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The Ras/Rac1/Cdc42/SEK/JNK/c-Jun Cascade Is a Key Pathway by Which Agonists Stimulate DNA Synthesis in Primary Cultures of Rat Hepatocytes

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The ability of signaling via the JNK (c-Jun NH_2-terminal kinase)/stress-activated protein kinase cascade to stimulate or inhibit DNA synthesis in primary cultures of adult rat hepatocytes was examined. Treatment of hepatocytes with media containing hyperosmotic glucose (75 mM final), tumor necrosis factor α (TNFα, 1 ng/ml final), and hepatocyte growth factor (HGF, 1 ng/ml final) caused activation of JNK1. Glucose, TNFα, or HGF treatments increased phosphorylation of c-Jun at serine 63 in the transactivation domain and stimulated hepatocyte DNA synthesis. Infection of hepatocytes with poly-L-lysine–coated adenoviruses coupled to constructs to express either dominant negatives Ras ^{N17}, Rac1 ^{N17}, Cdc42 ^{N17}, SEK1$^-$, or JNK1$^-$ blunted the abilities of glucose, TNFα, or HGF to increase JNK1 activity, to increase phosphorylation of c-Jun at serine 63, and to stimulate DNA synthesis. Furthermore, infection of hepatocytes by a recombinant adenovirus expressing a dominant-negative c-Jun mutant (TAM67) also blunted the abilities of glucose, TNFα, and HGF to stimulate DNA synthesis. These data demonstrate that multiple agonists stimulate DNA synthesis in primary cultures of hepatocytes via a Ras/Rac1/Cdc42/SEK/JNK/c-Jun pathway. Glucose and HGF treatments reduced glycogen synthase kinase 3 (GSK3) activity and increased c-Jun DNA binding. Co-infection of hepatocytes with recombinant adenoviruses to express dominant- negative forms of PI_3 kinase (p110α/p110γ) increased basal GSK3 activity, blocked the abilities of glucose and HGF treatments to inhibit GSK3 activity, and reduced basal c-Jun DNA binding. However, expression of dominant-negative PI_3 kinase (p110α/p110γ) neither significantly blunted the abilities of glucose and HGF treatments to increase c-Jun DNA binding, nor inhibited the ability of these agonists to stimulate DNA synthesis. These data suggest that signaling by the JNK/stress-activated protein kinase cascade, rather than by the PI_3 kinase cascade, plays the pivotal role in the ability of agonists to stimulate DNA synthesis in primary cultures of rat hepatocytes.

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INTRODUCTION

Data from several laboratories have suggested that signaling via the JNK (c-Jun NH_2-terminal kinase)/

stress-activated protein (SAP) kinase cascade plays a role in the transformation and proliferation of several types of cells, including epithelial cells such as hepatocytes (Liu *et al.*, 1994; Rai *et al.*, 1994; Westwick *et al.*, 1995; Boylan *et al.*, 1996; Bruccderi *et al.*, 1997; Michalopoulos and DeFrances, 1997). This is in contrast to other data using transformed and established fibroblast cell lines, which suggests that signaling by the mitogen-activated protein (MAP) kinase cascade plays the dominant role in stimulating cellular proliferation (Williams and Roberts, 1994). JNK1/2 have been shown to be activated by a variety of similar stimuli, such as by hepatocyte growth factor (HGF), the proinflammatory cytokine tumor necrosis factor α (TNFα), and hyperosmotic shock (Bagrodia *et al.*, 1995; Read *et al.*, 1997; Rodrigues *et al.*, 1997; Whitmarsh *et al.*, 1997). However, despite this evidence, the direct significance of JNK1/2 activation or c-Jun function in proliferative responses of nontransformed, nonestablished epithelial cells has not been definitively tested by molecular dissection of this pathway. To determine the significance of JNK/SAP kinase cascade signaling in the control of DNA synthesis in a nontransformed, nonestablished epithelial cell, we performed experiments in primary cultures of rat hepatocytes in vitro.

Stress-related signals have been shown to lead to the activation of the Ras/Rac1/Cdc42 small molecular weight G protein family, which plays a role in the activation of MEK kinase homologues (Clark *et al.*, 1997; Deak and Templeton, 1997). Downstream of MEK kinase is the stress-regulated equivalent of MEK1/2 in the MAP kinase pathway, SEK1 (also termed MKK4) (Yan *et al.*, 1994). SEK1 phosphorylates and activates JNK1/2. JNK1/2 phosphorylate the transcription factor c-Jun (AP-1 complex) by phosphorylation of serines 63 and 73 in the NH_2 terminus of the molecule, which positively regulates c-Jun function (Smeal *et al.*, 1991; Sanchez *et al.*, 1994; Karin, 1995; Kallunki *et al.*, 1996; Santana *et al.*, 1996; Verheij *et al.*, 1996). Phosphorylation of c-Jun in its NH_2 terminus has been shown to play an important role in the commitment to apoptosis in some transformed cells, but has also been suggested to be essential in the early proliferative response of hepatocytes after partial hepatectomy in vivo (Diehl *et al.*, 1994, 1995; Jarvis *et al.*, 1994; Westwick *et al.*, 1996). The function of c-Jun can also be negatively regulated by phosphorylation in the COOH terminus of the molecule, which inhibits c-Jun DNA binding (Boyle *et al.*, 1990). Phosphorylation of c-Jun in the COOH terminus has been proposed to be catalyzed by glycogen synthase kinase 3 (GSK3), a downstream effector of PI_3 kinase and c-Akt (Cross *et al.*, 1995; Jarvis *et al.*, 1996). Thus, to obtain full c-Jun activation potentially requires the coordinate actions of two separate signaling cascades.

Recent data from our laboratory have demonstrated that hepatocytes from regenerating livers in vivo and proliferating hepatocytes in vitro have increased basal activity of the JNK/SAP kinase cascade (Jarvis *et al.*, 1997a; Spector *et al.*, 1997). Other studies in hepatocytes have demonstrated that TNFα can enhance hepatocyte proliferation and activate JNK1 and c-Jun. Furthermore, it has also been shown that inhibition of TNFα function by use of neutralizing antibodies during the proliferative response of hepatocytes after partial hepatectomy blocks liver regeneration in vivo (Diehl *et al.*, 1994, 1995; Westwick *et al.*, 1996; Columbano *et al.*, 1997). Thus, evidence exists supporting the hypothesis that c-Jun function is important in the proliferation of hepatocytes in response to stress signals. We have tested whether hyperosmotic glucose treatment and the hepatocyte primary mitogens, TNFα and HGF, stimulate hepatocyte DNA synthesis via activation of the JNK/SAP kinase cascade and activation of c-Jun. The studies reported herein demonstrate that these agonists stimulate DNA synthesis in primary cultures of rat hepatocytes via a Ras/Rac1/Cdc42/SEK/JNK/c-Jun–dependent mechanism. Our data also demonstrate that the ability of cells to sense alterations in osmolarity lies at the level of the plasma membrane, above the level of the *Ras* proto-oncogene, suggesting that plasma membrane receptors may play a direct role in sensing changes in osmolarity.

MATERIALS AND METHODS

Materials

Male Sprague Dawley rats (200 g) had access to food and water ad libitum (Kunos *et al.*, 1995). Anti-p42$^{MAP\ kinase}$, Anti-JNK1, and anti-p70$^{S6\ kinase}$ antibodies were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Anti-GSK3α and anti-Rac1 antibodies and substrate peptide (YRRAAVPPSPSLSRHSSPHQpSEDEEE) were obtained from Upstate Biotechnologies (Lake Placid, NY). D-Glucose, HGF, and TNFα were purchased from Sigma Chemical (St. Louis, MO). AP-1 oligonucleotide for gel-shift assays (CGC TTG ATG ACT CAG CCG GAA) was purchased from Santa Cruz Biotechnology. Dominant-negative c-Jun (TAM67) was generated from wild-type c-Jun by deletion of residues 3–122 in the amino-terminal transcriptional activation domain (Jarvis *et al.*, 1997b). Radiolabeled [γ-^{32}P]ATP and [α-^{32}P]ATP, free ^{32}P-labeled inorganic phosphate, and [^{3}H]thymidine were purchased from New England Nuclear (Boston, MA). Glutathione *S*-transferase (GST)-c-Jun (aa1–169) was synthesized in *Escherichia coli* and purified on glutathione-Sepharose. Protein preparations and purchase of other reagents were as described previously (Dent *et al.*, 1995; Kunos *et al.*, 1995; Jarvis *et al.*, 1997a; Spector *et al.*, 1997).

Methods

Recombinant Adenoviral Vectors: Generation and Infection In Vitro

Studies herein are performed using two adenoviral technologies. First, replication-defective adenovirus was conjugated to poly-L-lysine (as described in Cristiano *et al.*, 1993; Nazareth and Weigel, 1996). Using polystyrene tubes, mixes of virus and a HEPES-buffered saline (HBS) solution were combined with 1 ng of mam-

[1] Abbreviations used: JNK, c-Jun NH_2-terminal kinase; MAP, mitogen-activated protein; SAP, stress-activated protein; SEK, stress/extracellular-regulated kinase.

malian expression plasmid (cytomegalovirus promoter) containing the gene of interest and incubated in the dark for 30 min. After 30 min, poly-L-lysine and HBS were mixed at 1.3 μg of lysine per μg DNA and incubated for another 30 min. The DNA-conjugated virus was added to hepatocytes at a multiplicity of infection (moi) of 250 and the cells were incubated for 4 h at 37°C. The cells were washed with media to remove virus. Cells expressed transduced gene products by 10–24 h after infection. Using a plasmid to express β-galactosidase under control of the cytomegalovirus promoter, we determined that 1 ng of plasmid conjugated to virus particles and infected into hepatocytes before plating at a moi of 250 gave 100% infection as judged by blue coloration after 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-gal) incubation 24 h after infection. Second, we have generated recombinant PI_3 kinase adenoviruses using a novel methodology developed by Drs. Matthias Paul Wymann and Stefano Brenz-Verca. In this procedure, the full-length recombinant adenovirus genome is cloned in a plasmid, flanked by a rare cutter (*Pac*I) restriction site, and is generated using a recombination proficient *E. coli* strain (*BJ5183*) with the genotype *recBC sbcBC* (Chartier *et al.*, 1996). Recombinant adenovirus to express dominant-negative c-Jun mutant (TAM67) was generated as described in the study by Valerie and Singhal (1995). To assess the effectiveness of recombinant adenoviral infection, we generated a recombinant adenovirus containing the gene for β-galactosidase. Primary hepatocytes were infected with this virus immediately after isolation in vitro (moi 250) and incubated at 37°C for another 24 h; cells were fixed and incubated with X-gal. A moi of 250 gave 100% infection after 24 h. To assess infection of other constructs used herein, we routinely performed Western immunoblots at 10–24 h after infection. Gene products were expressed approximately 18 h after infection.

Preparation of Hepatocytes

Rats were anesthetized by intraperitoneal injection of sodium pentobarbital (50 mg/kg), and the lower thorax and abdomen were shaved to remove fur. A small (3-cm) vertical midline incision was made in the abdominal wall from just below the costal margin/xiphoid process. Hepatocytes were prepared by cannulation of the portal vein and collagenase perfusion of the liver. A cannula was inserted into the portal vein and two ties were tightened to occlude the inferior vena cava and secure the cannula in the portal vein. Gassed Krebs-Henseleit buffer (95% air, 5% CO_2, 37°C) containing 0.3 mM EGTA (to prevent clotting) was passed through the liver (20 ml/min flow rate) to wash out erythrocytes; a second cannula was inserted into the inferior vena cava via the right atrium of the heart and also was secured with a tie, to act as a drain. Livers were washed sequentially with 200 ml of this buffer, followed by 200 ml of the same media omitting EGTA and including 0.5 mg/ml collagenase. The liver was then removed, filtered through muslin, and washed three times with DMEM. Hepatocytes were resuspended to 5.0×10^6 cells/ml in serum-free DMEM and placed into primary culture.

Primary Culture and Assay for DNA Synthesis in Hepatocytes

Hepatocytes were cultured on rat-tail collagen (Vitrogen)-coated plastic dishes (12 × 20 mm, 2×10^5 cells) in 1 μl of DMEM containing 100 nM insulin, 1 nM dexamethasone, and 1 nM thyroxine and were allowed to adhere to the dish. Cells were then infected with various adenoviruses, depending on which experiment was being performed. For cells undergoing acute assay, treatments occurred 4 h after plating. For adenoviral-infected cells, after 4 h of infection, media were replaced and hepatocytes were cultured (for DNA synthesis assays) in supplemented DMEM containing 2 μCi of [^{3}H]thymidine in 5% (vol/vol) CO_2. Hormonal treatments and/or protein kinase inhibitors were added 24 h after the media change. Cells were cultured for another 48 h, after which time cells were lysed with 0.5 M NaOH and DNA precipitated with 12.5% (wt/vol) trichloroacetic acid (final). Acid-precipitable material was transferred to glass fiber filters, washed with 5% (wt/vol) trichloroacetic acid, and [^{3}H]thymidine incorporation into DNA as quantified by liquid scintillation spectrometry (Spector *et al.*, 1997).

Treatment of Primary Cultures of Hepatocytes with Hormones and Cell Homogenization

Primary cultures of hepatocytes were cultured in 12-well plates or 20-mm dishes (2×10^5 cells) in DMEM as above (0.5 mg of total protein used per immunoprecipitate). D-Glucose, 2-deoxyglucose, and sorbitol (1 M stocks for each) in DMEM, TNFα, or HGF [in phosphate-buffered saline (PBS) as a 2 mg/ml stock] were added to give final specified concentrations (see text and figure legends), mixed, and incubated for the specified times at 37°C in a cell culture incubator. Cells were pretreated with protein kinase inhibitors (30 min) before hormonal additions. Twenty seconds before termination, plated cells were aspirated, followed by immediate homogenization. Cells were homogenized in 1 ml of ice-cold buffer A [25 mM HEPES, pH 7.4, at 4°C, 5 mM EDTA, 5 mM EGTA, 5 mM benzamidine, 1 mM phenylmethylsulfonyl fluoride, 1 mg/μl soybean trypsin inhibitor, 1.0 mM sodium *o*-vanadate, 1.0 mM sodium pyrophosphate, 0.05% (wt/vol) sodium deoxycholate, 1% (vol/vol) Triton X-100, and 0.1% (vol/vol) 2-mercaptoethanol], with trituration using a P1000 pipette to lyse the cells. Homogenates were clarified by centrifugation (14,000 × *g*, 4°C).

Immunoprecipitations from Homogenates

Fifty microliters of protein A-agarose (Ag) slurry (25-μl bead volume) were washed twice with 1 μl of PBS containing 0.1% (vol/vol) Tween 20 and were resuspended in 0.1 μl of the same buffer. Antibodies (2 μg, 20 μl) or serum (20 μl) were added to each tube and incubated (3 h, 4°C). Clarified hepatocyte homogenates (0.5 μl, 1 μg of total protein) were mixed with protein A-Ag–conjugated antibody in duplicate using gentle agitation (2.5 h, 4°C). Protein A-Ag was recovered by centrifugation and the supernatant was discarded and washed (10 min) sequentially with 0.5 μl of buffer A (twice), PBS, and buffer B [25 mM HEPES, pH 7.4, 15 mM $MgCl_2$, 0.1 mM Na_3VO_4 0.1% (vol/vol) 2-mercaptoEtOH].

Assay of $p42^{MAP\ kinase}$ Activity

Immunoprecipitates were incubated (final vol 50 μl) with 50 μl of buffer B containing 0.2 mM [[gamma]-^{32}P]ATP (5000 cpm/pmol), 1 mM Microcystin-LR, and 0.5 μg/μl myelin basic protein, which initiated reactions. After 20 min, 40 μl of the reaction mixtures were spotted onto a 2-cm circle of P81 paper (Whatman, Maidstone, England) and immediately placed into 180 mM phosphoric acid. Papers were washed four times (10 min each) with phosphoric acid and once with acetone, and ^{32}P-labeled incorporation into myelin basic protein was quantified by liquid scintillation spectroscopy. Preimmune controls were performed to ensure that myelin basic protein phosphorylation was dependent on specific immunoprecipitation of $p42^{MAP\ kinase}$ (Spector *et al.*, 1997).

Assay of JNK1 Activity

Immunoprecipitates were incubated (final vol 100 μl) with 2 μl (10 μg) of *GST-c-Jun* (*aa1–169*) and reactions were initiated with 98 μl of buffer B containing 0.2 mM [[gamma]-^{32}P]ATP (5000 cpm/pmol) and 1 μM Microcystin-LR. After 30 min, reactions were terminated with sample buffer and prepared for SDS-PAGE (10% gel) to quantify ^{32}P-labeled incorporation into excised, Coomassie blue-stained *GST-c-Jun* (*aa1–169*) bands by liquid scintillation spectroscopy. Preimmune control assays were performed to ensure that *GST-c-Jun* (*aa1–169*) phosphorylation was dependent on specific immunoprecipitation of JNK1 in the assay (Spector *et al.*, 1997).

Assay for GSK3 Activity

GSK3 was immunoprecipitated and assayed versus a phosphopeptide substrate (YRRAAVPPSPSLSRHSSPHQpSEDEEE). Immunoprecipitates were incubated (final vol 50 μl) with 50 μl of buffer B containing 0.2 mM [[gamma]-^{32}P]ATP (5000 cpm/pmol), 1 μM Microcystin-LR, and 200 μM phosphopeptide substrate, which initiated reactions. After 20 min, 40 μl of the reaction mixtures were spotted onto a 2-cm circle of P81 paper (Whatman) and immediately placed into 180 mM phosphoric acid. Papers were washed four times (10 min each) with phosphoric acid and once with acetone, and ^{32}P-labeled incorporation into phosphopeptide substrate compared with identical samples containing enzyme but without substrate (Welsh *et al.*, 1994).

Assay for p70$^{S6\ kinase}$ Activity

Immunoprecipitation of p70$^{S6\ kinase}$ was performed as above and assayed versus a peptide substrate (RRRLSSLA). Immunoprecipitates were incubated (final vol 50 μl) with 50 μl of buffer B containing 0.2 mM [[gamma]-^{32}P]ATP (5000 cpm/pmol), 1 μM Microcystin-LR, and peptide substrate (50 μM, final). After 20 min, 40 μl of the reaction mixtures were spotted onto a 2-cm circle of P81 paper (Whatman) and immediately placed into 180 mM phosphoric acid. Papers were washed four times (10 min each) with phosphoric acid and once with acetone, and ^{32}P-labeled incorporation into peptide substrate compared with identical samples containing enzyme but without substrate determined by liquid scintillation spectroscopy (Welsh *et al.*, 1994).

Transcription Factor DNA-binding Assay

Nuclear extracts were prepared as described (Stravitz *et al.*, 1996). Oligonucleotides were ^{32}P labeled with [[gamma]-^{32}P]ATP using polynucleotide kinase. Binding reaction mixtures (20 ml) were incubated at room temperature for 45 min in a mixture containing 1 ng of DNA probe and 5 μg of nuclear extract in 10 mM Tris/HCl (pH 7.5) at 25°C, 40 mM NaCl, 1 mM dithiothreitol, 1 mM EDTA, 5% (vol/vol) glycerol, and 2 μg poly(dl-dC) to inhibit nonspecific binding in the extract. DNA–protein complexes were resolved by electrophoresis through a 4%/8% nondenaturing polyacrylamide gel containing 50 mM Tris, 0.38 M glycine, and 2 mM EDTA. Gels were dried and quantified in the linear range (500–15,000 dpm) and processed using a phosphorimager.

Luciferase Assay

Luciferase assays were performed as described using a kit purchased from Promega (Madison, WI). Briefly, cells were scraped into PBS, pelleted by centrifugation, lysed, incubated on ice for 5 min, and clarified by centrifugation at 4°C for 10 min. Portions of the supernatant were added to assay mixture and the luminosity of each sample was determined in triplicate in a luminometer (20-s exposure; de Wet *et al.*, 1987).

Guanine Nucleotide-binding Assays

Determination of guanine nucleotide binding to Rac1 was determined as described in the report by Gibbs (1995). Briefly, hepatocytes (12-well plates, 2×10^5 cells) were cultured in phosphate-free DMEM and infected with plasmids to express Rac1^{N17}, Rac1^{V12}, Cdc42^{N17}, and Cdc42^{V12}. Twenty-four hours after infection, hepatocytes were incubated with 0.3 μCi of ^{32}P-labeled P_i for 90 min at 37°C. Radioactive media were aspirated, and the cells were washed once in ice-cold PBS and lysed with trituration for 1 h at 4°C in 50 mM Tris/HCl (pH 7.5), at 4°C, 20 mM $MgCl_2$, 150 mM NaCl, 0.5% (vol/vol) Nonidet P-40, 5 mM benzamidine, and 1.0 mM phenylmethylsulfonyl fluoride, each sample containing 4 μg of anti-Rac1 antibody. After 1 h, 0.1 ml of a bovine serum albumin-blocked 10% (wt/vol) charcoal slurry was added to the homogenate to remove unbound nucleotides; after 1 h at 4°C, samples were clarified by centrifugation. The radioactivity in a 2-μl aliquot of each sample was determined. Immunoprecipitation of Rac1 was completed by adding 50 μl of a 50% slurry of protein A-agarose (Bio-Rad, Richmond, CA) to a constant amount (0.5×10^7 cpm) of sample. Immunoprecipitates were heated to 85–90°C in 20 μl of 1 M KH_2PO_4 (pH 3.4), which releases coimmunoprecipitating nucleotides from Rac1. The solution was clarified and spotted onto a polyethyleneimine cellulose sheet. Chromatograms are developed with pH 3.4 1 M KH_2PO_4. Guanine nucleotides complexed to Rac1 were visualized by use of a phosphorimager, followed by scraping of each spot to quantify the amount of radioactivity with scintillant in a scintillation counter. A correction was made for the ratio of moles of phosphate to moles of guanosine (GTP cpm $\times$ 0.33; GDP cpm $\times$ 0.50) assuming uniform labeling of all phosphates. The data were expressed as percentage of GTP relative to the total GTP + GDP detected.

Data Analysis

Comparison of the effects of various hormone treatments was done using one-way analysis of variance and a two-tailed *t* test. Differences with a $p < 0.05$ were considered statistically significant. All bar graph fold values and means shown are $\pm$SE from 2 to 12 independent experiments (individual liver isolations).

RESULTS

Glucose, TNFα, and HGF Treatments of Primary Cultures of Hepatocytes Stimulate JNK1 Activity

Hepatocytes cultured in DMEM were hyperosmotically shocked with 50 mM glucose 4 h after plating, and the activities of various components within signal transduction cascades were examined (Figure 1). Ten minutes after the 50 mM glucose treatment, the activities of the SAP kinase JNK1 (Figure 1A) and p70$^{S6\ kinase}$ protein kinase (Figure 1B) were increased, whereas the activities of GSK3 (Figure 1C) and the p42 MAP kinase (Figure 1D) were reduced. Treatment of hepatocytes with TNFα (1 ng/μl) also caused activations of both JNK1 (Figure 1A) and p70$^{S6\ kinase}$ (Figure 1B) and an inhibition of GSK3 activity (Figure 1C). TNFα caused an acute transient increase in p42$^{MAP\ kinase}$ activity followed by a decrease in activity, which rebounded to give a small activation at later time points (Figure 1D). Treatment of hepatocytes with HGF (1 ng/μl) potently increased JNK1, p70$^{S6\ kinase}$, and p42$^{MAP\ kinase}$ activities (Figure 1, A, B, and D) and decreased GSK3 activity (Figure 1C). Thus, all agonists tested stimulated the JNK/SAP kinase cascade (Figure 1A) and the PI_3 kinase cascade (Figure 1, B and C), whereas only HGF caused significant acute and chronic increases in the activity of p42$^{MAP\ kinase}$ (Figure 1D).

Glucose, TNFα, and HGF Treatments of Hepatocytes Cause Phosphorylation of c-Jun at Serine 63 in the Transactivation Domain via the Ras/Rac1/Cdc42/SEK/JNK Pathway

Because hyperosmotic glucose, TNFα, and HGF treatments all activated JNK1, we next investigated

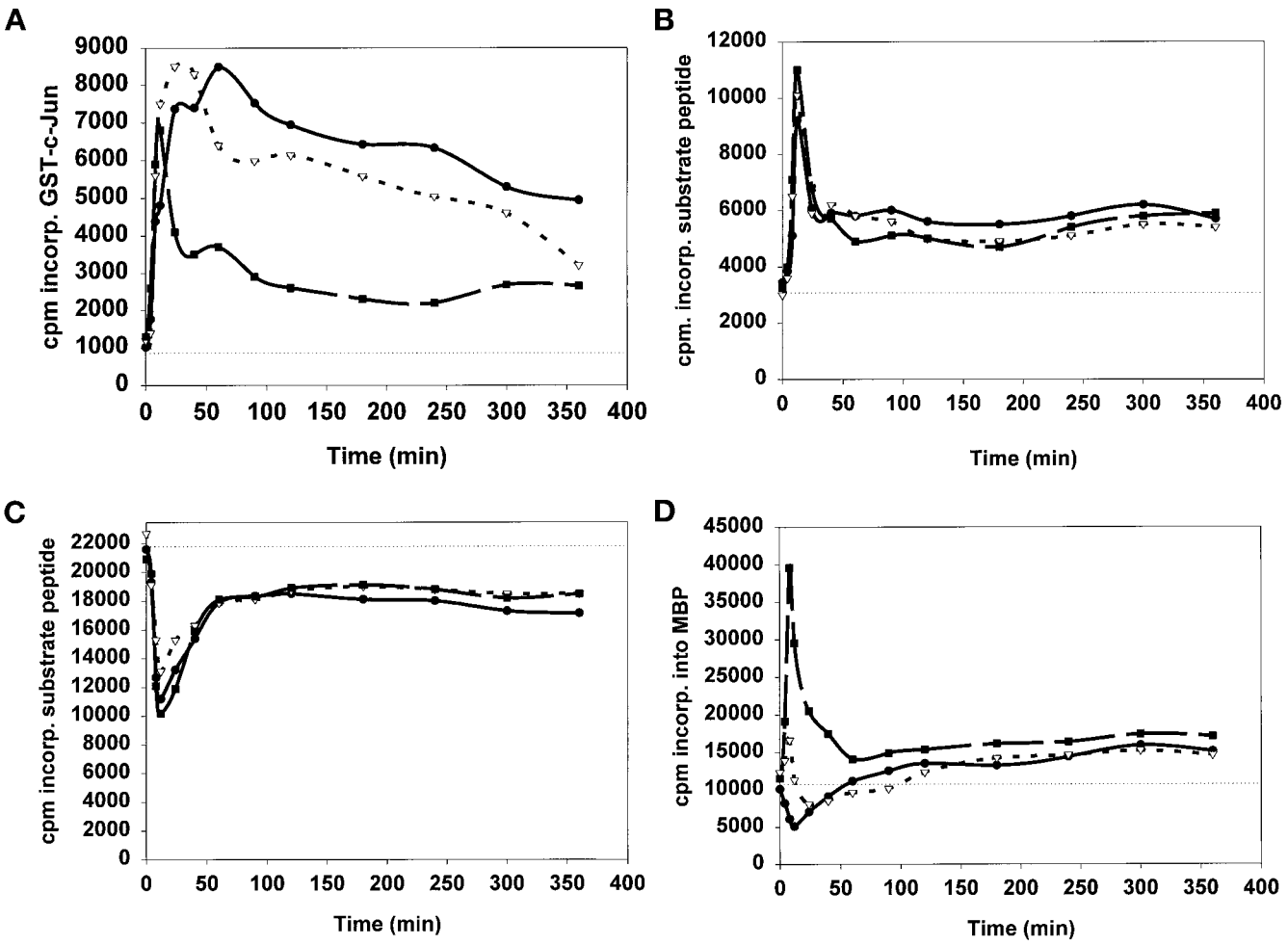

Figure 1. (A) Hyperosmotic glucose, TNFα, or HGF treatments activate JNK1. (B) Hyperosmotic glucose, TNFα, or HGF treatments activate $p70^{S6\ kinase}$. (C) Hyperosmotic glucose, TNFα, or HGF treatments inhibit GSK3. (D) Modulation of $p42^{MAP\ kinase}$ activity after hyperosmotic glucose, TNFα, or HGF treatments. Hepatocytes were cultured as described in MATERIALS AND METHODS. After 4 h, cells were treated for the indicated times with either media control, 50 mM glucose (closed circles), 1 ng/μl TNFα (open triangles), or 1 ng/μl HGF (closed squares) at 37°C, after which time media were aspirated and the cells frozen. Hepatocytes were lysed and subjected to immunoprecipitation followed by immune complex kinase assays for the indicated protein kinase. Activities shown are expressed as cpm incorporated into substrate versus media control treatment (5000 cpm/pmol) and are the means of triplicates from a representative of four experiments. Media control treatment did not alter the basal activities of protein kinases versus no treatment.

whether these agonists caused functional activation of the downstream effector of JNK1, the immediate early transcription factor c-Jun. To test whether JNK1 activation is modulating c-Jun phosphorylation at its NH_2 terminus in our system, plasmids containing control (null plasmid), dominant-negative Ras^{N17}, dominant-negative $Rac1^{N17}$, dominant-negative $Cdc42^{N17}$, dominant-negative JNK1, and dominant-negative SEK1 (Verheij *et al.*, 1996; Read *et al.*, 1997; Rodrigues *et al.*, 1997) were infected into hepatocytes at a high moi using a poly-L-lysine–conjugated adenovirus system. The infected hepatocytes were treated 24 h later with either 50 mM glucose, 1 ng/μl HGF, or 1 ng/μl TNFα for either 10 min (Table 1) or 20 min (Figure 2). In control-infected cells, glucose, HGF, and TNFα treatments stimulated JNK1 activity (Table 1) and phosphorylation of c-Jun at position serine 63 in the NH_2-terminal transactivation domain (Figure 2). Stimulation of JNK1 activity and c-Jun serine 63 phosphorylation were blocked in cells expressing dominant-negative Ras^{N17}, dominant-negative $Rac1^{N17}$, dominant-negative $Cdc42^{N17}$, dominant-negative JNK1, or dominant-negative SEK1 (Table 1 and Figure 2). Thus, treatment of hepatocytes with multiple agonists increases JNK1 activity and increases phosphorylation of serine 63 in the transactivation domain of c-Jun via a Ras/Rac1/Cdc42/SEK/JNK-dependent mechanism. These data also demonstrate that both

Table 1. Hyperosmotic glucose, HGF, and TNFα stimulate JNK1 activity, which is blocked by expression of dominant-negative Ras^{N17}, dominant-negative $Rac1^{N17}$, dominant-negative $Cdc42^{N17}$, dominant-negative SEK1, and dominant-negative JNK1, but not by expression of dominant-negative c-Jun(TAM67)

Glucose	null	Ras^{N17}	$Rac1^{N17}$	$Cdc42^{N17}$	$SEK1^{-}$	$JNK1^{-}$	c-Jun(TAM67)
Control	3,719 ± 570	1,627 ± 180[a]	833 ± 180[a]	533 ± 90[a]	1,605 ± 220[a]	2,172 ± 300[a]	4,438 ± 190
Glucose	10,168 ± 2,400[b]	1,377 ± 330[a]	929 ± 50[a]	1,184 ± 320[a]	2,567 ± 140[a]	2,107 ± 110[a]	11,313 ± 1,050[b]
HGF	12,585 ± 1,820[b]	1,711 ± 410[a]	1,263 ± 290[a]	1,517 ± 280[a]	2,564 ± 380[a]	1,873 ± 220[a]	11,647 ± 1,670[b]
TNFα	12,440 ± 1,750[b]	1,710 ± 240[a]	1,596 ± 300[a]	1,851 ± 60[a]	1,897 ± 180[a]	2,559 ± 145[a]	12,647 ± 770[b]

Hepatocytes were infected with either control plasmid poly-L-lysine adenovirus or dominant-negative Ras N17/Rac1 N17/Cdc42 N17/ $SEK1^{-}$/$JNK1^{-}$ poly-L-lysine adenovirus (250 moi), followed by culture as described in MATERIALS AND METHODS. In a parallel experiment, hepatocytes were infected with a recombinant adenovirus (250 moi) to express a nontransactivatable dominant-negative c-Jun(TAM67). After 24 h to allow expression of dominant-negative gene products, hepatocytes were treated for 10 min with either media control, 50 mM glucose, 1 ng/μl HGF, or 1 ng/μl TNFα. After 10 min, media were aspirated and cells frozen. Hepatocytes were lysed and subjected to immunoprecipitation followed by immune complex kinase assays for JNK1. Activities shown are expressed as cpm incorporated into substrate versus media control treatment (5000 cpm/pmol) and are the means ± SE from two to five experiments, each performed in duplicate.

[a] $p < 0.05$ decrease compared with corresponding value in null/control plasmid infected.

[b] $p < 0.05$ increase compared with control.

nontyrosine kinase receptor molecules (TNFα) and hyperosmotic agents (glucose) can activate the JNK/SAP kinase pathway via the *Ras* proto-oncogene.

Glucose Treatment of Hepatocytes Causes Activation of c-Jun Using an AP-1-Luciferase Reporter Assay

To test whether phosphorylation of c-Jun at serine 63 was functional for c-Jun transactivation in hepatocytes, cells were coinfected with poly-L-lysine–conjugated adenoviruses using constructs to express either dominant-negative SEK1 or dominant-negative JNK1 together with an *AP-1*–regulatable *luciferase* gene. Twenty-four hours later, hepatocytes were treated for 12 h with glucose and luciferase activity was measured (Figure 3). Glucose treatment of hepatocytes increased luciferase AP-1 activity, which was blocked by expression of either dominant-negative SEK1 or dominant-negative JNK1 (Figure 3). Similar data were obtained when hepatocytes were infected with a dominant negative c-Jun mutant (TAM67) construct. Thus, phosphorylation of serine 63 in hepatocyte c-Jun corresponds to a functional activation of transcription from AP-1–regulatable promoters.

Treatment of Hepatocytes with Glucose, TNFα, or HGF Increases DNA Synthesis via a Ras/Rac1/Cdc42/SEK/JNK/c-Jun–dependent Mechanism

Further experiments were performed to test whether stimulation of the JNK/SAP kinase pathway by glucose, TNFα, or HGF was causally related to the abilities of these agonists to stimulate hepatocyte DNA synthesis. Hepatocytes were infected with poly-L-lysine–conjugated adenoviruses using constructs to express either control (null plasmid), dominant-negative Ras^{N17}, dominant-negative $Rac1^{N17}$, dominant-negative $Cdc42^{N17}$, dominant-negative SEK1, dominant-negative JNK1, or dominant-negative SEK1 (Tables 2 and 3). In parallel experiments, hepatocytes were also infected with a recombinant adenovirus to express dominant-negative c-Jun (TAM67). TAM67 is an NH_2-deleted c-Jun molecule that is incapable of transactivation. Infected hepatocytes were treated 24 h after infection with either media control, 50 mM glucose, 1 ng/μl TNFα, or 1 ng/μl HGF, and the ability of each condition to synthesize DNA was determined 30 h later. Glucose treatment of control-infected hepatocytes stimulated DNA synthesis by ~225%. TNFα and HGF treatments of control-infected hepatocytes also increased DNA synthesis as judged by [^{3}H]thymidine incorporation into DNA, by ~250 and ~400%, respectively (Tables 2 and 3). However, expression of dominant-negative Ras^{N17}, dominant-negative $Rac1^{N17}$, dominant-negative $Cdc42^{N17}$, dominant-negative SEK1, dominant-negative JNK1, or dominant-negative c-Jun (TAM67) reduced basal DNA synthesis by ~70% and completely blocked the abilities of glucose, TNFα, or HGF to stimulate DNA synthesis (Tables 2 and 3).

In contrast to our data showing that inhibition of the JNK/SAP kinase pathway causes an ~70% reduction in the basal level of hepatocyte DNA synthesis, when we inhibited the MAP kinase pathway by expression of dominant- negative MEK1, basal hepatocyte DNA synthesis was reduced by only 20 ± 3%. Furthermore, expression of dominant-negative MEK1 blunted the abilities of hyperosmotic glucose, TNFα, and HGF to stimulate DNA synthesis by 46 ± 5%, 41 ± 3%, and 65 ± 6%, respectively. These data suggest that signaling by the MAP kinase pathway plays a lesser role in

stimulating hepatocyte DNA synthesis than does signaling by the JNK/SAP kinase pathway. The data presented in Tables 1–3 and Figures 1–3 strongly suggest that multiple stimuli increase DNA synthesis in primary cultures of hepatocytes by signaling via the Ras/Rac1/Cdc42/SEK/JNK/c-Jun pathway.

Dominant-Negative Cdc42 ^{N17} Does Not Block the Ability of HGF to Stimulate Rac1 GTP Association

Expression of both dominant-negatives Rac1^{N17} and Cdc42^{N17} blocked the ability of HGF to stimulate JNK1 activity and to increase hepatocyte DNA synthesis. To determine whether signaling by HGF to JNK1 progressed via Rac1 to Cdc42 or vice versa, we examined the GTP:GDP ratio of Rac1 after HGF stimulation in the presence or absence of dominant-negative Cdc42^{N17}. Under basal conditions, 11.1 ± 0.7% of Rac1 was in the GTP-associated state, which was stimulated 2 min after HGF treatment to 20.0 ± 1.3%. Expression of dominant-negative Cdc42^{N17} did not block the ability of HGF to increase the GTP-associated state of Rac1 (19.2 ± 1.2%), and expression of dominant-active Cdc42^{V12} also did not increase the GTP-associated state of Rac1 (13.5 ± 1.8%). These data suggest that HGF signaling progresses via Rac1 to Cdc42 and then to the JNK/SAP kinase cascade in hepatocytes.

Glucose and HGF Treatments Stimulate c-Jun DNA Binding in Primary Cultures of Hepatocytes Which Are Not Blocked by Dominant-Negative PI3 Kinase Mutants

We next tested whether either glucose or HGF treatments of hepatocytes could stimulate c-Jun DNA binding, and whether this binding correlated with the abilities of these agonists to modulate GSK3 activity. Hepatocytes were infected with either control virus or recombinant adenoviruses to express dominant-negative PI_3 kinase p110α and dominant-negative PI_3 kinase p110γ, and were treated 24 h later with either 50 mM glucose or 1 ng/μl HGF (Chung *et al.*, 1992; Ferrari and Thomas, 1994; Franke *et al.*, 1995; Vanhaesebroeck *et al.*, 1997). Glucose and HGF treatments of control-infected hepatocytes activated p70$^{S6\ kinase}$ and inhibited GSK3 activities, modulations that were both blocked by coexpression of dominant-negative PI_3 kinase p110α and dominant-negative PI_3 kinase p110γ (Figure 4). Glucose and HGF treatments of control-infected hepatocytes also stimulated c-Jun/AP-1 DNA binding (Figure 5). However, although expression of dominant-negative PI_3 kinase p110α and dominant-negative PI_3 kinase p110γ reduced the basal level c-Jun/AP-1 DNA binding by 30%, it did not block the abilities of either glucose or HGF treatments to stimulate c-Jun/AP-1 binding (Figure 5). These data suggest that inhibition of GSK3 may not be the only mechanism by which agonists can increase c-Jun/AP-1 DNA binding.

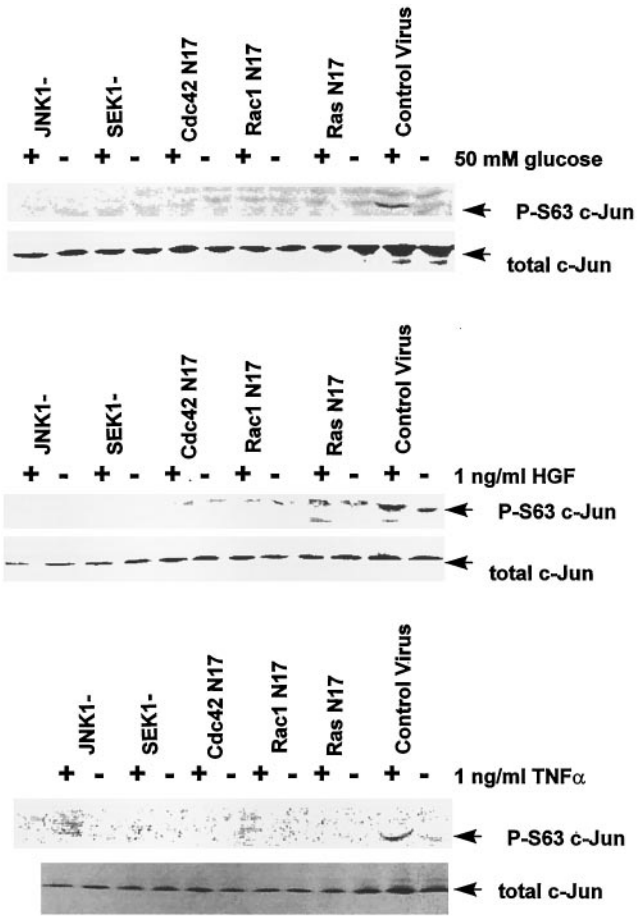

Figure 2. Hyperosmotic glucose (top panel), HGF (middle panel), and TNFα (bottom panel) stimulate phosphorylation of serine 63 in the transactivation domain of c-Jun, which is blocked by expression of dominant-negative Ras^{N17}, dominant-negative Rac^{N17}, dominant-negative Cdc42^{N17}, dominant-negative SEK1, and dominant-negative JNK1. Hepatocytes were infected with either control plasmid poly-L-lysine adenovirus or dominant-negative Ras N17/Rac1 N17/Cdc42 N17/SEK1/JNK1 poly-L-lysine adenovirus (250 moi), followed by culture as described in MATERIALS AND METHODS. After 24 h to allow expression of dominant-negative gene products, hepatocytes were treated for 20 min with either media control, 50 mM glucose, 1 ng/μl TNFα, or 1 ng/μl HGF. After 20 min, media were aspirated and cells frozen. Hepatocytes were lysed with 100 μl of 5× SDS-polyacrylamide sample buffer, diluted to 250 μl with distilled water, and placed in a 100°C dry bath for 15 min. One hundred-microliter aliquots of each time point were subjected to SDS-PAGE on two 10% gels. Gels were transferred to nitrocellulose and Western blotting versus either anti-serine 63 c-Jun or anti–c-Jun protein (performed as in MATERIALS AND METHODS). The top panels show blotting versus serine 63 c-Jun; the bottom panels show total c-Jun protein immunoblotting.

Additional experiments were performed to test whether dominant-negative PI_3 kinase p110α and dominant-negative PI_3 kinase p110γ blocked the abilities of glucose, TNFα, or HGF treatments to stimulate

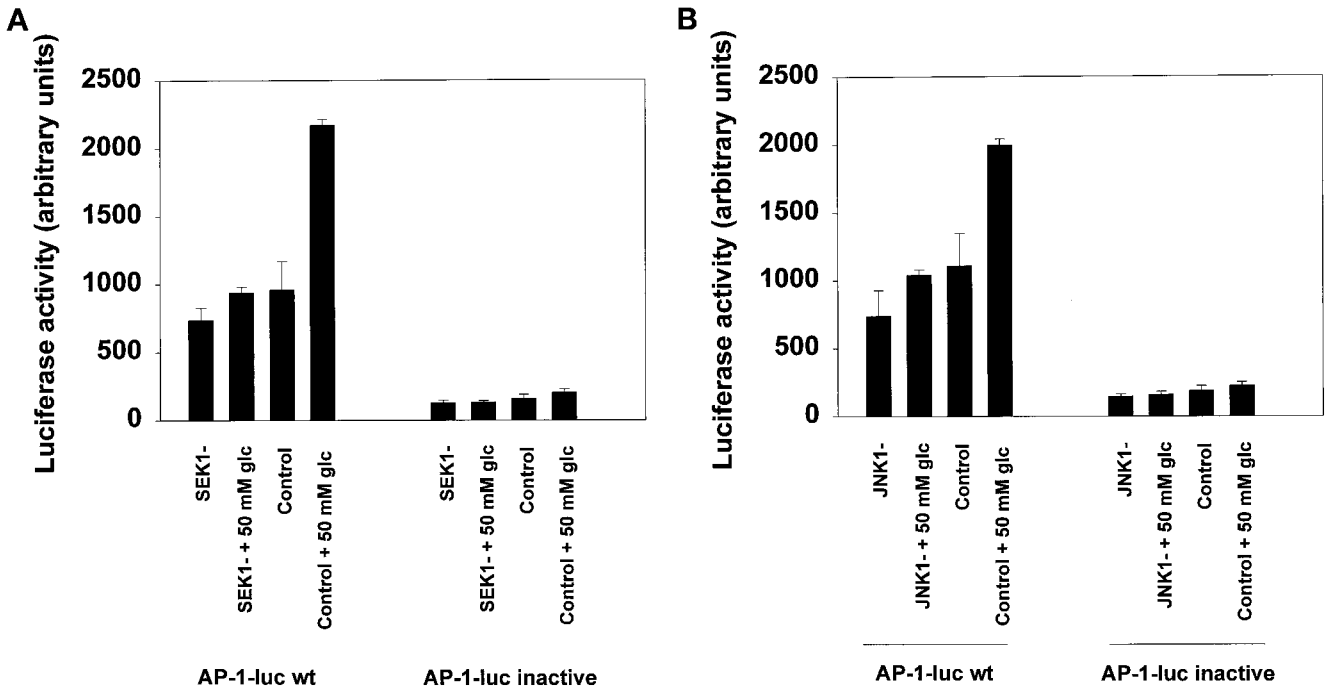

Figure 3. Hyperosmotic glucose transactivates c-Jun and stimulates luciferase activity from an AP-1-luciferase reporter construct, which is inhibited by expression of either dominant-negative SEK1 (A) or dominant-negative JNK1 (B). Hepatocytes were infected with either an AP-1-luciferase plasmid poly-L-lysine adenovirus or a mutant AP-1-luciferase poly-L-lysine adenovirus (100 moi) and coinfected in combination with either control plasmid poly-L-lysine adenovirus, dominant-negative SEK1 plasmid poly-L-lysine adenovirus, or dominant-negative JNK1 plasmid poly-L-lysine adenovirus (250 moi), followed by culture as described in MATERIALS AND METHODS. After 24 h to allow expression of dominant-negative SEK1 or dominant-negative JNK1, hepatocytes were treated for another 12 h with either media control or 50 mM glucose, after which media were aspirated and the cells frozen. Cells were prepared for assay of luciferase activity using a kit, as described in the manufacturer's instructions [see MATERIALS AND METHODS (de Wet *et al.*, 1987)]. Data are means ± SE from the averages of triplicates (six data points) from two experiments.

DNA synthesis (Table 4). Coexpression of dominant-negative PI_3 kinase p110α and dominant-negative PI_3 kinase p110γ alone caused a significant reduction in the ability of primary cultures of hepatocytes to synthesize DNA. However, this reduced level could be stimulated ~250% by 50 mM glucose treatment, 1 ng/μl TNFα treatment, or 1 ng/μl HGF treatment. Thus, coexpression of dominant-negative PI_3 kinase p110α and dominant-negative PI_3 kinase p110γ did not block the ability of multiple agonists to stimulate DNA synthesis in primary cultures of hepatocytes.

DISCUSSION

These studies were designed to test whether signaling by the JNK/SAP kinase cascade increased or decreased the ability of a nontransformed, nonestablished epithelial cell, the hepatocyte, to synthesize DNA in vitro. Treatments of hepatocytes with hyperosmotic glucose, TNFα, and HGF increased the activity of JNK1. Expression of dominant-negative Ras^{N17}, dominant-negative Rac^{N17}, dominant-negative $Cdc42^{N17}$, dominant-negative SEK1, and dominant-negative JNK1 blocked both JNK1 activation and c-Jun serine 63 phosphorylation after agonist treatments. Similarly, increased transactivation of c-Jun was dependent on activation of the JNK/SAP kinase cascade, as judged by the abilities of dominant-negative SEK1 and dominant-negative JNK1 to block glucose-induced luciferase activity from the AP-1-luciferase reporter construct. Glucose, TNFα, and HGF treatments increased DNA synthesis in primary cultures of hepatocytes, which was also blocked by expression of dominant-negative Ras^{N17}, dominant-negative Rac^{N17}, dominant-negative $Cdc42^{N17}$, dominant-negative SEK1, dominant-negative JNK1, and dominant-negative c-Jun (TAM67). Thus, molecular inhibition of the JNK/SAP kinase cascade results in a blunting of signals from proximal receptors to the distal transcription factor c-Jun, and these data strongly suggest that multiple agonists stimulate hepatocyte DNA synthesis via a Ras/Rac1/Cdc42/SEK/JNK/c-Jun cascade–dependent mechanism.

In a previous publication, we demonstrated that inhibition of the other known stress-activated protein

Table 2. Hyperosmotic glucose treatment, TNFα treatment, or HGF treatment increases DNA synthesis in primary cultures of rat hepatocytes, which is blocked by expression of either dominant-negative SEK1, dominant-negative JNK1, or dominant-negative c-Jun (TAM67)

Treatment	Control plasmid	Dominant-negative SEK1$^-$	Dominant-negative JNK1$^-$	Dominant-negative c-Jun (TAM67)
Media control	3,596 ± 640	1,236 ± 225[a]	994 ± 290[a]	1,119 ± 330[a]
50 mM glucose	12,189 ± 990[b]	2,007 ± 340[a]	1,325 ± 410[a]	1,600 ± 405[a]
1 ng/μl TNFα	12,274 ± 780[b]	1,614 ± 375[a]	—	1,826 ± 620[a]
1 ng/μl HGF	15,389 ± 1,120[b]	—	1,797 ± 630[a]	1,577 ± 380[a]

Hepatocytes were cultured as described in MATERIALS AND METHODS. Hepatocytes were infected with either control plasmid poly-L-lysine adenovirus, dominant-negative SEK1 poly-L-lysine adenovirus, dominant-negative JNK1 poly-L-lysine adenovirus, or recombinant dominant-negative c-Jun (TAM67) adenovirus (all at 250 moi each), followed by culture as described in MATERIALS AND METHODS. After 24 h to allow expression of dominant-negative SEK1, dominant-negative JNK1, and dominant-negative c-Jun (TAM67), hepatocytes were treated with either media control for 24 h, 50 mM glucose for 6 h followed by incubation with unsupplemented media for 18 h, or with 1 ng/μl TNFα for 24 h, or with 1 ng/μl HGF for 24 h (total time in primary culture 48 h). Hepatocytes under all conditions were continually incubated with 2 μCi of [^{3}H]thymidine throughout the final 24 h of culture, after which they were lysed and [^{3}H]thymidine incorporation into DNA was determined as in MATERIALS AND METHODS (n = 12).

[a] $p < 0.05$ decrease compared with corresponding value in control plasmid infected.

[b] $p < 0.05$ increase compared with media control.

kinase cascade, the p38-RK cascade, by the drug SB203580 also blunted the ability of primary cultures of hepatocytes to synthesize DNA (Spector *et al.*, 1997). These data, along with the data presented in this article and elsewhere (Westwick *et al.*, 1995; Bogoyevitch *et al.*, 1996; Loyer, *et al.*, 1996; Read *et al.*, 1997; Whitmarsh *et al.*, 1997) strongly suggest that the coordinate actions/activities of both the JNK/SAP kinase and p38-RK cascades play essential roles in the regulation of DNA synthesis in hepatocytes.

In contrast to the similar abilities of hyperosmotic glucose, TNFα, and HGF to activate JNK1, the abilities of these agonists to alter activity of the MAP kinase cascade were disparate. These data suggest that activation of the MAP kinase pathway plays a less important role in stimulating hepatocyte DNA synthesis than does activation of the SAP kinase pathway. This finding also correlates with previously published data demonstrating a reduction in MAP kinase basal activity when hepatocytes are proliferating and showing that an ~90% inhibition of basal MAP kinase activity caused only a small (~20%) decrease in DNA synthesis (Spector *et al.*, 1997). More recent data from this laboratory have demonstrated that chronic activation of the MAP kinase cascade in hepatocytes results in the expression of the cyclin-dependent kinase inhibitor proteins $p21^{Cip\text{-}1/WAF1}$ and $p16^{INK4a}$ and reduces DNA synthesis (Tombes *et al.*, 1998). Presumably the balance of activities between the MAP and SAP kinase pathways determines whether a

Table 3. Hyperosmotic glucose, HGF, and TNFα stimulate hepatocyte DNA synthesis via a Ras/Rac1/Cdc42/SEK/JNK/c-Jun–dependent mechanism

	Control plasmid	Dominant-negative Ras N17	Dominant-negative Rac N17	Dominant-negative Cdc42 N17
Media control	3,522 ± 400	1,127 ± 70[a]	1,808 ± 120[a]	985 ± 160[a]
50 mM glucose	9,763 ± 650[b]	1,995 ± 120[a]	1,517 ± 210[a]	810 ± 145[a]
1 ng/μl TNFα	12,385 ± 1,080[b]	868 ± 265[a]	1,389 ± 110[a]	1,287 ± 140[a]
1 ng/μl HGF	15,185 ± 870[b]	1,480 ± 440[a]	2,517 ± 405[a]	1,496 ± 105[a]

Hepatocytes were cultured as described in MATERIALS AND METHODS. Hepatocytes were infected with either control plasmid poly-L-lysine adenovirus, dominant-negative Ras N17 poly-L-lysine adenovirus, dominant-negative Rac1 N17 poly-L-lysine adenovirus, dominant-negative Cdc42 N17 poly-L-lysine adenovirus (all at 250 moi each), followed by culture as described in MATERIALS AND METHODS. After 24 h to allow expression of dominant-negative Ras N17, dominant-negative Rac1 N17, and dominant-negative Cdc42 N17, hepatocytes were treated with either media control for 24 h or with 50 mM glucose, 1 ng/μl HGF, or 1 ng/μl TNFα for 24 h (total time in primary culture 48 h). Hepatocytes under all conditions were continually incubated with 2 μCi of [^{3}H]thymidine throughout the final 24 h of culture, after which they were lysed and [^{3}H]thymidine incorporation into DNA was determined as in MATERIALS AND METHODS (n = 6). Under control conditions, HGF stimulated JNK1 activity 3.75 ± 0.25-fold after 8 min, which was completely blocked by expression of dominant-negatives Ras N17, Rac1 N17, and Cdc42 N17.

[a] $p < 0.05$ decrease compared with corresponding value in control plasmid infected.

[b] $p < 0.05$ increase compared with media contol.

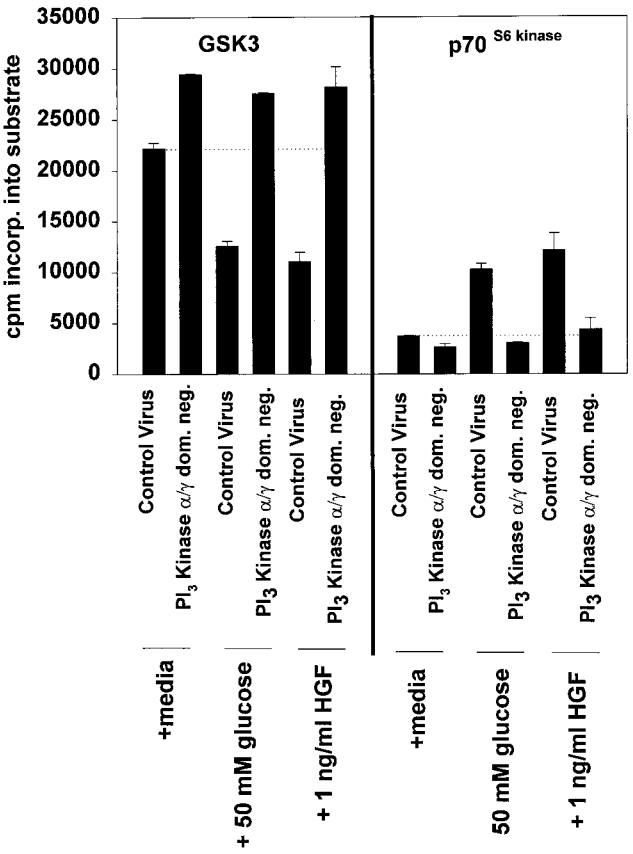

Figure 4. Coexpression of dominant-negative PI_3 kinase p110α and dominant-negative PI_3 kinase p110γ block the ability of hyperosmotic glucose treatment and HGF treatment to activate $p70^{S6\ kinase}$ and to inhibit GSK3 activities. Hepatocytes were infected with either control recombinant adenovirus (250 moi) or recombinant adenoviruses to express dominant-negative PI_3 kinase p110α and dominant-negative PI_3 kinase p110γ (125 moi each), followed by culture as described in MATERIALS AND METHODS. After 24 h to allow expression of dominant-negative PI_3 kinase p110α and dominant-negative PI_3 kinase p110γ, hepatocytes were treated for 10 min at 37°C with either media control, 50 mM glucose, or 1 ng/μl HGF. After 10 min, media were aspirated and the cells frozen. Hepatocytes were lysed and subjected to immunoprecipitation, followed by immune complex kinase assays for both GSK3 and $p70^{S6\ kinase}$. Activities shown are expressed as cpm incorporated into substrate versus media control treatment (5000 cpm/pmol) and are the means ± SE of duplicates from four experiments. Media control treatment did not alter the basal activities of protein kinases versus no treatment.

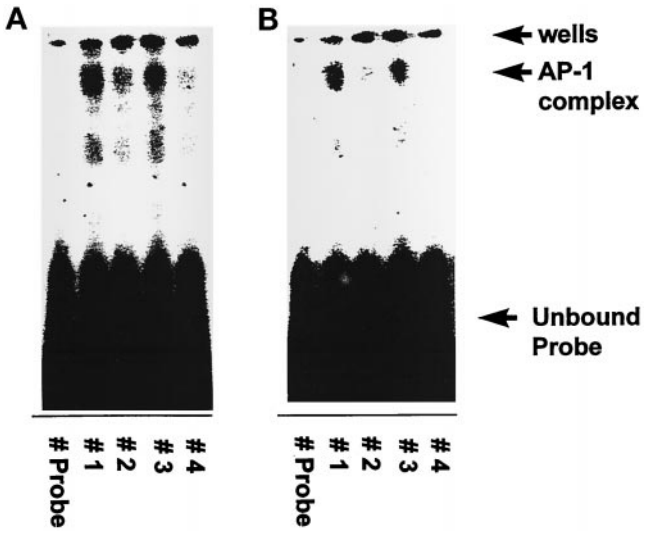

Figure 5. Hyperosmotic glucose (A) and HGF (B) treatments stimulate DNA binding of AP-1/c-Jun complex, which is not blocked by coexpression of dominant-negative PI_3 kinase p110α and dominant-negative PI_3 kinase p110γ. Hepatocytes were infected with either control recombinant adenovirus (250 moi) or recombinant adenoviruses to express dominant-negative PI_3 kinase p110α and dominant-negative PI_3 kinase p110γ (125 moi each), followed by culture as described in MATERIALS AND METHODS. After 24 h to allow expression of dominant-negative PI_3 kinase p110α and dominant-negative PI_3 kinase p110γ, hepatocytes were treated for 20 min at 37°C with either media control, 50 mM glucose, or 1 ng/μl HGF. After 20 min, media were aspirated and the cells frozen. Hepatocytes were lysed, nuclear extracts prepared, and AP-1 DNA-binding experiments performed as in MATERIALS AND METHODS. Data are representative of four independent experiments. No alteration in c-Jun/AP-1 DNA binding was observed in media control samples. Lane 1, control virus + 50 mM glucose; lane 2, control virus; lane 3, dominant-negative PI_3 kinase (p110α and p110γ) + 50 mM glucose; and lane 4, PI_3 kinase (p110α and p110γ).

hepatocyte will proliferate or senesce (Xia *et al.*, 1995; Gomez-Lechon *et al.*, 1996; Bogoyevitch *et al.*, 1997).

Hyperosmotic glucose and HGF treatments stimulated the activity of $p70^{S6\ kinase}$ and decreased the activity of GSK3. The regulation of $p70^{S6\ kinase}$ and GSK3 activities has been suggested to be downstream of signaling by PI_3 kinase (Franke *et al.*, 1995; Rahimi *et al.*, 1996), and we found that coexpression of dominant-negative PI_3 kinase (p110α and p110γ) inhibited the ability of these agonists to activate $p70^{S6\ kinase}$ and to inhibit GSK3. The ability of these agonists to modulate GSK3 activity is of interest because GSK3 has been proposed to be the protein kinase responsible for phosphorylating the DNA-binding domain of c-Jun, resulting in a reduction in the ability of c-Jun to associate with DNA (Boyle *et al.*, 1990). In agreement with this hypothesis, glucose and HGF treatments inhibited GSK3 activity and stimulated c-Jun DNA binding. In further agreement, expression of dominant-negative PI_3 kinase (p110α and p110γ) increased basal GSK3 activity and reduced basal c-Jun DNA binding. However, expression of dominant-negative PI_3 kinase (p110α and p110γ) did not block the abilities of glucose and HGF treatments to stimulate c-Jun DNA binding. These data suggest that inhibition of GSK3 may not be the only mechanism by which agonists can stimulate c-Jun/AP-1 DNA binding. Of note, however, is that the AP-1 transcription factor complex consists of both homodimers of c-Jun as well as heterodimers of c-Jun associated with several other JNK/SAP kinase and p38-RK effector molecules such as

Table 4. Hyperosmotic glucose increases DNA synthesis in primary cultures of rat hepatocytes, which is not blocked by inhibition of the $P1_3$ kinase/GSK3 pathway

Treatment	Control virus	Dominant-negative PI_3 kinase ($p110\alpha + \gamma$)
Media control	4,190 ± 470	3,149 ± 430
50 mM glucose	14,439 ± 1,245[a]	10,263 ± 1,060[a]
1 ng/μl TNFα	10,419 ± 640[a]	9,128 ± 685[a]
1 ng/μl HGF	14,670 ± 910[a]	11,636 ± 1,070[a]

Hepatocytes were cultured as described in MATERIALS AND METHODS. Hepatocytes were infected with either control recombinant adenovirus (250 moi) or dominant-negative PI_3 kinase p110α and dominant-negative PI_3 kinase p110γ recombinant adenoviruses (125 moi each), followed by culture as described in MATERIALS AND METHODS. After 24 h to allow expression of dominant-negative PI_3 kinase p110α and dominant-negative PI_3 kinase p110γ, hepatocytes were treated with either media control for 24 h, 50 mM glucose, 1 ng/μl TNFα, or 1 ng/μl HGF for 6 h, followed by incubation with unsupplemented media for 18 h (total time in primary culture 48 h). Hepatocytes under all conditions were continually incubated with 2 μCi of [^{3}H]thymidine throughout the final 24 h of culture, after which they were lysed and {^{3}H]thymidine incorporation into DNA was determined as in MATERIALS AND METHODS (n = 6-12).

[a] $p < 0.05$ versus media control.

ATF2, JunD, and c-Fos [through Elk1 (Karin, 1995; Kallunki *et al.*, 1996; Columbano *et al.*, 1997)]. Thus, inhibition of GSK3-mediated c-Jun COOH-terminal phosphorylation, and thereby c-Jun/AP-1 DNA binding, may be obscured by effects mediated by these other JNK/SAP kinase– and p38-RK–regulated transcription factors. To definitively prove whether GSK3 mediates both phosphorylation of c-Jun in its COOH-terminal sites and DNA binding in vivo, additional studies will need to be performed to directly examine the stoichiometry of phosphorylation within these sites.

Expression of dominant-negative PI_3 kinase (p110α and p110γ) reduced the basal ability of hepatocytes to synthesize DNA, but did not reduce the ability of agonist treatments to stimulate DNA synthesis. These data were surprising, based on the data of Rahimi *et al.* (1996), who demonstrated that blockade of PI_3 kinase function in transformed established epithelial cells abolished the abilities of agonists to stimulate DNA synthesis. It is unclear why blockade of PI_3 kinase function abolished stimulation of DNA synthesis in transformed, established epithelial cells but did not block stimulation of DNA synthesis in nontransformed, nonestablished epithelial cells. The most likely explanation for the difference between the data may be due to cellular transformation and the establishment in culture of the cells used by Rahimi *et al.*(1996).

It was recently demonstrated that a rapid reduction in glucose concentration also leads to a stimulation of JNK1 and $p42^{MAP\ kinase}$ activities in drug-resistant MCF-7 breast cancer cells (Gupta *et al.*, 1997, Liu *et al.*, 1997). Other studies, including the data presented in this article, have demonstrated that increases in osmolarity can also elevate JNK1/2 activity (Sanchez *et al.*, 1994; Bagrodia *et al.*, 1995; Xia *et al.*, 1995; Rosette and Karin, 1996). This suggests that positive and negative modulations in osmolarity can cause similar changes in activity within the same signal transduction cascade. Our data also demonstrated that the ability of hyperosmotic glucose to activate JNK1 (Table 1) is dependent on signaling downstream of the *Ras* proto-oncogene. Rosette and Karin (1996) demonstrated that increases in osmolarity cause clustering of plasma membrane receptors in PC12 cells, and it is likely that treatment of primary cultures of hepatocytes with hyperosmotic glucose will do likewise. They proposed that receptor clustering, via increased osmolarity, leads to the activation of signal transduction cascades. The key plasma membrane receptors upstream of the *Ras* proto-oncogene, by which positive and negative alterations in osmolarity regulate signaling pathways in primary cultures of hepatocytes, are yet to be determined.

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