Phosphorylation of pig brain diacylglycerol kinase by endogenous protein kinase

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Pig brain diacylglycerol kinase did not catalyze autophosphorylation. However, the kinase was phosphorylated on serine, when immunoprecipitated from the partially purified enzyme preparation preincubated with Mg^{2+} and [γ-^{32}P]ATP. The action of the endogenous protein kinase phosphorylating diacylglycerol kinase was independent of cyclic nucleotides and Ca^{2+}, and became maximum at pH 5.5. Although the extent of enzyme phosphorylation was limited (maximally about 0.25 mol P_i incorporated per mol kinase), the results show that diacylglycerol kinase can be a phosphoprotein.

Diacylglycerol kinase (Rabbit) Antibody Protein phosphorylation Protein kinase

1. INTRODUCTION

Diacylglycerol kinase, which also phosphorylates *sn*-2-monoacylglycerol [1], is responsible for a rapid accumulation of phosphatidic acid in agonist-stimulated cells (review [2,3]). Recent works employing an enzyme inhibitor [4] and diacylglycerol analogs (R.M. Bell, personal communication) showed that the action of diacylglycerol kinase is intimately involved in the control of protein kinase C [5] by regulating the intracellular level of diacylglycerol. It should be also noted that the reaction product of the kinase, phosphatidic acid [6–8] or lysophosphatidic acid [9,10], can be a potent cell agonist. These observations suggest the importance of the lipid kinase in regulating cell response phenomena.

Phosphorylation and dephosphorylation of proteins have been shown to be a major regulatory process for a number of enzymes [11,12]. In the case of glycerolipid synthesis, several enzymes have been described as being regulated by the covalent modification [13–16]. However, only circumstantial evidence has been available because of the difficulty of enzyme purification. Recently, we succeeded in raising rabbit antibody immunospecific toward pig brain diacylglycerol kinase [1]. This communication represents an application of the antibody to demonstrate that the kinase can be a substrate for endogenous protein kinase in the brain.

2. EXPERIMENTAL

[γ-^{32}P]ATP (4200 Ci/mmol) was bought from ICN. Sigma supplied cAMP, cGMP, protein kinase inhibitor, calmodulin and phosphoamino acid standards. Heat-killed, formalin-fixed *Staphylococcus aureus* cells (strain Cowan) from Calbiochem were heat-treated as described [17]. Preparation of rabbit antibody against diacylglycerol kinase has been described [1]. The kinase was partially purified (Step 4 [18]) and was used as a source of both diacylglycerol kinase and protein kinase after an extensive dialysis against ATP-free buffer.

Phosphorylation of diacylglycerol kinase was assessed in 50 μl of reaction mixture containing 25 mM 2-(*N*-morpholino)ethanesulfonic acid (pH 5.5), 5 mM $MgCl_2$, 1 mg/ml bovine serum albumin and 15 μg enzyme (5 nmol phosphatidate formed/min, 5 mU, when assayed in the presence

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of deoxycholate [18]). The reaction was initiated by adding 5 μM ATP (2–4 μCi per assay). The concentration of ATP was suboptimal, but was employed to save the radioactivity. The incubation was continued at 30°C for 30 min, and was stopped by adding 50 mM EDTA and 1 mM ATP in an ice-bath. Immunoprecipitation of diacylglycerol kinase was achieved by incubating the mixture in an ice-bath successively with 40 μg immune IgG and 30 μl *S. aureus* cell suspension (10%, w/v) [1]. The cells were collected by a brief centrifugation, and thoroughly washed as described [17]. The washed cells were boiled for 3 min in Laemmli sample buffer [19] and analyzed by SDS-polyacrylamide gel electrophoresis. The gels were dried and autoradiographed at −80°C with a DuPont Cronex Lightning Plus intensifying screen. The labeled proteins were cut out and counted in a toluene scintillator. Phosphoamino acid analysis was done according to Hunter and Sefton [20].

3. RESULTS

When the partially purified diacylglycerol kinase was immunoprecipitated after standard incubation, a major radioactive band (M_r 80000) was found in autoradiography (fig.1, lane b). No labeled proteins were detected when tested with preimmune IgG (not shown). Co-precipitation of a minor phosphoprotein (M_r 97000), which was not detected in immunoblotting [1], was reproducibly observed, in particular, when assayed at acidic pH. In view of the immunospecificity of the antibody [1], the labeling of the 80 kDa protein indicates phosphorylation of diacylglycerol kinase. The extent of enzyme phosphorylation was much less for further purified enzyme, and could not be detected for homogeneously purified enzyme by immunoprecipitation (fig.1, lane d) or trichloroacetic acid precipitation (not shown). Therefore, the enzyme phosphorylation was not due to autophosphorylation by the lipid kinase, but due to the action of endogenous protein kinase present in the partially purified enzyme preparation. Phosphoamino acid analysis showed that the kinase was phosphorylated exclusively at serine residues (fig.2).

The ^{32}P incorporation into diacylglycerol kinase was linear with respect to incubation time (up to

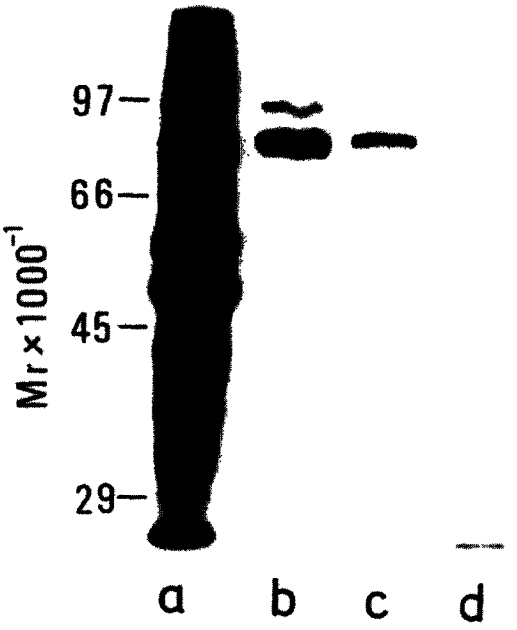

Fig.1. Immunoprecipitation of diacylglycerol kinase labeled with [γ-^{32}P]ATP. Diacylglycerol kinase obtained at different purification steps was labeled and immunoprecipitated as described in the text. Lanes: a,b, standard incubation of partially purified enzyme (15 μg); c, enzyme taken from an ATP-agarose column (0.5 μg, Step 5 in [18]); d, homogeneous enzyme from a hydroxyapatite column (0.28 μg [21]). In lane a, proteins were precipitated by 10% trichloroacetic acid and washed with 90% acetone containing 0.1 M HCl. The enzyme activity employed was 5 mU for all experiments, and equal aliquots (25 μl) from 50 μl of sample buffer were analyzed except for lane a, where only 5 μl was applied.

30 min) and the amount of enzyme (up to 30 μg). The enzyme phosphorylation became maximum at pH 5.5 (fig.3), and was completely dependent on Mg^{2+}, reaching a plateau at 5 mM. Other divalent cations, such as Mn^{2+}, Ca^{2+} and Co^{2+}, were ineffective. The apparent K_m value for ATP was estimated to be 7 μM (not shown). On the basis of molecular mass of the kinase and the specific enzyme activity of homogeneous enzyme (20 U/mg protein [21]), the amount of the kinase incubated (5 mU) corresponded to approx. 250 ng (3 pmol). In a prolonged incubation (for 2 h) in the presence of 20 μM ATP, maximally 0.25 pmol $^{32}P_i$ was incorporated per pmol diacylglycerol kinase.

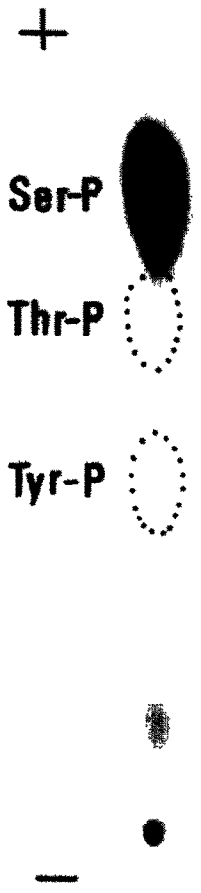

Fig.2. Phosphoamino acid analysis of diacylglycerol kinase. The enzyme was incubated with 25 μCi [γ-^{32}P]ATP in the standard assay condition. After immunoprecipitation, the kinase was detected by an autoradiography of undried gels. Proteins were extracted from the gels, partially acid hydrolyzed, and subjected to thin-layer electrophoresis at pH 3.5 [20]. Standards were localized by ninhydrin staining and are indicated by the broken lines. Ser-P, phosphoserine; Thr-P, phosphothreonine; Tyr-P, phosphotyrosine.

The enzyme phosphorylation was not affected by cyclic nucleotides and heat-stable protein kinase inhibitor (table 1). Addition of Ca^{2+} was inhibitory, and EGTA slightly enhanced the phosphorylation. No significant effects were noted with -SH blocking reagents. The effect of GTP suggests that the protein kinase can use GTP as phosphate donor. Polyamines (spermine and spermidine) tested at 1 mM did not affect the enzyme phosphorylation.

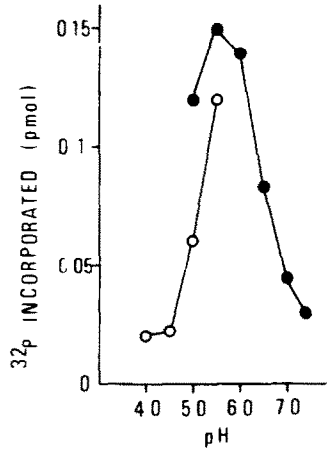

Fig.3. Effect of pH on the endogenous phosphorylation of diacylglycerol kinase. The partially purified enzyme was incubated in 25 mM sodium acetate (○) and 25 mM 2-(*N*-morpholino)ethanesulfonic acid (●) as described in the text.

Table 1

Effects of various additions on the phosphorylation of diacylglycerol kinase by endogenous protein kinase

Additions	^{32}P incorporated (fmol)
Standard incubation	121
Protein kinase inhibitor (5 μg)	120
Cyclic AMP (25 μM)	116
Cyclic GMP (25 μM)	120
GTP (5 μM)	73.6
EGTA (2 mM)	146
$CaCl_2$ (1 mM)	88.8
$CaCl_2$ (1 mM) and calmodulin (1 μg)	80.0
$CaCl_2$ (1 mM), phosphatidylserine (15 μg) and diolein (1 μg)[a]	69.6
N-Ethylmaleimide (2 mM)	100
Iodoacetic acid (2 mM)	105

[a] Lipids in chloroform were mixed, dried under nitrogen, and sonicated in 0.15 M NaCl

4. DISCUSSION

The phosphorylation of diacylglycerol kinase described here was possibly underestimated because of the low concentration (60 nM) of

substrate kinase in the assay mixture. We do not know whether the lipid kinase had already been phosphorylated before the experiments. Since numerous proteins were labeled in the enzyme preparation (fig.1, lane a), the inhibition by other protein substrates is also possible. Due to the lability of enzyme in the labeling condition, we could not assess the influence of protein phosphorylation on the enzyme activity. For unknown reasons, the incubation of crude cytosol and microsomes gave very little labeling of the kinase.

A separation from the lipid kinase is required to define further the properties of the endogenous protein kinase. It is premature to make a comparison with a variety of known protein kinases [5,11,12]. Although the partially purified enzyme was shown to contain an independent protein kinase, this work does not rule out the possibility that diacylglycerol kinase can be phosphorylated by well characterized multifunctional protein kinases, which are dependent on various second messengers [5,11,12]. Indeed, homogeneously purified diacylglycerol kinase was phosphorylated, though to a limited extent, by the catalytic subunit of cAMP-dependent protein kinase (not shown). Since diacylglycerol kinase now appears quite likely to be a phosphoprotein, experiments with intact cells or synaptosomes would be an interesting subject for further investigation to explore the functional significance of the protein phosphorylation.

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