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PRESENCE OF AN INSULIN-STIMULATED SERINE KINASE IN CELL EXTRACTS FROM IM-9 CELLS

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ABSTRACT: Insulin responsive protein kinase activities of wheat germ purified glycoproteins were examined. Glycoproteins were first incubated without or with insulin, and then exposed to a serum containing antibodies to insulin receptor. Thereafter, both immunoprecipitates and supernatants were studied for their kinase activity toward histone. Incubation with anti receptor antibodies promoted insulin receptor β subunit and histone phosphorylation. More important insulin receptor depleted extract contained a kinase activity toward histone, that was increased by preincubation with insulin. This stimulation was observed only when insulin was added before the immunoprecipitation of insulin receptors. Alkali treatment and phosphoamino acids analysis revealed that the kinase activity remaining in the supernatant is serine specific. These findings suggest, (i) that a serine kinase activity is associated with the insulin receptor, (ii) that it can be separated from the insulin receptor with anti receptor antibodies, (iii) that the serine kinase is activated by the hormone-receptor complex.

While the binding of insulin to its receptor is clearly the first step in the process of generating the diverse biological responses to insulin, the molecular events that underlie insulin action remain unclear. The current most promising hypothesis is that some of, or all, the effects of insulin are mediated through the tyrosine kinase activity of its receptor, a view supported by numerous observations detailed in recent reviews (1,2). While evidence has emerged to suggest that insulin receptor tyrosine kinase activity is involved in transmembrane signaling, the next link in the chain of events leading to insulin effects is missing. Many of those effects are caused by changes in the state of phosphorylation of various enzymes, which occur on serine or threonine residues (3-5). In some cell free systems, partially purified insulin receptor display an insulin-responsive serine

Abbreviation used: SDS/PAGE: sodium dodecylsulfate/polyacrylamide gel electrophoresis.

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kinase activity for both phosphorylation of receptor and substrates (6-12). In addition, in intact cells insulin promotes phosphorylation of its receptor on serine residues in addition to the initial tyrosine phosphorylation (6,13,14). This data favor the idea that insulin stimulates a serine kinase activity by a currently unknown mechanism. To further approach this mechanism we have studied serine-specific protein kinase activity found in wheat germ purified glycoproteins preparation derived from IM-9 cell line. By using auto-antibodies to insulin receptors this insulin responsive serine kinase activity could be separated from the tyrosine kinase activity carried out by the β subunit of the insulin receptor.

MATERIALS AND METHODS

Materials Triton X-100, N-acetyl-glucosamine, wheat-germ agglutinin-agarose, histone H2B, phosphoamino acids and bacitracin were purchased from Sigma (Saint Louis, MO, USA). Adenosine 5' (γ-^{32}P) triphosphate was from Amersham ; Porcine insulin was a gift from Novo Research Institute (Copenhagen, Denmark); protein A (Pansorbin) from Calbiochem. All reagents for SDS/PAGE were from BioRad Laboratories (Richmond, California). Serum from patient B2 containing autoantibodies to insulin receptors was a gift from Dr. C.R. Kahn (Joslin Research Laboratories, Boston, MA, USA).

Immunoprecipitation of insulin receptors. Insulin receptors partially purified from IM9 lymphocytes (15) were incubated for 15 hours at 4°C with antireceptor antibodies (serum B2 at a 1:100 dilution; immunoglobulin G concentration: 200 μg/ml). Immunoprecipitation was achieved by addition of protein A for 1 hour at 4°C.

Phosphorylation assay. Insulin receptors, partially purified or immunoprecipitated, and supernatants of the immunoprecipitation were incubated with the phosphorylation mixture containing $MnCl_2$ (4mM), $MgCl_2$ (8mM) and (γ-^{32}P)-ATP (15μM) after addition of 100μg histone. After 15 min at 18°C the reaction was terminated by addition of a boiling solution containing SDS* (3%, w/v), glycerol (10%, v/v), β-mercaptoethanol (2%, v/v) and bromophenol blue (w/v, 0.01%). The phosphoproteins were analyzed by one dimensional SDS/PAGE (16). Phosphoamino acids were analysed as previously described (17).

RESULTS

Partially purified insulin receptors (100-200 fmoles) were either directly used in a cell-free phosphorylation assay, or first immunoprecipitated and then subjected to a phosphorylation reaction. Insulin (10^{-7}M) led to autophosphorylation of the receptor β-subunit and to increased ^{32}P incorporation in histone (Fig. 1, lanes A and B). After immunoprecipitation with antireceptor serum at a 1/100 dilution, the insulin receptors have markedly increased kinase activity for both autophosphorylation and phosphorylation of

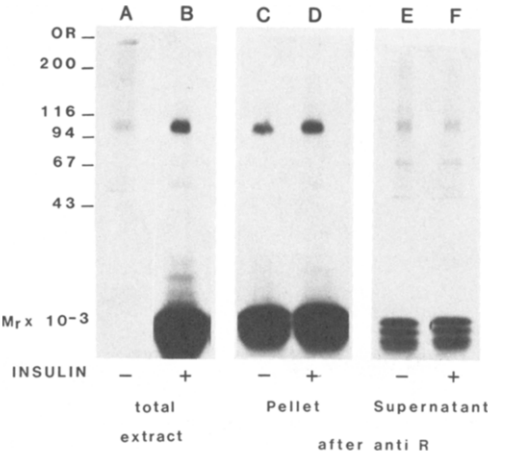

FIG. 1 Phosphorylation of histone H2B by insulin receptor depleted cell extract. Wheat germ purified glycoproteins from IM-9 lymphocytes were incubated without (lanes A,B) or with antireceptor serum (lanes C-F) for 15 hours at 4°C. The total cell extract and the supernatant were incubated without or with insulin for 1 hour at 18°C. Thereafter, histone H2B (100 µg) was added to the samples and the phosphorylation reaction was performed for 15 min at 18°C. Proteins were analyzed by SDS/PAGE under reducing conditions using a 5 to 15% linear gradient of acrylamide as resolving gel.

histone (Fig. 1, lane C). Further, insulin increased this receptor enzyme activity (Fig. 1, lanes C and D). Immunoprecipitation depleted the glycoprotein extract completely from insulin receptors as shown by the absence of phosphorylated receptor β-subunits in the supernatant. Histone phosphorylation by supernatant depleted from insulin receptor (Fig. 1, lanes E and F) was enhanced compared to the basal phosphorylation (Fig. 1, lane A). The increased kinase activity in the supernatant and in the pellet after exposure to anti-receptor serum is likely to be due to an insulinomimetic effect of the antibodies themselves rather than to insulin present in the serum. Indeed at a 1/100 dilution of this serum, the insulin concentration amounts only to 10^{-11}M, which does not induce a detectable increase in the receptor kinase activity.

We next wished to look for an insulin-sensitive protein kinase activity which is distinct from the receptor kinase itself, but which co-purifies with it. To this end a lectin-purified cell extract obtained from IM-9 lymphocytes was incubated without or with insulin, then exposed to antireceptor anti-

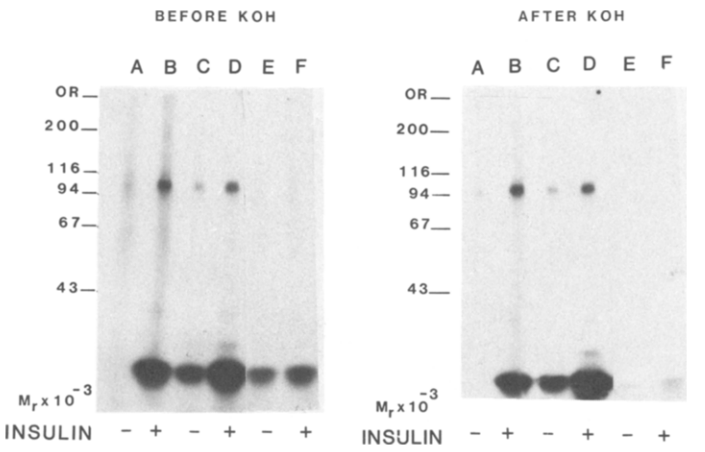

FIG. 2 Phosphorylation of histone H2B by an insulin-stimulated kinase distinct from the insulin receptor. Wheat germ purified glycoproteins were incubated without or with insulin for 1 hour at 18°C, and thereafter without (lanes A,B) or with anti-receptor serum (lanes C,F) for 15 hours at 4°C. The total extract (lanes A,B), the immune precipitates (lanes C,D) and the supernatants (lanes E,F) were phosphorylated in the presence of 100 µg of histone for 15 min at 18°C. The proteins were analyzed by SDS/PAGE (left panel). Following autoradiography, the gel was treated with KOH 1M for 2 hours at 55°C, dried and autoradiographied (right panel).

bodies, and finally both the immunoprecipitates and the supernatants were studied in a cell-free phosphorylation assay. In the supernatants, no phosphoprotein with an electrophoretic mobility appropriate for the receptor β-subunit (Mr 95000) could be identified, indicating complete depletion of insulin receptors by immunoprecipitation (Fig. 2, left panel lanes E and F). However, ^{32}P incorporation in histone was increased by approximately 40% when cells extract has been first incubated with insulin compared with a supernatant of an immunoprecipitation without prior incubation with insulin (Fig. 2, left panel, lanes E and F). To further document that this insulin-stimulated kinase activity found in supernatants was distinct from the tyrosine kinase activity of the insulin receptor itself, the gel was subjected to a KOH treatment, which breaks up phosphoserine bonds without affecting significantly phosphotyrosine bonds (17) (Fig. 2, right panel, lanes C and D). Alkali treatment of the gel led to a virtually complete disappearance of the labelling of histone induced by a supernatant from an immunoprecipitation of a cell extract which had been incubated with insulin

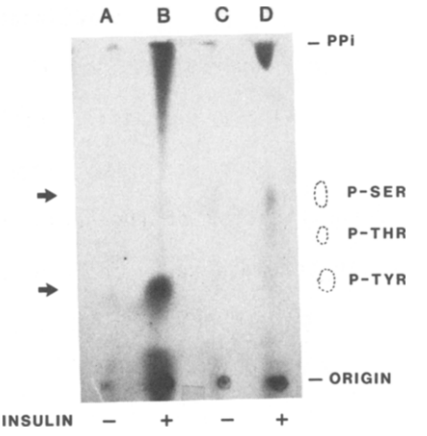

FIG. 3 Phosphoamino acids analysis of histone phosphorylated by the insulin receptor kinase and associated serine kinase. Partially purified insulin receptors were incubated for 1 hour at 18°C without insulin or with insulin. The samples were subjected to immunoprecipitation by anti-receptor serum. The immune precipitates (lanes A,B) and the supernatants (lanes C,D) were phosphorylated in the presence of 100 µg of histone for 15 min at 18°C. Samples were analyzed by SDS/PAGE. The labelled bands corresponding to the histone were excised from the gel and treated for phosphoamino acids analysis as described.

(Fig. 2, right panel, lanes E and F) without significantly modifying the labeling of the bands obtained with immunoprecipitated insulin receptors. This results were confirmed by phosphoamino acid analysis of histones. With immunoprecipitated receptors, previously stimulated by insulin, the phosphorylation of histone occured solely on tyrosine residues (Fig. 3, lane B). In contrast, with the supernatants of the cell extract incubated first with insulin, only phosphoserine could be detected in the histone preparation (Fig. 3, lane D).

DISCUSSION

In the present work we show that an insulin responsive serine kinase can be separated from the receptor by anti receptor antibodies. Interestingly the treatment of the glycoproteins by B2 serum led to the activation of a kinase remaining in the supernatant. More important, insulin was unable to stimulate this kinase activity, if the hormone was added to the supernatant depleted in insulin receptors. Nevertheless, when insulin was added prior immunoprecipitation, the kinase activity found in the supernatant was enhanced, indicating that insulin effect was mediated by the interaction of insulin

with its receptor. Alkali treatment and phosphoaminoacids analysis confirmed that the kinase remaining in supernatant is a serine specific kinase activity. Further this serine kinase copurified with the insulin receptor after wheat germ agarose column but was lost after purification by anti receptor antibodies. The copurification of the serine kinase with the insulin receptor by wheat germ lectin chromatography could be due to its glycoprotein nature, or to its close physical association to the receptor molecule, or both.

Two hypotheses for the activation of this serine kinase by insulin could be envisaged (i) the activation of the serine kinase could be dependent on its phosphorylation by the insulin receptor tyrosine kinase as it was previously described for the insulin receptor activation (18,19). However it appears that a phosphorylation of the serine kinase by receptor tyrosine kinase is not required. Indeed, the insulin-induced activation of the serine kinase is observed in the absence of ATP and divalent ions (Mn^{2+}, Mg^{2+}), which are expected to be essential for kinase functioning. (ii) the insulin receptor and the serine kinase could be physically associated. Binding of insulin to receptor would lead to separation and activation of the serine kinase. It should be noted that the separation of the two kinases and the activation of the serine kinase was also obtained when an insulinomimetic agent, such as the anti receptor serum, was used (Fig. 1). The above view of the regulation of the serine kinase upon insulin binding is reminiscent of the mechanism previously suggested to explain the activation of Acetyl CoA carboxylase in fat cells (5).

Finally, the nature and role of the insulin responsive serine kinase found in our cell-free system are entirely unknown. It is likely that further characterization of the serine kinase activity found here will permit to establish wether it is responsive in intact cells for serine phosphorylation of the insulin receptor, and/or other cellular proteins. This will help significantly to decipher the role of phosphorylation in receptor functioning and insulin action.

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