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Association of a myristoylated protein with a biological membrane and its increased phosphorylation by protein kinase C

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A hydrophilic enzyme, lysozyme, was myristoylated in vitro by the *N*-hydroxysuccinimide ester of myristic acid, and the monomyristoylated lysozyme was isolated by CM-cellulose cation-exchange column chromatography. The monomyristoylated lysozyme associated with phospholipid vesicles, whereas the association of native lysozyme was negligible. The membrane-associated monomyristoylated lysozyme was phosphorylated with partially purified rat brain Ca^{2+}- and phospholipid-dependent protein kinase (protein kinase C) in the presence of Ca^{2+}, phosphatidylserine and phorbolmyristate acetate. Thus, the myristoylated lysozyme became a substrate of protein kinase C through its hydrophobic association with the membrane. The present results suggest that the myristoylation of cytoplasmic proteins may have an important role in signal transduction.

Protein myristoylation; Protein phosphorylation; Protein kinase C; Lysozyme; Membrane-protein interaction

1. INTRODUCTION

A large number of proteins in eukaryotic cells are known to be covalently modified with myristic acid, but the physiological function of protein myristoylation remains to be elucidated. It was recently found by Aderem et al. that myristoylation of the 68 kDa protein of macrophages was promoted by bacterial lipopolysaccharide [1] and that the myristoylated 68 kDa protein was a major specific substrate for protein kinase C in brain and fibroblasts [2]. They also found that the myristoylated protein kinase C substrate was quantitatively associated with the membrane fraction [2]. These results may suggest that myristoylation of the 68 kDa protein directs its association with the plasma membrane as described by several investigators [3–6] and that the membrane-bound protein is preferentially phosphorylated by a membrane-bound active form of protein kinase C. In this report, we demonstrate that the hydrophilic enzyme, lysozyme, when myristoylated, associates readily with phospholipid vesicles and that the membrane-bound myristoylated enzyme is phosphorylated by partially purified rat brain protein kinase C.

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2. MATERIALS AND METHODS

2.1. *Chemicals*

Mercaptoethanol, *N*-hydroxysuccinimide, phenylmethylsulfonyl fluoride (PMSF), phorbol myristate acetate (PMA) and trinitrobenzenesulfonic acid (TNBS) were purchased from Sigma (St. Louis, MO). Phosphatidylserine (PS) and dipalmitoylphosphatidylcholine (DPPC) were obtained from Research Lab. (Canada). Lysozyme from chicken egg white was crystallized six times in our laboratory. CM-cellulose and Sephadex G-200 were obtained from Pharmacia (Uppsala). DEAE-cellulose (DE-52) and glass filters were obtained from GF/C, Whatman (Maidstone, England) and TSK-G3000 SW from Tosoh (Tokyo). [γ-^{32}P]ATP was obtained from ICN Radiochemicals (Irvine, CA). Leupeptin and other chemicals used were of analytical grade from Nakarai (Kyoto).

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2.2. *Preparation of protein kinase C from rat brain*

Protein kinase C was partially purified from cerebral tissue of male Dawley rats, weighing 200–250 g, by the method of Kikkawa et al. [7]. Rat brains were homogenized in a medium of 20 mM Tris-HCl (pH 7.5), 0.25 M sucrose, 5 mM EGTA, 2 mM PMSF, 0.01% leupeptin, 10 mM 2-mercaptoethanol, 0.1% Triton X-100 at 4°C, and then centrifuged at 100000 × *g* for 60 min. The supernatant was applied to a DE-52 column (0.7 × 7 cm) equilibrated with 20 mM Tris-HCl buffer (pH 7.5) containing 30 mM NaCl, 0.5 mM EGTA, 0.5 mM EDTA, 6 mM 2-mercaptoethanol, 1 mM PMSF and 0.01% leupeptin (buffer A). The enzyme was eluted from the column by the application of a one-step gradient of NaCl (30–100 mM) using buffer A containing 100 mM NaCl instead of 30 mM NaCl (buffer B). The crude fraction (0.2 ml) was then applied to a TSK-G3000 SW column (1 × 30 cm) and was eluted with 20 mM Tris-HCl buffer (pH 7.5) containing 100 mM NaCl, 0.5 mM EGTA, 0.5 mM EDTA and 6 mM 2-mercaptoethanol (buffer C).

2.3. *Myristoylation of lysozyme*

The *N*-hydroxysuccinimide ester of myristic acid (NHSM) was prepared by the method of Lapidot et al. [8]. 5 ml dimethyl sulfoxide containing 5.7 mg NHSM was added gently to 50 ml of 0.2% lysozyme in 1% $NaHCO_3$ solution. The mixture was stirred at 25°C for 12 h, and then dialyzed against 0.1 M phosphate buffer (pH 8.0) containing 0.1 M NaCl at 4°C for 24 h.

2.4. *Separation of membrane-bound protein from unbound protein by gel filtration*

Native or myristoylated lysozyme (250 μg) was mixed with 2.5 mg PS/DPPC vesicles (1 : 4 molar ratio) in 0.5 ml of 20 mM phosphate buffer (pH 7.4) containing 0.1 M NaCl and incubated at 20°C for 30 min. The incubation mixture was applied to a Sephadex G-200 column (1 × 25 cm) previously equilibrated with 20 mM phosphate buffer (pH 7.4) containing 0.1 M NaCl, and vesicle-associated and unassociated lysozyme fractionated by elution with the same saline buffer. Protein concentration in each fraction was assayed according to Lowry et al. [9].

2.5. *Assay of protein kinase C*

The activity of protein kinase C was routinely assayed by measuring the incorporation of ^{32}P from [γ-^{32}P]ATP into lysozyme or myristoylated lysozyme at 30°C for 3 min [10,11]. Phospholipids (PS/DPPC, 1 : 4 molar ratio) were first mixed in a small volume of chloroform and the solvent was evaporated in vacuo. The residue was suspended in 20 mM Tris-HCl buffer (pH 7.5) by sonication at 4°C for 30 min under nitrogen gas. The reaction was carried out in a mixture (250 μl) containing 20 mM Tris-HCl (pH 7.5), 19 mM magnesium acetate, 1 μM or 0.3 mM $CaCl_2$ and 10 μM (29–30 Ci/mol) [γ-^{32}P]ATP, in the presence or absence of 100 or 300 μM phospholipid (PS/DPPC, 1 : 4, molar ratio) and 150 nM PMA, and lysozyme (0.2 mg/ml) as substrate. After the incubation at 30°C for 3 min, the reaction was stopped by addition of 25% trichloroacetic acid. The acid-precipitable material was collected on a glass filter (GF/C, Whatman Ltd) [12].

2.6. *SDS-PAGE and autoradiography*

Materials were denatured by boiling for 2 min in 65 mM Tris-HCl buffer (pH 7.0) containing 2% SDS, 5% 2-mercaptoethanol, 15% glycerol and 0.01% bromophenol blue by the method of Laemmli [13]. Gels were stained with Coomassie brilliant blue R250 and then autoradiograms of the ^{32}P-labelled proteins were made as reported in our previous paper [11].

3. RESULTS AND DISCUSSION

3.1. *Purification of monomyristoylated lysozyme on a CM-cellulose column*

When lysozyme was reacted with a 5-fold molar excess of *N*-hydroxysuccinimide ester of myristic acid at alkaline pH, a fraction of modified lysozymes which showed a diminished mobility on non-denatured polyacrylamide gel electrophoresis (pH 4.3, 7.5% polyacrylamide) was produced (fig.1). To separate the modified lysozyme from the unmodified form, the reaction mixture was applied to a CM-cellulose column (3 × 40 cm) equilibrated with 0.1 M phosphate buffer (pH 8.0) containing 0.1 M NaCl, and eluted with the same buffer. Two fractions were obtained (fig.1), and non-denaturing PAGE reveals the former peak to be myristoylated lysozyme and the latter to be the native enzyme, respectively. Quantitative analysis of free amino groups using TNBS showed that the modified lysozyme thus obtained was monomyristoylated.

3.2. *Association of myristoylated lysozyme with biological membrane*

Lysozymes were incubated with PS/DPPC vesicles at 20°C for 30 min and then subjected to Sephadex G-200 column chromatography. Typical profiles of the separation of lysozyme-PS/DPPC vesicle complexes from unbound proteins are shown in fig.2. Phospholipid vesicles or protein-vesicle complexes were eluted at the void volume fraction. As shown in fig.2A, association of native lysozyme with the vesicles was negligible. Monomyristoylated lysozyme, in contrast, markedly associated with vesicles, and more than 50% of added protein was detected in the vesicle fraction (fig.2B). The association profiles of native and monomyristoylated lysozymes with vesicles were not changed by depletion of PS in DPPC vesicles. These results clearly show that protein myristoylation can confer the ability to bind to membrane on an otherwise soluble protein.

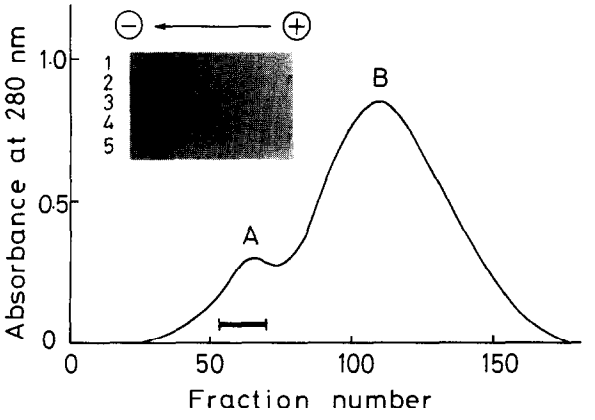

Fig.1. Fractionation of monomyristoylated lysozyme by CM-cellulose column chromatography. Lysozyme, myristoylated by *N*-hydroxysuccinimide ester of myristic acid, was fractionated on a CM-cellulose column. Inset shows non-denaturing PAGE (pH 4.3, 7.5% polyacrylamide). Lanes: (1,5) native lysozyme; (2) reaction mixture; (3) peak A; (4) peak B. The fraction indicated by the bar was used as monomyristoylated lysozyme.

3.3. *Phosphorylation of myristoylated lysozyme by protein kinase C*

As shown in figs 3 and 4, only weak phosphorylation by partially purified protein kinase C (a membrane-bound enzyme [14]) was

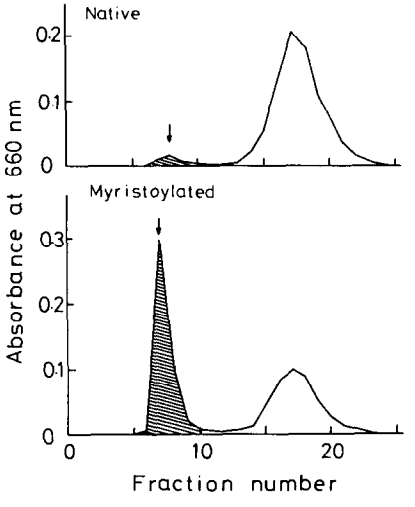

Fig.2. Gel filtration of PS/DPPC vesicles incubated with native or monomyristoylated lysozyme. Native or monomyristoylated lysozyme was incubated with PS/DPPC (1:4 in molar ratio) vesicles at 20°C for 30 min, and vesicle-associated and unassociated with lysozyme were fractionated on a Sephadex G-200 column. Arrows indicate the position of vesicles. A, native lysozyme; B, monomyristoylated lysozyme.

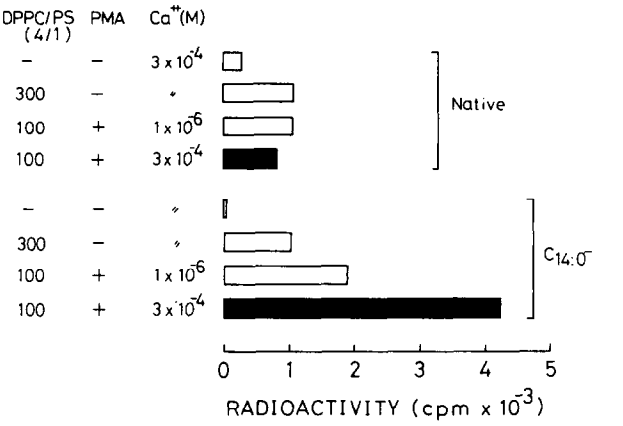

Fig.3. Phosphorylation of monomyristoylated lysozyme by protein kinase C. Native or monomyristoylated lysozyme was incubated with partially purified rat brain protein kinase C in a medium containing 1 μM or 300 μM Ca^{2+} in the presence or absence of 100 or 300 μM phospholipid vesicles (PS/DPPC, 1:4 in molar ratio), and 150 μM PMA at 30°C for 3 min.

detected in the case of native lysozyme and PS/DPPC vesicles even in the presence of Ca^{2+}, phosphatidylserine and PMA. In contrast, phosphorylation was strongly increased in the case of monomyristoylated lysozyme under the same conditions. This phosphorylation was entirely dependent on the existence of PMA and on the concentration of Ca^{2+} (fig.3). The phosphorylation of monomyristoylated lysozyme was confirmed by autoradiography of the protein on SDS-PAGE (fig.4). The degree of phosphorylation of the mono myristoylated lysozyme was very low

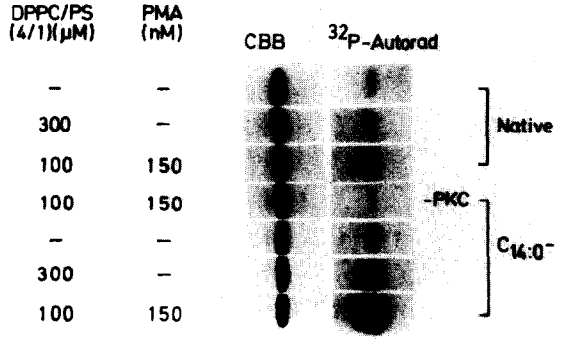

Fig.4. Autoradiogram of native and monomyristoylated lysozymes phosphorylated by partially purified rat brain protein kinase C. After phosphorylation, samples were analyzed by SDS-PAGE in 12% gel. Gels were stained with Coomassie brilliant blue R250 and then autoradiograms of the ^{32}P-labelled proteins were made.

as compared with that of histone, but was similar to that of cytoplasmic proteins [11,12].

In a preliminary experiment, we examined the effect of fatty acid chain length on membrane association and phosphorylation of the acylated lysozyme and found that lysozyme acylated with short-chain fatty acids such as C_6 or C_8 was neither associated nor phosphorylated. In contrast, both membrane association and phosphorylation were observed in the case of the acylated lysozyme with the longer chain fatty acids such as C_{10}–C_{14}. These data suggested that the enhancement of the phosphorylation of lysozyme by protein kinase C was not specific for the myristoylated lysozyme and was due to the increased association of the protein with the membrane.

To generalize the evidence obtained in the experiment described in this report, phosphorylation and membrane association of the other chemically acylated proteins are under investigation.

The results obtained here strongly support the hypothesis proposed by Aderem et al. which describes the sequence of events in stimulated macrophages [2]: (i) promotion of protein myristoylation, (ii) association of the myristoylated protein closely with protein kinase C on membrane, (iii) phosphorylation of the protein, and (iv) release of the protein into cytoplasm. Therefore, at least in part, myristoylation of certain cytoplasmic proteins will potentiate protein kinase C-dependent signal transduction.

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