Activation of cJun NH_2-Terminal Kinase/Stress-Activated Protein Kinase by Insulin†

Bradley S. Miller, Uma T. Shankavaram,‡ Mark J. Horney, Angela C. S. Gore, David T. Kurtz, and Steven A. Rosenzweig*

Department of Cell and Molecular Pharmacology and Experimental Therapeutics, Medical University of South Carolina, 171 Ashley Avenue, Charleston, South Carolina 29425

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ABSTRACT: One of insulin's many biological effects is the increased transcription of AP-1-regulated genes. cJun is the principal component of the AP-1 transcription complex, which is regulated by the newly discovered members of the MAPK superfamily referred to as cJun NH_2-terminal kinases (JNKs) or stress-activated protein kinases (SAPKs). We show that insulin stimulates a dose- and time-dependent increase in JNK activity in Rat 1 fibroblasts overexpressing human insulin receptors (Rat 1 HIR cells). Using two different polyclonal anti-JNK antibodies, JNK activity was measured after immunoprecipitation from whole cell extracts by phosphorylation of GSTcJun(1−79). Peak activation occurred 15 min after insulin addition, resulting in a 2.5-fold increase in GSTcJun(1−79) phosphorylation over unstimulated controls. Maximal JNK activation correlated with the onset of AP-1 DNA binding activity. Both insulin-stimulated JNK activity and insulin-induced AP-1 transcriptional activity were found to be Ras-dependent. These data suggest that in Rat 1 cells, JNK activation may play a role in insulin-regulated AP-1 transcriptional activity leading to a mitogenic response.

The insulin receptor (IR)[1] signal transduction cascade involves the integration of numerous cytoplasmic and nuclear effectors, the net effect of which leads to gene transcription regulated, in part, by the activator protein-1 (AP-1) transcription complex (White & Kahn, 1994). Early cytoplasmic events include the tyrosine phosphorylation of IRS-1 and Shc. Phosphorylated Shc binds Grb2•Sos complexes and activates Ras by increasing the rate of exchange of GDP for GTP (Baltensperger et al., 1993). Ras activation in turn leads to stimulation of the MAPK cascade involving Raf-1, MEK, and Erk. Once activated, Erk can translocate to the nucleus and phosphorylate TCF/Elk1 to regulate transcription at the serum response element (SRE; Gille et al., 1992). This results in the generation of cFos, thereby indirectly increasing AP-1 activity. Insulin has been shown to increase cFos production and the phosphorylation of a cFos-related protein (Kim & Kahn, 1994). A number of distinct cFos kinases have been identified, all of which are distinct from Erk and other members of the MAPK superfamily (Taylor et al., 1994; Deng & Karin, 1995).

The Erks may also phosphorylate cJun, a component of the AP-1 complex (Alvarez et al., 1991; Chen et al., 1992). However, this phosphorylation, in cJun's DNA binding domain, would inhibit AP-1 DNA binding (Alvarez et al., 1991; Minden et al., 1994a; Chou et al., 1992). The JNKs have been shown to phosphorylate cJun on sites which increase the transcriptional activity of the AP-1 complex (Hibi et al., 1993; Dérijard et al., 1994). The mechanisms involved in the regulation of JNK activity are currently under intense investigation. JNK is activated by Ras and several mitogens acting via the Ras pathway (Dérijard et al., 1994; Karin, 1995). It is also stimulated by agents that act through the Rho family of low molecular weight GTP-binding proteins (Vojtek & Cooper, 1995). Therefore, JNKs may play an important role in the regulation of AP-1 transcription in a Ras-dependent/-independent manner.

Using the well-characterized Rat 1 fibroblast cell line, stably overexpressing human insulin receptors (Rat 1 HIR; McClain et al., 1987), we have examined insulin-mediated activation of cJun/AP-1 transcription. Solid phase kinase assays and polyclonal anti-JNK antibodies in conjunction with immunoprecipitation kinase assays using GSTcJun(1−79) as substrate were used to demonstrate that insulin treatment results in JNK activation. Expression of dominant-negative N^{17} Ras blocked insulin-stimulated JNK activation and AP-1 transcriptional activity. These data suggest that the activation of JNK may serve as an ancillary signal in the pleiotropic actions of insulin.

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* To whom correspondence should be addressed. Telephone: (803) 792-5841. Fax: (803) 792-2475. E-mail: rosenzsa@smtpgw.musc.edu.

‡ Present address: Laboratory of Immunology, National Institute of Dental Research, National Institutes of Health, Bethesda, MD 20892.

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[1] Abbreviations: JNK, cJun NH_2-terminal kinase; SAPK, stress-activated protein kinase; GST, glutathione *S*-transferase; HIR, human insulin receptor; IRS-1, insulin receptor substrate-1; SHc, SH2-containing protein with collagen tail; SH2, src homology domain-2; Grb2, growth factor receptor binding protein-2; mSOS, mammalian homolog of son of sevenless; R1, Rat 1 HIR C cells; RJ, Rat 1 HIR C JNK transfectants; TCF, ternary complex factor; AP-1, activator protein-1; TRE, tumor responsive element; SRE, serum response element; RSV, Rous sarcoma virus; CMV, cytomegalovirus; Hyg, hygromycin; PMSF, phenylmethanesulfonylfluoride; DMEM, Dulbecco's modified Eagle's medium; MAPK, mitogen-activated protein kinase; Erk, extracellular signal-regulated kinase; MEKK-1, MAPK/Erk kinase (MEK) kinase; NGF, nerve growth factor; PDGF, platelet-derived growth factor; EGF, epidermal growth factor; DTT, dithiothreitol; SDS−PAGE, sodium dodecyl sulfate−polyacrylamide gel electrophoresis; HEPES, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid; PCR, polymerase chain reaction.

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EXPERIMENTAL PROCEDURES

Materials. The ECL Western blotting detection system was from Amersham (Arlington Heights, IL). [γ-^{32}P]ATP was from Dupont-NEN (Boston, MA). Porcine insulin was the gift of Eli Lilly and Co. (Indianapolis, IN). DMEM/F12 was obtained from GIBCO BRL (Grand Island, NY). PMSF, aprotinin, BSA, Triton X-100, sodium orthovanadate, and leupeptin were from Sigma Chemical Co. (St. Louis, MO). Restriction enzymes (*Eco*RI, *Bam*HI, *Xba*), Klenow enzyme, and DNA ligase were from Boehringer Mannheim (Indianapolis, IN). Protein G−Sepharose and glutathione−Sepharose were from Pharmacia (Piscataway, NJ). A double-stranded probe corresponding to the AP-1 binding sequence

5′-AGCTTGG**TGACTCA**TCCG-3′
3′-TCGAACC**ACTGAGT**AGGC-5′

a single-stranded oligonucleotide corresponding to the FLAG (Eastman Kodak Co., New Haven, CT) epitope (ATGGAC-TACAAGGACGACGATGAC), and the peptide -Cys-Asp^{362}-Leu-Glu-Glu-Arg-Thr-Lys-Asn-Gly^{370}, corresponding to the C-terminal end of JNK1 protein (Dérijard et al., 1994), were synthesized by the core facility at MUSC.

Cell Culture. Rat 1 HIR cells expressing 1.2×10^6 human insulin receptors/cell (the kind gift of Dr. Donald McClain, University of Mississippi) were maintained as detailed (McClain et al., 1987). Confluent monolayers were rinsed and incubated in serum-free medium containing 0.1% BSA for 30 min prior to experimental additions.

Antibodies. Polyclonal anti-JNK was raised against a keyhole limpet hemocyanin conjugate of the peptide, CD^{362}-LEERTKNG^{370} (Dérijard et al., 1994) according to Lerner et al. (1981). Antibodies were affinity-purified on columns of peptide−Sepharose (Rosenzweig et al., 1990) prior to use. Anti-SAPKβ generated against full-length GST-SAPKβ and rSAPK α and β were the kind gifts of Drs. John M. Kyriakis and Irma Sanchez (Massachusetts General Hospital, Charlestown, MA). Peroxidase-conjugated goat anti-rabbit IgG was from Chemicon International, Inc. (Temecula, CA).

Recombinant Protein Expression and Purification. FLAG-JNK1 (fJNK1) cDNA (Dérijard et al., 1994) and pGEX2T-GSTcJun(1−79) (Hibi et al., 1993) were the kind gifts of Dr. Roger Davis (HHMI, University of Massachusetts, Worcester, MA). GST-FLAG-JNK1 was prepared by amplification of fJNK1 by PCR using an oligo corresponding to the cDNA sequence of the FLAG epitope. The PCR product was digested with *Xba* and filled out with Klenow enzyme. After a subsequent digest with *Bam*HI, it was gel-purified and ligated into pGEX2T (Pharmacia) digested with *Eco*RI and *Bam*HI. The recombinant protein and solid phase affinity matrix, GSTcJun(1−79)−GSH−Sepharose, were generated and purified according to Hibi et al. (1993). The histidine-tagged chicken cJun expression vector pDSCJ8 (a gift from Dr. Peter Vogt, Scripps Institute, La Jolla, CA) was expressed in *E. coli*. The rcJun so obtained was purified by nickel chromatography (His·Bind Resin; Novagen, Madison, WI) in the presence of 6 M guanidine hydrochloride (Monteclaro & Vogt, 1993) and renatured by extensive dialysis.

Cell Lysate Preparation. Stimulated cells were washed and harvested in PBS with 1 mM sodium orthovanadate. After centrifugation for 8 min at 1500*g* at 4 °C, the cell pellet from one 10-cm dish was resuspended in 200 μL of lysis buffer (20 mM Tris, pH 7.4, 25 mM β-glycerophosphate, 137 mM NaCl, 2 mM EDTA, 2 mM tetrasodium pyrophosphate, 1 mM sodium orthovanadate, 1% Triton X-100, and 10% glycerol) and incubated at 4 °C for 30 min. The samples were microfuged for 30 min at 13 000 rpm at 4 °C. Supernatants were collected and their protein concentrations determined (Bradford, 1976). Samples were stored at −80 °C until use.

Immunoblotting. Lysates were boiled in SDS sample buffer, resolved by SDS−PAGE, and transferred onto nitrocellulose (Rosenzweig et al., 1990). After quenching in Tris-buffered saline with Tween (TBST: 10 mM Tris, pH 8.0, 150 mM NaCl, and 0.05% Tween 20) containing 5% nonfat dry milk (Johnson et al., 1984), membranes were incubated with 5 μg/mL anti-JNK in the same buffer for 18 h at 4 °C. After repeated washing in TBST with milk, the blots were incubated with peroxidase-conjugated goat anti-rabbit IgG (1:5000) for 1 h at 23 °C and washed in TBST. Immunoreactive proteins were visualized with the ECL reagent followed by exposure to Kodak XRP X-ray film for 10 min.

Immunoprecipitation Kinase Assay. Lysates (100 μg) were diluted with lysis buffer (200 μL) and incubated with anti-JNK (2.5 μg) for 30 min at 23 °C. Ten microliters of protein G−Sepharose (1:1 slurry) was added, and after 30 min at 23 °C, the beads were pelleted and washed 3 times with lysis buffer and once with kinase buffer (25 mM HEPES, pH 7.4, 25 mM β-glycerophosphate, 25 mM $MgCl_2$, 25 mM ATP, 1 mM sodium orthovanadate, and 0.5 mM DTT). Thirty microliters of kinase buffer containing 2 μCi of [γ-^{32}P]ATP and 10 μg of GSTcJun(1−79) (Hibi et al., 1993) was added, and the samples were incubated for 30 min at 30 °C. The reaction was terminated by addition of sample buffer and the proteins were resolved by SDS−PAGE (7.5% gel). Incorporation of [^{32}P]phosphate was visualized by autoradiography of the destained, dried gels and quantified by Cerenkov counting of the excised bands.

Solid-Phase Kinase Assay. Lysates (100 μg) were diluted with binding buffer (200 μL) (20 mM HEPES, pH 7.7, 50 mM NaCl, 2.5 mM $MgCl_2$, 0.1 mM EDTA, 0.05% Triton X-100, and 1 mM sodium orthovanadate) and mixed with 10 μL of GSTcJun(1−79)−GSH−Sepharose (2 μg/mL) at 4 °C for 3 h. The beads were pelleted and washed 3 times with binding buffer (200 μL). The kinase reaction was initiated by addition of 30 μL of kinase buffer containing 2 μCi of [γ-^{32}P]ATP and 10 μg of GSTcJun(1−79) and incubated for 30 min at 30 °C. Reactions were terminated by addition of sample buffer, and the samples were analyzed as detailed.

End Labeling. Twenty picomoles of oligonucleotide containing the TRE motif was 5′-end-labeled using polynucleotide kinase and [γ-^{32}P]ATP according to Maniatis et al. (1982). Labeled oligonucleotide was separated from the reaction mixture on a DE 52 cationic exchange column using 2 M triethylamine acetate as the mobile phase. Fractions containing radiolabeled probe were dried in tubes, resuspended in TNE (10 mM Tris-HCl, pH 8.0, 100 mM NaCl, and 1 mM EDTA), and stored at −20 °C until used.

Electrophoretic Mobility Shift Assay. Confluent 10 cm dishes were treated with 10^{-8} M insulin, and nuclear extracts were prepared (Andrews & Faller, 1991). Extracts were assayed for protein content and stored at −80 °C until use. Mobility shift assays were carried out (Singh et al., 1986)

using 5 μg of nuclear protein incubated in 20 μL of 50 mM HEPES, pH 7.4, 0.5 M NaCl, 0.5 mM PMSF, 0.5 mg/mL BSA, 20% v/v glycerol, 1 mM EDTA, 0.5 mM DTT, 50 mg/mL poly(dI-dC), and 2×10^5 cpm of the labeled oligo for 30 min at 30 °C. Binding was terminated by addition of 5 μL of sample buffer (10 mM DTT, 5% v/v glycerol, and 0.05% bromophenol blue). Samples were resolved on a 5% nondenaturing gel using 0.25× TBE (22 mM Tris, pH 8.0, 22 mM boric acid, and 50 mM EDTA) running buffer. DNA−protein complexes were visualized by autoradiography of the dried gel using Kodak XRP X-ray film and a Dupont Cronex Lightning Plus intensifying screen for 18−48 h.

Transient and Permanent Transfections. For transient transfections, subconfluent Rat 1-HIR cells in 60 mm dishes were transfected with the following cesium chloride-purified expression vectors using the calcium phosphate method (Sambrook et al., 1989): N^{17}Ras (Dr. Larry Feig, Tufts University) or pcDNA3 along with FLAG JNK1 (10 μg; Dérijard et al., 1994) for immunoprecipitation kinase assays or 3X-TRE-CAT (1 μg) for CAT assay. After 5 h, cells were shocked with DMEM/F12 containing 10% fetal bovine serum and 25% glycerol for 1 min. After removal of the glycerol and extensive washing with Puck's saline, the cells were refed with DMEM/F12 containing 1% FBS. For the preparation of permanent transfectants, the cells were transfected with CMV-JNK1 (10 μg, Dérijard et al., 1994) and RSV-Hyg (10 μg; a hygromycin B-resistance vector, Gritz & Davies, 1983) expression vectors as described above. The cells were then split into medium containing 400 μg/mL each of G418 and hygromycin B (Calbiochem) and maintained until colony formation. Clonal populations were maintained in the presence of 100 μg/mL of each selection agent and were periodically reselected with 400 μg/mL of each reagent. Verification of JNK1 overexpression was determined by immunoblot, immunocytochemical, and JNK activity assays.

CAT Assay. Eighteen hours after refeeding, cells were rinsed and refed with serum-free DMEM/F12 containing 0.1% BSA and experimental additions; 24 h later, the cells were rinsed with PBS, harvested in 1.4 mL of PBS, and pelleted by microcentrifugation. The pellet was resuspended in 60 μL of reporter lysis buffer (Promega) and incubated at 23 °C for 15 min. After microcentrifugation, 8.75 μL of the supernatant was removed and heat-inactivated by incubation at 70 °C for 15 min, followed by addition of 2.5 μL of *n*-butyrylCoA and 1.25 μL of [^{14}C]chloramphenicol (25 μCi/mL). This mixture was incubated for 18 h at 37 °C followed by extraction of *n*-butyryl-[^{14}C]chloramphenicol with tetramethylpentadecane/mixed xylenes (2:1) and quantification by liquid scintillation counting (Seed & Sheen, 1988). Variation in transfection efficiency was normalized by the protein assay.

RESULTS

To examine the role JNKs might play in insulin receptor signaling cascades, we developed a polyclonal antiserum based on a C-terminal peptide sequence of JNK1 (Figure 1A). On the basis of sequence homology, it was anticipated that this antiserum would recognize all members of the JNK/SAPK family. However, it would not recognize Erk1/Erk2 (Boulton et al., 1991) or p38/HOG1 (Boulton et al., 1991; Han et al., 1994) since they lack homologous regions. The antiserum generated recognized a JNK1 fusion protein and rSAPKα/JNK2 by immunoblot analysis (Figure 1B) and immunoprecipitation (data not shown). However, these antibodies did not interact with rSAPKβ in immunoblot analysis. The inability to recognize rSAPKβ may be due to the small difference in its primary sequence compared to JNK1, thereby altering the epitope conformation. In whole cell extracts from Rat 1 HIR cells, anti-JNK IgG labeled a 46 kDa doublet, a single band at 54 kDa, and a prominent band at 70 kDa. The 46 and 54 kDa proteins correspond to JNK1/SAPKγ and SAPKα/JNK2, respectively. The 70 kDa band is an unknown protein which lacked kinase activity toward GST-cJun(1−79) based on in-gel kinase assay (data not shown).

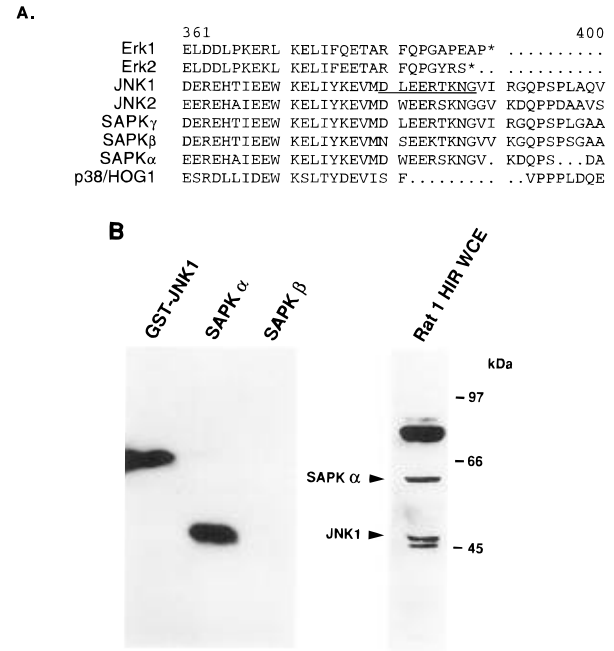

FIGURE 1: JNK anti-peptide IgG recognizes JNK1 and JNK2/SAPKα. (A) Primary sequence alignment of the JNK/SAPK family created by PILEUP program from Wisconsin Genetics Computer Group. Polyclonal anti-JNK was generated with the peptide sequence indicated by an underline and the addition of an NH_2-terminal cysteine residue. Sequences are aligned to Erks 1 and 2 (Boulton et al., 1991), SAPK α, β, and γ (Kyriakis et al., 1994), JNK1 (Dérijard et al., 1994), JNK2 (Kallunki et al., 1994), and p38/HOG1 (Han et al., 1994). (B) Antibody specificity was tested by immunoblotting recombinant standards and whole cell extract (WCE) with anti-JNK (5 μg/mL) as follows: 1 μg of GST-fJNK1, 1 μg of rSAPKα, 2.5 μg of rSAPKβ, and 100 μg of unstimulated Rat 1 HIR whole cell extract.

AP-1 transcriptional activity is positively regulated by the phosphorylation of cJun on Ser 63 and Ser 73 (Pulverer et al., 1991; Smeal et al., 1991). Hibi et al. (1993) demonstrated that JNKs specifically phosphorylate a GST fusion protein containing the amino-terminal portion of cJun(1−79) on Ser 63 and Ser 73. Alvarez et al. (1991) and Minden et al. (1994) further demonstrated that Erks 1/2 were unable to phosphorylate these N-terminal sites *in vitro*. Thus, GSTcJun(1−79) has been validated as a specific substrate for the measurement of JNK activity. The ability of JNK to bind tightly to the NH_2-terminus of cJun (Hibi et al., 1993; Kallunki et al., 1994) forms the basis of a solid-phase kinase assay for measuring all forms of activated JNK in cell extracts. Using the solid-phase kinase assay, we demon-

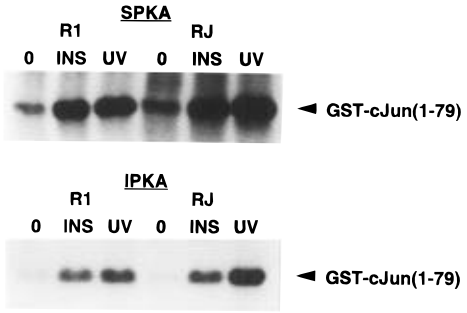

FIGURE 2: Comparison of insulin-induced JNK activation by solid-phase kinase assay and immune complex kinase assay. Rat 1 HIR (R1) cells or R1 cells permanently transfected with JNK1 (RJ) were stimulated with 10 nM insulin (15 min) or 30 s of UV. Equal aliquots of whole cell extracts (100 μg) were assayed for JNK activity by the solid-phase kinase assay (SPKA) or by the immune complex kinase assay (IPKA) using polyclonal anti-JNK IgG. In each assay, GST-cJun(1−79) was used as kinase substrate as described under Experimental Procedures. Shown is the autoradiogram of the dried gel.

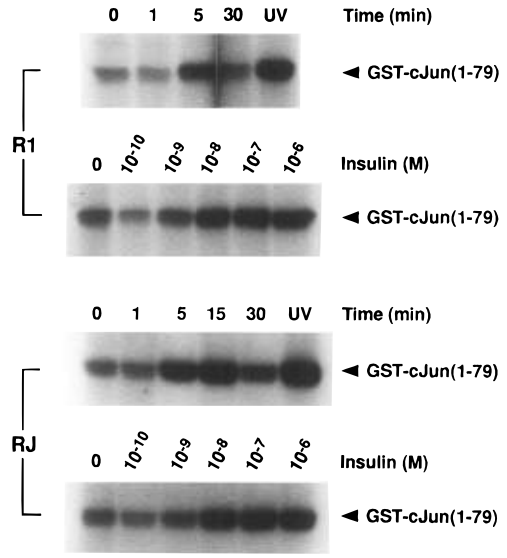

FIGURE 3: Time- and dose-dependent JNK activation by insulin. R1 and RJ cells were stimulated for the times indicated with 10^{-7} M insulin or for 15 min with the indicated doses of insulin. Whole cell extracts were prepared and assayed for JNK activity by the immune complex kinase assay.

strated that insulin stimulates JNK activation in Rat 1 HIR fibroblasts (R1; Figure 2). The activation of JNK by insulin was confirmed by immune complex kinase assay (Figure 2) using site-specific anti-JNK IgG. In both assays, insulin activated JNK to a level comparable to that obtained by UV treatment. Similar results were obtained with Rat 1 HIR cells stably overexpressing JNK1 (RJ). As shown in Figure 3, insulin stimulated a time- and dose-dependent increase in JNK activity in both the untransfected and transfected cell lines. Within 5 min of insulin addition, JNK activity was elevated 2-fold over unstimulated controls (Figure 3). JNK activation peaked at 15 min with a 2.5-fold stimulation and returned to near basal levels by 30 min. Maximal JNK activation was achieved by stimulation with 10 nM insulin in both cell lines. The JNK activation observed in response to insulin was equivalent to the increase in JNK activity induced by UV (2.5-fold in R1 cells and 3.5-fold in RJ cells).

Transcription of c*jun* mRNA occurs rapidly in response to insulin stimulation of fibroblasts overexpressing insulin receptors (Burgering et al., 1991; Draznin et al., 1993). Since

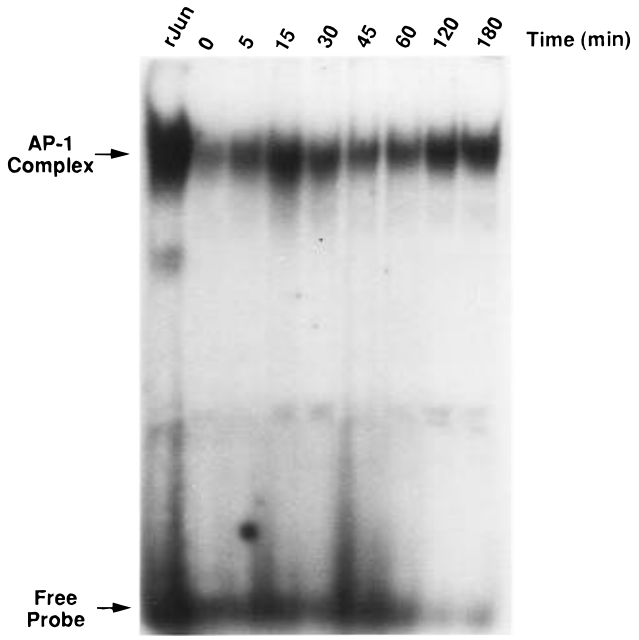

FIGURE 4: Time course of insulin-stimulated AP-1 DNA binding. Nuclear extracts (5 μg) from cells treated with 10^{-8} M insulin for the times indicated were assayed for AP-1 DNA binding activity using the electrophoretic mobility shift assay as described under Experimental Procedures.

c*jun* transcription has been shown to be autoregulated via the binding of AP-1 to the TPA-response element (Angel et al., 1988), we assessed the effect of insulin treatment on AP-1 function. Based on electrophoretic mobility shift assays, insulin increased AP-1 DNA binding activity in a biphasic manner (Figure 4). The first phase peaked within 15 min of stimulation and was followed by a second phase of binding which remained elevated for up to 180 min. The first phase of AP-1 DNA binding activity temporally correlated with the observed peak in insulin-stimulated JNK activity. The second phase of AP-1 DNA binding may be a reflection of newly synthesized AP-1 complexes resulting from the first phase of activation. This is consistent with the observation that the AP-1 DNA binding activity remained elevated beyond the point at which JNK activity returned to basal levels (Figure 3). The sustained increase in AP-1 DNA binding activity observed persisted for as long as insulin was present. The biphasic increase was a consistent finding and has also been observed with IGF-1 stimulation in other cells (Rosenzweig et al., 1993). These findings are in accord with the fact that phosphorylation of cJun's transcriptional activation domain has no effect on AP-1 DNA binding activity (Karin, 1995).

To determine whether the increased AP-1 DNA binding activity resulted in a transcriptional response, cells were transiently transfected with a 3xTRE-CAT reporter construct. As shown in Figure 5, the observed insulin-stimulated increase in AP-1 DNA binding activity resulted in AP-1-mediated transcriptional activation. This increase was ~50% of that induced by 10% fetal bovine serum. Both the insulin- and serum-stimulated increases in AP-1 transcriptional activity were blocked by cotransfection of dominant-negative N^{17}Ras (Figure 5). Although the JNK/SAPK family of MAPKs may be less dependent upon Ras for activation than the Erks (Minden et al., 1994b; Cano & Mahadevan, 1995; Vojtek & Cooper, 1995), both UV and tyrosine kinase activation of JNK activity have been shown to be mediated

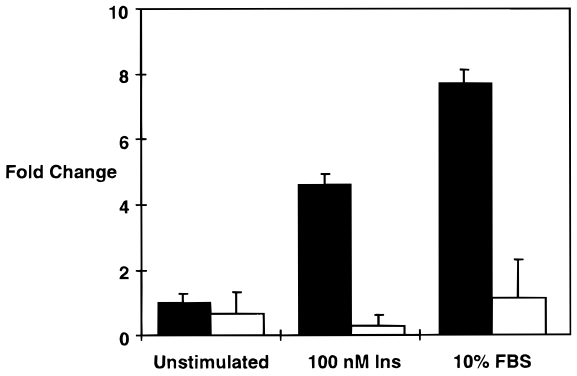

FIGURE 5: Coexpression of dominant-negative Ras abolishes insulin-stimulated AP-1 transcriptional activity. Rat 1 HIR cells were cotransfected with a 3xTRE-CAT reporter construct and N^{17}-Ras or pcDNA3. Cells were stimulated with 100 nM insulin or 10% FBS for 18 h at which time the cells were lysed and CAT activity was measured. (White bars) N^{17}Ras cotransfected; (black bars) pcDNA3 cotransfected. Stimulation by insulin or 10% fetal bovine serum was statistically significant compared to control at $p < 0.05$. The experiments shown were repeated twice with similar results.

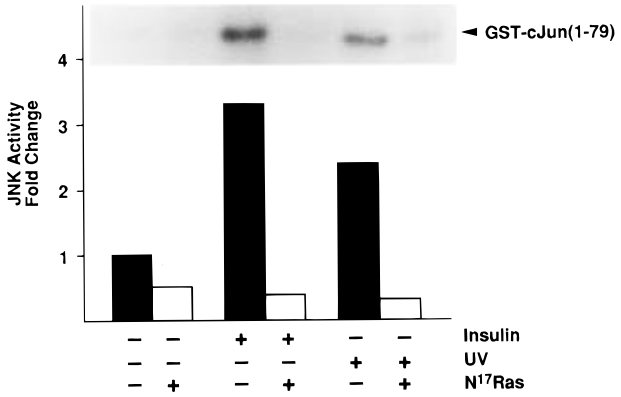

FIGURE 6: Insulin activation of JNK is blocked by expression of dominant-negative Ras. Rat 1 HIR fibroblasts were cotransfected with a pcDNA3 expression vector containing fJNK1 and either a pcDNA expression vector containing N^{17}Ras or pcDNA3 only. Cells were stimulated with insulin (100 nM) or UV light (30 s) followed by a 30 min incubation. Cells were then lysed, fJNK1 was immunoprecipitated with M2 monoclonal antibody (Kodak, New Haven, CT), and the immunoprecipitates were analyzed for JNK activity as described under Experimental Procedures. Inset: autoradiograph of the film, depicting the phosphorylated GST-cJun-(1−79); lower: quantification of the radioactivity present in the excised gel bands.

by Ras (Engelberg et al., 1994). On this basis, we tested the Ras-dependency of insulin-stimulated JNK activation in Rat 1 cells. As shown in Figure 6, cotransfection of dominant-negative N^{17}Ras blocked insulin- and UV-stimulated JNK activity. Taken together, these data suggest that the Ras-dependent activation of JNK contributes to insulin-stimulated transcription of AP-1-regulated genes in Rat 1 HIR cells.

DISCUSSION

Early response genes are defined as those transcripts rapidly appearing in response to extracellular stimulation that do not require protein synthesis for their production. c*jun* is an early response gene encoding a transcription factor whose protein product forms the AP-1 transcription complex either as a homodimer or as a heterodimer with cFos. The AP-1 complex regulates transcription at promoters containing the TPA-response element (TRE; $TGA^{C}_{G}TCA$; Lee et al., 1987). Since the c*jun* promoter contains a TRE site, c*jun* regulates the transcription of its own gene (Angel et al., 1988). In quiescent cells, little cFos protein is present due to its labile nature. However, cJun is generally present in quiescent cells. Thus, early activation of c*jun* transcription is likely regulated by AP-1 complexes largely composed of homodimers of preexisting cJun. Therefore, acute regulation of c*jun* transcription may have considerable impact on cell signaling events leading to mitogenesis.

As shown in Figure 4, DNA binding activity of the AP-1 complex in insulin-stimulated cells is increased in a manner consistent with an autoregulatory increase in cJun production. Since JNK is thought to be tightly bound to cJun, this allows for rapid activation of the basal transcription complex. In Rat 1 cells, insulin activation of JNK may contribute to the integration of signals in the nucleus leading to mitogenesis (Sasaoka et al., 1994). The role transcription factors play in the proliferative response following their regulatory phosphorylation induced by growth factors is typically examined over the course of hours to days, whereas the signaling events responsible for triggering transcription factor phosphorylation occur within seconds. It is in this early phase that the key regulatory events responsible for transcription of early response genes occur. These upstream effectors activate positive and negative regulatory processes which are integrated downstream at the level of transcription and lead the cell into a proliferative state.

Rat 1 HIR fibroblasts have been used as a model system to study the signal transduction pathways involved in insulin-regulated mitogenesis (McClain et al., 1987). In these cells, insulin receptor activation has been shown to be coupled to Ras activation resulting from the interaction of tyrosine-phosphorylated SHc with Grb2·mSOS complexes (Sasaoka et al., 1994). This model system was also used to demonstrate that insulin stimulates the rapid activation of the MAPKs, Erk 1 and Erk2 (Cobb et al., 1991). It is noteworthy that the present study demonstrates that insulin also stimulates JNK and that this stimulation is Ras-dependent.

The AP-1 transcription factor undergoes rapid transcriptional activation following the phosphorylation of preexisting complexes by JNK/SAPK (Karin, 1995). Indirect activation of AP-1, resulting from increased complex formation, occurs via the Erk pathway. These two pathways leading to AP-1 activation are not mutually exclusive. Rather, they may work synergistically to facilitate the net effect of the slowest link. Insulin stimulation of Rat 1 cells induces a number of signaling pathways and quantifiable biological outcomes, including proliferation (White & Kahn, 1995). The early activation of AP-1 via JNK can be viewed as a means of generating sufficient cJun protein to combine with *de novo* synthesized cFos protein resulting from Erk activation. An increase in cFos alone is not sufficient to drive the AP-1 complex, which has an absolute requirement for cJun (Karin, 1995). In addition to forming heterodimers with cJun, activating transcription factor-2 (ATF2) itself is a JNK substrate (Gupta et al., 1995). Thus, in addition to activating AP-1-mediated transcriptional events, JNK activation by insulin may result in ATF2-regulated gene expression.

Activation of the MAPK cascade through a Ras-dependent mechanism occurs in response to mitogenic stimuli acting

through receptor tyrosine kinases and G-protein-coupled receptors (Cano & Mahadevan, 1995; Luttrell et al., 1995). Activation of Erk has been shown to cause its translocation to the nucleus (Chen et al., 1992), where it was thought to stimulate AP-1-driven transcription by phosphorylating cJun. It has since been demonstrated that Erk phosphorylates cJun within its DNA binding domain (Ser 243), thereby inhibiting its ability to bind DNA (Alvarez et al., 1991; Minden et al., 1994a; Chou et al., 1992). Thus, Erk has no direct involvement in the transcriptional activation of cJun. Rather, Erk indirectly stimulates AP-1 activity via phosphorylation of the transcription factor Elk-1/p62^{TCF}, resulting in its enhanced binding to the serum response element (SRE) to induce c*fos* transcription (Gille et al., 1992).

The recently described JNKs/SAPKs have been shown to regulate AP-1-driven transcription by catalyzing the phosphorylation of the transcriptional activation domain of cJun (Dérijard et al., 1994; Kyriakis et al., 1994). Although JNKs/SAPKs were identified as stress-responsive kinases, they have since been shown to be activated by numerous ligands (Karin, 1995). In this study, we have demonstrated that insulin activates JNK in Rat 1 HIR fibroblasts. This activation preceded increased AP-1 DNA binding and transcriptional activation and was blocked by expression of dominant-interfering N^{17}Ras. The Ras-dependency of this activation is consistent with previous reports of JNK activation by MEKK-1 (Minden et al., 1994b) including studies supporting a more direct role for MEKK-1 regulation by the Rho family of low molecular weight GTP-binding proteins (Vojtek & Cooper, 1995).

Our results strongly suggest that in addition to being involved in acute responses to environmental stressors and signaling pathways leading to cell death (Xia et al., 1995), JNKs may also be involved in nondeleterious signaling cascades leading to mitogenesis or cell differentiation. In support of this notion, Coso et al. (1995) demonstrated that G-protein-coupled, m1 muscarinic receptors activate JNK in a model of proliferative signaling. JNKs have also been implicated in the integration of proliferative signals in T-lymphocytes (Su et al., 1994) and in IGF-1-stimulated AP-1 transcriptional activation and cell proliferation in IEC-6 intestinal cells (Simmons et al., 1995). Finally, Heasley et al. (1996) demonstrated JNK activation in PC12 cells by the expression of constitutively active members of the Gq family of heterotrimeric GTP-binding proteins or following the administration of NGF, PDGF-BB, and EGF. In these studies, JNK activation by constitutively active Gq family members was accompanied by PC12 cell differentiation; little or no Erk activation was detected in these experiments. This observation differs from a previous report demonstrating that JNK activation in PC12 cells leads to apoptosis while Erk activation leads to neuronal differentiation (Xia et al., 1995). Clearly, each cell line or clonal variant under investigation has the potential of a differential response to these signaling pathways. Taken together, these findings suggest that JNK activation and increased AP-1 activity may be regulated by ligands in a cell-specific or cell context-dependent manner. In the case of insulin signaling, activation of JNK may rapidly increase AP-1-mediated transcriptional events, serving to prime the cells for a mitogenic response. Alternatively, as insulin is a weak mitogen in fibroblasts, this priming may serve to blunt the mitogenic pathway. Further work is required to determine the precise involvement of this pathway in the insulin response, as well as the contributions of other members of the MAPK superfamily.

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