Signal Transduction Pathways Leading to Insulin-Induced Early Gene Induction†

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ABSTRACT: We examined the signal transduction pathway leading to insulin stimulation of two immediate early genes, c-*fos*, and the early growth response gene, *Egr*-1. In Rat 1 fibroblasts overexpressing normal human insulin receptors (HIRc-B), insulin and IGF-I rapidly and transiently induced the expression of both c-*fos* and *Egr*-1 mRNA with maximum accumulation at 30 min, declining to basal levels at 120 min. Insulin (100 ng/mL) increased c-*fos* and *Egr*-1 mRNA expression 10-fold (EC_{50} = 20 ng/mL), whereas IGF-I (100 ng/mL) and serum (20%) led to a 3- and 11.5-fold increase, respectively. Insulin-stimulated c-*fos* protein expression was maximal at 1 h postinduction and undetectable at 4 h. The effects of insulin and IGF-I on both c-*fos* mRNA and protein expression were absent in Rat 1 fibroblasts expressing tyrosine kinase-defective human insulin receptors (A/K1018). In cells expressing insulin receptors in which the two C-terminal tyrosines are mutated to phenylalanine (Y/F2 cells), the insulin stimulated increase in *Egr*-1 and c-*fos* mRNA was comparable to that of HIRc cells, whereas, in cells expressing C-terminal truncated receptors (ΔCT cells), the insulin induced increase in *Egr*-1 mRNA was normal, but the c-*fos* mRNA response was severely blunted. As expected, the insulin effect to increase ras GTP formation and MAP kinase activity was negligible in A/K1018 cells but normal, or supernormal, in Y/F2 cells. Importantly, stimulation of ras GTP was increased in ΔCT cells, whereas stimulation of MAP kinase activity was almost absent. Thus, the insulin signaling cascade was extinguished downstream of ras GTP formation and upstream of MAP kinase. In conclusion, (1) insulin and IGF-I induce a rapid and transient increase in c-*fos* and *Egr*-1 mRNA as detected by an RT/PCR assay in HIRc-B cells, (2) in the absence of a competent receptor tyrosine kinase, insulin does not mediate these biologic effects, and (3) insulin signaling is impaired downstream of p21rasGTP formation in ΔCT cells, and this may account for the differential regulation of *Egr*-1 vs c-*fos* mRNA.

Insulin stimulates a spectrum of physiologic effects, including transport of glucose and amino acids and increased synthesis of glycogen and certain metabolic enzymes as well as enhanced synthesis of DNA and RNA (Rosen, 1987). The initial step in insulin action is the binding of insulin to its cell surface receptor (Olefsky, 1990). The insulin receptor is a heterotetrameric glycoprotein consisting of two extracellular α-subunits and two transmembrane β-subunits (Ullrich et al., 1985). Insulin binds to the α-subunits and activates the tyrosine kinase of the β-subunit resulting in receptor autophosphorylation and phosphorylation of endogenous substrates. Although many studies suggest that receptor kinase activity is central to insulin signal transduction (McClain et al., 1987; Chou et al., 1987; Ebina et al., 1987; Ellis et al., 1986), the postreceptor processes mediating the signal from the receptor tyrosine kinase to intracellular target(s) are largely unknown.

In terms of acute effects, insulin increases the levels of specific mRNAs such as those of the *p33* gene, c-*fos*, c-*jun*, and the early growth response gene (*Egr*-1) (Stumpo & Blackshear, 1986, 1991; Burgering et al., 1991; Messina, 1990). The induction of c-*fos* mRNA expression is one of the earliest known transcriptional responses to mitogenic stimulation. In quiescent cells, the c-*fos* gene is expressed at relatively low levels, but treatment with growth factors, including platelet-derived growth factor (PDGF) (Kruijer et al., 1984; Muller et al., 1984), nerve growth factor (NGF) (Greenberg et al., 1985; Milbrandt, 1986; Kruijer et al., 1985), epidermal growth factor (EGF) (Greenberg et al., 1985; Milbrandt, 1986), insulin (Stumpo & Blackshear, 1986, 1991; Burgering et al., 1991; Messina, 1990), or IGF-I[1] (Stumpo & Blackshear, 1991), leads to a dramatic but transient induction of this gene. *Egr*-1 is also rapidly and transiently induced by a variety of mitogens (Stumpo &

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Blackshear, 1991; Sukhtme et al., 1987, 1988). Thus, both genes serve as convenient markers for mitogenic signal transduction.

In the present study, we utilized a quantitative reverse transcriptase/polymerase chain reaction (RT/PCR) assay in transfected cell lines expressing normal or various mutant insulin receptors to examine the effects of insulin and IGF-I on c-*fos* and *Egr*-1 gene expression. We have also analyzed the signaling pathway for insulin's effects on induction of c-*fos* and *Egr*-1 mRNA.

EXPERIMENTAL PROCEDURES

Cell Lines and Materials. The Rat 1 fibroblasts expressing wild-type or mutated human insulin receptors were maintained as previously described (McClain et al., 1987). The cell lines used in this study include Rat 1 fibroblasts overexpressing wild-type insulin receptors (HIRc-B cells), cells expressing insulin receptors in which the two C-terminal tyrosines at positions 1316 and 1322 are mutated to phenylalanine (Y/F2 cells), cells expressing insulin receptors truncated at position 1300 and, therefore, missing the C-terminal 43 amino acids (ΔCT cells), and cells expressing kinase inactive insulin receptors (A/K1018 cells). Pork insulin and IGF-I were generously provided by Eli Lilly, Inc. [α-^{32}P]dCTP (3000 Ci/mmol) and [^{3}H]thymidine (83 Ci/mmol) were from Du Pont-New England Nuclear. cDNA cycle kit was purchased from Invitrogen. 10× PCR amplification buffer and Taq polymerase (5 units/μL) were obtained from Perkin-Elmer/Cetus. Electrophoresis reagents were from Bio-Rad. All other reagents were purchased from Sigma.

Preparation of RNA. Total cellular RNA was purified from cells by the modified method of single-step guanidinium thiocyanate−phenol−chloroform RNA extraction (Nakanishi et al., 1985). A 35 mm plate of cells at 80−90% confluency was starved for 48 h in serum-free DMEM containing 1 mM L-glutamine, 100 units of penicillin/mL, and 100 μg of streptomycin sulfate/mL. After stimulation with insulin, IGF-I, or serum, cells were washed twice with ice-cold TD (140 mM NaCl, 5 mM KCl, 0.4 mM Na_2HPO_4, 25 mM Tris-HCl, pH 7.4) and solubilized with 250 μL of lysis buffer (4 M guanidine thiocyanate, 25 mM sodium citrate, 0.4% sodium lauroyl sarcosinate, antifoam, 100 mM β-mercaptoethanol, pH 7.0). The lysate was vortexed vigorously to shear DNA. Twenty-five microliters of 1 M sodium acetate (pH 4.0), 100 μL of chloroform, and 300 μL of water-saturated phenol were added. After vortexing, the samples were centrifuged at 12000*g* for 5 min. The aqueous phase was transferred to another tube, and the RNA was precipitated by addition of cold absolute ethanol (2.5 vol), incubated at −20 °C overnight, and then centrifuged at 12000*g* for 30 min. The RNA pellet was washed twice with ice-cold 70% ethanol, dried by evaporation, and dissolved in 100 μL of TE (10 mM Tris-HCl, 0.1 mM EDTA, pH 7.5). The RNA concentration was determined by measuring the OD at 260 nm.

Reverse Transcriptase (RT)/Polymerase Chain Reaction (PCR) and Northern Blot Analysis. cDNA was synthesized from 100 ng of total cellular RNA using a cDNA cycle kit and random hexamer primers. RT reactions were used directly for subsequent PCR amplification of specific cDNAs. One-tenth of the RT sample was added to a PCR mixture (30 μL final volume), which contained 1× PCR buffer (10 mM Tris-HCl, 50 mM KCl, 1.5 mM MgCl2, 0.1 mg/mL gelatin, pH 8.4), 0.2 mM each of dNTP, 2 μCi of [α-^{32}P]-dCTP, 15 pmol of each primer, and 0.75 unit of Taq polymerase. The $MgCl_2$ concentration was optimized for each set of primers. Primers were synthesized by a Cruachem DNA synthesizer. PCR cycles were performed in an automated DNA thermal cycler (Perkin-Elmer/Cetus) with the following temperature profile: denaturation at 94 °C for 30 s, primer annealing at 62 °C for 30 s, and primer extension at 72 °C for 60 s. Twenty-seven microliters of the PCR sample was electrophoresed on 10% polyacrylamide gels, and the PCR fragments were visualized by autoradiography. The relative product amounts were quantitated by measuring the amount of radioactivity incorporated into the bands with an AMBIS Radioanalytic Imaging System (AMBIS Inc., San Diego, CA).

For Northern blot analysis, 20 μg of total cellular RNA was fractionated on 2.2M formaldehyde/1% agarose gel electrophoresis and transferred to nitrocellulose membrane (Schleicher & Schull) in 10× SSC (1× SSC = 0.15 M NaCl/15 mM sodium citrate, pH 7.0) after staining with acridine orange (33 μg/mL) and destaining. The baked membrane was prehybridized with denatured salmon sperm DNA (100 μg/mL) in 5× SSC, 0.1% sodium dodecyl sulfate (SDS), and 5× Denhardt's solution (1× Denhardt's solution = 0.02% BSA/0.02% Ficoll/0.02% polyvinylpyrrolidone) for 4 h at 42 °C and then hybridized with ^{32}P-labeled v-*fos* cDNA (2×10^6 cpm/mL) for 16 h at 42 °C. The v-*fos* probe (1.9 kilobase *Sal*1 fragment) was purified using Gene Clean (Bio-Rad) and labeled with [^{32}P]dCTP using multiprimer DNA-labeling system (Amersham). The hybridized membrane was rinsed in 0.1× SSC/0.1% SDS at 55 °C and then autoradiographed.

PCR Primers. PCR primers were derived from rat c-*fos*, *Egr*-1, and ribosomal protein gene *L30* sequences. c-*fos* [position 140−309 (169 bp); Osterop et al., 1992]

sense: 5′-GTTCTCGGGTTTCAACGCGGACTACGAGGC-3′

antisense: 5′-GGCACTAGAGA-CGGACAGATCTGCGCAAAAGTCC-3′

Egr-1 [position 539−781 (252 bp); Porras et al., 1992]

sense: 5′-GAGCCGAGCGAACAA-CCCTACGAGCACCTG-3′

antisense: 5′-GCGCTGAGGATGAA-GAGGTTGGAGGGTTGG-3′

L-30 (Maassen et al., 1992)

sense: 5′-GAAAGTACGTGCTGGG-TACAAACAGACTC-3′

antisense: 5′-ATCGGAATCACCTGGGTCAA-TGATAGCCAG-3′

[position 71−309 (238 bp); used with c-fos primer]

[1] Abbreviations: BrdU, bromodeoxyuridine; BSA, bovine serum albumin; GNRF, guanine nucleotide releasing factors; IGF-I, insulin-like growth factor-I; N17 ras, dominant-negative mutant p21ras protein; PBS, phosphate buffer saline; RT/PCR, reverse transcriptase/polymerase chain reaction; T-24 ras, oncogenic p21ras protein.

antisense: 5′-CCACACGCTGTGCCCAA-
TTCAATGTTATTGC-3′
[position 71−254 (183 bp); used with Egr-1 primer]

Analysis of Guanine Nucleotide Bound to p21ras. GTP and GDP-bound p21ras were measured as described (Jhun et al., 1994). Briefly, cells grown to confluency were serum-starved for 16 h and phosphate-starved for an additional 1 h. The serum- and phosphate-starved cells were incubated for 4 h with [^{32}P]orthophosphate and then challenged with insulin for 10 min at 37 °C. p21ras was immunoprecipitated with anti-p21ras monoclonal antibody (Y13−259) from the cell lysates as described elsewhere (Gibbs et al., 1990; Downward et al., 1990). GTP- and GDP-bound p21ras were separated by thin layer chromatography and analyzed by autoradiography. Quantitation of the nucleotides was determined by cutting out and counting the labeled nucleotides in a liquid scintillation counter. The results were expressed as GTP/(GTP + GDP) × 100%, reflecting the amount of GTP-bound ras before and after exposure to insulin (Pang et al., 1994).

MAP Kinase Assay. MAP kinase activity was assayed as described (Pang et al., 1994). Briefly, cell lysates were prepared from cells stimulated with insulin for 10 min at 37 °C. Ten microliter aliquots of cell lysate were incubated with 0.2 mg/mL microtube-associated protein-2 (MAP-2) for 10 min at 30 °C in a final volume of 25 μL containing 50 mM Tris-HCl, pH 7.4, 2 mM EGTA, 10 mM $MgCl_2$, and 40 μM [γ-^{32}P]ATP (1 μCi). The reaction was stopped by the addition of 4× Laemmli SDS−PAGE sample buffer (Tominaga et al., 1994), and phosphorylated MAP-2 was resolved by SDS−PAGE (7.5% gel). Coomassie Blue-stained bands containing phospho-MAP-2 were excised from the gels, and incorporated radioactivity was measured by Cerenkov counting.

S6 Kinase Assay. S6 kinase activity was assayed as described (Takata et al., 1992). Briefly, cytosolic fraction was prepared from cells stimulated with insulin for 30 min at 37 °C. Kinase reactions were carried out for 30 min at 37 °C in a final volume of 25 μL containing 20 mM Hepes (pH 7.4), 10 mM $MgCl_2$, 50 μM unlabeled ATP, 2 μCi of [γ-^{32}P]ATP, 5 μL of 40S ribosomes (0.4 OD unit), and 5 μg of protein extract. Reactions were terminated by the addition of Laemmli sample buffer, analyzed on 5−15% linear gradient SDS−polyacrylamide gels, and subjected to autoradiography. Activity was quantified by counting the excised 32-kDa band.

RESULTS

Expression of c-fos and Egr-1 Genes: Measurement by Quantitative PCR. The addition of insulin, IGF-I, and serum to quiescent HIRc-B cells (a transfected Rat 1 fibroblast cell line expressing 1.4 × 10^6 normal human insulin receptors per cell) results in a rapid induction of c-*fos* mRNA (Figure 1A) as detected by Northern blot analysis. To further examine early transcriptional responses to insulin and IGF-I, we utilized a reverse transcriptase/polymerase chain reaction (RT/PCR) assay system (Figure 1B) (Tominaga et al., 1994). Using published sequences, primer pairs specific for c-*fos*, *Egr*-1, and the ribosomal protein gene, *L30*, were designed (Curran et al., 1987; Milbrandt 1987; Nakanishi et al., 1985). The ribosomal protein gene, *L30*, is expressed at invariant levels throughout the cell cycle and served as

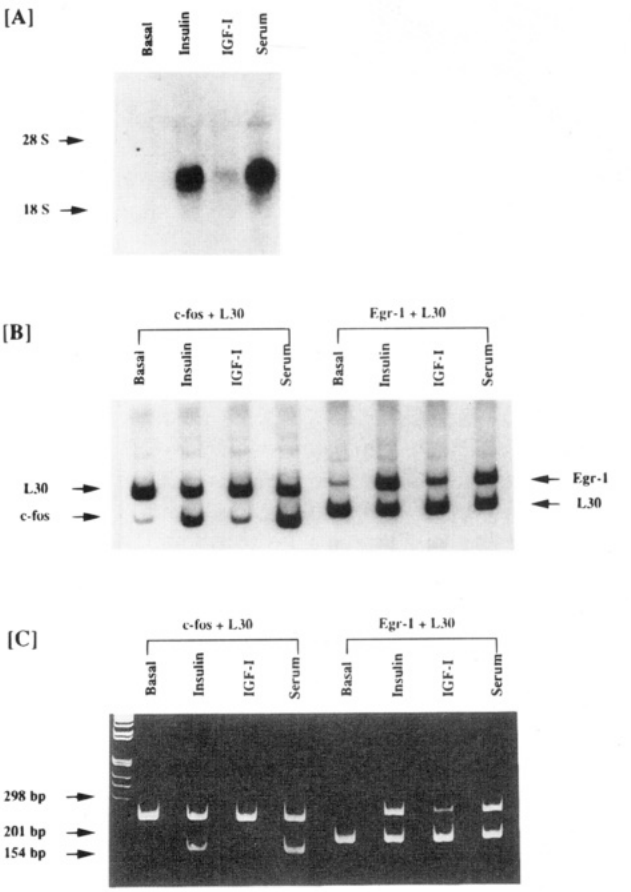

FIGURE 1: Comparison of Northern blot analysis and RT/PCR assay on insulin-, IGF-I-, and serum-induced gene expression in HIRc-B cells. Cells were deprived of serum for 48 h and subsequently stimulated with either insulin (100 ng/mL), IGF-I (100 ng/mL), or fetal calf serum (20%) for 30 min at 37 °C. Total cellular RNA was isolated as described in Experimental Procedures. In panel A, 20 μg of RNA was fractionated on 1% formaldehyde agarose gel and transferred to nitrocellulose. The Northern blot was hybridized with ^{32}P-labeled c-*fos* cDNA (2 × 10^6 cpm/mL), washed, and then subjected to autoradiography. The markers indicate the positions of the major ribosomal RNA species on the stained gel. In panel B, 0.1 μg of RNA was subjected to RT/PCR assay. cDNA equivalent to 0.01 μg of RNA was used for subsequent PCR amplification The larger *L30* primer pair was co-amplified with the c-*fos* primer pair, and the smaller *L30* primer pair was co-amplified with the *Egr*-1 primer pair. Resulting PCR samples were electrophoresed on 10% nondenaturing polyacrylamide gel and visualized by autoradiography. In panel C, after resolution by gel electrophoresis and staining with EtBr, DNA fragments of the predicted molecular weight were observed: c-*fos* (169 bp), *Egr*-1 (252 bp), and *L30* (238 and 193 bp).

an internal control for the amount of mRNA present during RT/PCR. Figure 1B shows that quiescent cells expressed a high level of *L30* mRNA and that neither insulin, IGF-I, nor serum detectably altered L30 mRNA levels. The PCR conditions were calibrated by assessing a series of different combinations of cycle number and RNA amount. One hundred nanograms of RNA and 30 cycles of amplification consistently defined a region of exponential amplification of the target templates during the PCR (data not shown).

When the PCR products were resolved on gels and stained with EtBr (Figure 1C), DNA fragments of the predicted molecular weight were observed: c-*fos* (169 bp), *Egr*-1 (252 bp), and *L30* (238 and 193 bp). The *L30* primer pair corresponding to the larger PCR product (238 bp) was co-amplified together with the c-*fos* primer pair (169 bp), and

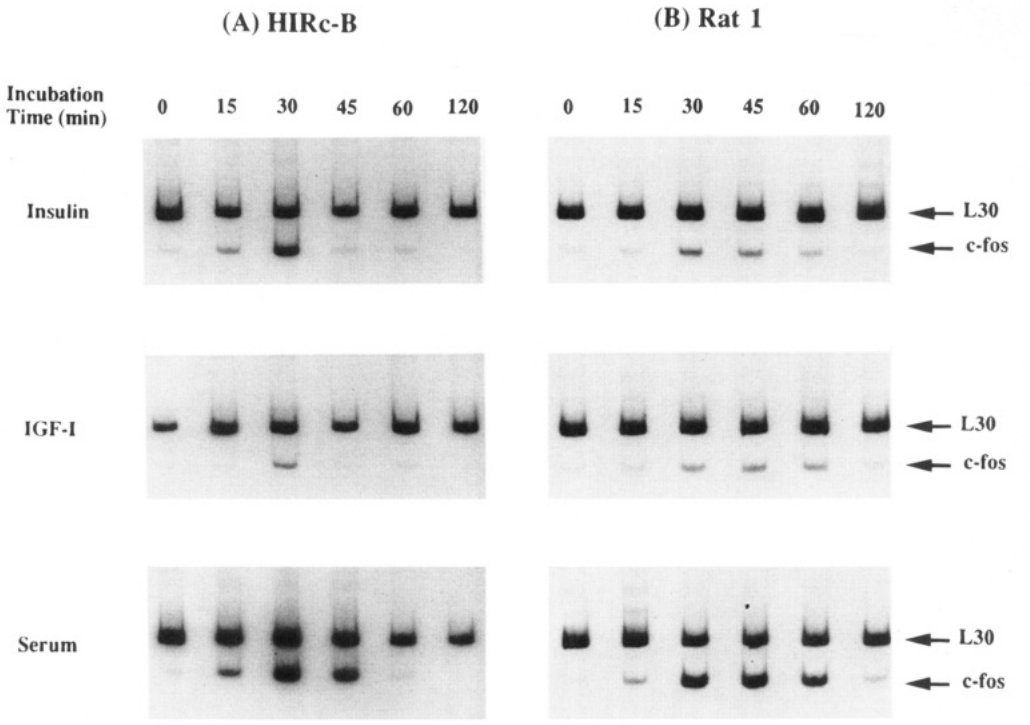

FIGURE 2: Time course of insulin-, IGF-I-, and serum-induced c-*fos* mRNA expression in HIRc-B and Rat 1 cells. Cells were deprived of serum for 48 h and then stimulated with either insulin (100 ng/mL), IGF-I (100 ng/mL), or fetal calf serum (20%) for the indicated times. Total cellular RNA was isolated, and 0.1 μg of RNA was subjected to RT/PCR assay as described in Experimental Procedures. Resulting PCR products (0.01 μg RNA equivalent) were subjected to electrophoresis on a 10% polyacrylamide gel followed by autoradiography. The resulting c-*fos* and *L30* PCR fragments are indicated.

the *L30* primer pair corresponding to the smaller PCR product (183 bp) was co-amplified with the *Egr-1* primer pair (252 bp).

Figure 1 compares the results of Northern blot and RT/PCR analyses derived from the same total RNA preparation from HIRc-B cells. As assessed by the RT/PCR assay, both c-*fos* and *Egr*-1 mRNA were increased 8-fold by insulin (100 ng/mL), 3-fold by IGF-1 (100 ng/mL), and 10-fold by serum (20%). A comparable induction of c-*fos* mRNA expression was detected by either the RT/PCR assay or Northern blot analysis. These results show that the RT/PCR assay can quantitatively assess c-*fos* and *Egr*-1 mRNA using extremely small amounts of total RNA (0.01 μg).

Time Course of the Induction of c-fos mRNA and c-fos Protein Expression. Serum induction of c-*fos* mRNA expression is rapid and transient in most cell lines (Stumpo & Blackshear, 1986; Kruijer et al., 1984; Muller et al., 1984). Figure 2 shows that insulin, IGF-I, and serum induction of c-*fos* mRNA expression in both Rat 1 and HIRc-B cells displays kinetics similar to those previously reported for other mitogens in various cell lines. c-*fos* mRNA expression was increased at 15 min after insulin or IGF-I stimulation, peaked at 30 min, and decreased almost to basal levels by 120 min. Serum also elicited a rapid, transient increase in c-*fos* mRNA, although the peak was somewhat broader as compared to insulin and IGF-I. Interestingly, in sharp contrast to the effect of insulin, the effect of serum was comparable in HIRc-B and Rat 1 cells. The level of *Egr*-1 mRNA was rapidly and transiently increased by insulin or IGF-I in a manner very similar to that observed for c-*fos* mRNA induction (data not shown).

As stimulation of HIRc-B cells with insulin and serum leads to a rapid and dramatic induction of c-*fos* mRNA, we investigated synthesis and localization of the c-fos gene product under these conditions using indirect immunofluorescence. As shown in Figure 3A, c-fos protein was almost undetectable in serum-starved HIRc-B cells but was rapidly induced 1 h after insulin stimulation where newly synthesized c-fos protein was primarily localized to the nucleus, consistent with previous reports in other cell lines (Kruijer et al., 1984; Muller et al., 1984). Little or no c-fos protein could be detected 4 h after induction. In parental Rat 1 cells, insulin did not lead to measurable c-fos protein induction, but serum stimulation led to a similar degree of induction as seen in HIRc-B cells (data not shown). This result suggests that the overexpressed competent insulin receptors efficiently couple the insulin signal to c-*fos* gene expression and protein synthesis, whereas the pathways mediating the serum response remain comparable between the parental and transfected cells.

Upon comparing the time courses of c-*fos* mRNA versus protein induction (Figure 3B), there was an approximate 30 min lag between appearance of c-*fos* mRNA compared to c-fos protein, indicating that the increased level of c-fos protein is the product of the newly synthesized c-*fos* mRNA.

To assess the relationship between c-*fos* mRNA induction and DNA synthesis, the insulin dose responsiveness for c-*fos* mRNA induction and [^{3}H]thymidine incorporation into DNA were compared in HIRc-B cells (Figure 4). Both biologic effects reveal similar EC_{50} values (approximately 20 ng/mL), suggesting that DNA synthesis and c-*fos* mRNA induction share the same signaling pathway.

Mechanisms of Insulin-Mediated c-fos and Egr-1 Induction. To begin an analysis of the signal transduction components which mediate the effects of insulin and IGF-I on c-*fos* and *Egr*-1 mRNA induction, we conducted dose–response studies using HIRc-B, Rat 1, and A/K1018 cells.

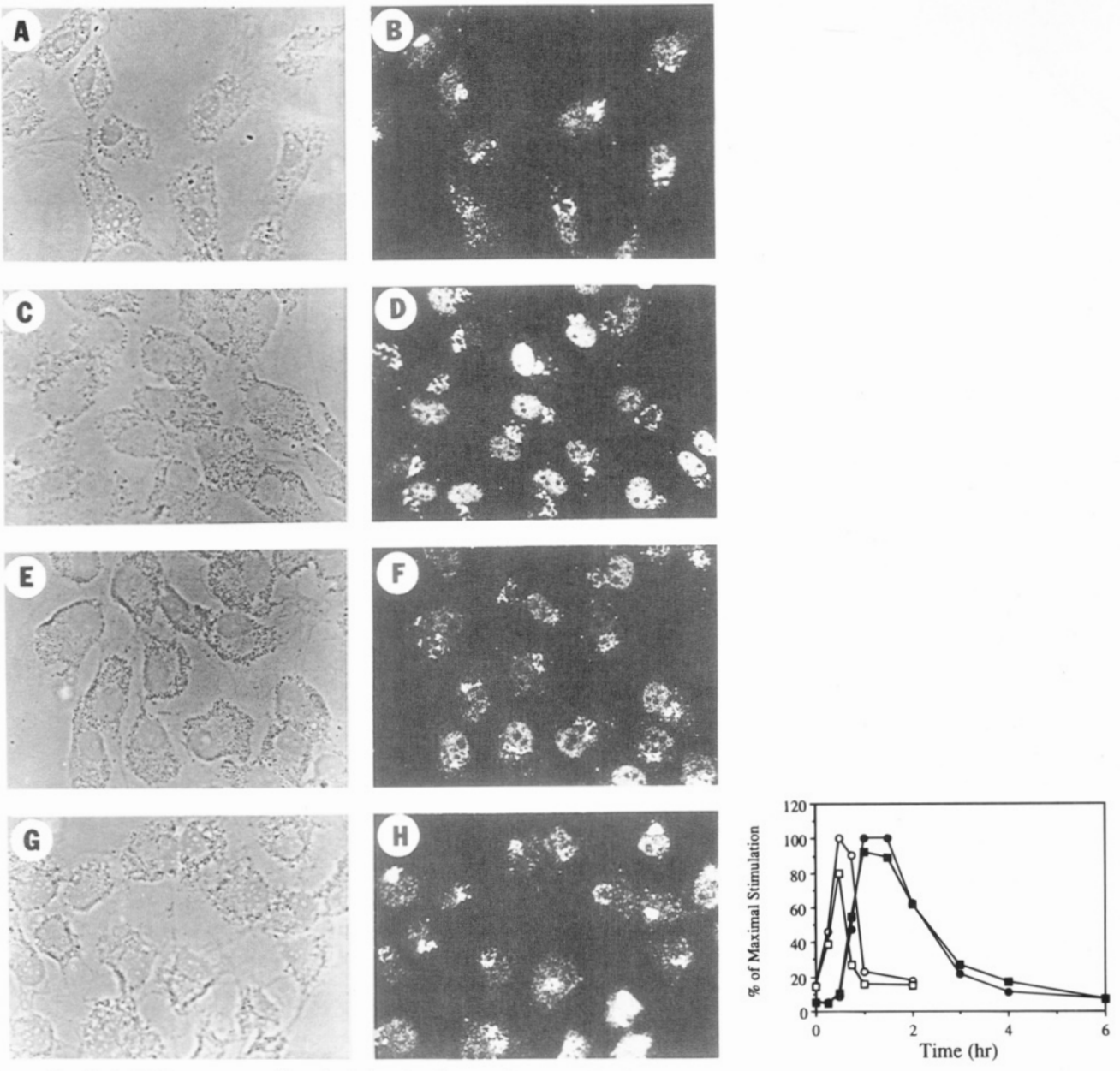

FIGURE 3: (A, left) Time course of insulin-induced c-fos protein expression in HIRc-B cells. Cells on coverslips were deprived of serum for 48 h and then stimulated with insulin (100 ng/mL) for 0 h (A, B), 1 h (C, D), 2 h (E, F), and 4 h (G, H). Cells were fixed and subsequently incubated with rabbit anti-c-fos antibody, biotinylated anti-rabbit IgG antibody, and Texas Red-conjugated streptavidin. Phase-contrast photomicrographs are shown on the left and fluorescence photomicrographs on the right. (B, right) Kinetics of c-*fos* mRNA and protein expression in HIRc-B cells. Serum-deprived cells were stimulated with insulin (100 ng/mL) (□, ■) or fetal calf serum (20%) (○, ●) for the indicated times. For c-*fos* mRNA expression (open symbols), RT/PCR was performed as in Figure 2. The radioactivity incorporated by the PCR products was quantitated by an AMBIS system. mRNA concentration during RT/PCR assay was calibrated using co-amplified *L30* mRNA amount as an internal control. The c-*fos* mRNA amount is expressed as the percentage of maximal serum stimulation of 30 min postinduction. For c-fos protein expression (closed symbols), the expressed c-fos protein was detected with anti-c-fos antibody as in panel A. The nuclear stained cells (approximately 150 cells) on the coverslips were counted using a Zeiss Axiophot fluorescence microscope. 85−90% of the cells exhibited fos-specific staining following serum stimulation for 1 h. The result is expressed as the percentage of maximal serum stimulation of 1 h postinduction. Each point represents the mean of three independent experiments.

A/K1018 cells are Rat 1 fibroblasts which overexpress (2.5×10^5 receptors/cell) human insulin receptors mutated at the ATP binding site (position 1018) of the β-subunit rendering the receptors completely devoid of tyrosine kinase activity (McClain et al., 1987).

Figure 5 summarizes the insulin and IGF-I dose response curves for c-*fos* and *Egr*-1 mRNA induction in HIRc-B, Rat 1, and A/K1018 cells. In the HIRc-B cells (closed circles), induction of c-*fos* and *Egr*-1 mRNA by insulin was evident at a concentration of 10 ng/mL with maximal induction occurring at 100 ng/ml (EC_{50} = 20 ng/mL, panels A and C). The level of c-*fos* mRNA was increased 10-fold by insulin (100 ng/mL) and 11.5-fold by serum (20%). HIRc-B cells contain high levels of receptors for IGF-I (1.5×10^5 receptors/cell). IGF-I stimulated a 3-fold increase in c-*fos* mRNA and 3.8-fold increase in *Egr*-1 mRNA levels in the HIRc-B cells with the same EC_{50} value of 25 ng/mL (panels B and D). In Rat 1 cells (open circles), the effect of insulin on c-*fos* and *Egr*-1 mRNA induction was slight compared to that in HIRc-B cells, demonstrating that overexpression of competent insulin receptors confers increased insulin signaling of gene expression.

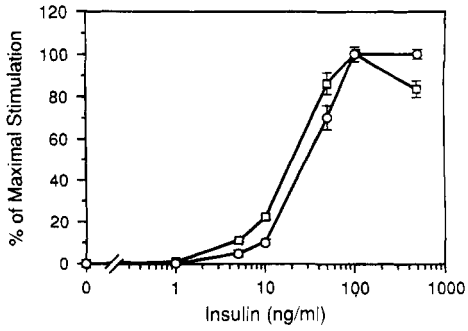

FIGURE 4: Comparison of insulin dose response of c-*fos* mRNA expression and DNA synthesis in HIRc-B cell. To measure insulin-stimulated DNA synthesis (○), cells were deprived of serum for 48 h and stimulated with various concentrations of insulin for 20 h at 37 °C. DNA synthesis was assayed by adding 1 μCi of [^{3}H]-thymidine for 4 h. The cells were rinsed, dissolved in 1 N NaOH, and counted in a β counter. Results are the mean of three experiments. The results of c-fos mRNA expression (□) are for panel A. Both results are presented as the percent of maximal insulin stimulation.

When the A/K1018 cells expressing kinase-incompetent human insulin receptors was studied (open squares), no effect of insulin on c-*fos* and *Egr*-1 mRNA levels was observed. In fact, the effect of insulin in A/K1018 cells was less than even the small effect seen in Rat 1 cells, consistent with a dominant negative effect of the kinase inactive insulin receptor to inhibit endogenous receptor function. Similar to the results with insulin stimulation, the effects of IGF-I on c-*fos* and *Egr*-1 mRNA expression were completely inhibited in A/K1018 cells compared to either the HIRc-B or parental Rat 1 fibroblasts. These results demonstrate that insulin receptor kinase activity is necessary for effective signaling and that the kinase inactive receptors inhibit the signaling of both endogenous insulin and IGF-I receptors.

We have conducted similar studies examining the ability of insulin to stimulate c-*fos* and *Egr*-1 mRNA induction in cells expressing other mutant insulin receptors. These include cells expressing a truncated insulin receptor, missing the C-terminal 43 amino acids (ΔCT cells) or a receptor with the two C-terminal tyrosine autophosphorylation sites mutated to phenylalanine (Y/F2 cells). As seen in Figure 6, stimulation of *Egr*-1 mRNA is comparable among HIRc, ΔCT, and Y/F2 cells (panel B). In contrast, c-*fos* stimulation in HIRc and Y/F2 cells shows a similar response, but a striking decrease in insulin stimulation of c-*fos* mRNA is observed in ΔCT cells (panel A).

We next considered the potential downstream signaling molecules which might couple insulin receptors to induction of these genes. For example, insulin increases cellular p21rasGTP content (Osterop et al., 1992; Porras et al., 1992; Maassen et al., 1992), and rasGTP plays a key role in mitogenic signaling by insulin (Jhun et al., 1994). Figure 7A summarizes the effect of insulin to enhance p21rasGTP concentration across the various cell lines. Insulin's effects on ras are equal or greater in ΔCT and Y/F2 cells, compared to HIRc cells, and markedly impaired in A/K1018 cells.

Current evidence indicates that ras is an upstream effector of raf kinase, which initiates a serine kinase cascade including MAP kinase kinase (MEK), MAP kinase (ERK), and S6 kinase (Porras et al., 1992; Rap, 1991; Rhomas, 1992; Wood

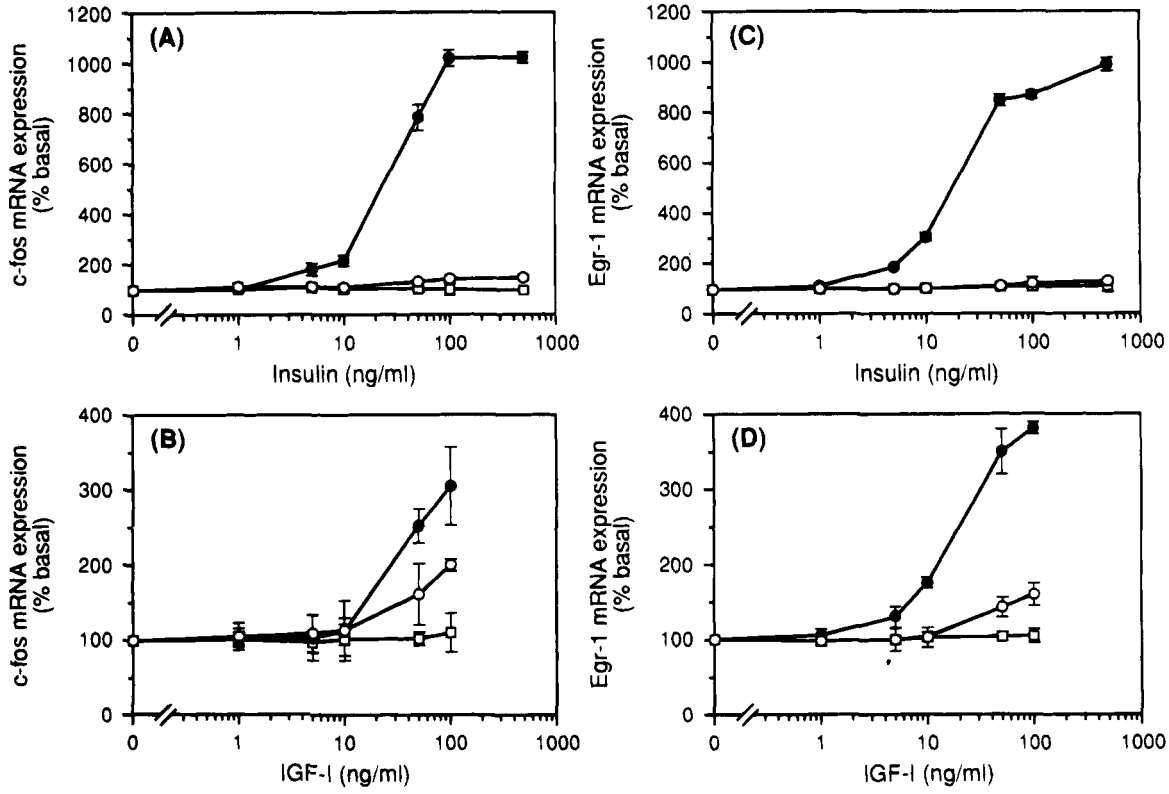

FIGURE 5: Dose–response of c-*fos* and *Egr*-1 mRNA expression after insulin and IGF-I stimulation in HIRc-B, A/K1018, and Rat 1 cells. HIRc-B (●), A/K1018 (□) and Rat 1 (○) cells were deprived of serum for 48 h and then stimulated for 30 min with either insulin (panels A and C) or IGF-I (panels B and D) at the indicated concentrations. RNA was isolated and 0.1 μg of RNA was used for RT/PCR assay. cDNA equivalent to 0.01 μg of RNA was subjected to PCR amplification. PCR products were resolved by polyacrylamide gel electrophoresis and incorporated radioactivity was quantitated by the AMBIS system. c-*fos* mRNA (panels A and B) and *Egr*-1 mRNA (panels C and D) expression were normalized as in legend of Figure 3B. Results are the mean of three independent experiments.

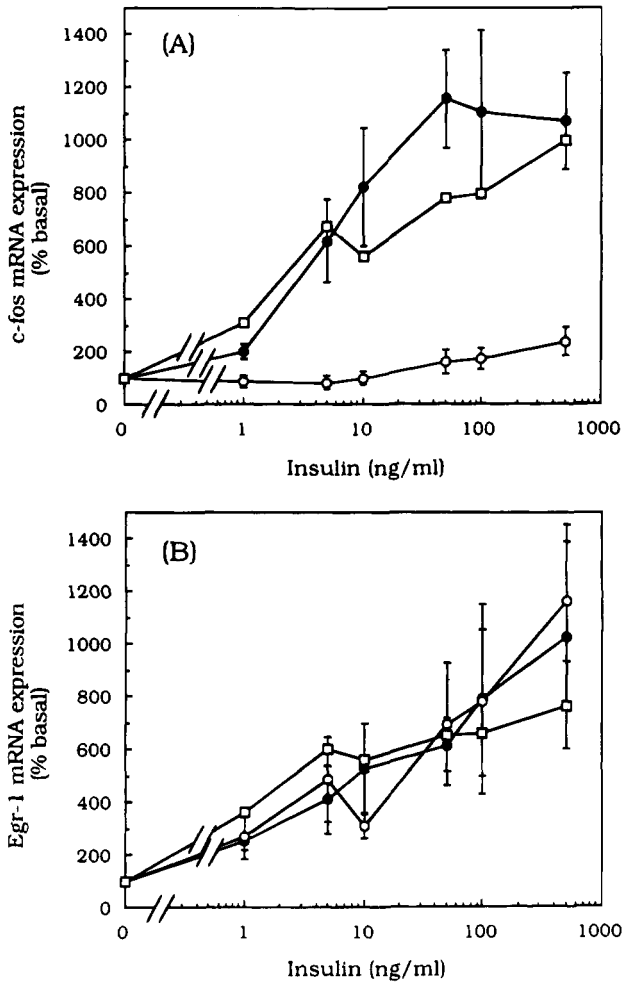

FIGURE 6: Dose–response of c-*fos* and *Egr*-1 mRNA expression after insulin stimulation in HIRc-B, ΔCT, and Y/F2 cells. HIRc-B (●), ΔCT (○), and Y/F2 (□) cells were deprived of serum for 48 h and then stimulated for 30 min with insulin at the indicated concentrations. RNA was isolated and 0.1 μg of RNA was used for RT/PCR assay. cDNA equivalent to 0.01 μg of RNA was subjected to PCR amplification. PCR products were resolved by polyacrylamide gel electrophoresis and incorporated radioactivity was quantitated by the AMBIS system. c-*fos* mRNA (panel A) and *Egr*-1 mRNA (panel B) expression were normalized as in legend to Figure 3B. Results are the mean of three (HIRc-B and ΔCT) or two (Y/F2) independent experiments.

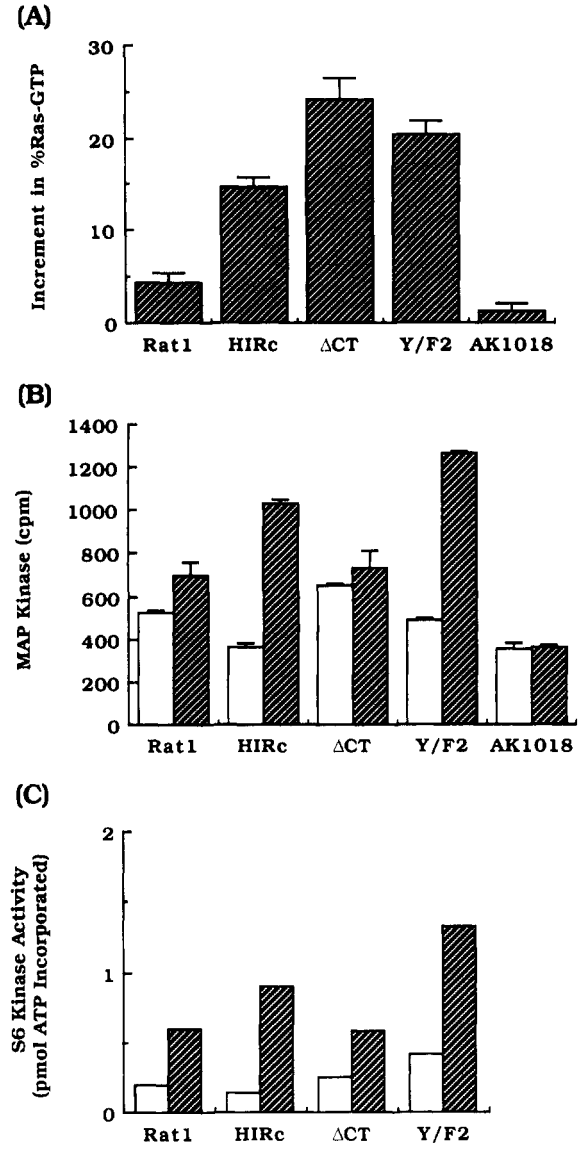

FIGURE 7: Activation of p21ras, MAP kinase, and S6 kinase by insulin. GTP-bound ras (panel A), MAP kinase (panel B), and S6 kinase (panel C) before (open bar) and after (shaded bar) insulin stimulation were assayed as described under Experimental Procedures. Results are from three (panels A and B) or two (panel C) independent experiments. The data in panel A represent the insulin stimulated absolute increment in percent ras GTP for each cell line. The basal and insulin stimulated values (mean ± SE) for percent ras in the GTP bound form were 26.9 ± 2.1 → 31.4 ± 1.8%, 36.7 ± 1.8 → 51.2 ± 3.4%, 28.5 ± 2.3 → 52.3 ± 2.8%, 33.3 ± 1.5 → 53.4 ± 2.7%, and 26.4 ± 1.9 → 27.6 ± 1.5%, for Rat 1, HIRc, ΔCT, Y/F2, and AK cells, respectively. Differences between ΔCT and HIRc and Y/F2 and HIRc are $p < 0.05$.

et al., 1992). Consequently, we measured MAP kinase and S6 kinase activity in these cells as shown in Figure 7B,C. Here we see that stimulation of both of these serine kinases is enhanced in HIRc and Y/F2 cells, compared to Rat 1 fibroblasts, consistent with the RT/PCR and p21rasGTP data. In contrast, insulin stimulated MAP kinase and S6 kinase activity is diminished in ΔCT cells. Thus, in these cells, the insulin signal appears to be inhibited, or extinguished, at some point distal to p21rasGTP. It should be noted that our measurement provides an assessment of overall S6 kinase activity and does not allow us to discriminate between p70S6K and $p90^{rsk}$. Therefore, we do not know the relative contribution of these kinases to the results in Figure 7C and to the defect of S6 kinase activity in ΔCT cells.

DISCUSSION

Insulin stimulation of quiescent cells expressing normal insulin receptors results in a rapid increase in specific gene expression within target cells (Stumpo & Blackshear, 1986, 1991; Burgering et al., 1991; Messina, 1990). In this paper, we examined the acute effects of insulin and IGF-I on two immediate early genes, c-*fos* and *Egr*-1, in cells overexpressing normal and kinase-defective human insulin receptors. Utilizing a rapid, sensitive, and quantitative RT/PCR assay, we demonstrate that insulin and IGF-I induce c-*fos* and *Egr*-1 gene expression and that receptor kinase activity is required for this induction.

The RT/PCR assay proved to be a sensitive and quantitative method to measure gene expression. It provided results equivalent to those of Northern blot analysis, yet required only 0.1% the amount of RNA and less time. Using the RT/PCR method, it was possible to show that insulin and

IGF-I induced c-*fos* and *Egr*-1 mRNA expression in a time- and dose-dependent fashion. Production of c-*fos* mRNA was followed by synthesis of new c-fos protein. Insulin action was much greater in the transfected HIRc-B cells than in the parental Rat 1 cells, demonstrating the role of competent insulin receptors in this effect. In contrast, the effects of serum on c-*fos* and *Egr*-1 mRNA were comparable between Rat 1 and HIRc-B cells, while IGF-I effects were only modestly increased in HIRc-B cells. The time courses of both c-*fos* and *Egr*-1 mRNA expression and c-fos protein synthesis in HIRc-B cells are similar to those previously reported for other cell types (Muller et al., 1984; Milbrandt, 1986; Kruijer et al., 1985; Sukhtme et al., 1987, 1988). The mRNA levels peak at 30 min, while maximal protein expression was more delayed, peaking at 1 h. Previous studies using mutated insulin receptors have employed transfection into those cells which are not classical insulin targets. Consequently, biologic responses in these stable cell lines tend to be rather anemic. Using the RT/PCR method, we were able to easily measure high amplitude (8−10-fold) effects of insulin on c-*fos* message within 30 min. This approach should have general applicability as a means to analyze biologic signaling by transfected mutant receptors in fibroblasts as well as other parental cell lines.

Dose−response studies were performed in A/K1018 cells expressing kinase inactive insulin receptors and showed no effect of insulin on c-*fos* and *Egr*-1 mRNA levels. Since a small stimulatory effect of insulin was present in the parental Rat 1 cells, this suggests that insulin action was inhibited in the AK1018 cells. This inhibitory effect on insulin action has been reported previously using insulin receptors with defective kinase activity in Rat 1 fibroblasts (Maegawa et al., 1988; McClain et al., 1987, 1990; Grako et al., 1992) and in CHO (Chou et al., 1987; Ebina et al., 1987) cells, for insulin stimulation of hexose transport, glycogen synthase activity, gene expression, and mitogenesis, and this has been termed a dominant negative effect (Begum et al., 1993; Mundshau et al., 1994; Grako et al., 1992). In the A/K1018 cell, the endogenous Rat 1 insulin receptors are expressed to the same extent as in untransfected Rat 1 cells (1.7×10^3 insulin receptors/cell), possess tyrosine kinase activity, and undergo autophosphorylation *in vitro* and *in vivo*. The dominant negative effect of A/K1018 receptors to inhibit endogenous receptor signaling has been ascribed to either formation of inactive hybrid rat:A/K1018 heterotetramers, substrate competition, or both (Grako et al., 1992).

Interestingly, as seen in Figure 4, IGF-I stimulation of both c-*fos* and *Egr*-1 mRNA was also strikingly inhibited in the A/K1018 cells. Thus, a "dominant negative" effect of the kinase inactive insulin receptor was also exhibited against endogenous IGF-I receptor signaling. Previous studies demonstrated that 50−60% of the endogenous IGF-I receptors form hybrid heterotetramers with the A/K1018 receptor (Grako et al., 1992), and it is reasonable to speculate that these hybrid receptors are inactive with respect to biologic signaling. On the other hand, 40−50% of the IGF-I receptors remain in the native heterodimer structure and should represent enough intact receptors to mediate ample IGF-I signaling. Nevertheless, as seen in Figure 5, IGF-I effects on the induction of immediate early gene expression were essentially obliterated in A/K1018 cells. This indicates that some mechanism, in addition to hybrid formation, must be operative to inhibit the remaining intact IGF-I receptors. One possible explanation for this inhibitory phenomenon is substrate competition; i.e., rate-limiting substrates may interact with the A/K1018 receptors in a nonproductive manner and thus are not available for interaction with the endogenous kinase competent IGF-I receptors. Indeed, it has been shown that, in A/K1018 cells, IGF-I stimulation of the endogenous substrate, pp185, is inhibited (Begum et al., 1993; Mundshau et al., 1994). Interestingly, IGF-I stimulated glucose transport is normal in A/K1018 cells, indicating divergent signaling pathways for IGF-I stimulated metabolic and mitogenic signaling.

The results in the Y/F2 and ΔCT cells were also of interest and provide additional insight into the signaling pathways which couple insulin receptors to stimulation of c-*fos* and *Egr*-1 mRNA. In all cell lines, there was a good correlation between the effects of insulin to stimulate p21rasGTP and Egr-1 mRNA, indicating that generation of p21rasGTP is an intermediary step in the insulin signaling pathway which results in mRNA induction from this gene. In contrast, while the effects of insulin on MAP kinase and S6 kinase activity were robust in Y/F2 cells, only minimal stimulation was seen in ΔCT cells. Current evidence is that following p21ras, the insulin signaling cascade involves p21rasGTP → raf kinase → MAP kinase kinase (MEK) → MAP kinase (ERK) → S6 kinase. In this event, it would appear that, in ΔCT cells, the insulin signal is poorly propagated downstream of p21ras. Whether this means that an additional action of insulin is necessary to carry the signal beyond p21ras, and that this additional insulin action is impaired in ΔCT cells, or whether an inhibitory effect downstream of p21ras exists in these cells is unknown. However, since stimulation of *Egr*-1 mRNA was preserved in these cells, the results suggest that MAP kinase and S6 kinase do not reside in the insulin signaling pathway leading to induction of *Egr*-1 mRNA. It is of interest that previous studies have also shown that not all actions of insulin are intact in ΔCT cells (Maegawa et al., 1988; Begum et al., 1993). Thus, this receptor displays impaired stimulation of glucose transport and PP-I activity in the rat fibroblast background, and it is possible that there is some common factor which impairs these actions in ΔCT cells. Insulin's effects to stimulate DNA synthesis are enhanced or normal in Y/F2 (Takata et al., 1992) and ΔCT (Maegawa et al., 1988) cells, consistent with the results of p21rasGTP and *Egr*-1 stimulation. The impaired c-*fos* induction in the ΔCT cells suggests that c-*fos* is poorly coupled to cell cycle progression.

In the ΔCT cells, c-*fos* mRNA was only poorly induced, indicating a striking dissociation between insulin stimulation of *Egr*-1 compared to c-*fos* genes. This suggests that the signaling pathways leading to these two biologic effects differ, and this is consistent with previous studies of PDGF action which showed a dissociation between *Egr*-1 and c-*fos* mRNA induction (Mundshau et al., 1994). It is known that MAP kinase leads to the phosphorylation of transcription factors such as ELK-1 and jun, which can stimulate c-fos transcription through the c-*fos* gene serum response element (SRE). Thus, the decrease in MAP kinase stimulation in ΔCT cells could account for the impaired c-*fos* induction. It is not clear why the regulation of *Egr*-1 mRNA is so different in ΔCT cells. The promoters of these genes contain many similar elements, including SRE, AP-1, and CArG motifs. Perhaps, at some point in the signaling cascade, there are parallel pathways for *Egr*-1 versus c-*fos* stimulation, and only

the c-*fos* pathway is impaired in ΔCT cells. For example, in ΔCT cells there is a decrease in insulin's effect to inhibit pp2A activity[2] and pp2A mediated dephosphorylation events could be important for transcriptional stimulation of c-*fos*, but not *Egr*-1. Clearly, more detailed information concerning the transcriptional regulation of these two early response genes will be necessary before this finding can be understood.

In summary, we have utilized an RT/PCR method to explore the signaling pathways leading from the insulin receptor to induction of two early response genes, *Egr*-1 and c-*fos*. Our findings show that insulin leads to a rapid time- and dose-dependent increase in c-*fos* and *Egr*-1 mRNA and that an intact tyrosine kinase activity of the insulin receptor is necessary for this response. In Y/F2 and ΔCT cells, the full response pathway is intact, with respect to stimulation of *Egr*-1 mRNA, despite the fact that the insulin signaling pathway is extinguished in ΔCT cells at some point distal to ras GTP generation, as manifested by impaired stimulation of MAP kinase and S6 kinase activity. The signaling events regulating *Egr*-1 and c-*fos* mRNA can be dissociated since induction of c-*fos* mRNA is blunted in ΔCT cells whereas stimulation of *Egr*-1 mRNA is enhanced.

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