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REGULATION OF PROTEIN KINASE FA (A TRANSMEMBRANE SIGNAL OF INSULIN AND EPIDERMAL GROWTH FACTOR) IN THE BRAIN

Shiaw-Der Yang* , Jau-Song Yu and Hua-Chien Chen

Institute of Molecular Cell Biology, Chang Gung Medical College, Tao-Yuan, Taiwan, Republic of China and Institute of Life Science, National Tsing Hua University Hsinchu, Taiwan, Republic of China

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Two forms of type-1 protein phosphatase activating factor (FA) termed FA1 and FA2 have been identified in plasma membranes of pig brain. FA1 is spontaneously active and trypsin-labile whereas FA2 is inactive and trypsin-resistant. Phospholipid reconstitution studies further indicate that the FA activity in the neutral phospholipids-reconstituted complex is spontaneously active and trypsin-labile whereas the FA activity in the acidic phospholipids-reconstituted complex is trypsin-resistant and inactive. The results indicate that inactive FA2 may have its catalytic domain interacted with negatively-charged phospholipids in brain membranes. This provides initial evidence for the regulation of protein kinase FA (a transmembrane signal of insulin and epidermal growth factor) in the central nervous system.

The ATP.Mg-dependent protein phosphatase (FcM) and its activating factor (kinase FA) were first identified in mammalian nonnervous tissues including liver, heart and skeletal muscle (1-3) but have subsequently been demonstrated in the central nervous system (4). In rabbit skeletal muscle, FA was further identified as a potent glycogen synthase kinase (5-7). The identity of glycogen synthase kinase and FA is unexpected, since they catalyze reactions which are antagonistic. Glycogen synthase kinase catalyzes phosphorylation and inactivation of glycogen synthase to terminate glycogenesis, while FA, by activating type-1 protein phosphatase, promotes dephosphorylation and activation of glycogen synthase to initiate glycogenesis. Because of this unique nature, FA may play an important role in the regulation of glycogen metabolism and has been demonstrated as a prime target for insulin action (8-10). In the central nervous system, glycogen synthase may not be a physiological substrate for FA. Brain FA appears to be analogous with

*To whom correspondence should be addressed.

Abbreviations : FA, activating factor of type-1 protein phosphatase; FcM, inactive type-1 phosphatase; MBP, myelin basic protein.

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FA/glycogen synthase kinase in rabbit muscle, in that it also catalyzes two antagonistic activities. As a myelin basic protein (MBP) kinase, FA promotes phosphorylation of MBP, while by activating FcM, which also exists in brain (4), FA promotes dephosphorylation of MBP phosphorylated by FA itself (11). Because of these unique features, there is growing interest in FA as a regulatory enzyme. However, there are no clues as to how this enzyme is regulated in the cell. In this report, two forms of membrane-associated FA (tentatively termed FA1 and FA2) have been identified in the brain. This is the first indication as to how kinase FA, a unique transmembrane signal of insulin and epidermal growth factor (8-10) is regulated in the central nervous system.

EXPERIMENTAL PROCEDURES

Materials --- [r-^{32}P] ATP was purchased from ICN. ATP, cAMP, phosphatidic acid, phosphatidylcholine, phosphatidylethanolamine, phosphatidylserine and phosphatidylinositol were obtained from Sigma. Sucrose, dithiothreitol, 2-mercaptoethanol and trichloroacetic acid were from Merck. Sepharose 4B and CNBr-activated Sepharose 4B were from pharmacia. DEAE-cellulose, CM-cellulose and phosphocellulose were from Whatman. Phosvitin-Sepharose 4B, casein-Sepharose 4B and histone-Sepharose 4B were prepared as previously described (1).

Protein purification --- Phosphorylase b (12) and phosphorylase b kinase (13) were purified from rabbit skeletal muscle. The catalytic subunit of cAMP-dependent protein kinase (14) was purified from pig heart. The ATP.Mg-dependent type-1 protein phosphatase FcM (4) and its activating factor (kinase FA) (11) and encephalitogenic myelin basic protein (MBP) (15) were purified from pig brain.

Preparation of ^{32}P-labeled protein substrates --- ^{32}P-phosphorylase a was prepared from phosphorylase b, phosphorylase b kinase and [r-^{32}P] ATP as described in Ref. 16. ^{32}P-MBP was prepared using 1 mg/ml MBP, 0.2 mM [r-^{32}P] ATP, 20 mM Mg^{++} ions and catalytic amount of cAMP-dependent protein kinase. The labeled ^{32}P-MBP was isolated as described in a previous report (11).

Preparations of brain plasma membranes --- Unless specifically indicated, all the operations were carried out at 4°C. Fresh pig brain was homogenized in 3 volumes of ice cold homogenization buffer containing 50 mM Tris-HCl at pH 7.4, 1.5 mM magnesium sulfate and 0.32 M sucrose. The homogenate was centrifuged at 1,000 x g for 10 min and the resulting pellet was washed two times and then dissolved in a final sucrose concentration of about 1.6 M in buffer A containing 20 mM Tris-HCl at pH 7.2 and 1 mM dithiothreitol. A discontinuous sucrose density gradient was formed by overlaying 10 ml of this suspension with 2 ml of 0.32 M sucrose in buffer A. This gradient was then centrifuged at 90,000 x g for 2 hr in a Beckman SW 41 swinging bucket rotor. The material at the 0.32 M to 1.6 M sucrose interface was harvested and further purified with the second discontinuous sucrose density gradient as described above. The purified fractions which were resuspended in buffer A were used as the source for brain plasma membranes throughout the whole text. The purification procedure for plasma membranes was carried out according to Gray and Whittaker (17). The mitochondria fractions which are located below the white layer that contains plasma membranes at the 0.32M to 1.6 M sucrose interface can be removed under this condition. The nuclear fractions which are located at the bottom of the sucrose gradient can also be separated under this condition.

Enzyme Assays --- The kinase FA/ATP.Mg-dependent type-1 protein phosphatase (FcM) and its activating factor (kinase FA) were determined by the methods as described in previous reports (1, 4). The activity of FcM was measured after a 10-min preincubation at 30°C with 0.1 mM ATP, 0.5 mM Mg^{++} ions and saturating amounts of FA required for full activation of FcM under the assay conditions. ^{32}P-phosphorylase a

and ^{32}P-MBP were simultaneously used as the substrates. Assay time was 5 min. The activity of FA as the activating factor of type-1 protein phosphatase was measured by the formation of activated FcM in a 1-min incubation at 30°C. The assay mixture contained appropriate dilutions of FA, 0.1 mM ATP, 0.5 mM Mg^{++} ions and excess amount of FcM to ensure linear activation of the phosphatase activity. Assay time was only 1 min and ^{32}P-phosphorylase a and ^{32}P-MBP were used as the protein substrates. For determination of the activity of inactive but trypsin-resistant FA2 in plasma membranes, membrane fractions were first pretreated with 0.02 mg/ml trypsin inhibitor to terminate the action of trypsin. The trypsinized membranes were next treated with 1% Triton X-100 followed by sonication at 25°C for 5 min prior to the assay for FA activity. A unit of protein phosphatase activity is that amount of enzyme that catalyzes the release of 1 nmol of phosphate/min. A unit of FA as type-1 protein phosphatase activator is that amount of enzyme that produces one unit of activated FcM after a 1-min preincubation.

Analytic Method --- Protein concentration was determined by the method of Lowry et al (18).

RESULTS AND DISCUSSION

As shown in Fig.1, brain membranes contained little protein phosphatase activity. Inclusion of 0.1 mM ATP and 0.5 mM Mg^{++} or exogenous purified FA together with ATP.Mg did not increase the phosphatase activity (Fig.1), indicating that

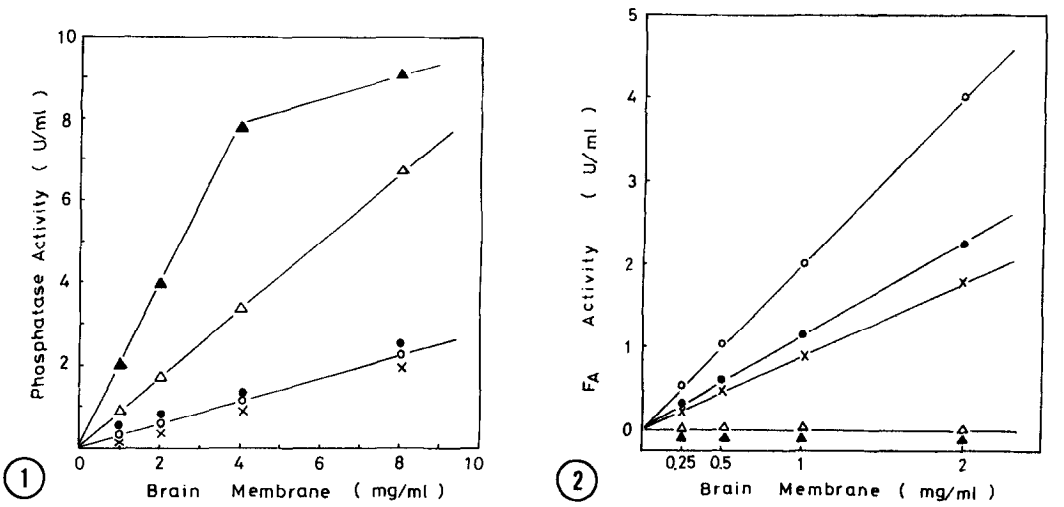

Fig. 1. Identification of two forms of FA in plasma membranes.

The protein phosphatase activities in brain plasma membranes were assayed under the following conditions : 1. buffer A containing 20 mM Tris-HCl at pH 7.2 and 1 mM dithiothreitol (○) . 2. buffer A + 0.1 mM ATP and 0.5 mM Mg^{++} ions (●). 3. 0.1 mM ATP, 0.5 mM Mg^{++} ions and 20 mU exogenous FA (x). 4. 0.1 mM ATP, 0.5 mM Mg^{++} ions and 20 mU exogenous FcM in the absence (△) and presence (▲) of 1% Triton X-100 followed by sonication at 25°C for 5 min. Assay conditions were as described under " EXPERIMENTAL PROCEDURES".

Fig. 2. Effect of trypsin on membrane-bound FA activities.

The FA activities in brain membranes were assayed under the following conditions : 1. 1% Triton X-100 treatment (○), 2. trypsinolysis followed by 1 % Triton X-100 treatment (●), 3. without any treatment (x) , 4. trypsinolysis only (△) and 5. 1% Triton X-100 treatment followed by trypsinolysis (▲) . Trypsinolysis was performed using 0.02 mg/ml of trypsin at 30°C for 3 min. Trypsin action was terminated by adding 0.2 mg/ml of trypsin inhibitor to the reaction mixture. Sonication and assay conditions were as described under " EXPERIMENTAL PROCEDURES ".

ATP.Mg-dependent type-1 protein phosphatase (FcM) (1-11) was not present in brain membranes. In contrast, when exogenously purified FcM and ATP.Mg were added to membranes, the phosphatase activity was stimulated very dramatically, demonstrating that substantial amount of FA was present in brain plasma membranes (Fig. 1). Moreover, when membranes were first treated with 1% Triton X-100 followed by sonication at 25°C for 5 min before the assay, this FA activity was further stimulated about 2.5-fold, suggesting that brain membranes may contain two forms of FA. One form of FA termed FA1 is active, whereas the other form termed FA2 is inactive unless treated with Triton X-100. Since FcM is not present in membranes whereas its activating factor (kinase FA) is substantially localized in membranes, the results further suggest that membrane-bound FA may be subject to regulation, and thus we decided to characterize these two FA's in brain membranes. As shown in Fig. 2, when brain plasma membranes were treated with 0.02 mg/ml of trypsin at 30°C for 3 min, the spontaneously active FA1 activity was completely destroyed. In contrast, the inactive FA2 activity was unaffected under the same conditions. However, in analogy with FA activity in the cytosol, when membranes were first sonicated in the presence of 1% Triton X-100 at 25°C for 5 min, both FA1 and FA2 activities, i.e. the total FA activities in membranes were completely destroyed by trypsin (Fig.2). The results indicate that as cytosolic FA, both FA1 and FA2 become trypsin-labile when they are in solubilized states. When they are associated with membranes, FA1 remains trypsin-labile whereas FA2 is trypsin-resistant. This suggests that FA1 is exposed outside membranes whereas FA2 is buried within membranes.

One of the possibilities for the distinctly different trypsin sensitivity and availability between these two membrane-associated FA's may be due to a specific interaction of FA with membrane phospholipids. For instance, the catalytic domain of inactive/trypsin-resistant FA2 may be specifically interacted with membrane phospholipids and buried inside membranes. In order to investigate this possibility, phospholipid reconstitution studies were performed. As illustrated in Table 1, various distribution of FA1 and FA2 activities in the phospholipid reconstituted complexes could be obtained depending on the mol percentage of neutral and acidic phospholipids being used for the formation of FA-phospholipid complex. Acidic phospholipids such as phosphatidylinositol

Table 1. Phospholipids and FA activity

Phospholipids used	FA1 activity	FA2 activity
PC	98 %	2 %
PE	98 %	2 %
PA	1 %	99 %
PS	2 %	98 %
PI	2 %	98 %
PC + PE	98 %	2 %
PC + PE + PS	65 %	35 %
PC + PE + PI	70 %	30 %
PC + PE + PS + PI	50 %	50 %

PC, phosphatidylcholin; PE, phosphatidylethanolamine; PA, phosphatidic acid; PS , phosphatidylserine and PI, phosphatidylinositol; concentrations used are 15 mM PC, 15 mM PE, 1.5 mM PS, 1.5 mM PI and 0.15 mM PA . Phospholipids were first mixed with purified free FA and then sonicated at 25°C for 10 min prior to the FA activity assay. Assay conditions were as described under " EXPERIMENTAL PROCEDURES " . Data were taken from the average of 5-time experiments. Total activity of FA was obtained from adding FA1 activity together with FA2 activity. FA1 activity was obtained from the direct assay of the reaction mixture. FA2 activity was obtained from the reaction mixture which was first trypsinized and then treated with 1 % Triton X-100 as described under "EXPERIMENTAL PROCEDURES ".

(PI) and phosphatidylserine (PS) appear to favor formation of inactive FA2 whereas neutral phospholipids such as phosphatidylethanolamine (PE) and phosphatidylcholine (PC) seem to favor formation of active FA1, suggesting that membrane phospholipids may play an important role in regulating membrane-bound FA activity towards activation of type-1 protein phosphatase in the brain.

FA has long been recognized as a cytosolic protein kinase involved in the activation of type-1 protein phosphatase (1-7). In order to investigate the physiological role of membrane-bound FA in regulating type-1 phosphatase which is present exclusively in the cytosol, quantitative study on the subcellular localization of FA in mammalian tissues was carried out. When brain tissue was homogenized in Polytron and /or blender, approximately 40% of FA was found in the cytosol whereas 60% of FA was associated with membranes. By contrast, when brain was homogenized in motor-driven Potter-Elvehjem glass homogenizer with a Teflon pestle, only 25% of FA was found in the cytosol. Moreover, when brain was homogenized in glass homogenizer with a Teflon

pestle using buffer A containing 0.32 M sucrose, the FA activity which could be detected in the cytosol was decreased to about 10% of the total activity in the cell. Conversely, during large scale preparation in which prolonged processing was unavoidable, more than 40% of FA could be found in the cytosol whereas the membrane-bound FA activity was proportionally decreased (data not shown). These results, taken together, strongly suggest that FA is a specific membrane-associated protein kinase in vivo and that the FA activity detected in the cytosol is likely an artifact which is artificially produced during tissue homogenization. The results provide initial evidence that interconversion between inactive/trypsin-resistant FA2 and active/trypsin-labile FA1 in brain membranes can be the physiologically relevant control mechanism in regulating type-1 protein phosphatase in vivo.

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