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pp-120: A COMMON ENDOGENOUS SUBSTRATE FOR INSULIN AND IGF-1 RECEPTOR-ASSOCIATED TYROSINE KINASE ACTIVITY IN THE HIGHLY MALIGNANT AS-30D RAT HEPATOMA CELLS

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Receptors for Insulin, Epidermal Growth Factor, Platelet-Derived Growth Factor and Insulin-like Growth Factor type 1 are tyrosine-specific protein kinases. This enzymatic activity may play a role in mediating the biological actions of these peptides. It has recently been identified a Mr 120 KDa glycoprotein in rat liver plasma membranes which can be phosphorylated by the insulin receptor and by the EGF receptor in a cell-free system and by the insulin receptor in intact cultured H-35 hepatoma cells. In the present report it is shown that the solubilized Insulin-like Growth Factor type 1 receptor can phosphorylate tyrosine residues in the same 120 KDa glycoprotein from the AS-30D rat hepatoma cells.

Receptors for insulin, Epidermal Growth Factor (EGF), Insulin-like Growth factor (IGF-1) and Platelet-Derived Growth Factor (PDGF) possess tyrosine specific protein kinase activity (1-4).

It has recently been demonstrated that the receptor-associated protein kinase activity plays an important role in mediating the biological action of these peptides (5).

Several laboratories have been looking for the physiological substrates for receptor-associated tyrosine kinase activity in liver or fat cells (6,7). In particular, the insulin and the EGF receptors have been shown to induce tyrosine phosphorylation of a 120 KDa membrane-associated phosphoprotein (pp-120) in a cell-free system (8-10).

The insulin receptor has been shown to induce tyrosine phosphorylation of the same protein in intact H-35 hepatoma cells (11).

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Since both normal hepatocytes as well as H-35 rat hepatoma cells do not express IGF-1 receptor (12,13), it has never been demonstrated an effect of this receptor on pp-120 phosphorylation.

In the present work the AS-30D highly glycolytic, highly malignant hepatoma cell line (14) was used to demonstrate that these cells express both insulin and IGF-1 receptors and that both receptors are able to induce tyrosine phosphorylation of pp-120 in a cell-free system.

METHODS

Microsome preparation.

Microsomes were prepared from livers of 100-150 g female Sprague-Dawley rats, as described previously (8). Microsomes were then solubilized in a buffer containing 50 mM Hepes, pH 7.80, 150 mM NaCl, in the presence of 1% Triton X-100.

The extracts were partially purified by chromatography over wheat germ agglutinin-agarose (Vector Laboratories, Burlington, CA.).

AS-30D rat hepatoma cell line was obtained from Dr. P.L. Pedersen, Department of Biological Chemistry, The Johns Hopkins University, School of Medicine, Baltimore, MD.

Cells were grown in ascitic form by intraperitoneal injection into 100-150 g female Sprague Dawley rats. AS-30D cells were pelleted from the ascitic fluid and membranes were prepared by differential centrifugation (15).

Protein phosphorylation.

Aliquots from the wheat germ agglutinin-agarose eluate from either rat liver or AS-30D hepatoma cells with comparable protein concentration were incubated in the presence of insulin or IGF-1 at concentrations ranging from 10^{-9} to 10^{-7} M. After incubation for 60 minutes at room temperature, phosphorylation was initiated by the addition of a reaction mixture containing γ [^{32}P] ATP (10 mCi/ml, 3000 Ci/mmole, New England Nuclear), 25 mM Mn-acetate, 0.025 mM unlabeled ATP, 5 mM CTP, as described previously (8).

Phosphorylation reaction was allowed to proceed for 20 minutes and was then terminated by the addition of a stopping solution (50 mM Hepes, pH 7.80, 16 mM EDTA, 320 mM NaF, 32 mM Na-pyrophosphate, 3.2 mM Na-orthovanadate, 0.1% Triton X-100.

In some experiments (Fig. 1, Panel A and B) samples were diluted with 4x electrophoresis sample buffer and directly analyzed by SDS polyacrilamide gel electrophoresis followed by autoradiography. In other experiments (Fig. 2, Panel A and B) pp-120 was immunoprecipitated with R2-6 rabbit anti pp-120 antiserum (8).

Immunoprecipitates were then analyzed by SDS-polyacrylamide gel electrophoresis and autoradiography. Porcine insulin was purchased by Sigma, St. Louis, MO., threo-59-IGF-1 was purchased by Amgen Biological, Thousand Oak, Ca.

Phosphoaminoacid analysis.

After pp-120 was identified by autoradiography the γ[^{32}P]-labeled bands were excised. Phosphoaminoacids were prepared by acid hydrolysis of the

protein and identified by high voltage electrophoresis at pH 3.5 followed by autoradiography (11).

RESULTS AND DISCUSSION

It has previously been shown that both the insulin and the EGF receptors are able to induce phosphorylation of a 120 KDa glycoprotein which is present in detergent-extracts of rat liver microsomes. The IGF-1 receptor shares a high degree of structural homology with the insulin receptor as well as with the EGF receptor (16).

A highly malignant, highly glycolytic cell line was used which expresses both insulin and IGF-1 receptors to show that both receptors independently induce phosphorylation of the same 120 KDa glycoprotein.

Insulin, but not IGF-1, is able to induce phosphorylation of the ß subunit of its own receptor in lectin-purified microsomal preparations from rat liver plasma membranes (Fig.1, panel A). Since insulin induces phosphorylation at very low concentrations (as low as 1 nM; Fig. 1, lane B) whereas IGF-1 is ineffective at high concentrations, we conclude that insulin receptors, but not IGF-1 receptors, are represented on normal rat liver membranes.

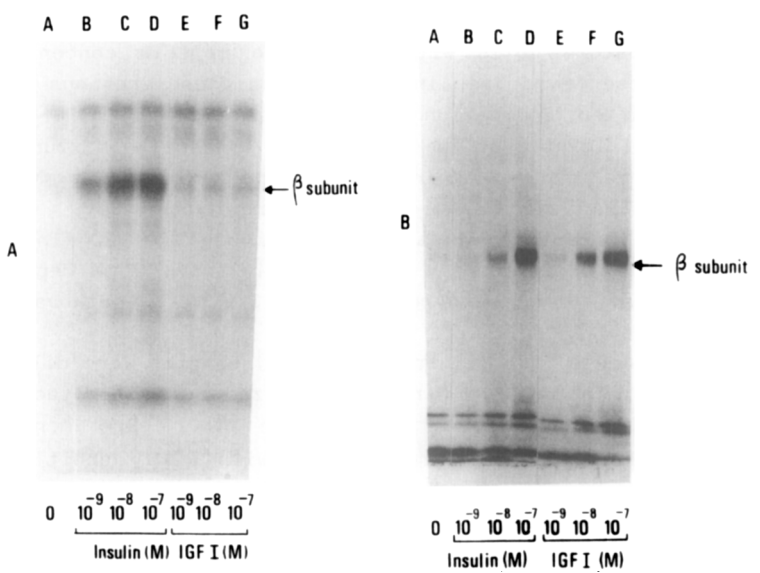

Fig.1. Stimulation of protein phosphorylation by insulin and IGF-1. Wheat germ agglutinin-purified solubilized plasma membrane preparations from normal rat liver (A) and AS-30 D cells (B),were incubated with $\gamma[^{32}P]$ATP in the absence or in the presence of increasing concentrations of insulin and IGF-1, as indicated. Samples were mixed with 4x electrophoresis sample buffer and analyzed by 7.5% SDS polyacrylamide gel electrophoresis, followed by autoradiography.

When lectin-purified plasma membrane preparations from AS-30D cells are used for phosphorylation experiments, it was observed that both insulin and IGF-1 are equally effective in inducing phosphorylation of the ß subunit of their own receptors. Since both the ligands seem to be effective at very low concentration (Fig 1, Panel B, lanes A-G), we presume that each ligand independently acts specifically upon its own receptor.

Similar results were obtained when the glycoproteins were subjected to immunoprecipitation with the specific antibody directed to pp-120 (R2-6).

Insulin, but not IGF-1, was able to induce phosphorylation of pp-120 in rat liver microsomal preparations (Fig 2, Panel A, lanes A-G), whereas both insulin and IGF-1 were able to induce phosphorylation of pp-120 when AS-30D cells were used as source of receptors (Fig. 2, panel B, lanes A-G). Phosphoaminoacid analysis of pp-120 phosphorylated in the presence of IGF-1 revealed that tyrosine is the main substrate for the IGF-1 receptor-associated kinase activity, as expected (Fig. 3).

Normal rat liver cells, as well as well differentiated rat hepatoma cells (H-35), do not express IGF-1 receptors. In the present report it has been showed that another hepatoma-derived cell line, the AS-30D cells, express

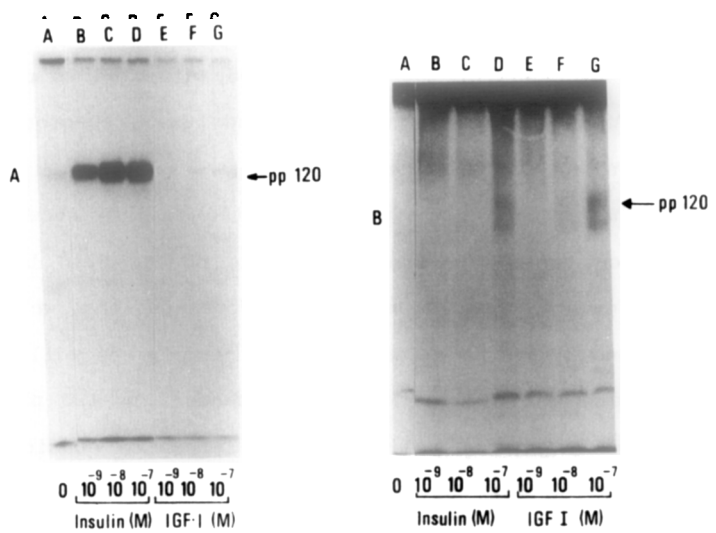

Fig. 2. Stimulation of protein phosphorylation by insulin and IGF-1. Lectin-purified plasma membrane glycoproteins from normal rat liver (A) and AS-30D cells (B) were labeled with $\gamma[^{32}P]ATP$, in the presence of increasing concentrations of insulin and IGF-1, as indicated. Phosphoproteins were then subjected to immunoprecipitation with anti-pp-120 antiserum. The immunoprecipitates were analyzed by 7.5% SDS polyacrylamide gel electrophoresis followed by autoradiography.

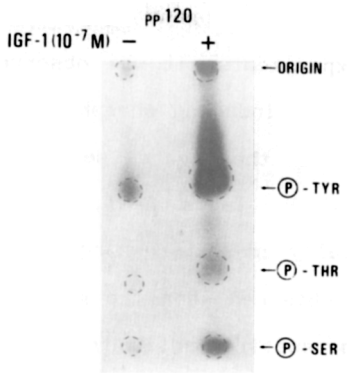

Fig. 3. Phosphoaminoacid analysis. The 120 KDa phosphoprotein obtained by specific immunoprecipitation of AS-30D derived, lectin purified plasma membranes, labeled with $\gamma[^{32}P]$ATP in the absence or in the presence of IGF-1, was cut from the gel, digested with HCl and applied to a high voltage, thin layer silica gel electrophoresis at pH 3.5, followed by autoradiography.

IGF-1 receptors. The expression of these receptors seems to be related to the degree of malignant transformation. As a matter of fact, another higly malignant hepatoma cell line, the BRL (Buffalo Rat Liver) cells, also show an enhanced expression of IGF-1 receptor (17).

When the lectin-purified glycoproteins are subjected to immunoprecipitation by means of the R2-6 antibody directed to pp-120, it was observed that, in AS-30D cells, both insulin and IGF-1 receptors are equally effective in inducing pp-120 phosphorylation.

Since IGF-1 has a very low affinity for the insulin receptor (15), so that no IGF-1-induced pp-120 phosphorylation is observed in the absence of IGF-1 receptors as occurs in rat liver membrane preparations, we conclude that pp-120 is an endogenous substrate for the IGF-1 receptor as well.

It has been demonstrated that insulin-dependent pp-120 phosphorylation is defective in type 2 diabetes mellitus (18). It can be considered of great interest the observation that pp-120 may serve as a substrate for insulin, IGF-1 and EGF receptor-associated tyrosine kinase activity, suggesting a possible common metabolic pathway for these receptors in determining cellular growth.

Recently pp-120 has been identified as an integral membrane glycoprotein of the bile canalicular domain (HA-4) (19).

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