Journal of Cellular Biochemistry 94:1190–1198 (2005)

Modulation of Elk-Dependent-Transcription by Gene33

Adam B. Keeton and Joseph L. Messina*

Department of Pathology, Division of Molecular and Cellular Pathology,
University of Alabama at Birmingham, Birmingham, Alabama, 35294-0019

Abstract Gene33 is a cytoplasmic protein expressed in many cell types, including those of renal and hepatic origin. Its expression is regulated by a large number of mitogenic and stressful stimuli, both in cultured cells and in vivo. Gene33 protein possesses binding domains for ErbB receptors, 14-3-3 proteins, SH-3 domains, and GTP bound Cdc42, suggesting that it may play a role in signal transduction. Indeed, these regions of Gene33 have been reported to modulate signaling through the ERK, JNK, and NFκB pathways. In the present work, epitope-tagged full-length and truncation mutants, as well as wild-type Gene33, were overexpressed in 293 cells. The expression of these proteins was compared to the level of endogenous Gene33 by Western blot using a newly developed polyclonal antibody. As proxies for activity of the ERK and JNK pathways, Elk- and c-Jun-dependent transcription were measured by a luciferase reporter gene. Moderate expression levels of full-length Gene33 caused a twofold increase in Elk-dependent transcription, while at higher levels, c-Jun-dependent transcription was partially inhibited. The C-terminal half of Gene33 significantly increased both Elk- and c-Jun-dependent transcription when expressed at approximately threefold above control levels. This effect on Elk-dependent transcription was lost at higher levels of Gene33 expression. In contrast, higher levels of the C-terminal half of Gene33 caused a progressively greater effect on c-Jun-dependent transcription. These findings suggest that Gene33 may increase ERK activity, and that the C-terminal half of Gene33 may act less specifically in the absence of the N-terminal half, inducing JNK activity. J. Cell. Biochem. 94: 1190–1198, 2005.

Key words: Gene33; Elk; c-Jun; ERK; JNK; transcription

Gene33 was originally cloned from a cDNA library prepared from liver of adrenalectomized, hydrocortisone treated rats. It encodes a protein of 459 amino acids with an apparent molecular weight of 53 KDa [Lee et al., 1985]. At the time it was cloned, Gene33 showed little homology to any known proteins, and the only clues to a possible function were several putative SH3 binding domains and consensus sequences for phosphorylation by numerous protein kinases. The human homolog of Gene33 has been identified separately by three groups and called alternately Mig-6 and RALT. The human Gene33 (Mig-6) was cloned as a mitogen-inducible gene that is elevated in the G_1 phase of the cell cycle [Wick et al., 1995], and Gene33 (RALT) was identified by two groups for its ability to associate with the intracellular portion of members of the ErbB family of tyrosine kinase receptors [Fiorentino et al., 2000; Hackel et al., 2001]. Gene33 also exhibits significant homology to the non-catalytic portion of activated Cdc42 associated kinase (Ack [Manser et al., 1995]).

Gene33 is widely, but not ubiquitously expressed. It is found in the liver, kidney, stomach, skeletal muscle, lung, brain, and intestine, but not in the spleen, heart, or bone marrow [Mohn et al., 1990]. Its expression in cultured cells is regulated by a wide array of mitogenic stimuli including insulin, phorbol esters, calcium ionophores, and ligands of the epidermal growth factor receptor family [Messina et al., 1987; Weinstock et al., 1992; Hackel et al., 2001; Fiorini et al., 2002]. Gene33 is also induced by a variety of cellular stresses, including partial hepatectomy, fulminant

Grant sponsor: American Diabetes Association; Grant sponsor: National Institute of Diabetes and Digestive and Kidney Diseases; Grant numbers: DK40456, DK62071; Grant sponsor: Diabetes Trust Fund (Boshell Scholarship in Medicine).

*Correspondence to: Joseph L. Messina, PhD, Department of Pathology, Division of Molecular and Cellular Pathology, Volker Hall, G019, 1670 University Blvd., University of Alabama at Birmingham, Birmingham, AL, 35294-0019.
E-mail: messina@path.uab.edu

Received 13 September 2004; Accepted 13 October 2004
DOI 10.1002/jcb.20370

hepatic failure, isolation of hepatocytes, experimental diabetic nephropathy, mechanical strain, and hypoxia [Haber et al., 1993; Varley et al., 1999; Makkinje et al., 2000; Saarikoski et al., 2002].

Recent studies have begun to address the question of Gene33's function. In 293 cells, Gene33 binds to 14-3-3 proteins. It also binds to, and functions cooperatively with, GTP-Cdc42 to specifically increase JNK activity [Makkinje et al., 2000]. Gene33 can increase NFκB dependent transcription through binding of its CRIB domain to IκBα [Tsunoda et al., 2002]. Gene33 is also able to bind both the ErbB2 receptor and Grb2 via motifs contained in its C-terminal region, resulting in inhibition of EGF-induced ERK1/2 activation [Fiorentino et al., 2000]. These findings were extended by work in Cos-7 and Rat-1 cells demonstrating the ability of Gene33 to bind to the EGF receptor and block EGF induced ERK1/2 activation and proliferation [Hackel et al., 2001]. Gene33 is able to inhibit ERK1/2 activation by all the members of the ErbB family of tyrosine kinases (ErbB1-4) via its ErbB-2 binding region (EBR) located between amino acids 323 and 372 (Fig. 1A [Anastasi et al., 2003]). Conversely, reduction of physiological levels of Gene33 expression by microinjection of anti-Gene33 antibodies enhances EGF-induced proliferation of fibroblasts [Fiorini et al., 2002].

In the present work, we examine the effect of exogenously expressed Gene33 on Elk-dependent-, as well as c-Jun-dependent-transcription in serum-starved 293 cells. We also report that the C-terminal portion of the Gene33 molecule is capable of affecting transcriptional regulation.

MATERIALS AND METHODS

Cell Culture

293HEK cells and Hep3B cells were maintained in subconfluent cultures in complete medium: Dulbecco's modification of Eagle's medium or Eagle's minimum essential medium, respectively, supplemented with 10% fetal bovine serum and 1% penicillin/streptomycin supplement, Mediatech/Cellgro, Herndon, VA. Phorbol 12-myristate 13-acetate (PMA, 0.1 μg/ml), insulin (I, 10 nM), and retinoic acid (RA, 1 μM) were obtained from Sigma-Aldrich (St. Louis, MO) and added to cultures for 5 h.

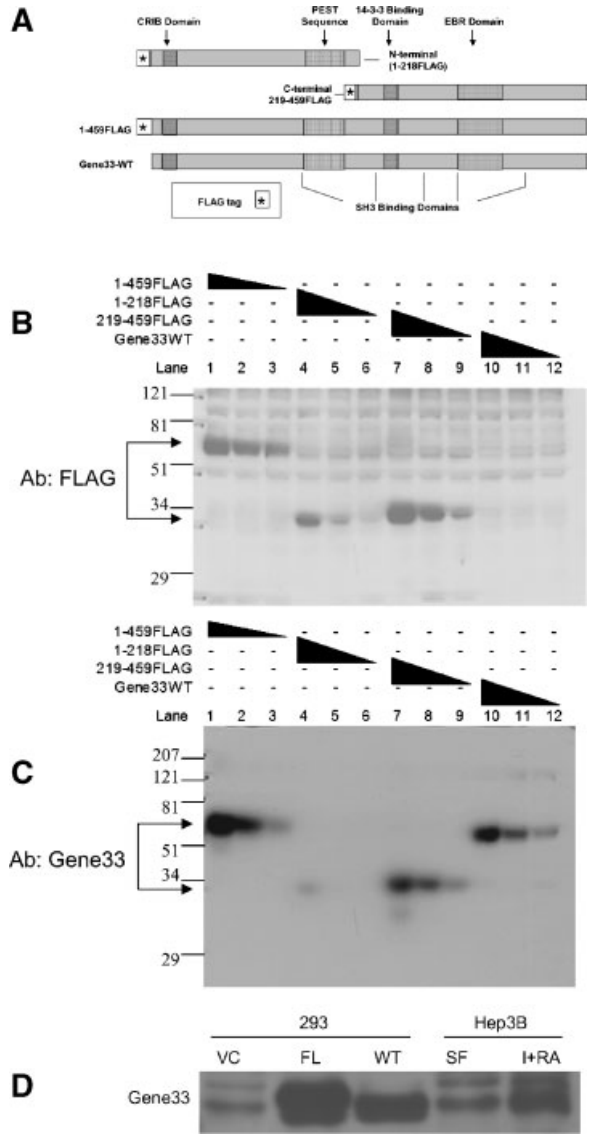

Fig. 1. Exogenous expression of Gene33. **A**: Schematic showing the motifs presents in the Gene33 constructs. Included are the Gene33 full length and deletion constructs with the location of the epitope tag were present. **B–D**: 293 cells were transfected with decreasing amounts of plasmid (40, 10, 4 ng, 1-459FLAG; 400,100, 40 ng, all others) expressing the FLAG tagged (FL) full-length (1–459), N-terminal (1–218), or C-terminal (219–459) or non-tagged (WT) Gene33 expression constructs as described in the Materials and Methods. Western blots of whole cell lysates from serum-deprived 293 transfectants were probed with anti-FLAG (B) or newly generated Gene33 (C, D) antiserum as indicated. D: Endogenous expression of Gene33 in 293 cells (VC), and Hep3B cells (basal and insulin + retinoic acid stimulated) is compared to overexpressed Gene33 in 293 cells (WT, 6.5-fold; FL, 14-fold).

Western Blot Analysis

Detergent (Igepal, Sigma-Aldrich, St. Louis, MO) soluble cell lysates were resolved by SDS–PAGE and transferred to Protran BA85

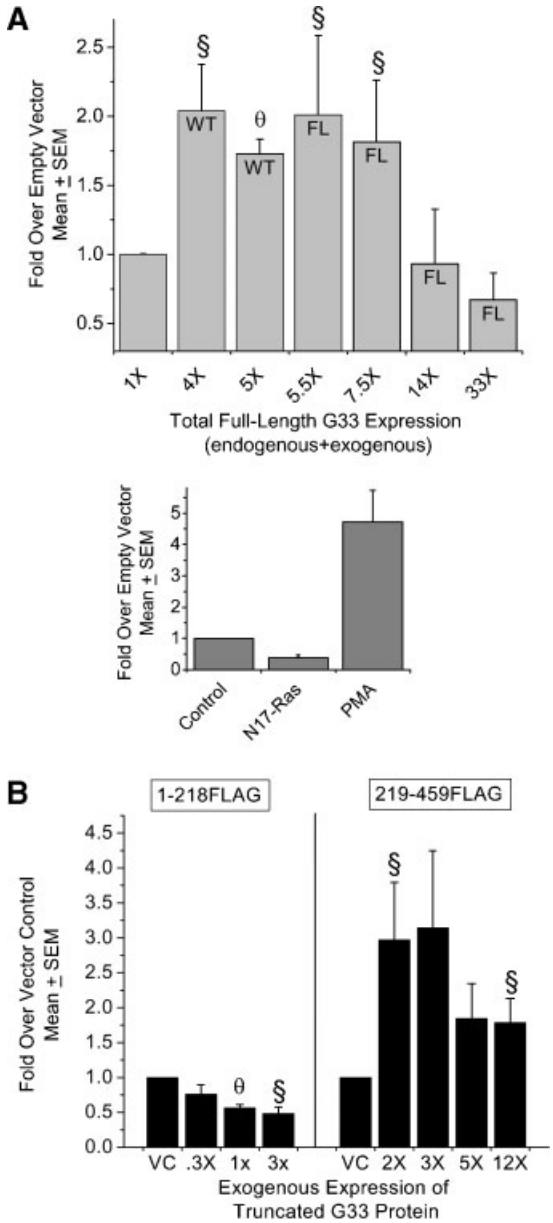

Fig. 2. Regulation of Elk-dependent-transcription by Gene33 protein expression. 293 cells were transfected with the pGal4-Elk and pGal4-Luc reporter constructs along with the (**A**) Gene33WT, 1-459FLAG (WT or FL, respectively), or N17-Ras expression constructs or (**B**) the 1-218FLAG or 219-459FLAG constructs. The relative expression level of Gene33 protein (derived from Western blot experiments) is indicated on the x-axis. In (A), x-axis value 1 indicates no exogenous expression of Gene33. PMA indicates treatment of VC cells with 0.1 μg/ml PMA for 5 h. In (B), vector control (VC) indicates no expression of truncated proteins. Mean changes in luciferase activity compared to empty vector controls (y-axis) from three or more separate experiments is presented along with SEM. $^{\S}P < 0.05$ versus vector control; $^{\theta}P < 0.001$ versus vector control (amount of total plasmid held constant by varying amounts of empty vector). In a typical experiment, values between $3–6 \times 10^5$ (A) and $5–22 \times 10^5$ (B) RLU/μg were obtained.

membranes (Schleicher & Schuell, Keene, NH), developed with ECL Plus (Amersham Biosciences, Buckinghamshire, England), and visualized by autoradiography [Reks et al., 1998; Ji et al., 1999; Keeton et al., 2002]. FLAG antiserum was obtained from Sigma-Aldrich, as were luciferin and ATP (St. Louis, MO). Polyclonal Gene33 antiserum was prepared as previously described [Keeton et al., 2004]. Briefly, a polyhistidine-Gene33 fusion protein was purified by metal affinity chromatography and used as an antigen for the production of polyclonal rabbit antiserum by Alpha Diagnostics, Inc. (Houston, TX).

Statistical Analysis

ANOVA and Student's *t*-test were performed using the Instat version 3.0 software package (Graphpad, Inc., San Diego, CA). Fold-changes in luciferase activity were compared to vector transfected control cultures.

Transient Transfections

The cationic lipid reagent Lipofectamine (Invitrogen/Life Technologies, Carlsbad, CA) was used according to the manufacturer's supplied protocol. Briefly, 24 h after cell seeding in 12-well plates, cells were transferred to serum-free medium. Plasmid DNA was used at the same total concentration (600 ng total plasmid combined) for all treatments by varying the ratio of empty vector to expression construct. Plasmid DNA complexed with the Lipofectamine reagent was incubated with cells for 5 h at 37°C in a 5% CO_2 incubator, then removed by media replacement. Serum was removed from cultures 48 h after transfection. Experiments were performed on transfected cells, 24 h after serum withdrawal. Gene33WT was expressed from the pCDNA3.1 vector using concentrations between 0 and 400 ng (Invitrogen Life Technologies, Carlsbad, CA). The V12-Cdc42, N17-Ras, and Gene33-FLAG tagged constructs (1-218FLAG, 219-459FLAG, and 1-459FLAG) were expressed from the pCMV-5 vector using concentrations between 0 and 300 ng (graciously provided by Dr. A. Makkinje). The Gal4-Elk, Gal4-c-Jun, and Gal4-luciferase plasmids were used at concentrations of 15, 15, and 150 ng, respectively (generously provided by Dr. A. Lin).

Luciferase Activity Assay

Briefly, cells were lysed in non-ionic detergent 24–48 h following transfection, and the

soluble supernatants were collected for assay in a luminometer (Autolumat LB953, Berthold, Bundoora, VIC, Australia). Relative light unit (RLU) measurements were normalized to the amount of protein and expressed as RLU per microgram of protein [Brasier et al., 1989]. Comparisons to equivalent empty vector controls are presented as fold-change in luciferase activity.

RESULTS

The schematic in Figure 1A represents the Gene33 overexpression constructs used in transfection experiments in the present work. As indicated, FLAG epitopes were fused to full-length, as well as N-terminal (1-218FLAG) and C-terminal (219-459FLAG) Gene33 truncation mutants. Additionally, a Gene33WT construct was used with no exogenous sequence added. Also shown are the relative locations of sequence motifs in Gene33 that have been reported to be necessary in Gene33 function, such as the Cdc42 Rac Interactive Binding (CRIB) domain [Makkinje et al., 2000; Tsunoda et al., 2002], ErbB-2 Binding Region (EBR [Anastasi et al., 2003]), internal PEST sequence [Fiorini et al., 2002], 14-3-3 binding domain [Makkinje et al., 2000], and SH3 binding sequences [Fiorentino et al., 2000].

Transient transfection of 293 cells with varying ratios of empty vector and Gene33 expression constructs (to maintain equal amounts of total plasmid DNA) were performed to produce defined changes in cellular Gene33 protein levels. Expression of each of the fusion proteins was determined by Western blotting with FLAG antiserum. Due to the different levels of expression of Gene33 protein from the various constructs, the amount of plasmid used for each was varied (Fig. 1B). The 1-459FLAG protein was expressed at high steady state levels, even from modest amounts of plasmid (4–40 ng). In contrast, a tenfold higher concentration of the Gene33WT and 219-459 FLAG plasmid (40–400 ng) was required to achieve similar levels of the expressed protein. Even the highest level of 1-218FLAG plasmid used (400 ng) gave only modest expression of the 1-218FLAG protein.

We have recently developed a polyclonal Gene33 antiserum, which is useful in studying endogenous Gene33 protein, as well as the level of exogenously expressed Gene33. Using this Gene33 antiserum, we examined levels of the exogenously expressed Gene33WT by Western blot (Fig. 1C). This protein was expressed at levels similar to the 219-459FLAG protein, and appeared significantly more abundant than the 1-218FLAG protein. The Gene33 specific antiserum also readily detected expression of the 1-459FLAG full-length protein. However, as observed with the FLAG antiserum, the level of 1-218FLAG protein was noticeably lower as measured with the Gene33 antiserum. These experiments allowed us to compare relative expression levels of transfected Gene33 proteins directly, and quantitation of Western blots have been used to estimate levels of Gene33 in subsequent experiments. For reference, basal and induced expression of endogenous Gene33 in a cell line of hepatic origin (Hep3B) was also compared to the levels of endogenous and overexpressed WT (6.5-fold) and FLAG-tagged (14-fold) Gene33 in 293 cells (Fig. 1D).

Recent reports have described a role for the human homolog of Gene33 (RALT/Mig-6) in downregulating the activation of MEK-ERK phosphorylation and MEK-ERK dependent processes. In the present work, we sought to understand the role of Gene33 in MAPK dependent transcription in the absence of external stimuli such as EGF. To eliminate the contribution of non-transfected cells, we have used a co-transfection system taking advantage of an exogenous promoter (yeast Gal4), which is not bound/activated by mammalian transcription factors. This Gal4-luciferase (Gal4-Luc) plasmid encodes the Gal4 promoter, which drives expression of the firefly luciferase reporter only when bound by ectopically expressed fusions of the Gal4 transcription factor. A second plasmid was co-transfected that encodes a fusion protein composed of the activation domain of mammalian ternary complex factor, Elk, and the DNA binding domain of the yeast transcription factor Gal4 (Gal4-Elk) [Sadowski and Ptashne, 1989; Marais et al., 1993]. This fusion protein binds only to the exogenous promoter of the Gal4 sequence encoded on the Gal4-Luc plasmid, which drives expression of the firefly luciferase gene [Lin et al., 1995]. Thus, luciferase activity is correlated with (MEK-ERK associated) Elk-dependent transcription in transfected cells only. In the luciferase assay, luciferase activity of untransfected cell extracts was not different ($<$10 RLU/μg) when compared to buffer added alone in the absence of cell extract. As an additional control, luciferase activity in cells

transfected with only the Gal4-Luc vector was less than 1% of co-transfection of Gal4-Luc and Gal4-Elk, confirming the requirement for the fusion protein transcription factor in this assay (data not shown). In these experiments, increasing amounts of Gene33 plasmid were transfected to yield overexpression of Gene33. These experiments were performed with either the non-tagged or FLAG-tagged Gene33 expression constructs. Levels of Gene33 from approximately 4- to 7.5-fold above control resulted in a 2-fold increase in basal Elk-dependent transcription (Fig. 2A). For comparison, the potent inducer of the MEK-ERK pathway, PMA, induced Elk-dependent transcription 4.7-fold (Fig. 2A lower panel). In contrast, inhibition of signaling via the MEK-ERK pathway by co-transfection with the inactive N17-Ras mutant resulted in a 62% inhibition of basal Elk-dependent transcription (0.38-fold). Further increases in Gene33 expression (greater than 14-fold above control) resulted in a return to a level equal to or even below the basal level of Elk-dependent transcription. Thus, there was a biphasic effect of Gene33 expression on basal Elk-dependent transcription.

In an attempt to identify the region(s) of Gene33 responsible for alterations in Elk-dependent transcription, co-transfections with the N-terminal and C-terminal truncation mutants were performed. Due to the low expression levels (or lability) of the 1-218FLAG protein, a maximum of threefold expression above the endogenous level of full-length Gene33 (vector control) was all that could be obtained, and this resulted in a significant decrease in Elk-dependent transcription as did expression of this truncated Gene33 at a level equivalent to the endogenous full-length Gene33 (onefold; Fig. 2B). A much higher level of 219-459FLAG was obtained, reaching maximum levels of 12-fold above the endogenous level of full-length Gene33. Moderate levels of 219-459FLAG overexpression (two- or threefold) resulted in substantial (4.1- and 4.6-fold, respectively) increases in Elk-dependent transcription, which decreased towards the basal level at higher levels of 219-459FLAG expression (greater or equal to fivefold).

It has also been reported that Gene33 can increase the activity of the related MAP Kinase, JNK in 293 cells [Makkinje et al., 2000]. Thus, we asked whether Gene33 could modulate JNK-dependent transcription. As with the studies of Elk-dependent transcription, effects of transfected Gene33 in the absence of external stimuli were examined. A similar reporter co-transfection system was used in these studies, in which luciferase expression was driven by a fusion protein consisting of the activation domain of the mammalian c-Jun transcription factor linked to the DNA binding domain of the GAL4 transcription factor [Lin et al., 1995]. As an additional control, luciferase activity in cells transfected with only the Gal4-Luc vector was less than 2% of co-transfection of Gal4-Luc and Gal4-c-Jun, again confirming the requirement for the fusion protein transcription factor in this assay (data not shown). The present work also indicates that there is no significant increase of basal c-Jun-dependent transcription following Gene33 co-transfection or PMA treatment (Fig. 3A). Instead, at higher levels of Gene33 expression, 7.5- and 14-fold above control, significant reductions in c-Jun dependent transcription (27% and 45%, respectively) were observed. At 33-fold increased Gene33 expression, there was a reduction of 20%. Although this was similar to the reductions at 7.5- and 14-fold Gene33 expression, due to experimental variability, it did not reach statistical significance. Unlike the findings where PMA induced Elk-dependent transcription (Fig. 2A), c-Jun dependent transcription was not induced by PMA (Fig. 3A, lower panel). Co-transfection with the constitutively active form of Cdc42 (V12-Cdc42) resulted in a modest stimulation of c-Jun dependent transcription.

In light of the lack of stimulatory effect of full-length Gene33 on c-Jun dependent transcription, our attempt to identify a portion of Gene33 responsible for alterations in c-Jun-dependent transcription yielded somewhat unexpected results. The modest overexpression of 1-218FLAG that was achievable caused a modest decrease in c-Jun-dependent transcription. However, expression of 219-459FLAG at levels from 2-fold to the maximum 12-fold above control level yielded significant increases in c-Jun-dependent transcription of 2.1- to 3.5-fold, respectively, above empty vector controls (Fig. 3B).

DISCUSSION

In the present work, we have demonstrated alterations in steady state levels of transfected, exogenously expressed Gene33. A specific polyclonal antiserum was used to compare the

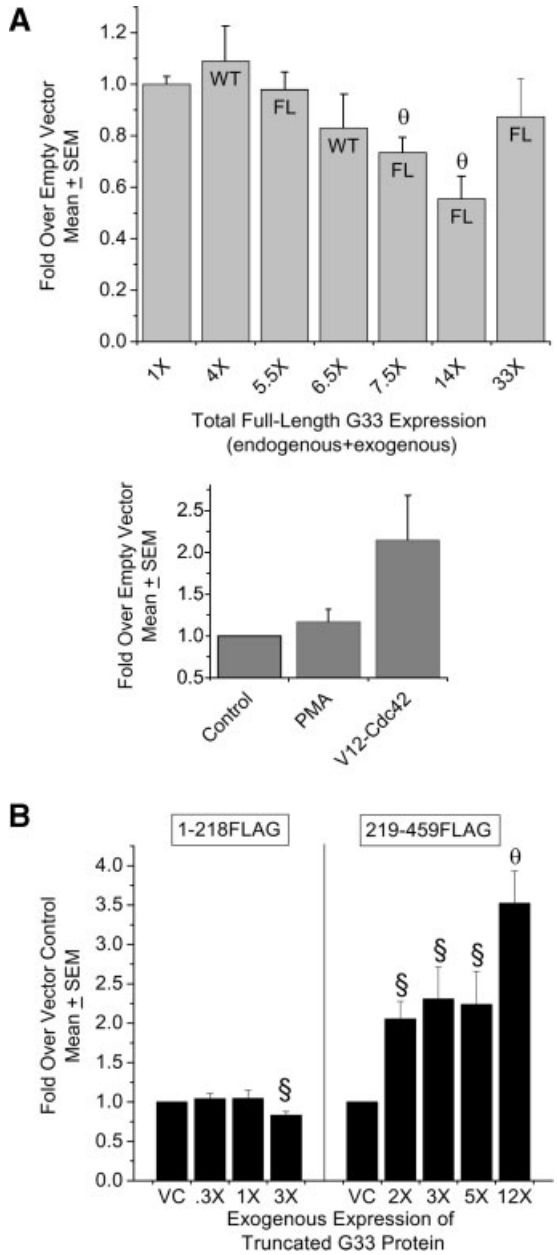

Fig. 3. Regulation of c-Jun-dependent-transcription by Gene33 protein expression. 293 cells were transfected with the pGal4-c-Jun and pGal4-Luc reporter constructs along with the (**A**) Gene33WT, 1-459FLAG (WT or FL, respectively), or V12-Cdc42 expression constructs or (**B**) the 1-218FLAG or 219-459FLAG constructs. The relative expression level of Gene33 protein (derived from Western blot experiments) is indicated on the x-axis. In (A), x-axis value 1 indicates no exogenous expression of Gene33. PMA indicates treatment of VC cells with 0.1 μg/ml PMA for 5 h. In (B), vector control (VC) indicates no expression of truncated proteins. Mean changes in luciferase activity compared to empty vector controls (y-axis) from three or more separate experiments is presented along with SEM. $^{\S}P < 0.05$ versus vector control; $^{\theta}P < 0.001$ versus vector control (amount of total plasmid held constant by varying amounts of empty vector). In a typical experiment, values between $2–5 \times 10^5$ (A) and $4–15 \times 10^5$ (B) RLU/μg were obtained.

levels of FLAG-tagged as well as non-tagged, Gene33WT proteins to the endogenously expressed Gene33 protein. Further, comparisons of the expression levels of FLAG-tagged truncation mutant constructs were made using anti-FLAG serum.

The levels of exogenously expressed Gene33 protein observed in the present work are well within the range of endogenous expression levels of this gene. The most pronounced effects were observed when the full-length Gene33 protein was expressed at levels 15-fold or less compared to basal levels. In previous studies, we have described changes in Gene33 mRNA of roughly 10–15-fold in response to calcium ionophores, glucocorticoids, insulin, or retinoic acid treatment of rat hepatoma cells, and combinations of inducers can result in over a 50-fold induction of Gene33 mRNA [Messina, 1989; Weinstock et al., 1992; Kent et al., 1994].

The reporter assay system used in the present studies allows for detection of subtle changes in MAPK pathway activity, resulting in expression of the luciferase enzyme. In support of this, Elk-dependent transcription was strongly activated by PMA, and inhibited by N17-Ras, corresponding with their well-established effects on ERK activation [Li et al., 1998]. Recent reports indicate that the human Gene33 homolog interacts with members of the ErbB family, interfering with their capacity to activate the MEK-ERK pathway in a ligand-dependent fashion [Hackel et al., 2001; Fiorini et al., 2002]. Our novel finding that increased Gene33 expression resulted in increased basal Elk-dependent transcription, in the absence of ligand stimulation, therefore differs from previous works. This indicates that increased levels of Gene33 result in an elevated basal (unstimulated) level of activity of the MEK-ERK pathway.

Previous studies in our laboratory have demonstrated that in rat hepatoma cells, an initial activation of the MEK-ERK pathway can prime this pathway and enhance the effect of a secondary cellular stimulation [Keeton et al., 2002, 2003]. Since treatments, such as insulin, glucocorticoids, or retinoic acid, can increase endogenous Gene33 expression, this may suggest that Gene33 plays a role in enhancing MEK-ERK dependent effects of some stimuli. In contrast, Gene33 limits MEK-ERK activation by ligands of the ErbB family of receptors in fibroblasts and human breast cancer cells

[Hackel et al., 2001]. Future studies will need to determine if Gene33 can affect the specificity of MEK-ERK activation by different stimuli or can alter MEK-ERK activation in a cell/tissue specific manner.

Our results indicate that Gene33's capacity to induce Elk-dependent transcription resides in the C-terminal domain, since the 219-459FLAG construct also induced Elk-dependent transcription. In contrast, expression of the N-terminal 1-218FLAG construct caused a slight reduction in Elk-dependent transcription. The C-terminal portion of Gene33 encodes binding domains for SH3 domain containing proteins and the 14-3-3 protein [Fiorentino et al., 2000; Makkinje et al., 2000]. We suspect that the presence of these motifs may account for Gene33's activation of the MEK-ERK pathway. Specifically, it has been reported that 14-3-3 proteins interact with Raf-1, an upstream activator of the MEK-ERK pathway, stabilizing an inactive (phosphorylated) form of the protein. Disruption of this Raf-1:14-3-3 interaction exposes an inhibitory domain to dephosphorylation, in turn allowing full activation of the kinase [Dhillon et al., 2002]. Thus, at moderate levels, Gene33 may compete with Raf-1 to bind 14-3-3 proteins, allowing a greater proportion of Raf-1 to be active, in turn increasing basal MEK-ERK activity and Elk-dependent transcription. However, when expression of either full-length or the C-terminal portion of Gene33 is very high, the presence of an excess of SH3-binding domains may interfere with productive interactions of SH3 domain containing proteins in the MEK-ERK cascade [Cohen et al., 1995]. Due to the lack of availability of antisera, there has been little data published on the expression level of the Gene33 protein. In certain circumstances (with multiple inducers of the gene), Gene33 mRNA can be increased greater than 30-fold. Significantly, the more normal induction is 5- to 15-fold by a single hormone or growth factor, in the range of changes in protein being discussed in the present experiments [Messina, 1994].

Makkinje et al. [2000] observed a significant increase in activation of exogenously expressed JNK when Gene33 was co-expressed. Our data seem to contradict these findings, in that overexpression of Gene33 at modest or even very high physiologic levels did not increase c-Jun-dependent transcription. In fact, in the present work, intermediate Gene33 levels significantly inhibit transcriptional output from this pathway. This discrepancy may be due to the fact that in the present work, levels of JNK were not altered by exogenous expression, and therefore suggest that relative levels of Gene33 may have a different effect on endogenous JNK.

This finding is consistent with the observation that activities of the MEK-ERK and JNK pathways may be reciprocally regulated by a given stimulus [Robinson and Cobb, 1997]. For example, withdrawal of trophic factors or expression of SHP-2 causes inhibition of the ERK pathway and enhanced activity of the JNK and p38 pathways [Xia et al., 1995; Shi et al., 1998]. Thus, our findings that expression of Gene33 resulted in different effects on processes downstream of ERK and JNK are consistent with the hypothesis that alterations in the balance of activity of the MAPK pathways determine cellular responses to stimuli [Robinson and Cobb, 1997; Keeton et al., 2002].

The CRIB and SH3 binding domains present in Gene33's N-terminal domain imply that it could activate signaling through the JNK pathway. The CRIB domain containing N-terminal portion of Gene33 then might function as a dominant negative protein, reducing activation of this pathway, because Gene33 has been previously demonstrated to bind small GTPases [Makkinje et al., 2000]. However, our finding that the N-terminal portion of Gene33 only modestly altered c-Jun-dependent transcription was consistent with a previous report in which the N-terminal portion of Gene33 did not activate JNK [Makkinje et al., 2000]. Thus, the role, if any, of the CRIB domain containing N-terminal portion of Gene33 in the JNK pathway is not likely to be of great importance. In contrast to the lack of stimulatory effects of the full length or 1-218FLAG constructs, the C-terminal 219-459FLAG protein induced significant increases in c-Jun-dependent transcription. This finding extends those in previous work, which demonstrated that the C-terminal portion of Gene33 is able to increase the activity of JNK [Makkinje et al., 2000]. Thus, further studies are required to clarify if the C-terminal portion of Gene33 functions as a JNK activation domain as previously proposed and how this function is modified in the intact protein.

In summary, we have presented evidence that alterations in Gene33 expression regulate MEK-ERK pathway-directed Elk-dependent transcription. This property of Gene33 appears

to reside in its C-terminal region. Our results further indicate that no such functional (transcriptional) output is exerted by full-length Gene33 on basal JNK-associated c-Jun-dependent-transcription.

ACKNOWLEDGMENTS

This work was supported by a grant from the American Diabetes Association and by an NIH Grant (DK40456) to J.L.M. Partial support for A.B.K. was provided by the Boshell Scholarship in Medicine from the Diabetes Trust Fund. We thank Dr. Anning Lin for providing the pGal4-c-Jun, pGal4-Elk, and pGal4-Luciferase plasmid constructs. We are also grateful to Dr. Anthony Makkinje for generously providing the 1-218FLAG, 219-459FLAG, and 1-459FLAG expression plasmids. We also thank Jie Xu, William L. Bennett, LaWanda T. Holland, and Dr. Yuchen Ma for their helpful and insightful discussions and suggestions in preparation of this manuscript.

REFERENCES

Anastasi S, Florentino L, Fiorini M, Fraioli R, Sala G, Castellani L, Alema S, Alimandi M, Segatto O. 2003. Feedback inhibition by RALT controls signal output by the ErbB network. Oncogene 22:4221–4234.

Brasier AR, Tate JE, Habener JF. 1989. Optimized use of the firefly luciferase assay as a reporter gene in mammalian cell lines. Biotechniques 7:1116–1122.

Cohen GB, Ren R, Baltimore D. 1995. Modular binding domains in signal transduction proteins. Cell 80:237–248.

Dhillon AS, Meikle S, Yazici Z, Eulitz M, Kolch W. 2002. Regulation of Raf-1 activation and signalling by dephosphorylation. EMBO J 21:64–71.

Fiorentino L, Pertica C, Fiorini M, Talora C, Crescenzi M, Castellani L, Alema S, Benedetti P, Segatto O. 2000. Inhibition of ErbB-2 mitogenic and transforming activity by RALT (Gene33), a mitogen-induced signal transducer which binds to the ErbB-2 kinase domain. Mol Cell Biol 20:7735–7750.

Fiorini M, Ballaro C, Sala G, Falcone G, Alema S, Segatto O. 2002. Expression of RALT, a feedback inhibitor of ErbB receptors, is subjected to an integrated transcriptional and post-translational control. Oncogene 21:6530–6539.

Haber BA, Mohn KL, Diamond RH, Taub R. 1993. Induction patterns of 70 genes during nine days after hepatectomy define the temporal course of liver regeneration. J Clin Invest 91:1319–1326.

Hackel PO, Gishizky M, Ullrich A. 2001. Mig-6 is a negative regulator of the epidermal growth factor receptor signal. Biol Chem 382:1649–1662.

Ji S, Guan R, Frank SJ, Messina JL. 1999. Insulin inhibits growth hormone signaling via the growth hormone receptor/JAK2/STAT5B pathway. J Biol Chem 274: 13434–13442.

Keeton AB, Amsler MO, Venable DY, Messina JL. 2002. Insulin signal transduction pathways and insulin-induced gene expression. J Biol Chem 277:48565–48573.

Keeton AB, Bortoff KD, Bennett WL, Franklin JL, Venable DY, Messina JL. 2003. Insulin-regulated expression of Egr-1 and Krox20: Dependence on ERK1/2 and interaction with p38 and PI3-kinase pathways. Endocrinology 144:5402–5410.

Keeton AB, Xu J, Franklin JL, Messina JL. 2004. Regulation of Gene33 expression by insulin requires MEK-ERK activation. Biochim Biophys Acta 1679:248–255.

Kent TA, Messina JL, Weinstock RS, Stein JP. 1994. Synergistic induction of gene 33 expression by retinoic acid and insulin. Endocrinology 134:2237–2244.

Lee KL, Isham KR, Stringfellow L, Rothrock R, Kenney FT. 1985. Molecular cloning of cDNAs cognate to genes sensitive to hormonal control in rat liver. J Biol Chem 260:16433–16438.

Li X, Lee JW, Graves LM, Earp HS. 1998. Angiotensin II stimulates ERK via two pathways in epithelial cells: Protein kinase C suppresses a G-protein coupled receptor-EGF receptor transactivation pathway. EMBO J 17: 2574–2583.

Lin A, Minden A, Martinetto H, Claret F-X, Lange-Carter C, Mercurio F, Johnson GL, Karin M. 1995. Identification of a dual specificity kinase that activates the Jun kinases and p38-Mpk2. Science 268:286–290.

Makkinje A, Quinn DA, Chen A, Cadilla CL, Force T, Bonventre JV, Kyriakis JM. 2000. 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. J Biol Chem 275:17838–17847.

Manser E, Chong C, Zhao ZS, Leung T, Michael G, Hall C, Lim L. 1995. Molecular cloning of a new member of the p21-Cdc42/Rac-activated kinase (PAK) family. J Biol Chem 270:25070–25078.

Marais R, Wynne J, Treisman R. 1993. The SRF accessory protein Elk-1 contains a growth factor-regulated transcriptional activation domain. Cell 73:381–393.

Messina JL. 1989. Insulin and dexamethasone regulation of a rat hepatoma messenger ribonucleic acid: Insulin has a transcriptional and a posttranscriptional effect. Endocrinology 124:754–761.

Messina JL. 1994. Regulation of gene 33 expression by insulin. In: Draznin B, Leroith D, editors. Molecular biology of diabetes. Part II. Insulin actions, effects on gene expression and regulation and glucose transport. Totowa, NJ: The Humana Press Inc. pp 263–281.

Messina JL, Hamlin J, Larner J. 1987. Positive interaction between insulin and phorbol esters on the regulation of a specific messenger ribonucleic acid in rat hepatoma cells. Endocrinology 121:1227–1232.

Mohn KL, Laz TM, Melby AE, Taub R. 1990. Immediate-early gene expression differs between regenerating liver, insulin-stimulated H-35 cells, and mitogen-stimulated Balb/c 3T3 cells. Liver-specific induction patterns of gene 33, phosphoenolpyruvate carboxykinase, and the *Jun, Fos*, and *Egr* families. J Biol Chem 265:21914–21921.

Reks SE, Smith PH, Messina JL, Weinstock RS. 1998. Activation of PKC delta by insulin in a rat hepatoma cell line. Endocrine 8:161–167.

Robinson MJ, Cobb MH. 1997. Mitogen-activated protein kinase pathways. Curr Opin Cell Biol 9:180–186.

Saarikoski ST, Rivera SP, Hankinson O. 2002. Mitogen-inducible gene 6 (MIG-6), adipophilin and tuftelin are inducible by hypoxia. FEBS Lett 530:186–190.

Sadowski I, Ptashne M. 1989. A vector for expressing GAL4(1-147) fusions in mammalian cells. Nucleic Acids Res 17:7539.

Shi ZQ, Lu W, Feng GS. 1998. The Shp-2 tyrosine phosphatase has opposite effects in mediating the activation of extracellular signal-regulated and c-Jun NH2-terminal mitogen-activated protein kinases. J Biol Chem 273:4904–4908.

Tsunoda T, Inokuchi J, Baba I, Okumura K, Naito S, Sasazuki T, Shirasawa S. 2002. A novel mechanism of nuclear factor kappaB activation through the binding between inhibitor of nuclear factor-kappaBalpha and the processed NH(2)-terminal region of Mig-6. Cancer Res 62:5668–5671.

Varley CL, Armitage S, Dickson AJ. 1999. Activation of stress-activated protein kinases by hepatocyte isolation induces gene 33 expression. Biochem Biophys Res Comm 254:728–733.

Weinstock RS, Manning CA, Messina JL. 1992. The regulation of p33 gene expression by insulin and calcium ionophores. Endocrinology 130:616–624.

Wick M, Burger C, Funk M, Muller R. 1995. Identification of a novel mitogen-inducible gene (mig-6): Regulation during G1 progression and differentiation. Exp Cell Res 219:527–535.

Xia ZG, Dickens M, Raingeaud J, Davis RJ, Greenberg ME. 1995. Opposing effects of ERK and JNK-p38 MAP kinases on apoptosis. Science 270:1326–1331.