Vol. 151, No. 1, 1988
February 29, 1988

BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
Pages 61-69

INSULIN INDUCES ACTIVATION AND TRANSLOCATION OF PROTEIN KINASE FA (A MULTIFUNCTIONAL PROTEIN PHOSPHATASE ACTIVATOR) IN HUMAN PLATELET

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Received January 7, 1988

Protein kinase FA (an activator of the ATP.Mg-dependent multifunctional protein phosphatase) has been identified in both cytosol and plasma membrane isolated from human platelets. The FA activity in the cytosol is active whereas the FA activity in the membrane is inactive. Quantitative analysis further indicates that approximately 90% of total FA is present in the membrane whereas only 10% of FA is localized in the cytosol, suggesting that the inactive membrane-associated FA might be regulated. This notion has subsequently been demonstrated that exposure of platelets to physiological concentrations of insulin for only 1 min resulted in an increase in cytosolic FA activity to about 300% of control values in the absence of insulin and in a corresponding decrease in FA activity in the membrane. It is concluded that the molecular basis for insulin action on cellular metabolism may partly be mediated through the activation and translocation of protein kinase FA in the membrane. It is suggested that redistribution of protein kinase FA may represent a transmembrane signal of insulin.

The molecular basis for insulin action on cellular metabolism involved the modulation of the phosphorylation states of many key regulated enzymes (1-5). It is thought that insulin may regulate certain multifunctional protein kinase(s) and/or phosphatase(s) in order to mediate such actions. Part of this hypothesis has been demonstrated by reports that phosphorylation of ribosomal S6 protein is markedly increased by insulin in 3T3-L1 fibroblasts (6-8). However , the molecular mechanism by which insulin may possibly modify a

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0006-291X/88 $1.50

multifunctional protein phosphatase activity has never been put forward.

An ATP.Mg-dependent multifunctional protein phosphatase has been identified in mammalian nervous and non-nervous tissues (9,10). The enzyme exists in an inactive form consisting of a catalytic subunit termed Fc and a modulator subunit termed M, a phosphatase inhibitor (11). This inactive FcM complex can be activated in the presence of ATP.Mg and an activating factor termed FA, a cAMP- and Ca^{2+}-independent protein kinase (12-17). The activated phosphatase becomes capable of modifying activities of many key regulated enzymes involved in the regulation of diverse cell metabolism and functions (2-4, 18-20). Due to its multifunctional nature, the activation of the ATP.Mg-dependent protein phosphatase might be a target for insulin action. However, since the control mechanism for the ATP.Mg-dependent activation by kinase FA is not yet fully understood, the proposed hormonal regulation of this multifunctional protein phosphatase remains a subject for speculation.

In a recent report (21), we have presented initial evidence that a substantial amount of protein kinase FA is localized in mammalian plasma membranes in spite of the fact that FA has long been recognized as a cytosolic enzyme (9-20). Since the ATP.Mg-dependent phosphatase is present exclusively in the cytosol, the membrane-associated FA should be subjected to regulation in order to mediate the activation of the inactive phosphatase (21). In this report, we have used human platelet to demonstrate that insulin indeed can induce activation and translocation of membrane-bound FA to the cytosol in a time- and concentration-dependent manner. The molecular mechanism by which insulin may modify a multifunctional protein phosphatase activity is therefore proposed.

MATERIALS AND METHODS

Materials : [γ-^{32}P]ATP was purchased from ICN. Human platelets were provided by Veterans General Hospital in Taipei, Taiwan, Republic of China. Porcine insulin was obtained from Lilly.

Human Platelet Incubation and Preparation of Cytosol and Membrane fractions : Human platelets (1 x 10^9 cells/ml) were incubated in isotonic buffer containing 250 mM sucrose, 1 mM magnesium chloride, 1 mM calcium chloride, 4 mM EDTA and 20 mM Tris-HCl pH 7.0 in the absence and presence of physiological concentrations of insulin at 30°C for various periods of time. The platelets were then homogenized in 10 volumes of buffer A containing 20 mM Tris-HCl at pH 7.4, 200 mM sucrose, 0.5 mM dithiothreitol, 4 mM EDTA and 0.5 mM phenylmethylsulfonyl fluoride. The homogenate was centrifuged at 100,000 x g for 30 min and the resulting supernatant was used as the cytosolic fraction. The pellets were used as the particulate fractions. For further purification of the plasma membrane, the pellets after extensive washing were resuspended in a final concentration of about 1.6 M sucrose in buffer B containing 20 mM Tris-HCl 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 B. This gradient was then centrifuged at 90,000 x g for 2 h. in a Beckmam SW 41 swinging bucket rotor. The plasma membranes which were located at the sucrose interface was harvested and further purified with the second sucrose gradient as described above. The purified fractions were finally resuspended in buffer B and used as the plasma membrane. Platelet membranes were purified basically according to Barber and Jamieson (22).

Protein Purification : Phosphorylase b (23), phosphorylase b kinase (24) and ^{32}P-phosphorylase a (25) were prepared from rabbit skeletal muscle. The kinase FA/ATP.Mg-dependent protein phosphatase (10) and its activator kinase FA (17) were purified from pig brain.

Enzyme assays : The activities of the ATP.Mg-dependent protein phosphatase and its activator FA were determined by methods described in previous reports (10,12,17). Briefly, the activity of ATP.Mg-dependent phosphatase was typically measured after a 10-min preincubation at 30°C with 0.1 mM ATP, 0.5 mM magnesium ions and a saturating amount of FA required for full activation of the inactive phosphatase under the assay conditions. ^{32}P-phosphorylase a was used as the substrate. Assay time was 5 min. The activity of FA as the activator of ATP.Mg-dependent phosphatase was measured by the formation of activated phosphatase in a 1-min incubation at 30°C. The assay mixture contained appropriate dilutions of FA, 0.1 mM ATP, 0.5 mM magnesium ions and excess amount of inactive phosphatase to ensure linear activation of the phosphatase 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 the activator of the ATP.Mg-dependent phosphatase is that amount of enzyme that produces one unit of activated phosphatase after 1-min preincubation

RESULTS AND DISCUSSION

As shown in Fig. 1A, the cytosolic fraction of human platelet contains a substantial amount of FA activity assayed as an activator

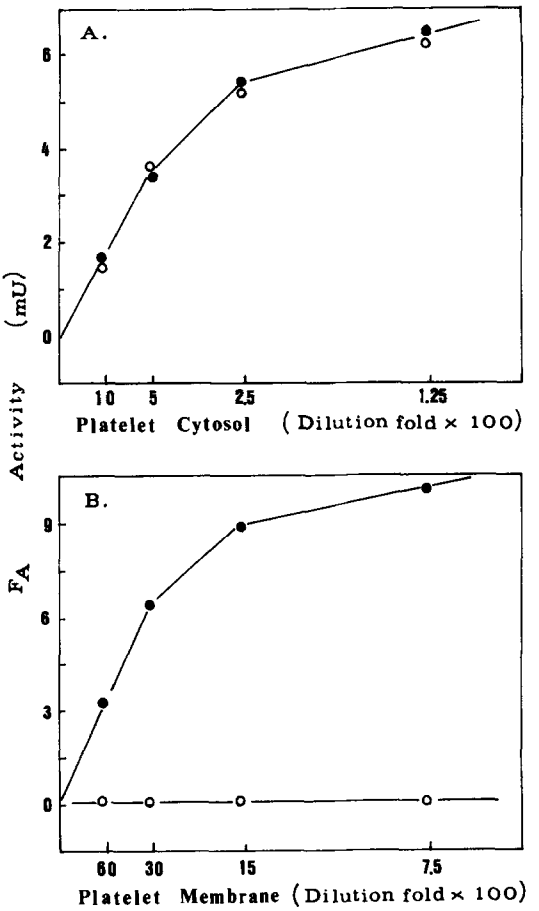

Fig. 1. Identification and subcellular localization of protein kinase FA in human platelets.

A. The cytosolic fractions of human platelets were first sonicated in the presence (●) and absence (o) of 1% Triton X-100 at 25°C. The fractions of 0.01 ml after appropriate dilutions were next treated with 0.1 mM ATP, 0.5 mM magnesium chloride and 100 mU_{32} of pure ATP.Mg-dependent phosphatase at 30°C for another 5 min. ^{32}P-phosphorylase a was used as the substrate. The total assay volume was 0.03 ml. Definition of FA and detailed assay conditions were as described in Method. The final volume of the cytosolic fraction prepared from 100,000 human platelets was 1 ml.

B. The same as in A except that the cytosolic fractions were replaced by the particulate fractions isolated from human platelets. The particulate fractions isolated from 100,000 human platelets was routinely resuspended in 1 ml of buffer B.

of ATP.Mg-dependent protein phosphatase (9-13). Treatment of the platelet cytosol with 1% Triton X-100 followed by sonication at 25°C did not cause any stimulatory effect on the FA activity, indicating that FA exists in an active state in the platelet cytosol (see Fig. 1A). In sharp contrast, the platelet pellets do not

contain any FA activity which is detectable when assayed under the same conditions. However, when platelet pellets were first sonicated in 1% Triton X-100 prior to the assay, the FA activity was very dramatically stimulated, demonstrating that FA exists mostly in an inactive form in the particulate fractions of the human platelet. This is illustrated in Fig. 1B. It is noted that 1% Triton X-100 could not bring out any direct effect on FA when the enzyme was in a detergent-solubilized state. In order to rule out the possibility that factors other than FA might be involved in the phosphatase activation and thereby interfered with the determination of the FA activity in the crude cellular fractions, we had measured the phosphatase activation in the absence of ATP and/or Mg^{2+} ions. We found that both cellular fractions could not bring out any phosphatase activation under these conditions (not shown). Taken together, the results support the notion that the phosphatase activation in the platelet subcellular fractions as described above is a very specific reflection of the FA activity. Quantitative analysis further indicates that approximately 90% of FA is associated with the platelet pellet fraction whereas only 10% of FA is localized in the cytosolic fraction (see Fig. 1). Although protein kinase FA has long been recognized as a cytosolic enzyme (3-4, 9-20), the current data strongly suggested that the majority of FA is associated with the membrane fractions of the human platelet. To further establish the point that FA is indeed a membrane-associated protein, the platelet pellets were extensively purified and analyzed by 8-30% sucrose gradient. FA was found to co-migrate with membranes at a high molecular weight in contrast to free FA which has a low molecular weight of about 45,000 (Fig. 2), demonstrating that FA is indeed a membrane-associated protein. Since ATP.Mg-dependent protein phosphatase is present exclusively in the cytosol, the results further suggested that the membrane-bound FA might be regulated.

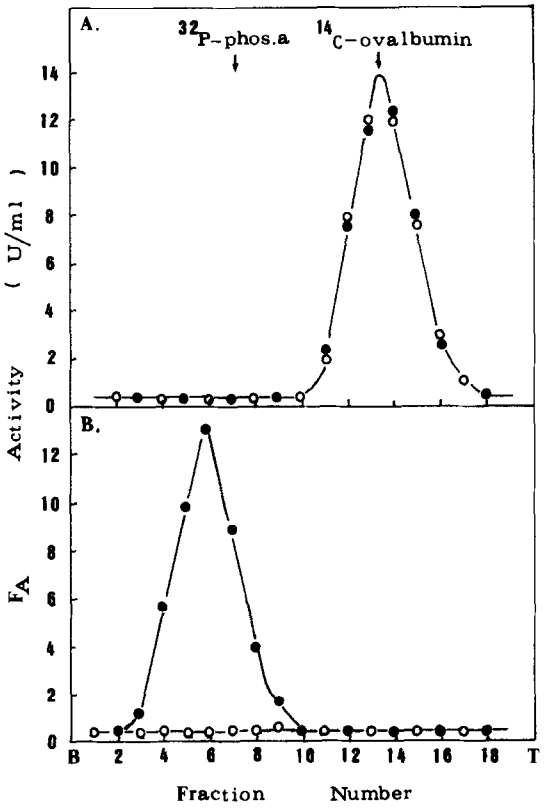

Fig. 2. Identification of FA-membrane complex on 8-30% sucrose density gradient ultracentrifugation.
8-30% sucrose density gradient ultracentrifugation was performed using the following samples : A. 20 units of purified free FA, and B. the purified human platelet plasma membrane containing about 20 units of membrane-bound FA. The samples were centrifuged at 4°C in SW 50.1 rotor at 45,000 rpm. for 12 h. Fractions of 0.2 ml were collected. The FA activity in each fraction was assayed in the absence (o) and presence (●) of 1% Triton X-100. Assay conditions were as described in Method.

Since protein kinase FA might be a target for insulin action, we therefore measured the FA activity in both cytosol and membrane fractions of platelets that were treated or untreated with insulin. As shown in Fig. 3, exposure of platelets to physiological concentrations of insulin resulted in an increase in cytosolic FA activity to 300-400% of control values and in a corresponding decrease in FA activity in the membrane. The insulin effect was dose-dependent. Further, this activation-translocation process induced by insulin occurred very rapidly, reaching the maximal effects within 1 min, suggesting that the redistribution of FA is an early step in the

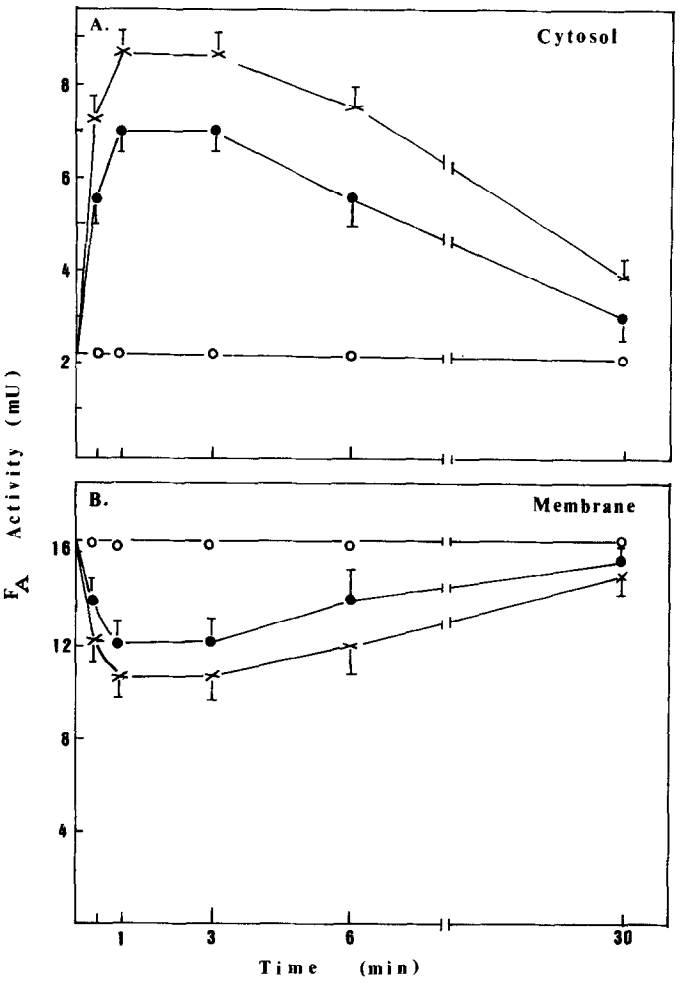

Fig. 3. Time course and concentration dependence of insulin effects on the activation and redistribution of protein kinase FA in human platelets.
Isolated human platelets were treated with 10^{-9}-10^{-8} M insulin for the indicated time points. The FA activities in the cytosolic fractions (A) and in the Triton X-100-solubilized membrane fractions (B) were determined as described in Method. Data are pooled from five independent experiments, each point using a separate tube and expressed as the mean and S.E. (bars) of the net amount of the FA activity assayed the activator of the ATP.Mg-dependent protein phosphatase. Symbols are as follows : (o) control values without insulin, (●) with 10^{-9} M insulin and (x) with 10^{-8} M insulin. At each point, 20,000 platelets were used and both cellular fractions were routinely suspended in 0.2 ml of buffer B. The resuspended fractions of 0.01 ml after appropriate dilutions were used per assay. The total assay volume was 0.03 ml.

post-receptor activation cascade following insulin. After prolonged treatment of the cells with insulin, the FA activities in both cytosol and membrane gradually and simultaneously returned to control levels

(see Fig. 3), indicating that the subcellular redistribution of FA by insulin is a transient process. When insulin was directly added to the cytosolic fractions of the platelets, the FA activity was not affected (not shown), indicating that insulin does not have any direct effect on the activation of the cytosolic version of the enzyme. Taken together, the results further support the notion that indeed it is the FA protein that is activated in the membrane and translocated to the cytosol by insulin.

Although the physiological function of protein kinase FA in human platelets remains to be elucidated, the present study clearly demonstrates that protein kinase FA, an activator of a multifunctional protein phosphatase is present mainly in the plasma membrane and that its activity can be modulated by insulin. Further, FA has recently been identified as a multisubstrate protein kinase. In addition to ATP.Mg-dependent protein phosphatase as its substrate, FA is capable of acting on many substrate proteins including glycogen synthase (13,14), the R subunit of cAMP-dependent protein kinase (26) , the nerve growth factor receptor proteins (27) , myelin basic protein (17) and many endogenous substrate proteins in brain and platelet (Yang, unpublished observations). Involvement of FA in the action of insulin may eventually explain both an increased and a decreased phosphorylation in the cell (1-5). By its dual role as a multifunctional protein kinase and as a multifunctional protein phosphatase activator, FA could simultaneously modulate phosphorylation and dephosphorylation states of many diverse substrates in mammalian tissues. The generation of protein kinase FA from plasma membrane may represent a transmembrane signal which will set into motion a cascade of phosphorylation-dephosphorylation reactions and may eventually lead to the expression of some or all of the metabolic effects of insulin.

ACKNOWLEDGEMENTS

This work was supported in part by the National Science Council in Taiwan, Republic of China.

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