Insulin Activates the Raf-1 Protein Kinase*

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Several growth factors and mitogens have been shown to activate the proto-oncogene product Raf-1 protein kinase in murine fibroblasts, apparently through a direct agonist-stimulated tyrosine phosphorylation of the Raf-1 protein. We investigated the possibility that insulin could also activate the Raf-1 kinase, since its receptor also contains an intrinsic insulin-activated protein tyrosine kinase activity. In several cell lines expressing relatively large numbers of insulin receptors, insulin rapidly stimulated the phosphorylation ofimmunoreactive Raf-1 protein. In H35 cells, a line of well differentiated rat hepatoma cells, the effect of insulin was maximal by 6 min and at 7 ns insulin and occurred normally in cells virtually completely depleted of protein kinase C activity. The insulin-stimulated increase in Raf-1 protein phosphorylation occurred concurrently with a 3-fold increase in Raf-1 protein kinase activity. However, phosphoamino acid analysis showed that only phosphoserine and a trace of phosphothreonine were present in the Raf-1 protein with the PDGF receptor in intact cells and that phosphorylation of the protein on tyrosine residues. Finally, the phosphorylation of the Raf-1 kinase in vitro led to a marked increase in Raf-1 kinase activity (4). Thus, Raf-1 was proposed as a protein kinase whose activation in cells was a direct result of phosphorylation on tyrosine residues by the PDGF receptor kinase (3-5).

Although Morrison et al. (3) found no activation or phosphorylation of Raf-1 in response to insulin, we evaluated this possibility in cells that express many more insulin receptors than the 3T3 cells used in their study. This approach had led to the discovery of several insulin effects that are not found in 3T3 fibroblasts (6-8). The studies described here demonstrate that insulin can rapidly activate the Raf-1 kinase in various insulin-responsive cell types; however, we could find no evidence that this activation was accompanied by tyrosine phosphorylation of the Raf-1 protein.

MATERIALS AND METHODS

Cells—The cell lines used in this study were: NIH EC, an NIH-3T3 cell derivative that overexpresses Raf-1 (9); HIR R, a rat 1 cell derivative that overexpresses the normal human insulin receptor (10); H35, a well differentiated rat hepatoma cell line (11, 12); and HIR 3.5, an NIH-3T3 cell derivative that overexpresses normal human insulin receptors (13). Cells were grown to confluence, changed to serum-free medium for 16 h, and incubated with 0.1 mCi/ml ^32P for 2 h as described (8, 14).

Immunoprecipitation after ^32P Cell Labeling—Serum-deprived cells were incubated with ^32P for 2 h; after exposure to insulin or other agents for the times specified, they were then washed three times with ice-cold Krebs buffer (14), scraped into 0.7 ml of a buffer containing 50 mM Tris-HCl (pH 8.3), 1.0% (v/v) Nonidet P-40, 5 mM EDTA, 150 mM NaCl, sonicated for 5 s (Branson sonifier, setting 2.5), incubated on ice for 15-30 min, and then centrifuged at 70,000 X g for 30 min at 4 °C. The supernatants were then diluted to achieve equal amounts of trichloroacetic acid-precipitable radioactivity in samples from a given cell line and incubated with 4 ml of normal rabbit serum/500 ml of sample at 4 °C for 1 h. To the samples was then added 25 ml of a 10% (v/v) suspension of Pansorbin that had been washed three times in 50 mM Tris-HCl (pH 8.3), 0.15 mM NaCl, 1 mM EDTA, and 0.6% (v/v) Nonidet P-40. The samples were then incubated with Pansorbin for a further 1 h at 4 °C and then centrifuged at approximately 13,000 X g for 5 min. To the supernatants was then added 4 ml of an antiserum (GF009) raised against a carboxyl-terminal dodecapeptide that is conserved in both the v-rap and c-Raf-1 proteins (15, 16). The samples were then incubated for 4 h at 4 °C, followed by a further 1-h incubation with Pansorbin, followed again by centrifugation for 13,000 X g. The resulting pellets were washed four times in the wash buffer described above for Pansorbin

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The abbreviations used are: PDGF, platelet-derived growth factor; SDS, sodium dodecyl sulfate; PAO, phenylarsine oxide.
and then resuspended in 100 μl of phosphate-buffered saline containing 1% (v/v) sodium dodecyl sulfate (SDS), 83 mM dithiothreitol, 10 mM EDTA, and 0.25 mM sucrose. The samples were placed in boiling water for 5 min, centrifuged at 12,000 × g for 5 min, and then 50 μl of the supernatants were separated by SDS-polyacrylamide gel electrophoresis (7.2% acrylamide), followed by autoradiography of the gel at −70°C.

Immunoprecipitation Kinase Assays—Serum-deprived H35 cells were exposed to control conditions or insulin (10 nM for 10 min). The cells were then washed four times with ice-cold phosphate-buffered saline and lysed in a buffer containing 50 mM Tris-HCl (pH 7.5), 150 mM NaCl, 1% (v/v) Triton X-100, 0.5% (w/v) sodium deoxycholate, 0.1% (w/v) sodium dodecyl sulfate, 5 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, 1 mM dithiothreitol, and 0.2 mM sodium orthovanadate. Insoluble material was removed by centrifugation at 4°C for 30 min at 12,000 × g. The samples were diluted to equal protein concentrations (approximately 5.5 mg/ml); protein was measured by the method of Bradford (17). The lysates were then incubated for 3 h at 4°C with a 1:250 dilution of antisera SP683 and a suspension of protein A-Sepharose; the immunoprecipitated pellets were then washed three times at 4°C in the same lysis buffer and twice with kinase buffer (50 mM Tris-HCl (pH 7.3), 150 mM NaCl, 12.5 mM MnCl₂, 1 mM dithiothreitol, and 0.1% (v/v) polyoxyethylene sorbitan monolaurate (Tween 20)). Immune complex kinase assays were performed by incubating immunoprecipitates from 1.25 mg of lysate protein (from approximately 3 million cells) in a total reaction volume of 0.1 ml containing kinase buffer, 20 μCi of γ-[³²P]ATP, and 1 mg/ml Raf-1 substrate peptide VQQQFYV(Thr)GRKASDDPFKTLTD. The reactions were linear for at least 40 min; both forms of the peptide gave identical results. At the end of 30 min, samples (15 μl, in duplicate) were spotted onto Whatman P81 phosphocellulose paper; washed 4 times for 30 min each in 0.5% (w/v) phosphoric acid, dried, and subjected to Cerenkov scintillation counting.

Phosphoamino Acid Analysis—Serum-deprived cells were labeled for 3 h with approximately 1 mCi/ml [³²P]P for 2 h and then exposed to control conditions or 70 nM insulin for 10 min. The cells were then processed for immunoprecipitation, electrophoresis, and autoradiography as described above. The radiolabeled Raf-1 protein was eluted from the gel and processed for phosphoamino acid analysis as described (18).

RESULTS AND DISCUSSION

We first evaluated the ability of insulin to stimulate the phosphorylation of the Raf-1 protein in several insulin-sensitive cell lines. Because of a relatively high level of apparent basal phosphorylation of the Raf-1 protein, stimulated phosphorylation has been demonstrated in previous studies by a characteristic decrease in Raf-1 migration in SDS-polyacrylamide gels, accompanied by a modest increase in net phosphorylation. In three different insulin-sensitive cell lines, characteristic decrease in Raf-1 migration in SDS-polyacrylamide gels, accompanied by a modest increase in net phosphorylation. In three different insulin-sensitive cell lines, a synthetic Raf-1 peptide as an exogenous substrate (Fig. 2). We conclude from these studies that insulin can activate the Raf-1 protein whose phosphorylation was stimulated by insulin (B-D) or serum (A) in these cells. The positions of molecular weight standards are indicated.

Although activation of protein kinase C can lead to phosphorylation of the Raf-1 protein (3, 4), insulin appeared to stimulate Raf-1 phosphorylation normally in cells preincubated for 16 h with 16 μM phorbol 12-myristate 13-acetate (data not shown). This treatment has been shown to deplete these cells of more than 93% of cytosolic and 96% of particulate protein kinase C activity (8).

An assessed by measuring Raf-1 kinase activity in immunoprecipitates prepared from control and insulin-treated cells, insulin treatment (70 nM for 10 min) resulted in an approximately 3-fold increase in Raf-1 kinase activity directed toward a synthetic Raf-1 peptide as an exogenous substrate (Fig. 2). We conclude from these studies that insulin can activate the Raf-1 kinase in these well differentiated rat hepatoma cells and in other insulin-sensitive cell lines.

We next attempted to determine whether the insulin effect occurred as the result of tyrosine phosphorylation of the Raf-1 protein. Phosphoamino acid analysis of the Raf-1 protein from cells exposed to either control conditions or insulin (70 nM for 15 min) showed that phosphorylation was by far the predominant phosphoamino acid present, with a trace of phosphorylated serine being detected after long exposure of the autoradiograph (Fig. 3). No phosphothreonine was detected, even after much longer autoradiographic exposure. In other experiments, we pretreated the cells with phenylarsine oxide (PAO), an inhibitor of protein tyrosine phosphatases that has been used to uncover cryptic insulin-stimulated tyrosine phosphorylation in cells (20, 23). PAO pretreatment (35 μM for 10
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Serum-deprived H35 cells were exposed to control conditions (C) or insulin (I, 70 nM for 10 min). The cells were then washed four times with ice-cold phosphate-buffered saline, lysed, and used in immunoprecipitation kinase assays as described under "Materials and Methods." Shown are the means ± S.D. of duplicate values taken from six separate kinase assays; \( p < 0.0005 \) when comparing the means by the Student's \( t \) test for paired comparisons.

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