Insulin-induced egr-1 and c-fos Expression in 32D Cells Requires Insulin Receptor, Shc, and Mitogen-activated Protein Kinase, but Not Insulin Receptor Substrate-1 and Phosphatidylinositol 3-Kinase Activation*

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Many studies suggest that insulin utilizes multiple signal transduction pathways. Insulin's effects are initiated by insulin binding to the insulin receptor, resulting in tyrosine phosphorylation of insulin receptor and intracellular substrates, such as insulin receptor substrate-1 (IRS-1), IRS-2, or Shc. We recently demonstrated that immediate-early gene egr-1 transcription was fully induced without phosphorylation of IRS-1 in Chinese hamster ovary cells (Harada, S., Smith, R. M., Smith, J. A., Shah, N., Hu, D.-Q. & Jarett, L. (1995) J. Biol. Chem. 270, 26632–26638). In the present study, we examined the effects of insulin on immediate-early gene egr-1 and c-fos expression in 32D cells overexpressing the insulin receptor (32D/IR), IRS-1 (32D/IRS), or both (32D/IR+IRS) and compared these effects with insulin-induced tyrosine phosphorylation. Insulin (17 nM) increased egr-1 and c-fos expression in 32D/IR and 32D/IR+IRS cells, but not in parental cells or 32D/IRS cells, as determined by Northern blot analysis. Insulin treatment (5 min at 37°C) markedly increased tyrosine phosphorylation of several proteins, including the insulin receptor, IRS-1, and Shc, in 32D/IR+IRS cells as determined by immunoprecipitation and Western blot analysis with anti-phosphotyrosine antibody. In contrast, only two tyrosine-phosphorylated proteins, i.e., insulin receptor and Shc, were detected in 32D/IR cells. These data suggest that insulin receptor and Shc phosphorylation is necessary for insulin-induced egr-1 and c-fos expression, but IRS-1 phosphorylation is not necessary or sufficient for the expression of these genes. Furthermore, the effect of specific inhibitors on insulin-induced egr-1 expression was examined. Wortmannin (25 nM), a phosphatidylinositol 3-kinase inhibitor, had no effect on insulin-induced egr-1 expression. In contrast, PD 98059 (30 μM), a mitogen-activated protein kinase kinase inhibitor, totally blocked egr-1 expression induced by insulin. These data indicate that mitogen-activated protein kinase activation, but not phosphatidylinositol 3-kinase activation, is involved in insulin-induced egr-1 expression. Taken together, insulin receptor tyrosine phosphorylation, Shc tyrosine phosphorylation, and mitogen-activated protein kinase activation appear to be the signal transduction pathway responsible for insulin-induced egr-1 expression in 32D cells. These data demonstrate that insulin has multiple signal transduction pathways that vary from cell to cell.

Insulin’s effects are initiated by insulin binding to its plasma membrane receptor and the sequential tyrosine phosphorylation of the insulin receptor and intracellular substrates, such as insulin receptor substrate-1 (IRS-1), IRS-2, or Shc, mainly through phosphotyrosine binding domains (reviewed in Ref. 1). These substrates bind to SH2 domains of several cytoplasmic signal proteins through their tyrosine phosphorylation sites. These proteins include the 85-kDa subunit of phosphatidylinositol (PI) 3′-kinase, GRB-2, or Syp (tyrosine phosphatase) (1). Activation of these molecules and the following activation of other intracellular molecules, such as p21ras, Raf-1, mitogen-activated protein (MAP) kinase, or S6 kinase, are believed to be responsible for many of insulin’s biological responses. However, many studies suggest that insulin utilizes multiple signal transduction pathways. The insulin signaling network is more complex than was thought a decade ago.

Insulin has mitogenic effects as well as metabolic effects and affects nuclear events such as gene expression or cell growth (reviewed in Ref. 2). One of insulin’s effects on nuclear events is the stimulation or inhibition of a number of immediate-early genes (3, 4). The immediate-early genes are a large and diverse group, and the mechanisms involved in their regulation are complex. The induction of c-fos transcription, one of the well characterized immediate-early genes, by insulin or other growth factors is believed to require receptor phosphorylation and p21ras activation (5, 6). However, recent reports suggested that induction of expression of some immediate-early genes was independent of growth factor receptor autophosphorylation. For instance, Eldredge et al. (7) reported that epidermal growth factor induced c-fos expression in cells expressing kinase-deficient epidermal growth factor receptors. Mundschau et al. (8) showed that induction of egr-1, but not c-fos, c-myc, and JEB, was independent of platelet-derived growth factor receptor autophosphorylation using three different conditions in which platelet-derived growth factor receptor autophosphorylation was blocked. The early growth response gene egr-1, also known as NGFIA, Krox-24, zif 268, and TIS-8, encodes a protein with three zinc finger motifs, structures that are present in many DNA-binding transcription factors (9). We recently demonstrated that insulin induced egr-1 mRNA transcription

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1 The abbreviations used are: IRS-1, insulin receptor substrate-1; PI, phosphatidylinositol; MAP, mitogen-activated protein; CHO, Chinese hamster ovary; MEK, mitogen-activated protein kinase kinase.
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A potential problem in interpreting the results with CHO cells is their low levels of endogenous insulin receptor or IRS-1. The argument could be made that undetectable levels of insulin receptor or IRS-1 phosphorylation could account for insulin’s effects in the CHOneo and CHO1018K cells, despite data to the contrary. 32D cells are mouse myeloid progenitor cells and are insensitive to insulin because they have very low levels of insulin receptors and insulin-like growth factor-1 receptors and no detectable IRS-1 or related molecules, e.g. IRS-2/4PS (11). 32D cells overexpressing insulin receptors, IRS-1, or both have been investigated to rule out the occurrence of these molecules in insulin signaling mechanisms. Previous studies using 32D cells demonstrated that insulin receptors or IRS-1 alone is not sufficient for insulin-stimulated mitogenesis (11). IRS-1 is essential for insulin stimulation of PI 3-kinase and p70S6K (12), whereas insulin receptors alone are sufficient to mediate insulin-stimulated tyrosine phosphorylation of Shc and activation of p21ras and MAP kinase (13). In this study, we assessed the effects of insulin on immediate-early gene expression in 32D cells overexpressing the insulin receptor (32D/IR), IRS-1 (32D/IRS), or both (32D/IR+IRS) and compared these effects with insulin-induced tyrosine phosphorylation. Our data demonstrate that insulin-induced egr-1 and c-fos mRNA expression in 32D cell clones requires the insulin receptor and its phosphorylation, but not IRS-1 phosphorylation. Shc phosphorylation, Shc-GRB-2 association, and MAP kinase activation seem to be a pathway responsible for insulin-induced egr-1 and c-fos expression.

EXPERIMENTAL PROCEDURES

Materials—Mouse monoclonal antibody against phosphotyrosine (4G10) was obtained from Upstate Biotechnology, Inc. Rabbit polyclonal antibodies against phosphotyrosine, the insulin receptor β-subunit, or Shc were obtained from Transduction Laboratories. Rabbit polyclonal antibody against GRB-2 (C-23) was from Santa Cruz Biotechnology Inc. Rabbit anti-mouse immunoglobulin was from Rockland Inc. Porcine insulin was a gift from Dr. R. E. Chance (Eli Lilly Research Laboratory, Indianapolis, IN). PD 98059 was kindly provided by Dr. Alan R. Saltiel (Parke-Davis Pharmaceutical Research, Ann Arbor, MI). The plasmid DNAs for c-fos, egr-1, and α-tubulin were obtained from Drs. R. Taub, J. G. Monroe, and J. L. Swain (all at the University of Pennsylvania), respectively. 125I-Protein A (≥30 μCi/μg) was from ICN, and [α-32P]dCTP (370 MBq/mmol, 10 μCi/mmol, 3000 Ci/mmol) was from Amersham Corp.

Cell Culture and Treatments—32D mouse myeloid progenitor cell clones that express no insulin receptors or IRS-1 (32D), insulin receptors but no IRS-1 (32D/IR), IRS-1 but no insulin receptors (32D/IRS), and both insulin receptors and IRS-1 (32D/IR+IRS) were cloned as described previously (11, 12). The 32D cell clones were cultured in RPMI 1640 medium supplemented with 10% fetal bovine serum and 5% WEHI conditioned medium that contained interleukin-3 under 5% CO2 (12). The cells expressing IRS-1 were cultured in the presence of 2.5 mM histidinol. The cells were cultured in Dulbecco’s modified Eagle’s medium with 0.1% bovine serum albumin for 5 h (serum deprivation) and then incubated with 0–100 nM insulin for 0–90 min at 37°C. In some experiments, the cells were preincubated with inhibitors, PD 98059 or Wortmannin (Sigma) before addition of insulin.

Northern Blot Analysis—After the cells were washed with ice-cold phosphate-buffered saline, total cellular RNA was extracted, isolated, and applied to 0.8% agarose gels, and transferred onto nylon membranes (Hybond-N, Amersham Corp.) as described previously (10). The membranes were hybridized with [α-32P]dCTP-labeled cDNA probes for c-fos, egr-1, and α-tubulin, and the 32P-labeled bands were detected by a PhosphorImager and analyzed by ImageQuant software (Molecular Dynamics, Inc.).

Immunoprecipitation and Western Blot Analysis—The cells were incubated with or without insulin for 1–5 min, washed with ice-cold phosphate-buffered saline, and lysed in lysis buffer (50 mM HEPES, pH 7.4, 150 mM NaCl, 1% Nonidet P-40, 5 mM EDTA, 5 mM EGTA, 20 mM Na3P2O7, 20 mM NaF, 1 mM Na3VO4, 1 mg/ml bacitracin, 1 mM phenylmethylsulfonyl fluoride, 8 μg/ml aprotinin, and leupeptin). The insoluble material was removed by centrifugation, and the lysates (0.7 mg) were incubated with specific antibody (2 μg) for 18 h at 4°C. The immunocomplex was precipitated with protein A beads (Trisacryl, Pierce). For anti-phosphotyrosine antibody (4G10), rabbit anti-mouse antibody was added before adding protein A beads. Immunoprecipitated proteins were washed, solubilized in Laemmlli buffer (24), and subjected to SDS-polyacrylamide gel electrophoresis and electrophoresis to polyvinylidene difluoride membrane (Immobilon-P, Millipore Corp.) using a Bio-Rad miniature slab gel apparatus (Mini-Protean II). Western blot analysis with antibodies against phosphotyrosine (1:500), the insulin receptor β-subunit (1:250), Shc (1:250), or GRB-2 (1:250) was performed as described previously (10), and the labeled proteins were detected by a PhosphorImager.

RESULTS

Effect of Insulin on egr-1 and c-fos mRNA Expression—To determine the requirement of the insulin receptor or IRS-1 in insulin signaling mechanisms that lead to immediate-early gene expression, we examined the effect of insulin on immediate-early gene egr-1 and c-fos mRNA expression in 32D cell clones. 32D, 32D/IR, 32D/IRS, or 32D/IR+IRS cells were incubated with 17 nM insulin for 0–90 min at 37°C. Total cellular RNA was extracted for Northern blot analysis with α-32P-labeled probes for egr-1, c-fos, and α-tubulin. α-Tubulin, an insulin-insensitive gene, was used as an internal control. As shown in Fig. 1, insulin increased egr-1 mRNA expression in 32D/IR and 32D/IR+IRS cells with a similar time course. Insulin increased c-fos mRNA expression also, but more transiently, and it decreased to the basal level in 90 min. In contrast, insulin had no effect on egr-1 and c-fos mRNA expression in 32D and 32D/IRS cells. These results suggest that insulin-induced egr-1 and c-fos mRNA expression requires the insulin receptor, but not IRS-1.

To determine the sensitivity to insulin, we next examined the effect of different concentrations of insulin on immediate-early gene expression. 32D/IR or 32D/IR+IRS cells were incubated with 0–100 nM insulin for 30 min at 37°C. The level of egr-1 and c-fos mRNAs in Northern blot analysis was quantified by a PhosphorImager and ImageQuant software, and was expressed as a percentage of maximum stimulation (at 100 nM). The level of α-tubulin RNA did not change significantly under the conditions. As shown in Fig. 2, both 32D/IR and 32D/IR+IRS cells showed a similar insulin concentration-dependent response of egr-1 and c-fos expression; the response reached close to the maximum at a concentration of 1 nM. These results indicate that both 32D/IR and 32D/IR+IRS cells have a similar sensitivity to insulin, i.e. IRS-1 does not contribute to immediate-early gene expression.
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**Effect of Insulin on Tyrosine Phosphorylation Cascade**—To determine the pathways by which insulin induces immediate-early gene expression, we examined the effect of insulin on the tyrosine phosphorylation cascade in 32D/IR or 32D/IR+IRS cells by immunoprecipitation followed by Western blot analysis, both with anti-phosphotyrosine antibody. As shown in Fig. 3, insulin increased tyrosine phosphorylation of several proteins, including the insulin receptor β-subunit (95 kDa), IRS-1 (180 kDa), and Shc (52 kDa), in 32D/IR+IRS cells. In contrast, in 32D/IR cells, insulin increased tyrosine phosphorylation of two proteins, the insulin receptor β-subunit and 52-kDa Shc. We confirmed that the 95- and 52-kDa phosphoproteins were the insulin receptor β-subunit and Shc, respectively, by stripping the membrane and reblotting with specific antibodies. Neither the insulin receptor nor Shc was phosphorylated in 32D/IRS cells (data not shown). Interestingly, phosphorylation of the insulin receptor was less and slower in 32D/IR cells, although they have equal or greater numbers of the insulin receptor as determined by binding studies (data not shown). These data suggest that Shc phosphorylation is independent of IRS-1 phosphorylation and that Shc seems to be the only detectable tyrosine-phosphorylated substrate of the insulin receptor in 32D/IR cells. In 32D/IR+IRS cells, IRS-1 contributes to a phosphorylation cascade, resulting in tyrosine phosphorylation of several additional substrates.

**Shc Phosphorylation and Association with GRB-2**—We next examined the effect of insulin on Shc phosphorylation and Shc-GRB-2 association. 32D/IR or 32D/IR+IRS cells were incubated with or without 100 nM insulin for 5 min at 37 °C. The cells were lysed, and tyrosine-phosphorylated proteins were immunoprecipitated with anti-phosphotyrosine antibody (4G10) and subjected to SDS-polyacrylamide gel electrophoresis and Western blot analysis with antibodies against phosphotyrosine (aPY), the insulin receptor (IR) β-subunit (aIR), or Shc (aShc).

**Effect of PI 3-Kinase or MEK Inhibitors on Insulin-induced Immediate-early Gene Expression**—Last, to determine the downstream pathways involved in insulin-induced immediate-early gene expression, we examined the effect of wortmannin, a PI 3-kinase inhibitor, or PD 98059, a MEK inhibitor, on egr-1 expression. 32D/IR or 32D/IR+IRS cells were preincubated with no addition or with 25 nM wortmannin or 30 μM PD 98059 for 15–30 min; then 17 nM insulin was added, and the cells were further incubated for 45 min at 37 °C. Total RNA was extracted for Northern blot analysis with α-32P-labeled cDNA probes for egr-1, c-fos, and α-tubulin. The quantitative data were expressed as a percentage of maximum stimulation in samples with no inhibitor. α-Tubulin mRNA levels were not affected by either insulin or inhibitors (data not shown). 25 nM wortmannin had no inhibitory effect on insulin-induced egr-1 expression as shown in Fig. 5. Even with 100 nM wortmannin, egr-1 expression was not inhibited, but actually slightly increased in 32D/IR+IRS cells (data not shown). In contrast, PD 98059 almost completely inhibited insulin-induced egr-1 ex-
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3D/IR 3D/IR+IRS

Fig. 5. 32D/IR or 32D/IR+IRS cells were incubated with no addition (open bars) or with 25 nM wortmannin (hatched bars) or 30 µM PD 98059 (closed bars) for 15–30 min; then 17 nM insulin was added, and the cells were incubated for 45 min at 37°C. Total RNA was extracted, and Northern blot analysis with egr-1 was performed as described under “Experimental Procedures.” The quantitative data are expressed as a percentage of maximum stimulation in samples with no inhibitor.

expression (Fig. 5). Similar results were obtained with c-fos expression (data not shown). These results suggest that MEK and MAP kinase activation, but not PI 3-kinase activation, is involved in insulin-induced immediate-early gene expression.

DISCUSSION

The insulin signaling network is complex and involves molecules that regulate each other. Adding to the complexity are the observations that different cell types may have different and cell-specific concentrations of these signaling molecules. Recently, several studies have shown that IRS-1 phosphorylation and PI 3-kinase activation, but not p21<sup>ras</sup> or MAP kinase activation, are necessary for insulin’s metabolic effects, such as glucose transporter GLUT4 translocation in 3T3-L1 adipocytes (14–16). Regulation of glycogen synthase activity by insulin involves a MAP kinase-independent and rapamycin-sensitive pathway (17, 18). In contrast, p21<sup>ras</sup> or MAP kinase activation seems to be related to insulin’s mitogenic effects (19, 20). In this study, we have demonstrated that insulin-induced immediate-early gene egr-1 and c-fos mRNA expression requires insulin receptor phosphorylation, Shc phosphorylation, Shc-GRB-2 association, and MAP kinase activation. These effects appear to be independent of IRS-1 phosphorylation or PI 3-kinase activation. Previous studies with 32D cells showed that insulin receptors alone are sufficient to mediate insulin-stimulated tyrosine phosphorylation of Shc and activation of p21<sup>ras</sup> and MAP kinase (12). Taken together, these data suggest that the insulin receptor, Shc-GRB-2-SOS, p21<sup>ras</sup>, MEK, and MAP kinase constitute the pathway that leads to insulin-induced immediate-early gene expression.

Others have shown that insulin-induced mitogenesis, measured by thymidine incorporation, requires IRS-1 phosphorylation (11, 21), suggesting that an IRS-1 pathway is necessary for complete mitogenesis, which requires many other events, such as translation and activation of transcription factors. The mechanisms involved in translation are not completely understood. In one of the best-characterized systems, phosphorylation of eukaryotic translation initiation factor 4E and its binding protein, PHAS-I (phosphorylated heat- and cold-stable protein), correlates with an increase in the rate of protein synthesis under a variety of in vivo conditions including stimulation by insulin. Recently, it has been shown that phosphorylation of eukaryotic translation initiation factor 4E and PHAS-I requires IRS-1-mediated stimulation of PI 3-kinase and p70<sup>S6K</sup> (18, 22). Regulation of PHAS-I seems to be independent of MAP kinase activation or SH2 domain-containing protein-tyrosine phosphatase (18, 22). These results suggest that insulin utilizes different pathways depending on the different actions of insulin. Induction of immediate-early genes, one of the earliest steps for mitogenesis, requires Shc and MAP kinase activation, whereas PI 3-kinase and p70<sup>S6K</sup> are necessary in protein synthesis.

Interestingly, we saw more Shc phosphorylation in 32D/IR cells than in 32D/IR+IRS cells, although phosphorylation of the insulin receptor β-subunit was less in the former. These results suggest that Shc and IRS-1 may compete for the binding site on the insulin receptor. This speculation is supported by the data obtained with a yeast two-hybrid system showing that both IRS-1 and Shc bind to the same region of the insulin receptor β-subunit (23). Therefore, less insulin receptor is available for Shc to bind to in the cells overexpressing IRS-1. Another possibility is that an IRS-1 pathway and a Shc pathway may negatively regulate each other. A recent study demonstrated that constitutively active MEK (MAP kinase kinase) inhibited GLUT4 translocation by negative regulation of PI 3-kinase, suggesting that a MAP kinase pathway and a PI 3-kinase pathway negatively regulate each other. These interactions appear to be important for regulation of cell-specific insulin action. Our data showing that inhibition of PI 3-kinase in 32D/IR+IRS cells actually increased egr-1 expression are consistent with these findings.

Finally, the results with 32D cells demonstrated in this study are different from our previous report with CHO cells, which demonstrated that insulin-induced egr-1, but not c-fos, mRNA expression was independent of phosphorylation of the insulin receptor or IRS-1 (10). The reason may be because each cell type utilizes different and cell-specific signaling mechanisms. We saw an increase in tyrosine phosphorylation of 120-kDa proteins in CHO cells, whereas phosphorylation of these 120-kDa proteins was not regulated by insulin in 32D cells. Alternatively, CHO cells, even CHO<sub>neo</sub> cells, have considerable amounts of insulin receptor or insulin-like growth factor-1 receptor compared with 32D cells. So even though we could not detect receptor or IRS-1 phosphorylation, we cannot rule out the possibility that the downstream substrate may be more sensitive, and minute phosphorylation of the receptor may be enough to conduct insulin signaling.

In summary, we demonstrated that insulin induced egr-1 and c-fos mRNA expression in a similar manner in 32D/IR cells and 32D/IR+IRS cells, but not in 32D cells or 32D/IRS cells. The signaling mechanisms involved seem to be insulin receptor phosphorylation, Shc phosphorylation, Shc-GRB-2 association, and MAP kinase activation. IRS-1 phosphorylation and PI 3-kinase activation were not involved in the pathway. These results clearly separate and identify the signaling mechanism for one of the many actions of insulin. Whether or not each cell type has different and cell-specific pathways and how to regulate this complicated insulin network will require further study.

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