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Gene 33/Mig-6, a Transcriptionally Inducible Adapter Protein That Binds GTP-Cdc42 and Activates SAPK/JNK*:

A POTENTIAL MARKER TRANSCRIPT FOR CHRONIC PATHOLOGIC CONDITIONS, SUCH AS DIABETIC NEPHROPATHY. POSSIBLE ROLE IN THE RESPONSE TO PERSISTENT STRESS*

Anthony Makkinje, **Deborah A. Quinn**[‡], **Ang Chen**[§], **Carmen L. Cadilla**[¶], **Thomas Force**[||], **Joseph V. Bonventre**[§], and **John M. Kyriakis**[**]

[‡]Diabetes, Pulmonary Critical Care, Medical Services, Massachusetts General Hospital, Charlestown, Massachusetts 02129

[§]Diabetes, Renal, Medical Services, Massachusetts General Hospital, Charlestown, Massachusetts 02129

[||]Diabetes, Cardiology Units, Medical Services, Massachusetts General Hospital and the Department of Medicine, Harvard Medical School, Charlestown, Massachusetts 02129

[¶]Department of Biochemistry, University of Puerto Rico School of Medicine, San Juan, Puerto Rico 00936

Abstract

Chronic stresses, including the mechanical strain caused by hypertension or excess pulmonary ventilation pressure, lead to important clinical consequences, including hypertrophy and acute respiratory distress syndrome. Pathologic hypertrophy contributes to decreased organ function and, ultimately, organ failure; and cardiac and diabetic renal hypertrophy are major causes of morbidity and morality in the developed world. Likewise, acute respiratory distress syndrome is a serious potential side effect of mechanical pulmonary ventilation. Whereas the deleterious effects of chronic stress are well established, the molecular mechanisms by which these stresses affect cell function are still poorly characterized. *gene 33* (also called mitogen-inducible gene-6, *mig-6*) is an immediate early gene that is transcriptionally induced by a divergent array of extra-cellular stimuli. The physiologic function of Gene 33 is unknown. Here we show that *gene 33* mRNA levels increase sharply in response to a set of commonly occurring chronic stress stimuli: mechanical strain, vasoactive peptides, and diabetic nephropathy. Induction of *gene 33* requires the stress-activated protein kinases (SAPKs)/c-Jun NH_2-terminal kinases. This expression pattern suggests that *gene 33* is a potential marker for diabetic nephropathy and other pathologic responses to persistent sublethal stress. The structure of Gene 33 indicates an adapter protein capable of binding monomeric GTPases of the Rho subfamily. Consistent with this, Gene 33 interacts *in vivo* and, in a GTP-dependent manner, *in vitro* with Cdc42Hs; and transient expression of Gene 33 results in the selective activation of the SAPKs. These results imply a reciprocal, positive feedback relationship between Gene 33 expression and SAPK activation. Expression of

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**To whom all correspondence should be addressed: Diabetes Research Laboratory, Massachusetts General Hospital East, 149 13th St., Charlestown, MA 02129. Tel.: 617-726-9451; Fax: 617-726-9452; kyriakis@helix.mgh.harvard.edu.

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Gene 33 at sufficient levels may enable a compensatory reprogramming of cellular function in response to chronic stress, which may have pathophysiological consequences.

The molecular basis of organ system failure arising from chronic stresses is poorly understood. A number of clinically important chronic pathologic conditions, ranging from diabetes to hypertension and chronic inflammation, induce hypertrophy, an overall increase in the protein mass of the affected tissue that is due predominantly to cell enlargement and excess matrix deposition, without an increase in cell numbers. At the cellular level, hypertrophy is marked by an increase in overall protein synthesis, new gene expression (notably of immediate early genes), and, in some cases, reorganization of the actin cytoskeleton and the expression of embryonic genes and extra-cellular matrix proteins. Among the agonists thought to have a role in hypertrophy are vasoactive peptides (*e.g.* endothelin-1 and angiotensin-II), mitogens, and inflammatory mediators (*e.g.* platelet-derived growth factor, interleukin-1, transforming growth factor-β, and gp130-coupled cytokines such as cardiotropin), diabetic hyperglycemia, and mechanical strain (1, 2). While modest compensatory hypertrophy, such as that observed upon unilateral nephrectomy or in the heart in response to regular vigorous exercise, can occur without major clinical repercussions, excessive and/or eccentric hypertrophy arising as a consequence of sustained stress generally results in the physiologic insufficiency and ultimate failure of organ systems, as occurs in cardiac and diabetic renal failure, two major causes of morbidity and mortality in the developed world (1, 2).

Mechanical strain not only causes hypertrophy in the heart and kidney but has been implicated in precipitating acute respiratory distress syndrome (ARDS),[1] as occurs during mechanical ventilation, especially if the ventilation pressure is not adequately controlled. Ameliorating the consequences of excess ventilation pressure is a significant challenge in dealing with pulmonary disease (3). Accordingly, dissection of the molecular mechanisms that underlie mechanical and hypertension-induced cell stress responses is of general importance.

Signal transduction pathways recruited in response to the persistent presence of pro-hypertrophic and other chronic stress agonists may participate in the initiation of pathologic hypertrophy and other deleterious effects. The stress-activated protein kinases (SAPKs; also called c-Jun NH_2-terminal kinases) and the p38s, two mitogen-activated protein kinase (MAPK) subgroups that are activated strongly by environmental stresses, are among several signaling mechanisms that have been implicated in the progression to hypertrophy. In particular, both kinase groups can be activated by mechanical strain and vasoactive peptides (4–8). As with all MAPK signaling pathways, SAPKs and p38s are activated as part of three-tiered MAPK kinase kinase (MAP3K) → MAPK kinase (MKK) → MAPK "core" signaling modules (5). These core modules, in turn, are subject to multiple forms of regulation, including activation by the trimeric G protein α-subunits $G_{\alpha 12}$, $G_{\alpha 13}$, $G_{\alpha 16}$, and $G_{\alpha q}$; trimeric G protein $\beta\gamma$ subunits ($\beta_1\gamma_1$); and Rac and Cdc42, members of the Rho subfamily of Ras type GTPases. $G_{\alpha 12}$ and $G_{\alpha 13}$, along with $G_{\alpha o}$, are putative effectors for angiotensin-II, a potent pro-hypertrophic vasoactive peptide (9–17).

[1]The abbreviations used are: ARDS, acute respiratory distress syndrome; AP-1, activator protein-1; CRIB, Cdc42/Rac interaction and binding; ERK, extracellular signal-regulated kinase; GST, glutathione *S*-transferase; HA, hemagglutinin; MAPK, mitogen-activated protein kinase; MEKK1, MAPK/ERK kinase kinase-1; Mig, mitogen-inducible gene; MKK, MAPK kinase; MAP3K, MKK kinase; PDZ, postsynaptic density-95 (PSD95)/disks large (Dlg)/Z0–1; SAPK, stress-activated protein kinase; SEK1, SAPK/ERK kinase-1; aa, amino acids; PAGE, poly-acrylamide gel electrophoresis; SH3, Src homology 3; AH, Ack homology; PAK, p21-activated kinase; GTPγS, guanosine 5′-3-*O*-(thio)-triphosphate; GDPβS, guanyl-5′-yl thiophosphate.

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Support for the idea that the SAPKs and p38s are involved in hypertrophy comes from studies showing that expression in cardiomyocytes of constitutively active mutants of MKKs upstream of the SAPKs (SAPK/extracellular signal-regulated kinase (ERK) kinase-1, SEK1, and MKK7) or p38s (MKK-3 and -6) can induce hypertrophy in the absence of external stimulus (6, 7). Moreover, expression of a dominant inhibitory, kinase-inactive form of SEK1 can completely block neonatal rat cardiomyocyte hypertrophy stimulated by endothelin, one of the most potent pro-hypertrophic agonists known (4). Although the SAPK and p38 pathways may indeed be involved in the progression to hypertrophy, there is no clear picture of the molecular basis by which these stress pathways reprogram cells adapt to chronic stresses, including those that cause hypertrophy.

The ability of the SAPKs and p38s to phosphorylate transcription factors and thereby trigger changes in gene expression is thought to underlie a substantial part of the mechanism by which these pathways affect cell function. Thus, the SAPKs and p38s are largely responsible for recruiting of the activator protein-1 (AP-1) transcription factor in response to stress (5, 18). Key to dissecting the molecular mechanisms responsible for pathogenic stress responses such as hypertrophy is the identification of stress-induced genes that can sustain the prolonged activation of stress pathways and/or initiate the phenotypic changes characteristic of the responses to chronic stresses.

gene 33 encodes a polypeptide of previously unknown function. It has been established for some time that rat *gene 33*, like c-*fos* and *c-jun*, is an immediate early gene that is rapidly induced by a heterologous array of mitogenic and stressful stimuli. The human isoform of *gene 33*, *mig-6*, behaves in a similar manner (19–21). Nevertheless, despite the extensive analysis of stimuli that induce *gene 33*, a possible physiologic function for Gene 33 has heretofore not been identified. Given the reported induction of *gene 33* by stress, we sought to determine if pro-hypertrophic and chronic stress agonists could induce *gene 33* transcription. In addition, we wished to begin to identify potential biochemical functions for the Gene 33 polypeptide. Our results show that *gene 33* mRNA levels increase dramatically soon after the onset of diabetes and continue to increase throughout the progression to diabetic nephropathy. By contrast, c-*fos* and c-*jun* transcripts are detected in the kidney early in diabetes but return to basal levels well before the onset of nephropathy. We also show that angioten-sin-II and endothelin-1, potent pro-hypertrophic agonists in both the hypertensive heart and the diabetic kidney, can induce *gene 33* and that mechanical strain, which is important to the pathology of cardiac and renal hypertrophy as well as ARDS (1–3), induces *gene 33* expression in a SAPK-dependent manner. This raises the possibility that expression of *gene 33* could serve as a marker for chronic stress conditions such as incipient diabetic renal failure and, perhaps, the response to persistent environmental stresses such as those that precipitate ARDS.

The structure of the Gene 33 protein is suggestive of a molecular adapter that couples to Rho family GTPases. Two forms of Gene 33 are generated as a consequence of differential RNA splicing, a long form and a short form missing aa 67–142. We demonstrate that the long Gene 33 polypeptide is cytosolic and can interact with Cdc42Hs *in vivo* and in a GTP-dependent manner *in vitro*. Moreover, transient expression of the long form of Gene 33 results in the substantial and selective activation of the SAPKs. Our findings are the first to identify potential functions for the Gene 33 protein and suggest that stress-induced expression of Gene 33 may serve to reprogram the cellular signaling machinery in response to chronic stress, resulting in sustained activation of the SAPKs and, consequently, SAPK-dependent gene expression.

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EXPERIMENTAL PROCEDURES

Plasmids, Constructs, Cells, and Transfections

We used the following vectors: pEBG, a mammalian expression vector that expresses a glutathione *S*-transferase (GST)-tagged polypeptide; pCMV5-FLAG, a mammalian expression vector that expresses an M2-FLAG-tagged polypeptide; pCMV5-Myc, a mammalian expression vector that expresses a Myc-tagged polypeptide; pMT3, a mammalian expression vector that expresses a hemagglutinin (HA)-tagged polypeptide; and pGEX-KG, a bacterial expression vector that expresses a GST-tagged polypeptide. pCMV5-FLAG- and pGEX-KG-Cdc42Hs, pMT3-SAPK-p46β1, pMT3-p38α, and pMT3-ERK1 have been described (22, 23). The recombinant adenoviral construct encoding SEK1(K129R) has been described (4). cDNA encoding 14-3-3ζ was kindly provided by Drs. Guri Tzivion and Joseph Avruch (Massachusetts General Hospital). cDNA for ASK1 was kindly provided by Dr. Hidenori Ichijo (Tokyo Medical and Dental University). Gene 33 deletion constructs were generated by polymerase chain reaction and cloned, using standard methods (24), into various expression plasmids.

Human embryonic kidney 293 cells and murine NIH3T3 fibroblasts were transfected by the calcium phosphate method. Rat renal mesangial cells were prepared and cultivated as described (25). Cells used were at passage 3. A549 cells, a human pulmonary alveolar epithelial tumor cell line, were infected with the SEK1(K129R) adenovirus at the multiplicities of infection indicated in the figures. Mesangial cells were treated with human endothelin-1 (100 nM, 60 min), human angiotensin-II (500 nM, 60 min), or calcium ionophore (A23187, 1 μM, 60 min). For mechanical strain experiments, A549 cells were cultured on elastic surfaces. A flat piston, which is pushed upward in a cyclic fashion against the bottom of the elastic surface, produces uniform biaxial strain (26). The rate of cyclic stretch was 12 cycles/min, and the degree of stretch was 5% for 2 h.

Streptozotocin Induction of Diabetes in Rats

Male Harlan Sprague-Dawley rats (150–200 g) were made diabetic with an intraperitoneal injection of streptozotocin (50 mg/kg). Control animals were injected with water. Early diabetes was defined as the first detectable onset of glucosuria (250 mg/dl), which generally occurred within 15–20 h of injection. Diabetic nephropathy was defined as the onset of frank proteinuria and was apparent within 5 weeks of injection. Unilateral nephrectomy was performed as described (27).

Protein Kinase Assays

SAPK, p38, and ERK1 immune complex kinase assays were performed as described (23). 293 cells were transfected with 1 μg of pMT3-SAPK, p38, or ERK1 and either empty plasmid or pCMV5-FLAG-Gene 33. After cell lysis, kinases were immunoprecipitated with anti-HA.

Coimmunoprecipitation Assays

In vivo associations between Gene 33 and various proteins were performed as described (28). *In vitro* association of Gene 33 and Cdc42Hs was performed as follows. GST-Cdc42Hs was expressed in *E. coli*, purified on GSH-agarose, eluted with free GSH, and stored as described (29). For reimmobilization and nucleotide charging, purified GST-Cdc42Hs was then normalized to 1 mg/ml in GSH-Sepharose binding buffer (20 mM Hepes, pH 7.4, 100 mM NaCl, 5 mM $MgCl_2$, 2 mM dithiothreitol, 20% (v/v) glycerol), and 500 μl of protein was added per 50 μl (settled) aliquot of glutathione-agarose. After 1 h of rotating at 4 °C, the slurry was washed twice in nucleotide depletion buffer (20 mM Hepes, pH 7.4, 10 mM EDTA, 1 mM dithiothreitol, 50 mM NaCl, 5% (v/v) glycerol, 0.1% (w/v) Triton X-100).

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GST-Cdc42Hs beads were then loaded with GTPγS or GDPβS as follows. 50-μl beads were incubated with 1 ml of nucleotide loading buffer (20 mM Hepes, pH 7.4, 50 mM NaCl, 10 mM $MgCl_2$, 1 mM dithiothreitol, 5% (v/v) glycerol, 0.1% (w/v) Triton X-100) containing 0.2 mM freshly added GTPγS or GDPβS. Twenty-four hours prior to the experiment, 293 cells were transfected with M2-FLAG-Gene 33 (1 μg of plasmid/10-cm dish) or, as a positive control, M2-FLAG-p21-activated kinase-1 (PAK1; 10 μg of plasmid/10-cm dish). Twenty-four hours later, extracts were prepared from transfected cells by scraping into extraction buffer (20 mM Hepes, pH 7.4, 10% (v/v) glycerol, 50 mM NaCl, 50 mM β-glycerophosphate, 1 mM EGTA, 1% (w/v) Triton X-100, 1 mM dithiothreitol, 2 μM leupeptin, 10 milliunits/ml aprotinin, 400 μM phenylmethylsulfonyl fluoride, 20 μM Na_3VO_4). Extracts were normalized to 2 mg/ml protein and supplemented with 10 mM $MgCl_2$ plus either GTPγS or GDPβS at a final concentration of 0.2 mM. Charged Cdc42Hs was pelleted by centrifugation, the supernatant was removed, and 1 ml of the cognate (GTPγS- or GDPβS-supplemented) cell extract was added. After 2 h at 4 °C, the beads were washed with the relevant (GTPγS- or GDPβS-supplemented) nucleotide loading buffer. Polypeptides bound to the GST-Cdc42Hs were detected by SDS-PAGE and immunoblotting with anti-FLAG.

Miscellaneous Methods

Northern blotting was performed as described (24). Immunofluorescence analysis of Gene 33 localization was performed as described (30).

RESULTS

Transcriptional Induction of gene 33 in Response to Environmental Stress

Transcription of *gene 33* is known to occur in response to insulin, growth factors, and some stresses (19). We wished to confirm and extend the hypothesis that *gene 33* was induced in response to environmental stress. Cultures of rat glomerular mesangial cells were subjected to hypertonic shock (500 mM sorbitol) for 20 min, at which time the medium was returned to iso-osmotic conditions. Total cellular RNA was prepared immediately after the 20-min stimulus and for various times up to 4 h after the cessation of stimulus and used for Northern analysis of *gene 33* expression. As is apparent in Fig. 1, *gene 33* expression is detectable first at 40 min after sorbitol removal, achieving a maximum at 1 h and declining slightly thereafter; however, expression is still detectable at 4 h. By contrast, SAPK is activated within the first 20 min of osmotic shock and remains active up to 2 h after the return to iso-osmotic conditions (data not shown). c-*jun* expression is similarly transient, appearing before *gene 33* (20 min), peaking at 40 min, and declining to base line by 2 h. Thus, *gene 33* expression follows activation of the SAPKs and c-*jun* induction and remains elevated long after c-*jun* expression returns to control levels.

Induction of Gene 33 Expression in Unilateral Nephrectomy and Diabetes: Gene 33 Expression as a Potential Marker for Chronic Stresses, Including Diabetic Nephropathy

The induction of *gene 33* by hypertonicity suggested that Gene 33 might serve as a general stress-responsive element and prompted us to ask if the transcriptional induction of *gene 33* might occur in response to physiologically significant, chronic, pathologic stresses. Diabetes is the single largest cause of end stage renal failure in the United States and Europe, and nephropathy accounts for a substantial fraction of the mortality associated with diabetes. Diabetic nephropathy is a chronic stress response, characterized by glomerular mesangial cell hypertrophy. Hypertension in the renal microvasculature and elevated plasma glucose are believed to contribute to this hypertrophy (1). In order to ascertain the effect of diabetes on *gene 33* expression in the kidney, we employed a well established technique, the administration of streptozotocin, a genotoxin that targets selectively the β cells of the

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pancreas, to render rats diabetic. At various times, kidneys were removed, and the mRNA was extracted and subjected to Northern analysis using *gene 33*, c-*jun*, and c-*fos* cDNAs as probes.

Within 24 h of streptozotocin administration, diabetes (glucosuria and elevated blood glucose) was evident in the injected animals. At this time, there was a significant increase in *gene 33* mRNA, consistent with the characterization (19–21) of *gene 33* as an immediate early gene. Similarly, expression of two other immediate early genes, c-*jun* and c-*fos*, was stimulated at this early time point (Fig. 2*A*). Expression of c-*fos* continued to increase and was maximal by 41 h after the onset of diabetes, declining thereafter; by contrast, c-*jun* expression remained constant for up to 46 h after the onset of diabetes.

Unilateral nephrectomy is associated with immediate early gene expression and correlates with a transient compensatory hypertrophy in the remaining kidney (1). Unilateral nephrectomy, in nondiabetic animals, produced a striking induction of *gene 33*, within 17 h, in the remaining kidney. In parallel, c-*jun* expression in the remaining kidney was also strongly stimulated. c-*fos* induction was observed but was less robust than that incurred by diabetes. *gene 33* and c-*jun*, but not c-*fos*, expression was greater in unilaterally nephrectomized, diabetic animals than in animals subjected to either treatment alone (Fig. 2*A*).

We allowed some of the streptozotocin-injected animals to progress, over 5 weeks, to frank diabetic nephropathy, a point at which they displayed overt proteinuria and, as determined by gross examination of the kidneys, renal hypertrophy. We performed Northern analysis of mRNA from control and diabetic rat kidneys at 5 weeks post-streptozotocin administration; again, *gene 33*, c-*jun*, and c-*fos* cDNAs were used as probes. Northern blots of total RNA from rat kidneys at 23 h and 5 weeks post-streptozotocin injection revealed a continuous increase in *gene 33* mRNA levels (Fig. 2*B*). By contrast, c-*jun* and c-*fos* mRNAs, which were markedly induced at 23 h, had returned to basal levels by 5 weeks (Fig. 2, *A* and *B*). Thus, the onset of diabetic nephropathy correlates closely with a progressively increasing induction of *gene 33*, an expression pattern that differs significantly from the comparatively transient expression of c-*jun* and c-*fos*, two other immediate early genes, the expression of which has been associated with diabetic nephropathy (1, 2, 25). This persistent expression, tracking with the pathogenesis of a chronic disease, suggests that *gene 33* may be a good marker for the cellular response to persistent stress. Although a role for Gene 33 in the pathogenesis of diabetes has yet to be established, the expression pattern of *gene 33* corresponds closely with that of the known increase in extracellular matrix protein expression that is characteristic of diabetic nephropathy (1), and this persistent expression of *gene 33* is consistent with the hypothesis that *gene 33* expression is a marker for diabetic nephropathy. By contrast, c-*fos* and c-*jun* expression, although possibly necessary for triggering renal hypertrophy, appear not to correlate with the progression of diabetic renal disease and may be more general markers for inducible immediate early gene expression.

Induction of gene 33 by Vasoactive Peptides and Mechanical Strain in Renal and Pulmonary Cells Points to a Role in the General Stress Response: Expression of gene 33 in Response to Mechanical Strain Is SAPK-dependent

The renal disease characteristic of diabetes is particularly evident in glomerular mesangial cells. In the diabetic kidney, elevated plasma glucose stimulates the secretion, by glomerular mesangial cells, of excess extracellular matrix and the production of transforming growth factor-*β*. In the diabetic kidney, there is also a striking increase in circulating vasoactive peptides (angiotensin-II and endothelin-1). Hyperglycemia, along with elevated matrix deposition, transforming growth factor-*β* production, and vasoactive peptide levels, in turn, elicit immediate early gene expression and are thought to contribute to glomerular

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hypertension. Hypertension also subjects the mesangial cells of the diabetic kidney to mechanical strain. A positive feedback cycle is thought to ensue, with elevated cytokines and vasoactive peptides triggering immediate early gene and matrix protein expression which, in turn, increases glomerular hypertension, resulting in continued cytokine and vasoactive peptide release (1, 25). It has been proposed that these events lead ultimately to glomerular mesangial cell hypertrophy and glomerulosclerosis, resulting eventually in renal failure (1, 8, 25).

Angiotensin-II, endothelin-1, and mechanical strain have also been implicated in pressure overload cardiomyocyte hypertrophy (2). Because these stresses are thought to be important to hypertrophy and other stress responses, we sought to determine if any of these stimuli could induce *gene 33* expression as a means of establishing if, at the cellular level, *gene 33* expression was a general stress response.

Treatment of primary cultures of renal mesangial cells with endothelin-1 or angiotensin-II results in striking induction of *gene 33* (Fig. 3*A*). Interestingly, although endothelin-1 induces strong expression of *gene 33*, induction of c-*jun* expression is much weaker (Fig. 3*A*). Both endothelin-1 and angiotensin-II stimulate a transient increase in intracellular calcium concentrations. This calcium influx is important to signaling by these vasoactive peptides (31). The calcium ionophore A23187 also vigorously induces expression of *gene 33* and c-*jun*, suggesting that agonists that induce increases in intracellular calcium also induce *gene 33* expression (Fig. 3*A*).

Cyclical mechanical stretching of A549 pulmonary epithelial cells cultured on an elastic surface (see “Experimental Procedures”) is a facile cellular model for the mechanical strain incurred by inappropriately controlled mechanical ventilation (3). Mechanical stretching also mimics the features of the strain that may occur at the mesangial cell membrane with glomerular hypertension or in cardiomyocytes with pressure overload (7, 31). Cyclical mechanical stretching of A549 pulmonary epithelial cells also causes a substantial induction of *gene 33* (Fig. 3*B*).

The promoter for *gene 33* contains a consensus tetradecanoyl phorbol ester response element at position −476 relative to the transcriptional start site (19, 20). The tetradecanoyl phorbol ester response element is a *cis*-acting element that binds AP-1 and *trans*-activates genes in response to stimuli that recruit AP-1 (18). The SAPKs recruit AP-1 both by phosphorylating and activating the *trans*-activating activity of c-Jun and activating transcription factor-2 as well as by phosphorylating and activating Elk-1, which participates in c-*fos* induction (5, 18). In addition, there is a cyclic AMP-response element at position −55 in the *gene 33* promoter (19, 20). Mitogens and stresses (acting through activating transcription factor-2 and cAMP-response element modulator-τ) as well as stimuli that elevate cAMP (acting through cAMP response element binding protein) can *trans*-activate genes containing a cyclic AMP-response element (32). The observed increases in *gene 33* mRNA could also be post-transcriptional. In this regard, the SAPKs have been implicated in the stabilization of some rapidly turning over mRNAs (33).

With these observations in mind, we sought to begin to ascertain if induction of *gene 33* was SAPK-dependent. Mechanical stretching of renal mesangial cells and pulmonary A549 cells results in substantial SAPK activation.[2] A549 cells are easily susceptible to expression of genes of interest from recombinant adenoviral constructs. Infection of A549 cells with a recombinant adenovirus encoding a dominant inhibitory form of the SAPK-specific MKK SEK1 prevents mechanical stretch induction of *gene 33* in A549 cells (Fig. 3*B*) in a dose-

[2]A. Makkinje and D. A. Quinn, unpublished observations.

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dependent manner. From the results in Figs. 2 and 3, we conclude that stimuli associated with hypertrophy and other pathogenic stresses can induce expression of *gene 33* and that a component of this induction is SAPK-dependent. These results are consistent with the findings in Fig. 1 indicating that stress induction of *gene 33* reaches a maximum after peak induction of c-*jun* is observed.

The Gene 33 Polypeptide Is a Cytosolic Adapter Protein That Binds 14-3-3ζ and, in a GTP-dependent Manner, Binds Cdc42Hs

The existence of Gene 33 as an immediate early gene has been known for some time (reviewed in Ref. 19); however, until recently, no clues as to its biochemical function have emerged. Initial examinations of the structure of the Gene 33 polypeptide were uninformative because, at the time, there was little information available concerning protein domain structure and function. More recent examination of Gene 33 has, however, proved illuminating.

Fig. 4 is a diagram of the Gene 33 polypeptide. The cDNA for Gene 33 is predicted to encode a polypeptide of 459 amino acids with a calculated molecular mass of 49,909 Da. At the amino terminus of Gene 33 is a motif significantly homologous to the consensus for a Cdc42/Rac interaction and binding (CRIB) domain (34) (aa 1–38). Comparison of the putative Gene 33 CRIB domain with that of other proteins with CRIB domains indicates that the closest similarity is with that of the Tyr kinase Ack-1, a known effector for Cdc42Hs (35). Gene 33 also contains a polyproline putative binding site for proteins with SH3 domains (36) (aa 148–158) and a consensus binding site for proteins of the 14-3-3 family (37) (aa 246–253). 14-3-3 proteins can homodimerize and heteromerize *in vivo* and *in vitro* with numerous signaling proteins including the MAP3Ks Raf-1 and MAPK/ERK kinase kinase-1 (MEKK1) (37–40). Finally, the carboxyl terminus of Gene 33 (aa 264–424) is strikingly homologous to the noncatalytic carboxyl terminus of Ack-1. We refer to this domain as the Ack homology domain (AH domain). At the extreme carboxyl terminus of Gene 33 is a putative binding site for proteins containing postsynaptic density-95 (PSD95)/ disks large (Dlg)/Z0–1 domains (PDZ domains) (41). PDZ domains are involved in the assembly of ion channels, signaling proteins, and cytoskeletal polypeptides into multiprotein complexes at synapses, cell junctions, and polarized membrane domains (42).

A second, alternatively spliced Gene 33 transcript has also been identified, but the shorter transcript represents only 5–10% of the total hybridizable pool of *gene 33* mRNA. The protein coded by the second, shorter cDNA is missing aa 67–142 and is predicted to have a molecular mass of 42,218 Da (see Ref. 20; reviewed in Ref. 19). The regions deleted in the short form of Gene 33 reside well carboxyl-terminal to the CRIB domain and extend just upstream of the SH3 binding region (Fig. 4). Both the long and short Gene 33 polypeptides migrate aberrantly slowly upon SDS-PAGE (Figs. 5 and 6).[3]

Thus, although Gene 33 possesses no apparent catalytic motifs, there are several possible binding sites for proteins implicated in signal transduction. Moreover, there is remarkable conservation of the Gene 33 CRIB and carboxyl-terminal domains with those of Ack-1. The presence of protein interaction domains on Gene 33 that are selective for signaling proteins, coupled with an apparent lack of catalytic function, is suggestive of a molecular adapter protein involved in signal transduction.

Gene 33 contains no consensus nuclear localization signal. Expression of FLAG-tagged Gene 33 in NIH3T3 cells reveals a punctate cytosolic localization (Fig. 5*A*). Polypeptides of

[3] A. Makkinje, unpublished observations.

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the 14-3-3 family bind to motifs with the consensus sequence RS*X*-S*X*P, wherein at least one of the Ser residues is phosphorylated (37). There is evidence that the binding of 14-3-3 proteins to their targets is involved in retaining these target proteins in the cytosol. In addition, 14-3-3 proteins participate in the nucleation of signaling complexes and in the controlled regulation of signal transduction (37–40). The presence of a putative binding site for 14-3-3 prompted us to investigate if 14-3-3 could interact *in vivo* with Gene 33. From Fig. 5*B* it is clear that recombinant 14-3-3ζ can interact *in vivo* with Gene 33. Thus, coexpression of GST-14-3-3ζ and FLAG-Gene 33 permits the isolation of detectable levels of FLAG-Gene 33 immunoreactivity in GST-14-3-3ζ isolates (Fig. 5*B*).

The significance of the Gene 33/14-3-3ζ interaction is still unclear. 14-3-3 proteins typically exist as dimers *in vivo* and are thought to foster the homo- and heteromerization of some of their target proteins (37, 38). It will be important to determine if 14-3-3 proteins affect the oligomerization state of Gene 33 or couple it to effectors.

CRIB domains are required for the binding and regulation of some, but not all, effectors for Rac and Cdc42, monomeric GTPases of the Rho subfamily that have been implicated in mitogenic and stress signaling as well as cytoskeletal regulation. Ack1 is a Tyr kinase that selectively binds Cdc42Hs *in vivo* and *in vitro* (15–17, 34, 35, 43). The marked similarity between the CRIB motif of Gene 33 and that of Ack1 led us to ask if Gene 33 could interact *in vivo* with human Cdc42 (Cdc42Hs). Accordingly, 293 cells were transfected with GST-Gene 33 and FLAG-V12-Cdc42Hs. The Cdc42Hs was immunoprecipitated with anti-FLAG and subjected to SDS-PAGE and immunoblotting with anti-GST to detect bound Gene 33. From Fig. 5*C*, it is clear that Gene 33 can associate *in vivo* with Cdc42Hs. This association is quite strong and is resistant to washing with 1% Triton X-100 and 1 M LiCl.

The binding of Cdc42Hs to Gene 33 requires the Gene 33 CRIB motif. We expressed FLAG-Cdc42Hs with either GST-wt Gene 33 or HA-Gene 33 (aa 61–459), wherein the CRIB motif was deleted (see Fig. 4). Cdc42Hs was immunoprecipitated with anti-FLAG and subjected to SDS-PAGE and immunoblotting with anti-GST or -HA to detect associated Gene 33. The results in Fig. 5*D* indicate that HA-Gene 33 (aa 61–459), while expressed, is unable to associate with Cdc42Hs under conditions wherein GST-wild type Gene 33 does.

The interaction between Cdc42Hs and Gene 33 can be recapitulated *in vitro* and is GTP-dependent. Thus, we produced GST-Cdc42Hs in bacteria, immobilized it on GSH-agarose, and charged it with either GTPγS or GDPβS, nonhydrolyzable analogues of GTP and GDP, respectively. The Cdc42Hs preparations were incubated with extracts from cells transfected with either FLAG-tagged Gene 33 or, as a positive control, p21-activated kinase-1 (PAK1), a known effector for Cdc42Hs (44). The GSH beads were then washed and subjected to SDS-PAGE and immunoblotting with anti-FLAG. From Fig. 5*E*, it is evident that the binding of Gene 33 to Cdc42Hs is GTP-dependent. Thus, greater levels of Gene 33 associate with GTP-Cdc42Hs than with GDP-Cdc42Hs. GTPases of the Ras superfamily such as Cdc42Hs are inactive when in the GDP-bound state and are competent to interact with their effectors when in the GTP-bound state. The GTP-dependent association between Cdc42Hs and Gene 33 (Fig. 5*E*) supports the contention that Gene 33 might be a Cdc42 effector. Similar results are seen for the interaction between PAK1 and Cdc42Hs. By contrast, although we were also able, in overexpression experiments, to detect *in vivo* associations between Gene 33 and both Rac1 and Ras (but not RhoA), these interactions either did not occur or were not GTP-dependent *in vitro* (data not shown).

From Fig. 5*E* it is also evident that PAK1 binds more strongly to Cdc42Hs than does Gene 33. In the experiments shown in Fig. 5*E*, transfection conditions were adjusted such that equal levels of FLAG-Gene 33 and FLAG-PAK1 were expressed (Fig. 5*E*, *left*) or high

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levels of Gene 33, relative to PAK1, were expressed. Thus, when Gene 33 and PAK1 are expressed at comparable levels, greater amounts of PAK1 than Gene 33 are associated with Cdc42Hs. Only when the level of Gene 33 exceeds that of PAK1 (Fig. 5*E*, *right*) is the binding of Gene 33 to Cdc42Hs equal to that of PAK1. It is noteworthy that the levels of Gene 33 present in the cell are subject to change, in response to environmental stimuli (Figs. 1–4 and Ref. 19), and Gene 33 levels become quite substantial as animals progress to diabetic nephropathy (Fig. 2*B*).

The functional significance of the Gene 33/Cdc42 interaction is still unclear. It is possible that Gene 33 interacts with Rho family GTPases that have yet to be identified. However, the significance of the comparatively modest binding of Gene 33 to Cdc42 might be to prevent Gene 33 binding to Cdc42 (possibly resulting in activation of Gene 33's downstream targets) until the level of Gene 33 protein reaches a threshold at which it can effectively compete with other Cdc42 effectors. Such levels might only be attained after prolonged stress (Fig. 2*B*).

Selective Activation of the SAPKs by Gene 33

The SAPKs and p38s are downstream targets of Cdc42, and several polypeptides upstream of the SAPKs, most notably the SAPK-specific MAP3Ks MEKK1 and mixed lineage kinase-3, bind in a GTP-dependent manner and are possibly regulated by Cdc42 (15–17, 34, 45, 46). The results in Fig. 5 point to the possibility that Gene 33 is a Cdc42 effector, and, accordingly, we wished to determine if Gene 33 could recruit the SAPKs and p38s, known Cdc42 targets (15–17). Thus, 293 cells were cotransfected with FLAG-Gene 33 and either HA-SAPK-p46β1, HA-p38α, or HA-ERK1. FLAG-V12-Ha-Ras served as a positive control for ERK1 activation (47), while the MAP3K apoptosis signal-regulating kinase-1 (untagged) served as a positive control for p38 activation and FLAG-V12-Cdc42Hs served as a positive control for SAPK activation (15–17, 48). From Fig. 6*A*, it is clear that expression of Gene 33 activates the SAPKs to a degree comparable with that incurred by V12-Cdc42Hs alone (~5–8-fold). By contrast, under conditions wherein Ha-Ras induced massive (~20-fold) activation of ERK1, Gene 33 was able to induce only ~1.5-fold ERK1 activation. Gene 33 failed to recruit p38 under conditions in which apoptosis signal-regulating kinase-1 vigorously activated p38 (Fig. 6*A*). We conclude, therefore, that with regard to mammalian MAPK pathways, Gene 33 is a selective activator of the SAPKs.

Deletion studies of the Gene 33 polypeptide reveal that expression of the carboxyl-terminal half of the molecule (aa 819–459), devoid of the CRIB motif but containing the 14-3-3 binding sites as well as the AH domain and the putative PDZ binding site (Fig. 6*B*), is both necessary and sufficient for activation of the SAPK pathway (Fig. 6*C*). Thus, we refer to this portion of the Gene 33 polypeptide as the effector region.

By contrast, the amino-terminal half of the protein, including the CRIB and SH3 binding domains (Fig. 6*B*) is insufficient for SAPK pathway activation (Fig. 6*C*). We also observe that Gene 33 (or the carboxyl-terminal half of Gene 33) and V12-Cdc42Hs, when coexpressed, activate SAPK synergistically (Fig. 6*C*). Curiously, however, expression of the Gene 33 CRIB motif does not block SAPK activation by V12-Cdc42Hs (Fig. 6*C*), perhaps due to the redundancy of mechanisms by which Cdc42 is thought to recruit the SAPKs (44, 46) and the comparatively low affinity of Gene 33 for Cdc42 (Fig. 5*E*).

The experiments in Figs. 5 and 6*C* employed the long form of Gene 33 and deletions thereof. We next wished to ascertain the biochemical properties of the short Gene 33 polypeptide encoded by the alternatively spliced *gene 33* mRNA. This deletion does not remove any of the Gene 33 effector sequences shown in Fig. 6*C* to be necessary for coupling to the SAPKs. To our surprise, however, this construct was unable to activate coexpressed

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SAPK under conditions wherein the long Gene 33 polypeptide and Cdc42 engendered strong SAPK activation (Fig. 6*D*). It is conceivable that the short form of Gene 33 adopts a conformation wherein the carboxyl-terminal effector sequences are inaccessible to downstream targets. Thus, although the Gene 33 amino terminus is unable to trigger SAPK activation *per se*, it seems to exert a positive or, perhaps, a disinhibiting effect on Gene 33 signaling.

DISCUSSION

Our results suggest a model for Gene 33 regulation and function (Fig. 7) wherein Gene 33 serves to enable the cell to respond persistently to chronic stress. In this model, there is a reciprocal regulatory relationship between the SAPKs and Gene 33. Thus, chronic stresses such as those associated with the onset of hypertrophy activate the SAPKs and perhaps other pathways that contribute to the transcriptional induction of *gene 33*. When Gene 33 polypeptide is sufficiently abundant in the cell, it recruits the SAPKs (possibly as a consequence of its association with Cdc42, but this remains to be determined), contributing to further *gene 33* expression. If indeed Gene 33 is a Cdc42 effector (and the GTP-dependent association between Gene 33 and Cdc42 supports this idea), recruitment of and signaling through Gene 33 would require not only the accumulation of sufficient levels of Gene 33 polypeptide but also conditions in which Cdc42 is activated. While we have detected Gene 33 protein expression,[3] we still do not know the conditions under which maximal Gene 33 protein levels are achieved. An overall consequence of the complex combination of processive expression of *gene 33* and activation of Gene 33-dependent mechanisms might be to redirect cell function to respond to ongoing persistent stress. It will be important to identify the combination of cellular factors that enable signaling by Gene 33.

We observe that *gene 33* mRNA increases in response to a subset of chronic stresses and agonists associated with mechanical strain and hypertrophy. Thus, *gene 33* expression in the kidney occurs with the onset of diabetes. In contrast to c-*jun* and c-*fos*, which are expressed transiently, *gene 33* expression increases throughout the progression to diabetic nephropathy. Moreover, *gene 33* is induced by endothelin-1, angiotensin-II, and mechanical strain, specific stimuli known to elicit hypertrophy (1, 2, 7). Mechanical strain is also implicated in the triggering of ARDS. The induction of *gene 33* expression by mechanical strain apparently requires the SAPKs inasmuch as expression of a dominant inhibitory construct of SEK1 abrogates significantly mechanical stress-induced *gene 33* expression. These increases in *gene 33* mRNA be a result of both increased transcription as well as increases in *gene 33* mRNA stability (33); however, it has been shown that the bulk of the regulation of *gene 33* mRNA levels is at the transcriptional level (19).

The persistent, comparatively selective up-regulation of *gene 33* expression during the progression to diabetic nephropathy (which mirrors the persistent up-regulation of extracellular matrix genes, an established marker for diabetic nephropathy (1)) coupled with the ability of known pro-hypertrophic stimuli to induce *gene 33*, make *gene 33* a candidate transcriptional marker for diabetic nephropathy and other long term chronic conditions associated with the recruitment of stress-activated signaling pathways. Accordingly, a detailed study of the function of Gene 33 in the pathology of diabetic nephropathy is warranted.

We present the first biochemical characterizations of the Gene 33 protein. Our results suggest the possibility that Gene 33 functions as an adapter protein that recruits machinery involved in stress-activated signal transduction. Thus, Gene 33 binds 14-3-3. Moreover, Gene 33 appears to be a candidate effector for Cdc42 inasmuch as Gene 33 and Cdc42Hs interact in a GTP-dependent manner. A role for Gene 33 in the recruitment of signaling

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cascades is evident from the observation that expression of the long form of Gene 33 results in substantial activation of the SAPKs. The ability of Gene 33 to activate the SAPKs apparently resides in a carboxyl-terminal effector region consisting of the 14-3-3 binding and AH domains. By contrast, the short splicing isoform of Gene 33, in which an amino-terminal segment between the CRIB and SH3 binding regions (aa 67–142) is deleted, appears inactive with regard to recruitment of the SAPKs.

The conformation of the short form of Gene 33 may render the Gene 33 effector region unable to recruit downstream targets coupled to the SAPKs. Thus, with regard to SAPK activation, the overexpressed long form of Gene 33 may adopt an active conformation *in vivo* in which aa 67–142 serve in an activating capacity to disinhibit the carboxyl-terminal effector region. The short form, missing this activating function, would accordingly be unable to recruit the SAPKs. It is conceivable that the short form of Gene 33 may serve to recruit different pathways or cellular functions that have yet to be identified. These findings establish a framework for future investigations of the biological function of Gene 33 protein.

Expression of active Cdc42Hs is known to activate the SAPK and p38 pathways (15–17). Still, it is unclear from our results if Gene 33 provides a mechanism whereby Cdc42Hs could recruit the SAPKs. Although Gene 33 binding to Cdc42Hs is GTP-dependent, the strength of this interaction is considerably lower than that between Cdc42Hs and PAK1, and expression of the Gene 33 CRIB motif does not significantly hamper activation of the SAPKs by V12-Cdc42Hs. Taken together, these results suggest that for Gene 33 to be a true Cdc42Hs effector with regard to SAPK activation, its levels in the cell must be sufficiently high to permit appreciable binding to GTP-Cdc42. Accordingly, one would expect the biochemical functions of Gene 33 to be most apparent when expression of Gene 33 is maximum, as in diabetic renal disease. By contrast, basal or modest levels of Gene 33 would not compete effectively against other Cdc42Hs targets. Alternatively, it is possible that the Cdc42Hs-dependent functions of Gene 33 and the SAPK-activating function of Gene 33 are exclusive, and SAPK activation by Gene 33 may represent a mechanism regulated by an element other than Cdc42Hs.

Although the carboxyl-terminal effector region of Gene 33 is necessary and sufficient for SAPK activation, the biochemical mechanism by which Gene 33 activates the SAPKs is still nebulous. The domain structure of Gene 33 is indicative of an adapter protein. It is plausible to speculate that Gene 33 binds other polypeptides, such as MAP3Ks, which are more directly coupled to the SAPKs, and delivers these to GTP-loaded Cdc42Hs and/or other upstream activators. In this regard, we do not detect binding of Gene 33 to MEKK1 or mixed lineage kinase-3, MAP3Ks that, like gene 33, recruit the SAPKs selectively and interact with GTP-Cdc42Hs (5, 45, 46). Mixed lineage kinase-3 also possesses an SH3 domain (5), which could bind the SH3 binding site of Gene 33. However, given the lack of an observed interaction between Gene 33 and mixed lineage kinase-3, coupled with the fact that deletion of the SH3 binding site has no effect on Gene 33 recruitment of the SAPKs, it is doubtful that mixed lineage kinase-3 is a Gene 33 target. We have observed, however, that Gene 33 is associated quite stably with an as yet unidentified protein Ser/Thr kinase activity.[3] While this activity cannot directly activate SEK1 *in vitro*, it will be important to determine if this Gene 33-associated kinase is involved in regulation of the SAPKs.

Given that the fragment of Gene 33 consisting of the 14-3-3-binding, AH, and putative PDZ-binding sites is sufficient to signal to the SAPKs, polypeptides that associate with these regions may relay signals to the SAPKs. MEKK1 can associate with 14-3-3 (40); however, as described above, we do not observe an *in vivo* interaction between Gene 33 and full-length MEKK1. The mammalian Wnt pathway component disheveled-1 (Dsh-1) contains a PDZ domain and, in overexpression experiments, can recruit the SAPKs (49, 50). However,

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we do not reliably observe an interaction between Dsh-1 and Gene 33.[4] Moreover, the PDZ domain of Dsh is dispensable for SAPK activation (49–51). The homology between the Gene 33 AH domain and the noncatalytic carboxyl terminus of Ack1 is striking, and it is possible that similar target recruitment mechanisms are employed by Ack and Gene 33. However, little is known of the regulation and function of Ack1. Ack2, a Tyr kinase related to Ack1, can activate SAPK modestly but does not contain the conserved AH domain present in Gene 33 and Ack1 (35, 52).

Most MAPK signaling pathways are activated transiently in cells, due in part to the activity of phosphatases that are either constitutively expressed and active in cells or are transcriptionally up-regulated in response to MAPK-activating stimuli. In the absence of a counterbalancing activating input, these phosphatases can swiftly deactivate MAPKs even if the stimulus persists for some time (5, 47). An unanswered question then is how very long term, sublethal stress signals can, once inhibitory mechanisms are recruited, permit the MAPK-dependent reprogramming of cell function to respond appropriately. Our model for the regulation of Gene 33 expression and function (Fig. 7) suggests that when the level of Gene 33 polypeptide is sufficient to allow it to interact with its upstream activators (possibly including Cdc42Hs), Gene 33 then triggers further activation of the SAPKs, which, in a positive feedback cycle, stimulates more *gene 33* transcription. This processive activation of SAPK and *gene 33* expression maintains chronic expression of SAPK-dependent genes (including *gene 33* itself) and activation of SAPK-dependent mechanisms even against a background of inhibitory signals. Thus, Gene 33 converts what is initially a transient event (SAPK activation by extracellular stimuli) to a continuous one. Gene 33 may activate additional pathways that have yet to be identified. Given that the SAPK pathway has been implicated in hypertrophy (4, 6), it is plausible to speculate that sustained Gene 33-dependent SAPK pathway activation, arising as a consequence of hypertension or diabetes, may trigger the cell to initiate hypertrophy.

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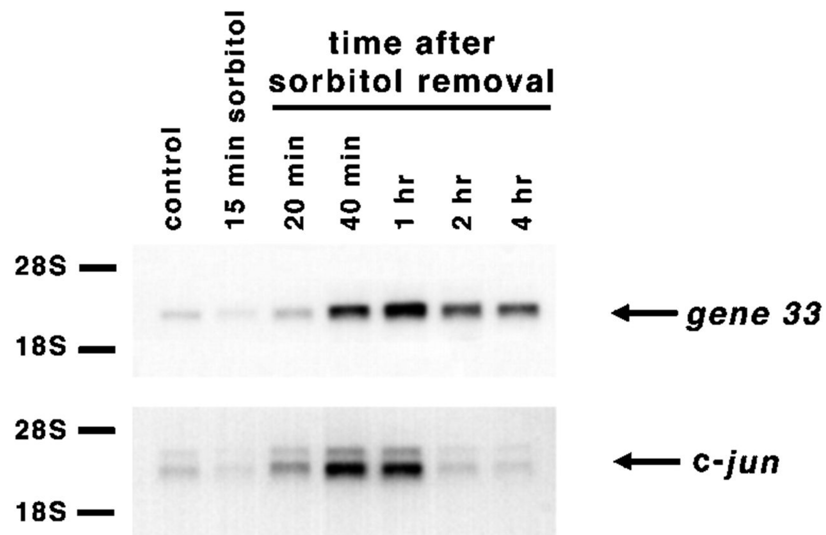

Fig. 1. Transcriptional induction of *gene 33* in response to osmotic stress

Cultured (passage 3) rat renal mesangial cells were treated with 500 mM sorbitol for 15 min, at which time the medium was returned to iso-osmotic conditions. Samples were removed after the sorbitol treatment or for various times after restoration of normal conditions and assayed for *gene 33* expression by Northern blot.

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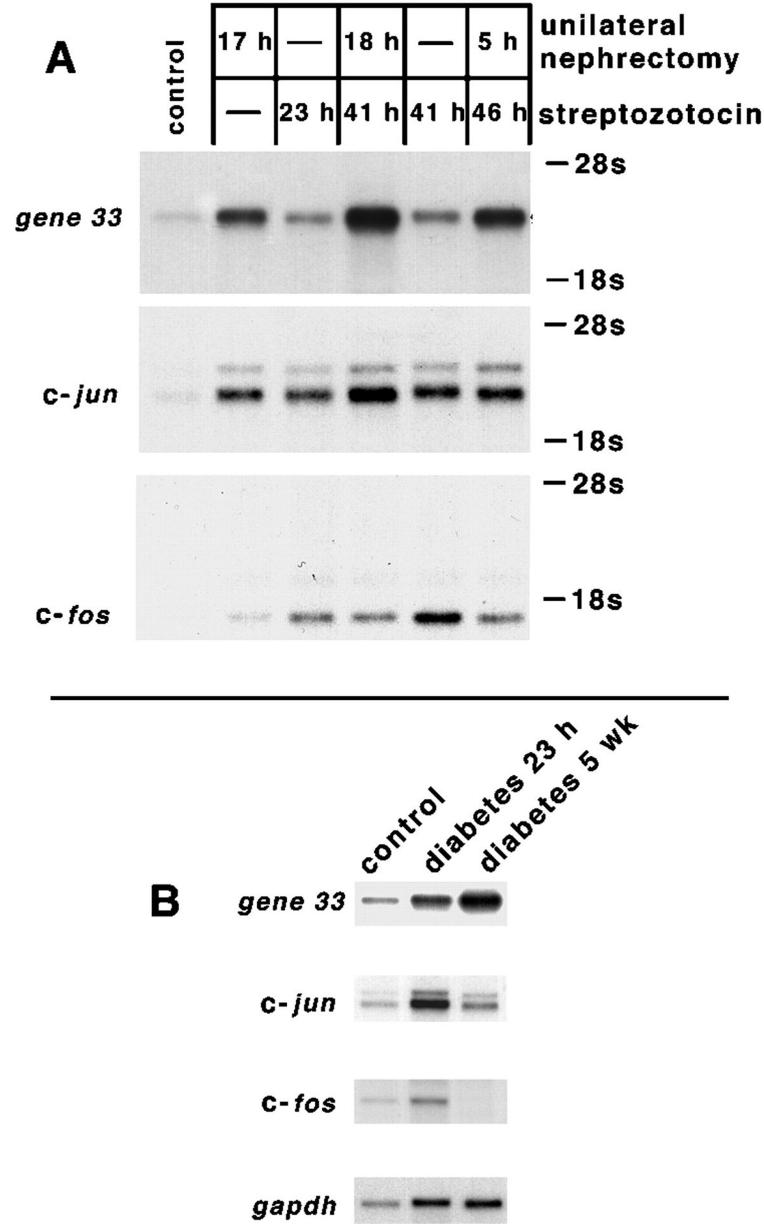

Fig. 2. Transcriptional induction of *gene 33* in response to unilateral nephrectomy and streptozotocin-induced diabetes: *gene 33* expression as a marker for diabetic nephropathy

Male rats were made diabetic upon injection of streptozotocin as described under "Experimental Procedures." Control animals were injected with water. *A*, transcription pattern in early diabetes. At the times indicated after streptozotocin injection, one kidney was removed from control or diabetic animals, and RNA was prepared and analyzed for *gene 33*, c-*jun*, and c-*fos* expression by Northern blot. The remaining contralateral kidney was removed later, at the indicated time, to assess the effects of unilateral nephrectomy on *gene 33* expression in an identical manner. The *numbers* indicate the hours elapsed between the treatment (nephrectomy, streptozotocin, or both) and harvest of the kidney. *B*, same as *A*, except that the animals were allowed to proceed to frank diabetic nephropathy (5 weeks post-streptozotocin injection). In these assays, unilateral nephrectomy was not performed,

and only the effects of diabetes on gene expression were tested. The blots were probed for glyceraldehyde-3-phosphate dehydrogenase (*gapdh*) expression as a loading control.

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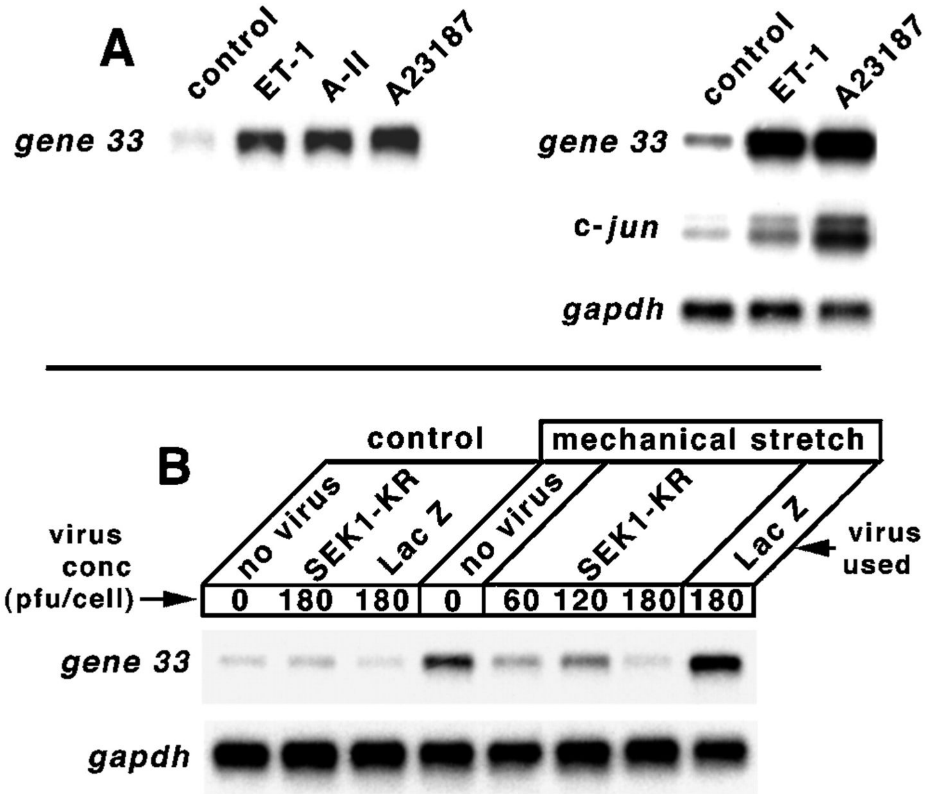

Fig. 3. Transcriptional induction of *gene 33* in response to vasoactive peptides, calcium ionophore, and mechanical strain: Induction by mechanical strain requires SAPK activity

A, rat renal mesangial cells were treated with endothelin (*ET-1*), angiotensin-II (*A-II*), or calcium ionophore (*A23187*) as indicated (see "Experimental Procedures"). *gene 33* induction was determined by Northern blot. *gapdh* expression served as a loading control. *B*, pulmonary A549 cells were infected with the indicated recombinant adenoviruses at the indicated *Pfu*/cell and, after 48 h, subjected to mechanical strain (see "Experimental Procedures"). *gene 33* induction was determined by Northern blot. *gapdh* expression served as a loading control.

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Fig. 4. Structure of the Gene 33 polypeptide

The *top diagram* indicates the modular structure of Gene 33 which is suggestive of an adapter protein, since no apparent catalytic sequences are present. The *bottom panel* compares the putative domains of Gene 33 with the cognate domains in established signaling proteins. Conserved amino acid residues are indicated with *colons; vertical lines* indicate identical residues. Inferences concerning possible functional characteristics are based on sequence homology.

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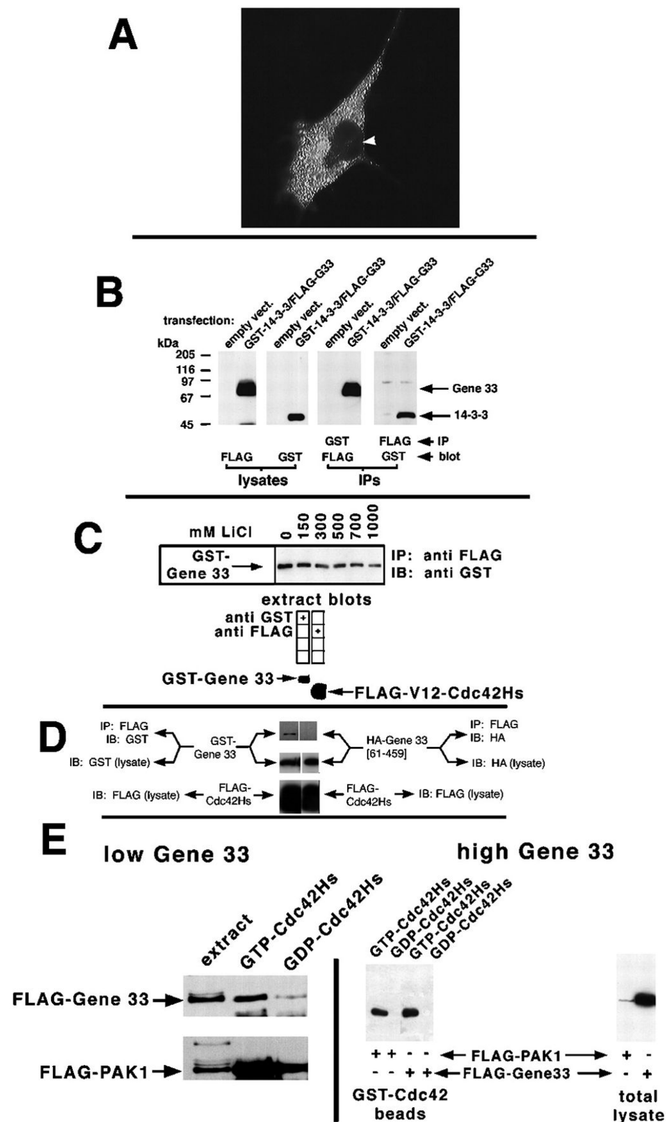

Fig. 5. Gene 33 is a cytosolic protein that interacts with 14-3-3ζ and, in a GTP-dependent manner, with Cdc42Hs

A, cytosolic localization of Gene 33. NIH3T3 cells were transfected with FLAG-Gene 33. Cells were stained with anti-FLAG and fluorescein isothiocyanate-labeled anti-mouse. Gene 33 was detected by immunofluorescence. The *arrowhead* indicates the nucleus. *B*, interaction with 14-3-3ζ. 293 cells were transfected with GST-14-3-3ζ and FLAG-Gene as indicated. GST pull-downs and anti-FLAG immunoprecipitates (*IPs*) were prepared and subjected to SDS-PAGE and reciprocal immunoblotting with anti-GST and anti-FLAG to detect coprecipitated proteins. *C*, *in vivo* binding of Gene 33 to Cdc42Hs. 293 cells were transfected with GST-Gene 33 and FLAG-V12-Cdc42Hs. Anti-FLAG-Cdc42Hs immunoprecipitates (*IP*) were prepared and subjected to washing with progressively higher concentrations of LiCl as indicated in the *top panel*, followed by SDS-PAGE and immunoblotting (*IB*) with anti-GST to detect bound Gene 33. Expression blots are shown in the *bottom panel*. *D*, the Gene 33/Cdc42Hs interaction requires the Gene 33 CRIB motif. Cells were transfected with FLAG-Cdc42Hs (V12) plus either GST-Gene 33 or HA-Gene 33 (aa 61–459). Cdc42Hs was immunoprecipitated with anti-FLAG and subjected to SDS-PAGE and immunoblotting with anti-GST or anti-HA as indicated. Crude lysates were

blotted with the indicated antibodies to judge expression of the transfected proteins. *E*, *in vitro* GTP-dependent binding of Gene 33 to Cdc42Hs. Bacterially expressed GST-Cdc42Hs was loaded with either GTP-γS or GDP-βS as indicated (GTP or GDP, respectively) and incubated with extracts of 293 cells that had been transfected with Gene 33 or PAK1 (FLAG-tagged) expressed at equal levels (*left*) or with Gene 33 in excess (*right*). Crude 293 cell extracts, indicated in the *figure*, were subjected to SDS-PAGE and blotted with anti-FLAG to detect expression of Gene 33 or PAK1. Cdc42Hs beads were washed and subjected to SDS-PAGE and immunoblotting with anti-FLAG to detect bound Gene 33 or PAK.

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Fig. 6. Selective activation of the SAPK pathway by the long form of Gene 33: Requirement for the C-terminal half of Gene 33 consisting of the 14-3-3-binding, PDZ-binding, and AH domains (the short form of Gene 33 cannot activate the SAPKs)

A, activation of MAPK pathways by Gene 33. 293 cells were transfected with HA-SAPK, ERK1, or p38 as indicated plus vector or FLAG-Gene 33. As indicated, FLAG-V12-Cdc42Hs served as a positive control for SAPK activation, FLAG-V12-Ha-Ras was a positive control for ERK1, and untagged apoptosis signal-regulating kinase-1 was a positive control for p38. HA-MAPKs were immunoprecipitated with anti-HA and assayed with GST-c-Jun (SAPK), myelin basic protein (MBP, ERK assays), or GST-activating transcription factor-2 (p38) (*right panels*). In the *left panels*, crude lysates were probed with the cognate antibodies to determine expression of the transfected constructs. *B*, schematic diagram of Gene 33 truncation mutants. *wt*, wild type; *BD*, binding domain; *C*, activation of SAPK by

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Gene 33 deletion constructs alone or in combination with V12-Cdc42Hs. 293 cells were cotransfected with HA-SAPK and the indicated FLAG-tagged Gene 33 and/or Cdc42Hs constructs. HA-SAPK was immunoprecipitated and assayed for GST-c-Jun kinase (*top panel*). Crude lysates were probed with anti-HA to detect SAPK levels (*middle panel*) or anti-FLAG to detect Gene 33 and Cdc42Hs levels (*bottom panel*). *D*, the short form of Gene 33 cannot activate coexpressed SAPK. The *top diagram* shows the structure of the long and short forms of Gene 33 (*Gene 33 L* and *Gene 33 S*, respectively). The *bottom panel* indicates the effect of these Gene 33 constructs on the activity of coexpressed SAPK. 293 cells were cotransfected with GST-SAPK and the indicated FLAG-tagged Gene 33 and/or V12-Cdc42Hs constructs. GST-SAPK was isolated on GSH-agarose and assayed for GST-c-Jun kinase (*top panel*). Crude lysates were probed with anti-GST to detect SAPK levels (*middle panel*) or anti-FLAG to detect Gene 33 and Cdc42Hs levels (*bottom panel*). IB, *immunoblot*.

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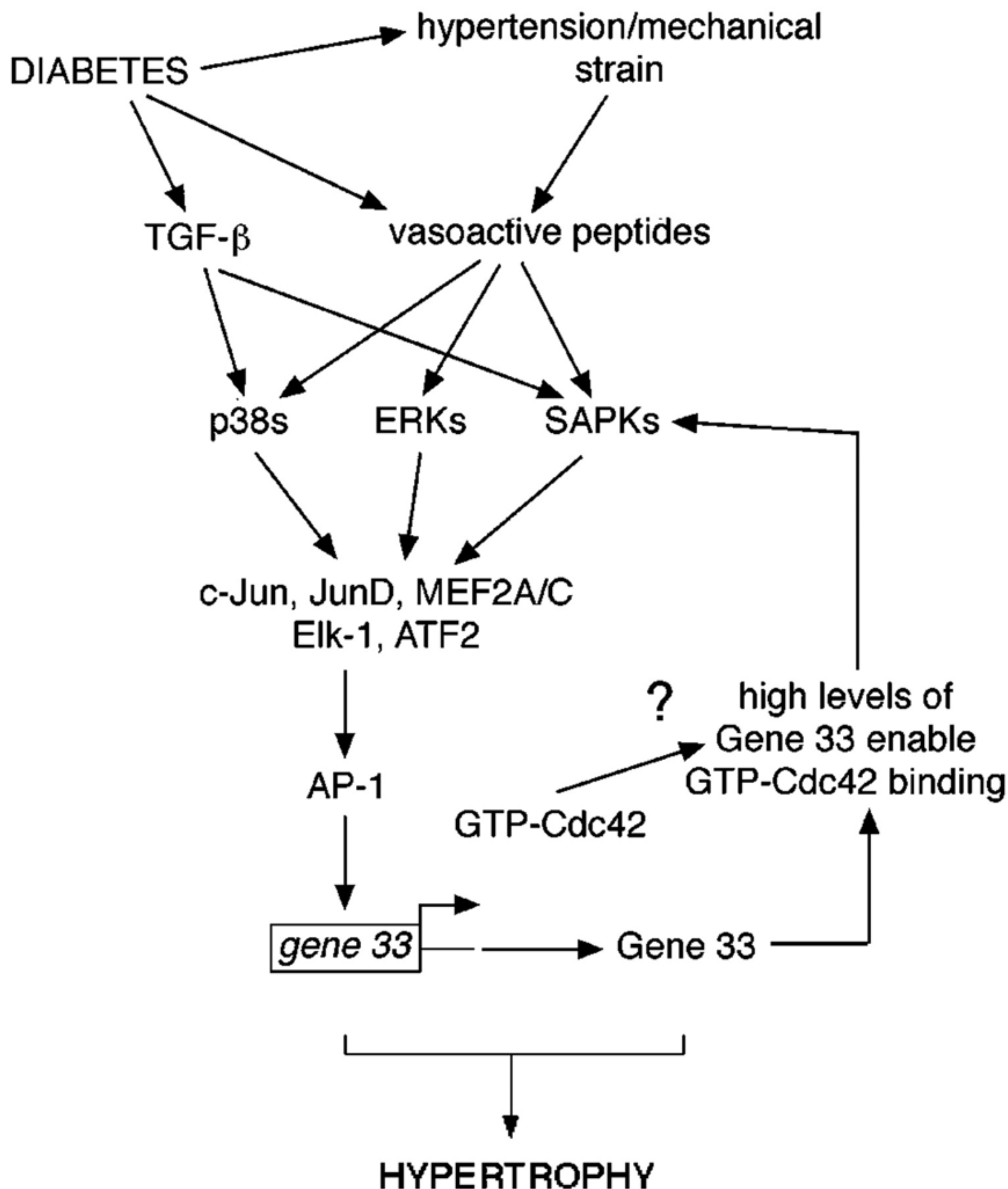

Fig. 7. Model for the role of Gene 33 in reprogramming the cell to respond to chronic stress

Stress-induced *gene 33* expression acts to maintain responses under conditions of sustained stress. The model is not intended to imply that Gene 33 represents the sole mechanism signaling hypertrophy; instead, the potential role for Gene 33 in the progression to hypertrophy is highlighted. The diagram suggests that Gene 33 might couple Cdc42 to the SAPKs under certain conditions. For this to be true, sufficient levels of Gene 33 would need to be present so as to permit appreciable binding to Cdc42. In addition, the cell would need to be treated with agonists that stimulate Cdc42 activation. Details are discussed under "Results" and "Discussion."