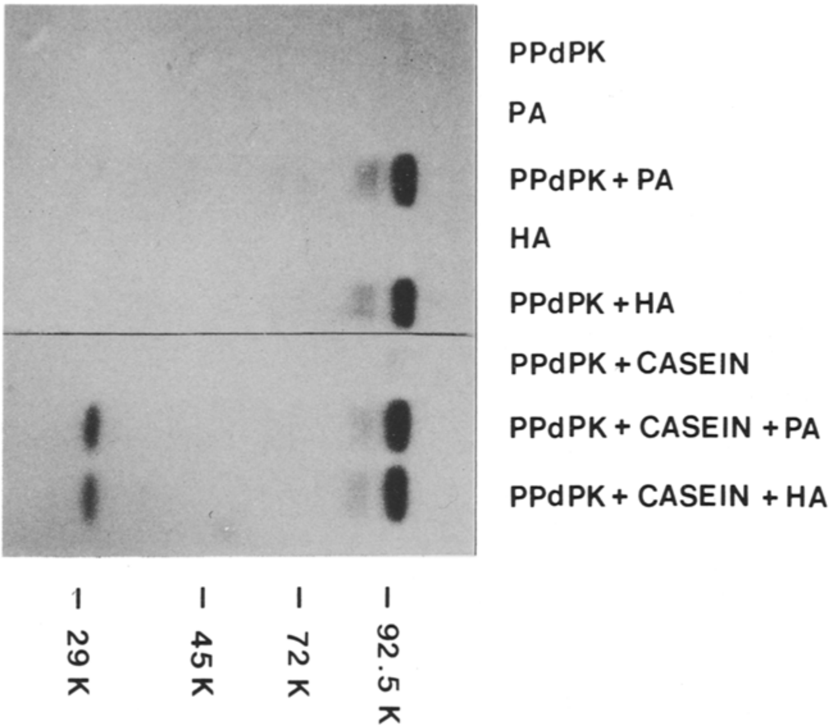

Fig. 2. SDS-PAGE and autoradiography of PPdPK. PPdPK (1.2 μg) obtained from peak fraction of the TSK column (Fig. 1) was incubated for 1 h at room temperature with and without crude PA (12 μg) or HA (12 μg) and casein βA-3 (0.2 μg) under the conditions of protein kinase assay and then analyzed by SDS-PAGE autoradiography as described under 'Materials and Methods'.

Lane 1 PPdPK alone
Lane 2 PA alone
Lane 3 PPdPK + PA
Lane 4 HA alone
Lane 5 PPdPK + HA
Lane 6 PPdPK + casein
Lane 7 PPdPK + casein + PA
Lane 8 PPdPK + casein + HA

Properties of PPdPK

The activity of PPdPK was completely dependent on addition of Mg^{2+} and was linear for 60 min (Fig. 3). Mg^{2+} at 35 mM was optimal, and neither Mn^{2+} nor Ca^{2+} substituted for Mg^{2+}. Phosphorylation by PPdPK with PA or HA alone was very low (autophosphorylation), but with casein alone in the absence of PA variable phosphorylation was observed. PA alone was completely inactive. Phosphorylation was substantially stimulated when PPdPK was supplemented with both PA (C-18) (the polypeptide activator isolated on the C-18 h.p.l.c. column) and casein.

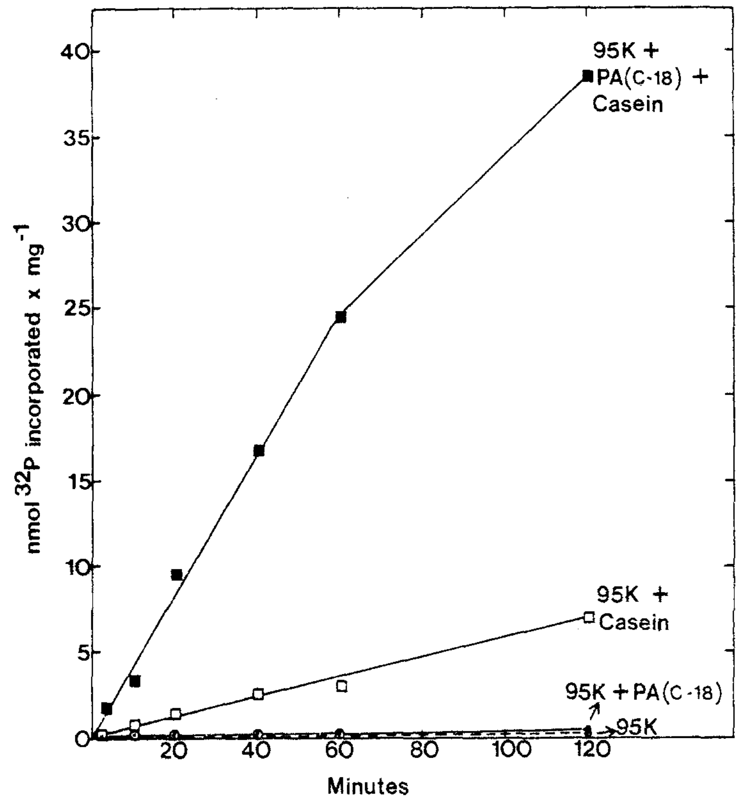

Fig. 3. Protein phosphorylation catalyzed by purified PPdPK. The reaction was performed with casein (50 μg), PPdPK (1 μg of protein from the 95-K peak of TSK column), and PA (1.3 μg of h.p.l.c.-C-18-purified polypeptide) as indicated in the figure. Quantitation of incorporation of ^{32}P into proteins was carried out as described under 'Materials and Methods'.

As shown in Fig. 4, serine was the amino acid on casein phosphorylated by PPdPK. Table 1 shows that among commonly used substrates for protein kinase, β casein was by far the best.

Several activators of protein kinases, including cAMP, Ca^{2+}, EGF, insulin, calmodulin, and phospholipids, did not substitute for PA as an activator of PPdPK. However, polypeptides prepared (6) from other sources, including kidney, brain, salivary glands, and lung, did. When assayed as PPdPK activator PA was found to be non-dialyzable, heat-stable, and trypsin-sensitive, properties shared with the transforming growth factors (6). Indeed, fractions of PA obtained from h.p.l.c. C-18 columns eluted at about 40% acetonitrile concentration had the highest specific activity as protein kinase activator and also contained transforming-growth-factor activity in the soft agar assay. However, conclusion with respect to a possible relationship between the growth factors and protein kinase activators will require purification to homogeneity.

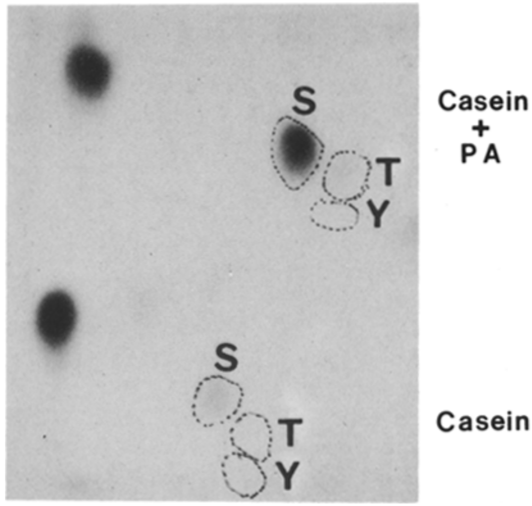

Fig. 4. Phosphoamino acid analysis of casein phosphorylation with and without PA. The phosphorylation reaction was carried out with PPdPK (0.55 µg) and casein (50 µg) in the absence and in the presence of PA (0.24 µg of h.p.l.c.-C-18-purified protein) as described under 'Materials and Methods'. Phosphoamino acid analysis was performed as described elsewhere (13). The dark spot on the left is $^{32}P_i$. S = phosphoserine; T = phosphothreonine; Y = phosphotyrosine.

Table 1. Substrate specificity of PPdPK

The assay was performed with 1.8 µg of PPdPK (after hydroxylapatite purification) as described under 'Materials and Methods' in the presence of 50 µg of various casein samples. The other protein substrates were tested at the indicated concentrations.

Substrate added	Protein kinase activity pmol x min^{-1} x mg protein $^{-1}$ - PA	+PA
None	12	37
Casein (Sigma)	28	232
βA-2	73	270
βA-3	51	155
βB	47	214
αS,A	64	122
αS,B	37	61
Casein K	62	55
Phosvitin (20 µg)	22	41
(50 µg)	23	45
(100 µg)	22	35
α-Lactalbumin (20 µg)	20	25
(40 µg)	26	46
(100 µg)	42	43

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References

1. Ushiro H & Cohen S (1980) J. Biol. Chem. **255**, 8363-8365.
2. Reynolds FH Jr, Todaro GJ, Fryling C & Stephenson JR (1981) Nature **292**, 259-262.
3. Kasuga M, Zick Y, Blithe DL, Crettaz M & Kahn CR (1982) Nature **298**, 667-669.
4. Bo E & Heldin C-H (1982) J. Biol. Chem. **257**, 10486-10492.
5. Nishimura J, Huang JS & Deuel TF (1982) Proc. Natl. Acad. Sci. U.S.A. **79**, 4303-4307.
6. Roberts AB, Lamb LC, Newton DL, Sporn MB, DeLarco JE & Todaro GJ (1980) Proc. Natl. Acad. Sci. U.S.A. **77**, 3494-3498.
7. Savage CR & Cohen S (1972) J. Biol. Chem. **247**, 7609-7611.
8. Hock RA & Hollenberg MD (1980) J. Biol. Chem. **255**, 10731-10736.
9. Carpenter G, Poliner L & King L Jr (1980) Molec. and Cell. Endocrinol. **18**, 189-199.
10. Lowry OH, Rosebrough NJ, Farr AL & Randall RJ (1951) J. Biol. Chem. **193**, 265-275.
11. Bradford MM (1976) Anal. Biochem. **72**, 248-254.
12. Laemmli UK (1970) Nature **227**, 680-685.
13. Hunter T & Sefton BM (1980) Proc. Natl. Acad. Sci. U.S.A. **77**, 1311-1315.