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THE TYPE-1 PROTEIN PHOSPHATASE ACTIVATING FACTOR F_A IS A MEMBRANE-ASSOCIATED PROTEIN KINASE IN BRAIN, LIVER, HEART AND MUSCLES

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Although the activating factor F_A of the type-1 protein phosphatase has long been recognized as a cytosolic enzyme involved in the regulation of cell metabolism and nervous functions, strong indications have been obtained that F_A is in fact a membrane-bound protein kinase in most mammalian tissues. For instance, direct treatment of the tissue extracts including brain, liver, cardiac, smooth and skeletal muscles with 1% Triton X-100 can cause several fold stimulation of the F_A activity. Moreover, at least 50% of the F_A can be detected in the particulate fractions of the extracts. Chromatography of the extracts in the presence and absence of Triton X-100 further demonstrate these results. The data can now explain the reason why most people can not isolate reasonable amount of F_A from mammalian tissues. It is recommended that Triton X-100 should be used for purification of F_A from most mammalian tissue extracts. The results also suggest that most previous studies on the action of F_A involved in the regulation of cell functions should be re-evaluated and the membrane-associated F_A should be taken into consideration.

The activating factor F_A of the type-1 protein phosphatase has been identified in both mammalian nervous and non-nervous tissues . In the non-nervous tissues including liver and skeletal muscle, F_A has further been identified as a potent glycogen synthase kinase. (1-7). The identity of glycogen synthase kinase and factor F_A is unexpected, since they catalyze reactions which are antagonistic. As a glycogen synthase kinase, F_A promotes phosphorylation and inactivation of glycogen synthase to initiate glycogenolyis while by activating the type-1 phosphatase, F_A promotes dephosphorylation and activation of glycogen synthase to initiate glycogenesis. In the central nervous system where glycogen metabolism is of

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little importance, F_A has also been identified as a key enzyme possessing two antagonistic activities. As a myelin basic protein kinase in the brain myelin, F_A is supposed to be an enzyme to promote phosphorylation of brain myelin while by activating the type-1 phosphatase which also exists in myelin, F_A promotes the dephosphorylation of myelin basic protein in the central nervous system (1,8). Because of its unique nature, F_A is likely a prime target for hormonal and neural control (8-12). However, progress on the work of F_A has been greatly hampered for the past 5 years, since Cohen and coworkers (4,13) reported that only 0.04 to 0.1 mg of F_A could be isolated from 5 kg of rabbit skeletal muscles. Although part of the reasons for this low quantity of F_A protein in mammalian tissue has recently been pointed out by Yang (8) that this is simply due to the insolubility of the enzyme during purification using ammonium sulfate fractionations, the quantity of F_A in most mammalian tissues may possibly be still underestimated (8), which remains to be further established.

In the present report, we have tried to make an extensive study on the localization of this unique bifunctional protein kinase F_A in most mammalian tissues including brain, liver, heart and skeletal muscle. Amazingly, we found that tremendous amount of F_A is in fact associated with the particulate fractions of the tissues which previously have been totally ignored (1-8,13). Since this membrane-associated protein kinase F_A not only exists in nervous tissue but also in non-nervous tissues including rabbit skeletal muscle, it is strongly suggested that the previous work on F_A protein for the past years should be completely re-evaluated. The new strategy to get high yield of this unique bifunctional key enzyme from mammalian tissues is also proposed in this report.

MATERIALS AND METHODS

Materials : Most materials are as described in previous reports (1-3,8). [γ-^{32}P] ATP was either purchased from ICN or

kindly provided by Institute of Nuclear Energy Research, Atomic Energy Council, Taiwan, ROC.

Protein Purifications : Phosphorylase b (14), phosphorylase b kinase (15) and ^{32}P-phosphorylase a (16) were prepared from rabbit skeletal muscle. Kinase F_A/ATP.Mg-dependent type-1 protein phosphatase (1) and kinase F_A(8) were purified to near homogeneity from pig brain. Myelin basic protein (17) and ^{32}P-labeled myelin basic protein (MBP) (1) were prepared from pig brain.

Preparation of Crude Tissue Extracts : Fresh tissues were homogenized in 2.5 volumes of buffer containing 50 mM Tris-HCl pH 7.8, 1 mM dithiothreitol, 1 mM ethylene glycol-bis-(2-amino-ethyl ether) N,N,N',N',-tetraacetic acid, 4 mM EDTA, 0.1 mM phenyl-methanesulfonyl fluoride, 0.1 mM 1-chloro-3-tosylamido-7-amino-L-2-heptanone and 0.1 mM L-1-tosylamido-2-phenylethylchloromethyl-ketone. The homogenate was centrifuged and the resulting super-natant was used as the cytosolic fraction. The pellets were used as the particulate fractions. Homogeniztion of the tissues were performed in the presence and absence of 1% Triton X-100. Before enzyme assay or chromatography of the extracts, Triton was routin-ely diluted below 0.1%.

Enzyme Assays : Unless specifically indicated, F_A activity as the activating factor of the type-1 protein phosphatase was commonly measured after the tissue extracts were extensively diluted in 1 mg/ml bovine serum albumin in order to rule out the interference of the endogenous factors. The enzyme activity was measured by the formation of the activated phosphatase in a 2-min incubation at 30°C. The assay mixture contained appropriate dilu-tions of inactive type-1 phosphatase, 0.1 mM ATP, 0.5 mM magnesium acetate and tissue extracts. Assay time was 2 min. A unit of F_A as phosphatase activator is that amount of enzyme that produces 1 unit of phosphatase after a 1-min preincubation. Detailed assay procedures were essentially as described in the previous reports (1-3,8).

Analytical Method : Protein concentrations were determined by the method of Lowry (18).

RESULTS AND DISCUSSION

In agreement with the previous reports (1-3,8), pig brain and rabbit skeletal muscle extracts indeed contain lots of F_A activities when measured as the type-1 protein phosphatase acti-vating factor and using ^{32}P-labeled myelin basic protein as the substrate (see Fig. 1A and 1B). However, when tissues were homo-genized in the presence of 1% Triton X-100, F_A activities were found to be stimulated several-fold under the same assay condition used (Fig. 1A and 1B). This is the first indication that F_A may be a membrane-associated protein kinase in mammalian brain and skeletal muscle tissues. In order to rule out the possibility that

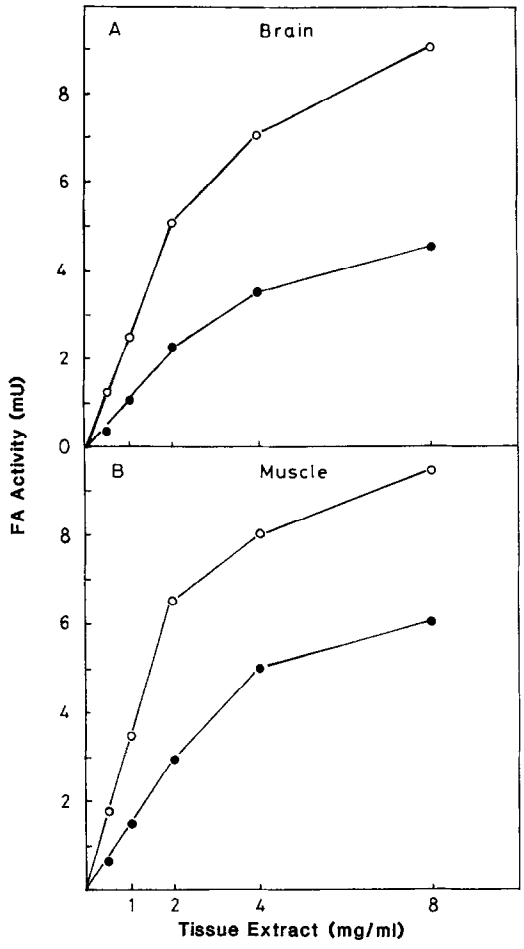


Fig. 1. Triton X-100 effect on the activity of the activating factor F_A of type-1 protein phosphatase in tissue extracts. 20 grams of pig brain or rabbit skeltal muscle were homogenized in the presence (o) and absence (●) of 1% Triton X-100 and the resulting supernatants after appropriate dilutions were assayed for F_A activities under the same conditons as described in " Methods." ^{32}P-MBP was used as the substrate and protein concentrations were determined by the method of Lowry.

the stimulation of Triton X-100 on F_A activity in the crude extract is simply due to a direct effect on the F_A/ATP.Mg-mediated activation of the inactive type-1 protein phosphatase (1-3,8), we have tried using the exogenous pure F_A and inactive phosphatase isolated from pig brain or rabbit skeletal muscle to test this Triton's effect. It turned out that Triton X-100 in fact can not cause any slight effect on this F_A/ATP.Mg-mediated activation process (data not illustrated). The results further support the notion that F_A

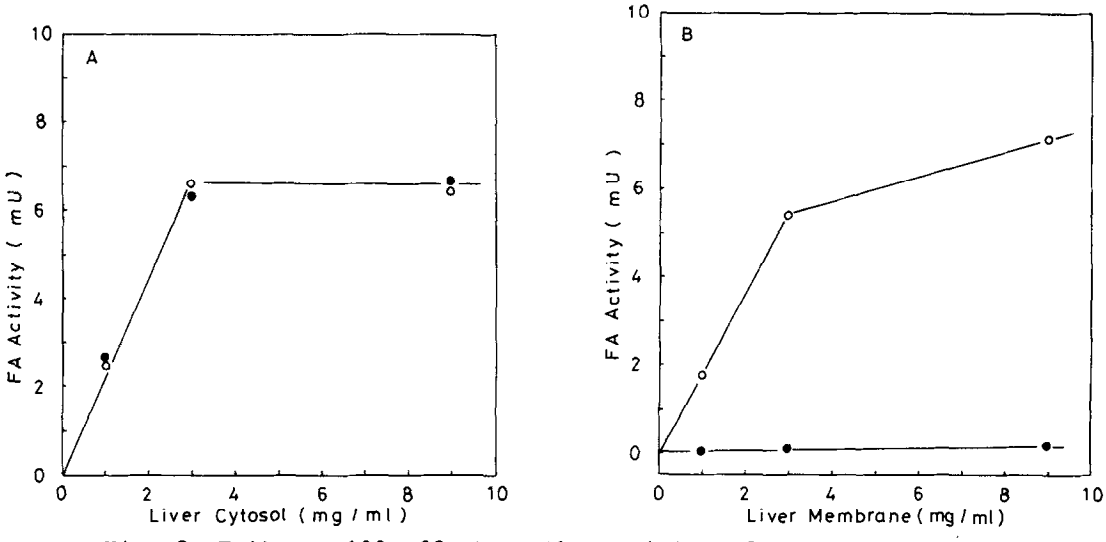


Fig. 2. Triton X-100 effect on the activity of the activating factor F_A of type-1 protein phosphatase in liver cytosolic and particulate fractions.
The liver homogenate was first separated into cytosolic and particulate fractions by differential centrifugations. Both fractions were next divided into two equal parts and treated with (o) and without (●) 1% Triton X-100 at 4°C for 30 min. After extensive dilutions, the total four fractions were assayed for F_A activities and ^{32}P-MBP was used as the substrate. Protein concentrations were determined by the method of Lowry.

is a membrane-associated protein kinase which can be dissociated after the treatment with Triton X-100.

In order to further establish this point, the pig liver homogenate was next separated into two fractions namely the clear cytosolic fractions and the particulate membrane fractions. When both fractions were treated with 1% Triton X-100 at 4°C for 30 min, the F_A activities which are already detectable in the liver cytosolic fractions were not slightly influenced by the treatment (see Fig. 2A). In very sharp contrast, the liver particulate membrane fractions did not contain any detectable F_A activities. However, after the Triton X-100 treatment, tremendous amount of F_A activity could be very dramatically produced. This is illustrated in Fig. 2B. The same observations could also be extended to pig brain, heart and rabbit skeletal and smooth muscles (data not illustrated). Taken together, the results strongly indicate that a major part of F_A in most mammalian tissues is in fact associated with

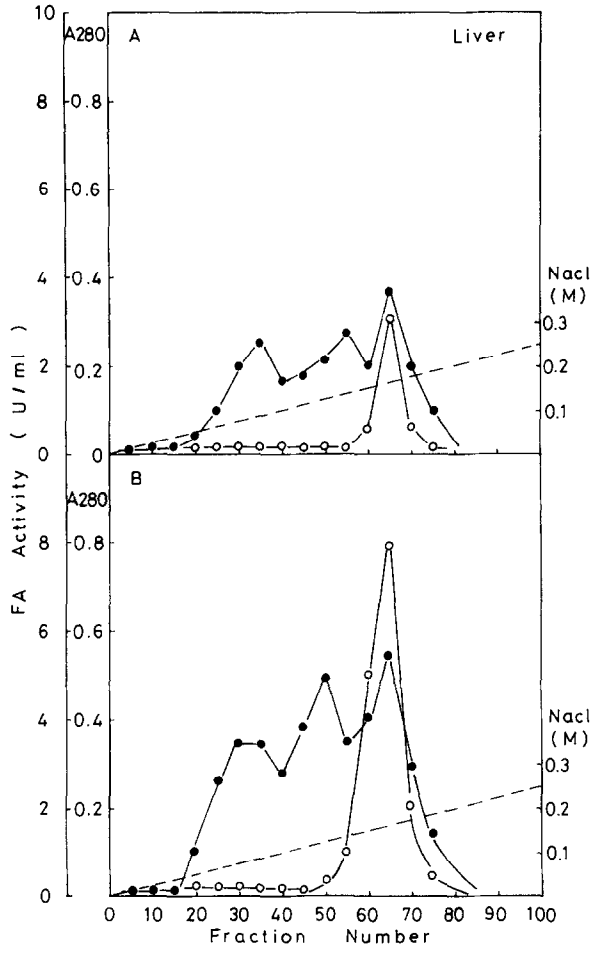


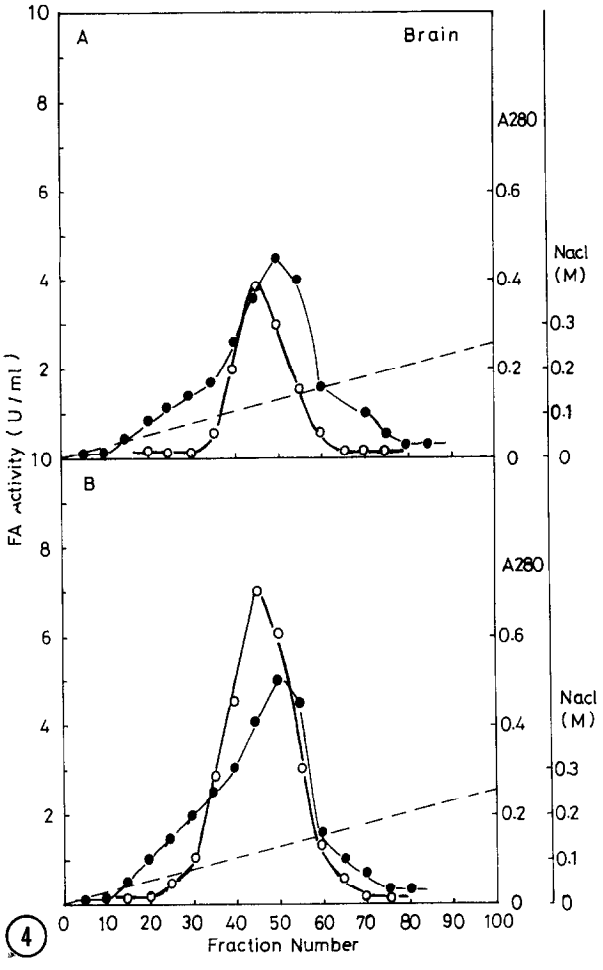
Fig. 3. CM-cellulose chromatography of kinase F_A activities in the liver extracts in the presence and absence of Triton X-100.
40 grams of fresh pig liver were homogenized in the absence (A) and presence (B) of 1% Triton X-100. The resulting supernatants after glass wool filtration were directly absorbed onto a CM-cellulose column (2.5 x 25 cm) and eluted with a 0 to 0.25 M salt gradient (---). The F_A activities measured as the type-1 phosphatase activator and using phosphorylase a as the substrate (o) were assayed in the collected fractions basically as described in " Methods ". Protein concentrations were estimated (●) at the absorbance of 280 nm.

membrane which can not be detected unless treated with Triton X-100 (see Figs 1 and 2).

In order to further demonstrate that the stimulated F_A activity by Triton X-100 is indeed the kinase F_A itself, we next applied the tissues extracts to a CM-cellulose column (2.5 x 25 cm) and eluted the enzyme with a linear salt gradient going from 0 to 0.25 M NaCl. As shown in Fig. 3A, a small sharp symmetric activity

peak of F_A could be detected in the CM-cellulose column when liver tissue was homogenized in the absence of Triton X-100. In contrast, when liver was homogenized in the presence of 1% Triton X-100 before applied to the same CM-cellulose column and processed in an identical manner, this activity peak of F_A was found to be enhanced many-fold and eluted at the same place on the CM-cellulose chromatography (see Fig. 3A and 3B). Figs. 4 and 5 further show that this observation also exists in pig brain and rabbit cardiac muscle. As a matter of fact, these observations could also be extended to rabbit smooth muscle and skeletal muscle (not shown). All the results taken together demonstrate that F_A is a membrane-associated protein kinase in most mammalian tissues.

The findings that the activating factor F_A of the multi-substrate protein phosphatase and glycogen synthase kinase-3 are the same protein in the non-nervous tissues (4,5) and the identity of F_A and myelin basic protein kinase in the nervous tissues (8) have presented an intriguing problem. Because of its unique nature, F_A have been proposed to play a pivotal role in the regulation of diverse cell functions and is likely a prime target for hormonal and neural action (1-12). The new discovery presented in this report that F_A is in fact a membrane-associated protein kinase further supports the notion. It is highly possible that hormonal and/or neural actions may be mediated through the translocation of this membrane-associated F_A into the cytosol to carry out the biochemical reactions and fulfill the physiological response of the extracellular stimuli. The relationship between the membrane-associated F_A and cytosolic F_A is clearly an intriguing problem which needs further investigations. The results presented here also provide concrete evidence that during purification and isolation of this enzyme from mammalian tissues, Triton X-100 is a crucial factor which should be included in order to get good



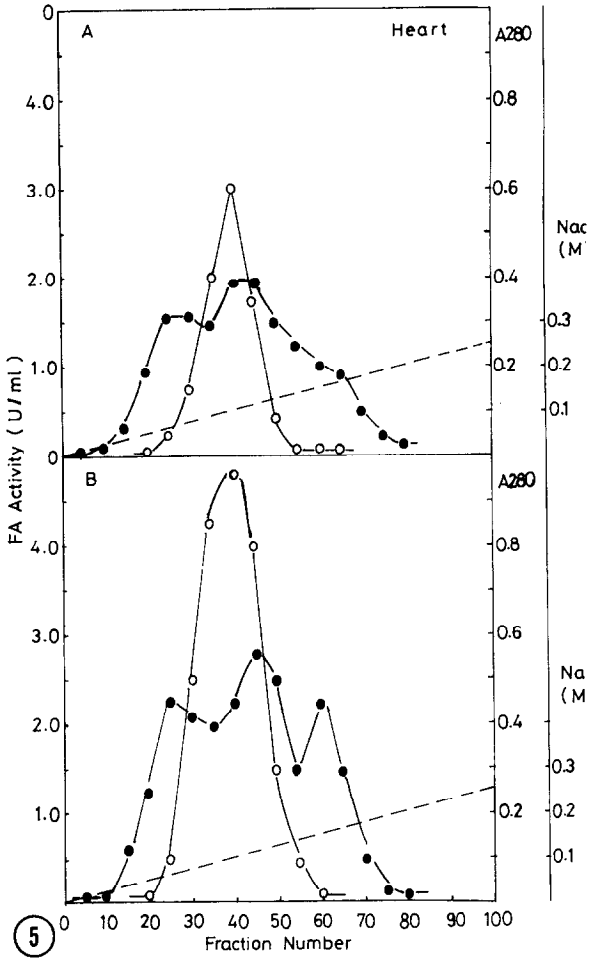


Fig. 4. CM-cellulose chromatography of kinase F_A activities in the brain extracts in the presence and absence of Triton X-100. 40 grams of fresh pig brain were homogenized in the absence (A) and presence (B) of 1% Triton X-100. The resulting extracts were processed in an idetical manner basically as described in the legend of Fig. 3. (---) salt gradient, (o) F_A activity, (●) optical density at 280 nm.

Fig. 5. CM-cellulose chromatography of kinase F_A activities in pig heart extracts in the presence and absence of Triton X-100.
40 grams of fresh pig heart were homogenized in the absence (A) and presence (B) of 1% Triton X-100. The resulting supernatants were next processed in an identical manner as described in the legend of Fig. 3. (---) salt gradient, (o) F_A activity and (●) optical density at 280 nm.

yield of this unique protein kinase for biochemical and biological studies.

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