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THE JOURNAL OF BIOLOGICAL CHEMISTRY Vol. 279, No. 24, Issue of June 11, pp. 25898–25904, 2004
Printed in U.S.A.

TDAG51 Mediates the Effects of Insulin-like Growth Factor I (IGF-I) on Cell Survival*

Received for publication, January 20, 2004, and in revised form, March 19, 2004
Published, JBC Papers in Press, March 22, 2004, DOI 10.1074/jbc.M400661200

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Insulin-like growth factor-I (IGF-I) receptors and insulin receptors belong to the same subfamily of receptor tyrosine kinases and share a similar set of intracellular signaling pathways, despite their distinct biological actions. In the present study, we evaluated T cell death-associated gene 51 (*TDAG51*), which we previously identified by cDNA microarray analysis as a gene specifically induced by IGF-I. We characterized the signaling pathways by which IGF-I induces *TDAG51* gene expression and the functional role of TDAG51 in IGF-I signaling in NIH-3T3 (NWTb3) cells, which overexpress the human IGF-I receptor. Treatment with IGF-I increased *TDAG51* mRNA and protein levels in NWTb3 cells. This effect of IGF-I was specifically mediated by the IGF-IR, because IGF-I did not induce *TDAG51* expression in NIH-3T3 cells overexpressing a dominant-negative IGF-I receptor. Through the use of specific inhibitors of various protein kinases, we found that IGF-I induced *TDAG51* expression via the p38 MAPK pathway. The ERK, JNK, and phosphatidylinositol 3-kinase pathways were not involved in IGF-I-induced regulation of TDAG51. To assess the role of TDAG51 in IGF-I signaling, we used small interfering RNA (siRNA) expression vectors directed at two different target sites to reduce the level of TDAG51 protein. In cells expressing these siRNA vectors, TDAG51 protein levels were decreased by 75–80%. Furthermore, *TDAG51* siRNA expression abolished the ability of IGF-I to rescue cells from serum starvation-induced apoptosis. These findings suggest that TDAG51 plays an important role in the anti-apoptotic effects of IGF-I.

Insulin-like growth factor-I (IGF-I)[1] is a polypeptide hormone that is structurally homologous to insulin. The pleiotrophic effects of IGF-I and insulin on cell proliferation, cell survival, and metabolism are mediated by a complex network of intracellular signaling pathways. The biological and physiological functions of IGF-I and insulin are initiated when these ligands bind to their receptors. The structures of the IGF-I receptor (IGF-IR) and insulin receptor (IR) are similar, each consisting of two extracellular α-subunits and two transmembrane β-subunits. Although both ligands interact with each receptor, the receptors bind their own ligands with 100–1000-fold higher affinity than that of the heterologous peptides. After ligand binding, each receptor becomes autophosphorylated and the intrinsic tyrosine kinase activity of these receptors becomes activated. Various substrate proteins, including Shc, Gab-1, and the insulin receptor substrate proteins, are then phosphorylated on tyrosine residues by the activated receptors. Tyrosine-phosphorylated insulin receptor substrate and Shc molecules interact with specific downstream signaling molecules containing Src homology 2 domains, including the p85 regulatory subunit of phosphatidylinositol 3-kinase (PI3K) and Grb2, which lead to activation of the PI3K pathway and Ras/Raf/MAPK signaling pathways, respectively (1–3). Although the IGF-IR and IR signaling pathways generally overlap, IGF-I and insulin exhibit distinct physiological functions. Whereas insulin generally regulates metabolism, IGF-I controls cell growth, differentiation, and protects cells against apoptosis (4, 5). The differences between IGF-I and insulin receptor signaling that mediate these distinct biological effects remain to be elucidated.

In a previous study, we stimulated NIH-3T3 fibroblasts either with IGF-I or with insulin and then evaluated changes in gene expression patterns by cDNA microarray analysis to identify genes that are differentially regulated by IGF-I and insulin (6). We found 30 genes that were specifically responsive to IGF-I. Most of these were related to mitogenesis and differentiation. We characterized *Twist*, which is one of the genes that are specifically responsive to IGF-I, and showed that *Twist* is positively involved in the anti-apoptotic effects of IGF-I (7). Here, we have evaluated *TDAG51* (T cell death-associated gene 51), another gene that is specifically regulated by IGF-I.

Mouse *TDAG51* was originally isolated and shown to regulate the expression of Fas and T cell receptor activation-induced apoptosis in mouse T cell hybridomas (8). *TDAG51* is ubiquitously expressed in mice, and strong expression is found in brain, lung, liver, and thymus (8). Subsequently, the rat and human homologues were readily identified. The rat homologue was isolated as an immediate early gene induced by fibroblast growth factor in neuronal cells and was shown to promote cell death (9). The human homologue was shown to be down-regulated in metastatic melanoma cells, as compared with primary melanoma cells (10). The TDAG51 protein has highly repeated sequences in its carboxyl-terminal region, including proline-glutamine (PQ) repeats and proline-histidine (PH) repeats. It

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[1] The abbreviations used are: IGF-I, insulin-like growth factor-I; IGF-IR, IGF-I receptor; IR, insulin receptor; PI3K, phosphatidylinositol 3-kinase; JNK, c-Jun NH_2-terminal kinase; ERK, extracellular signal-regulated kinase; MAPK, mitogen-activated protein kinase; MEK, mitogen-activated protein kinase/extracellular signal-regulated kinase kinase; PARP, poly(ADP-ribose) polymerase; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; siRNA, small interfering RNA; FBS, fetal bovine serum; 7-AAD, 7-aminoactinomycin D.

has been shown that proteins containing PQ-rich domains may function as transcriptional activators and mediate apoptosis in various neurodegenerative diseases, such as Huntington's disease (11). Taken together, several lines of evidence suggest that TDAG51 may be associated with enhanced apoptosis.

It has been well established that IGF-I can protect cells from apoptosis under a variety of circumstances. For example, IGF-I prevents apoptosis induced by overexpression of c-*myc* in fibroblasts (12), by interleukin-3 withdrawal in interleukin-3-dependent hemopoietic cells (13, 14), by the topoisomerase I inhibitor, etoposide (15), by anti-cancer drugs (16), by irradiation with UV-B (17), and by serum deprivation in PC12 cells (18). While the anti-apoptotic effect of IGF-I has been clearly demonstrated, the molecular mechanisms by which IGF-I inhibits apoptosis induced by these various stimuli remain unknown.

In this study, we set out to determine the signaling pathways involved in IGF-I-induced expression of TDAG51 in NIH-3T3 cells and to determine the role of TDAG51 in the functions of IGF-I in these cells.

EXPERIMENTAL PROCEDURES

Materials—Human recombinant IGF-I was a gift from Genentech (South San Francisco, CA). LY294002, which is a PI3K-specific inhibitor, was purchased from Sigma. U0126, a MEK1/2-specific inhibitor and SB202190, a p38 MAPK-specific inhibitor were obtained from Calbiochem. SP600215, a JNK-specific inhibitor, was from Biomol Research Laboratories Inc. (Plymouth Meeting, MA). The stock solutions of these inhibitors were prepared in Me_2SO at a 1000-fold concentration, such that the concentration of Me_2SO was below 0.1% when the compounds were added to the culture medium. Polyclonal antibodies to TDAG51 (M-20) and to the IGF-IR β-subunit (C-20) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). A monoclonal antibody to actin was obtained from Sigma. Anti-phospho-p38 MAPK, anti-p38 MAPK, anti-phospho-Akt (Ser-473), anti-Akt, anti-ERK1/2, anti-phospho-ERK, and anti-cleaved poly(ADP-ribose) polymerase (PARP) antibodies were purchased from Cell Signaling Technology (Beverly, MA). A monoclonal antibody to the human IGF-IR (αIR-3) was obtained from Oncogene Research Products (Cambridge, MA). Mouse IgG was from Pierce. The pTRI-GAPDH-mouse antisense control template was purchased from Ambion (Austin, TX). The radionuclide [α-^{32}P]dCTP (6000 Ci/mmol) was from PerkinElmer Life Sciences.

Construction of Expression Plasmids—The p*Silencer*™ 1.0-U6 siRNA expression vector was purchased from Ambion (Austin, TX). As the inserts for expressing short hairpin RNA, two inserts were selected: 21-sense (5′-GCAGCTACAACAGCAGCAGTTCAAGAGACTGCTGCTGTTGTAGCTGCTTTTTT-3′) and 21-antisense (5′-AATTAAAAAAGCAGCTACAACAGCAGCAGTCTCTTGAACTGCTGCTGTTGTAGCTGCGGCC-3′) and 37-sense (5′-GTCTACCAGGCAGAAGCAGTTCAAGAGACTGCTTCTGCCTGGTAGACTTTTTT-3′) and 37-antisense (5′-AATTAAAAAAGTCTACCAGGCAGAAGCAGTCTCTTGAACTGCTTCTGCCTGGTAGACGGCC-3′). Each insert was annealed and subcloned with p*Silencer*, which was linearized with ApaI and EcoRI. The construct containing siRNA insert 21 or 37 was designated psi21 or psi37. We determined whether the transfected clones contained the siRNA expression vectors by PCR analysis. The forward and reverse primers were 5′-GATCTTGTGGGAGAAGCTCGGCT-3′ and 5′-ACAAAAGCTGGAGCTCCACCGC-3′, respectively. Genomic DNA was isolated from each cell line using DNeasy Tissue Kit (Qiagen, Valencia, CA).

Cell Culture and Stable Transfection—The NWTb3 cell line expresses the human IGF-IR at a level of $\sim 4 \times 10^5$ receptors per cell (19). Two cell lines expressing dominant-negative forms of the human IGF-IR (~ 3–7×10^5 receptors/cell) were also used. These include the NKA8 cell line, in which the Lys-1003 residue at the ATP-binding site was substituted with Ala (NKA8 mutant), and the NKR1 cell line, in which Lys-1003 was substituted with Arg (NKR1 mutant) (20). The IR cell line, a gift from Dr. S. Taylor (National Institues of Health, Bethesda, MD), expresses the human IR at a level of about 2×10^6 receptor/cell (21). NWTb3, NKR1, NKA8, and IR cells were cultured in Dulbecco's modified Eagle's medium supplemented with 10% heat-inactivated fetal bovine serum (FBS), 100 units/ml penicillin, 100 μg/ml streptomycin, 300 mg/ml L-glutamine, and Geneticin (0.5 g/liter, Invitrogen) in a humidified atmosphere of 95% air and 5% CO_2 at 37 °C.

For stable transfections, NWTb3 cells were grown to 70–80% confluence in complete culture medium. The cells were harvested by trypsinization and suspended in complete culture medium at 1×10^7 cells/ml. In a 0.4-cm cuvette, 0.4 ml of the cell suspension was mixed with the *TDGA51* siRNA expression vector (psi21 and psi37) or p*Silencer* (30 μg) and pcDNA3.1-hygro (+) (10 μg, Invitrogen). The samples were then electroporated in a Bio-Rad Gene Pulsar (Bio-Rad) at 950 microfarads and 0.22 kV/cm (t = 20–30 ms). After incubation at room temperature for 10 min, the electroporated cells were diluted (1:100) in complete culture medium and plated into 100-mm dishes (22). Beginning 48 h after transfection, 0.2 g/liter of hygromycin (Clontech) was added to the cultures to select for clones expressing siRNA. Two weeks later, independent colonies were picked using coloning disks (Scienceware). The resulting stable clones (siHygro1, si21-3, and si37-57) were cultured in complete culture medium with Hygromycin (0.2 g/liter).

Northern Blot Analysis—The template for the *TDAG51* cDNA probe was obtained by RT-PCR. The position of the amplified cDNA was as follows: mouse *TDAG51* (GenBank™ accession number NM_009344) 552–851. The amplified cDNA was subcloned into the pCR®II-TOPO vector (Invitrogen), and the resulting plasmid was subjected to DNA sequencing analysis to confirm the sequence. The *TDAG51* cDNA template was labeled using the Rediprime labeling kit (Amersham Biosciences).

Total RNA was isolated using TRIzol reagent (Invitrogen) according to the manufacturer's recommended instructions. Total RNA (20 μg) was resolved on 1.25% denaturing agarose gels. The integrity and amount of RNA were confirmed by visualization of ribosomal RNA. After electrophoresis, RNA was transferred to Nytran nylon membranes (Schleicher & Schüll) by capillary action overnight and immobilized by UV exposure. Blots were prehybridized for 2 h at 42 °C in a buffer containing 50% formamide, 5× Denhardt's, 1% SDS, 5× SSC, and 100 μl/ml salmon sperm. Blots were then hybridized overnight at the same temperature with 5×10^6 cpm/ml [^{32}P]dCTP-labeled DNA probe in a buffer containing 50% formamide, 2.5× Denhardt's, 1% SDS, 5× SSC, and 100 μl/ml salmon sperm. The blots were washed at high stringency and the hybridized radioactivity was measured using Fuji BAS1800II instrument (FujiFilm, Stamford, CT). *TDAG51* mRNA levels were quantified and normalized to GAPDH levels, using the Image Reader software and Image Gauge software together with a Fuji BAS1800II instrument.

Western Blot Analysis—Cell lysates were prepared in lysis buffer (10 mM Tris, pH 7.4, 150 mM NaCl, 1 mM EDTA, 1% Triton-X, 100 mM sodium fluoride, 10 mM sodium orthovanadate, 10 mM sodium pyrophosphate) to which a protease inhibitor mixture was added (Complete Mini EDTA-free, Roche Applied Science). Lysates were centrifuged at $12{,}000 \times g$ for 30 min at 4 °C to remove insoluble materials. The protein concentration in the supernatants was determined with the BCA protein assay kit (Pierce). The extracted protein samples were subjected to SDS-PAGE and transferred to nitrocellulose membranes (Invitrogen). The membranes were blocked with 5% nonfat milk in Tris-buffered saline with 0.1% Tween 20 (TBS-T) for 1 h at room temperature. The membranes were then incubated with various antibodies overnight, as indicated in the figure legends. After washing with TBS-T, the membranes were incubated with the appropriate horseradish peroxidase-conjugated secondary antibodies (Amersham Biosciences) for 1 h and washed again. Immunoreactivity was detected with an enhanced chemiluminescence kit (PerkinElmer Life Sciences) and quantified by densitometry, using Mac Bas V2.52 software (FujiFilm, Stamford, CT).

Analysis of Apoptosis by Flow Cytometry—Cells were plated on 100-mm dishes in the culture medium. After 18 h of incubation, the medium was changed to serum-free medium with or without IGF-I (50 nM), and the cells were incubated for another 48 h. For the treatment with mouse IgG or αIR3 antibody, the cells were preincubated in serum-free medium with mouse IgG or αIR-3 antibody (1.0 μg/ml) for 2 h and then FBS (10%) and IGF-I (10 nM) was added or not to the medium for another 48 h. The cells were then collected and washed twice with HEPES Buffer (10 mM HEPES/NaOH, pH 7.4, 140 mM NaCl, and 2.5 mM $CaCl_2$) at room temperature as described previously (23). Cells were resuspended in 0.2 ml of HEPES Buffer that included 3 μl of Annexin V-FITC (Pharmingen) and 5 μl of 7-aminoactinomycin D (7-AAD) (Pharmingen) and were incubated for 15 min at room temperature in the dark. Finally, the stained cells were analyzed by FACSCalibur using CellQuest Software (BD Biosciences).

Analysis of the Cleaved Caspase-3 and PARP—Cells were plated on 100-mm dishes in the culture medium. After 18 h, the medium was changed to serum-free Dulbecco's modified Eagle's medium with or without IGF-I (50 nM), and the cells were incubated for another 48 h. The cells were then collected and subjected to lysis as described above. The cleavage of caspase-3 and PARP was analyzed by subjecting cell

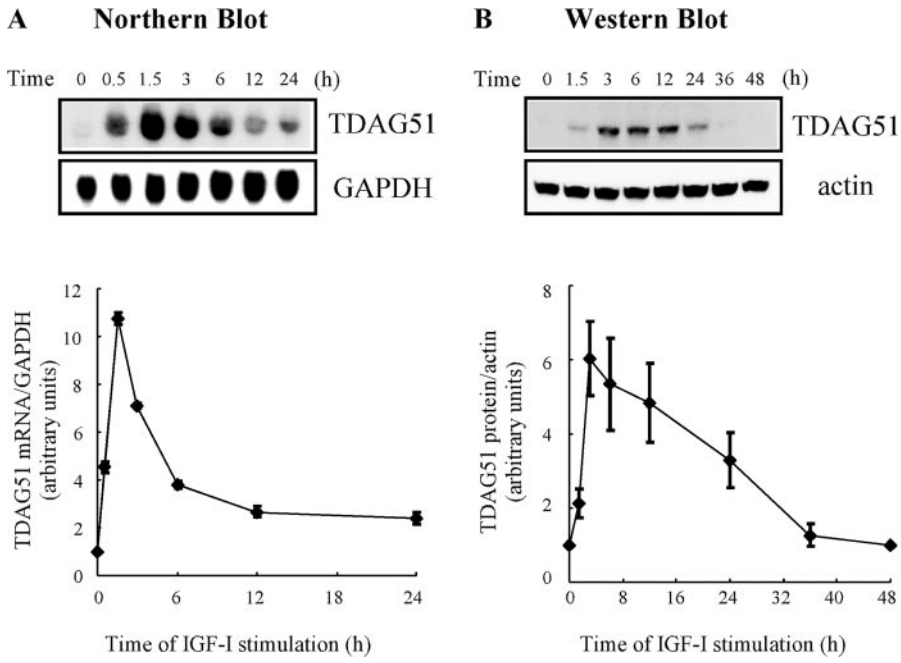

FIG. 1. **Time course of effects of IGF-I on TDAG51 mRNA and protein levels in NWTb3 cells.** NWTb3 cells (70–80% confluent) were serum-starved overnight and then incubated with IGF-I (50 nM) for the indicated times. *A*, total RNA was isolated and analyzed by Northern blot analysis to measure *TDAG51* mRNA levels (*upper panel*). After autoradiography and quantification, membranes were stripped and reprobed with a mouse *GAPDH* probe, as an internal control. Data are expressed as the mean ± S.E. for three separate experiments (*lower panel*). *B*, cells were homogenized in lysis buffer and the resulting protein extracts were subjected to SDS-PAGE and immunoblot analysis with an anti-TDAG51 antibody (*upper panel*). Immunoreactivity was detected by ECL and quantified by densitometric scanning. Membranes were then stripped and reprobed with an anti-actin antibody, as an internal control. Data are expressed as the mean ± S.E. for three separate experiments (*lower panel*).

lysates to immunoblotting with anti-cleaved caspase-3 and PARP antibodies.

Statistical Analyses—All values are expressed as the mean ± S.E. The statistical significance was determined by unpaired Student's *t* test using Statview 5.0 software (SAS Institute Inc., Cary, NC). Differences were considered to be statistically significant at $p < 0.05$.

RESULTS

TDAG51 Expression Is Induced by IGF-I in NWTb3 Cells—In a previous cDNA microarray analysis study, we showed that TDAG51 gene expression was specifically induced by IGF-I (6). To confirm this finding, we tested the effects of various durations of IGF-I treatment (50 nM) on *TDAG51* mRNA levels in NWTb3 cells. The effect of IGF-I on *TDAG51* mRNA was maximal after 1.5 h of stimulation, which increased *TDAG51* levels by 10-fold. After longer incubation times (3–24 h), *TDAG51* gene expression progressively decreased (Fig. 1*A*). We also analyzed TDAG51 protein levels and showed TDAG51 protein was increased ~6-fold by IGF-I after 3–6 h of stimulation. This high level of protein expression persisted through 12 h of IGF-I stimulation (Fig. 1*B*). TDAG51 protein level gradually decreased after 12 h of IGF-I stimulation, and the IGF-I effect was abolished after 36 h of IGF-I treatment. Thus, IGF-I strongly stimulates the expression of both *TDAG51* mRNA and protein in NWTb3 cells. On the other hand, in NIH-3T3 cells overexpressing human insulin receptors (IR cells), insulin's effect (50 nM) on *TDAG51* gene expression was only 2-fold after 1.5 h of stimulation (data not shown).

To confirm that IGF-I induced *TDAG51* expression was mediated through the IGF-IR, we used NKR1 and NKA8 cells, which overexpress dominant-negative versions of the human IGF-IR in NIH-3T3 cells. NKR1 and NKA8 cells were stimulated with IGF-I (50 nM) for 1.5 h, and *TDAG51* gene expression was determined by Northern blot analysis. Fig. 2 shows that the increase in *TDAG51* induced by IGF-I was only observed in NWTb3 cells (which overexpress the wild-type human IGF-IR) and not in either the NKR1 or NKA cell lines. These results suggest that activation of the IGF-IR is essential for IGF-I-induced TDAG51 expression.

The p38 MAPK Pathway Is Involved in IGF-I-induced TDAG51 Expression in NWTb3 Cells—To delineate the signaling pathways involved in IGF-I-induced expression of TDAG51, we used specific inhibitors for various protein ki-

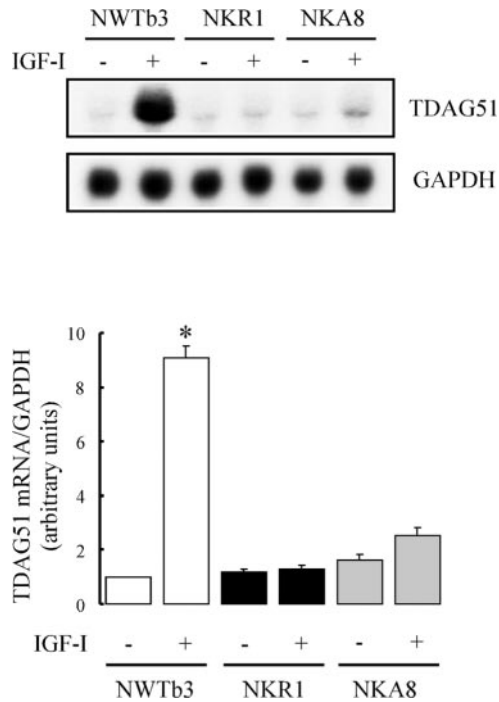

FIG. 2. **The effect of IGF-IR activation on *TDAG51* expression in mouse fibroblasts.** NWTb3, NKR1, and NKA8 cells (70–80% confluent) were serum-starved overnight and then incubated with or without IGF-I (50 nM) for 1.5 h, as indicated. Total RNA was isolated and analyzed by Northern blot analysis to measure *TDAG51* mRNA levels (*upper panel*). After autoradiography and quantification, membranes were stripped and reprobed with a mouse *GAPDH* probe, as an internal control. Data are expressed as the mean ± S.E. for three separate experiments (*lower panel*).

nases known to be activated by IGF-I, including PI3K, p38 MAPK, ERK1/2, and JNK1/2. We first evaluated the activity of these inhibitors in our system. NWTb3 cells were treated with the following inhibitors: 50 μM LY294002, a specific inhibitor of PI3K, 50 μM SB202190, a specific inhibitor of p38 MAPK, 2 μM U0126, a specific inhibitor of MEK, or 20 μM SP600125, a specific inhibitor of JNK1/2, for 1 h prior to stimulation with IGF-I (50 nM, 10 min). Western blot analysis was performed using antibodies against the phospho- and

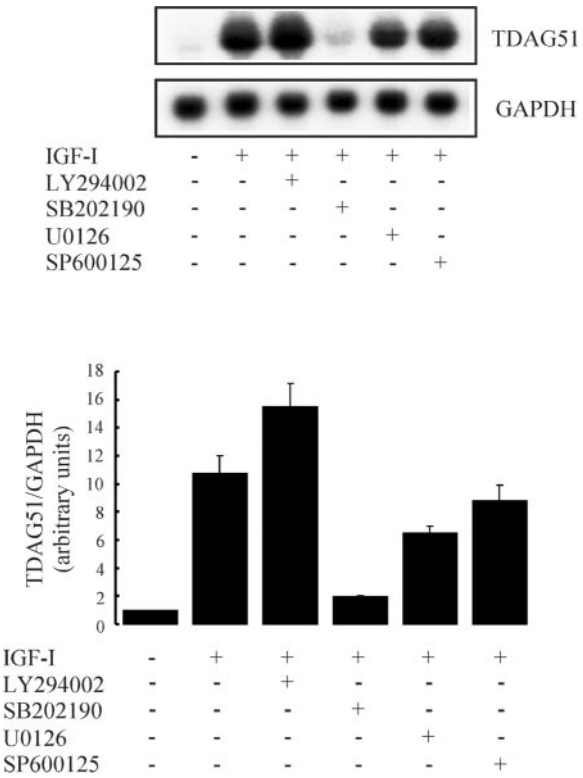

FIG. 3. **The effect of several protein kinase inhibitors on IGF-I-induced *TDAG51* expression in NWTb3 cells.** Serum-starved NWTb3 cells were preincubated for 1 h with the following inhibitors: 50 μM LY294002, 50 μM SB202190, 2 μM U0126, or 20 μM SP600125. Cells were then stimulated with or without IGF-I (50 nM) for 1.5 h, as indicated. Total RNA was isolated and analyzed by Northern blot analysis to measure *TDAG51* mRNA levels (*upper panel*). After autoradiography and quantification, membranes were stripped and reprobed with a mouse *GAPDH* probe, as an internal control. Data are expressed as the mean ± S.E. for three separate experiments (*lower panel*). *, $p < 0.05$ *versus* non-IGF-I-simulated cells in NWTb3 cells.

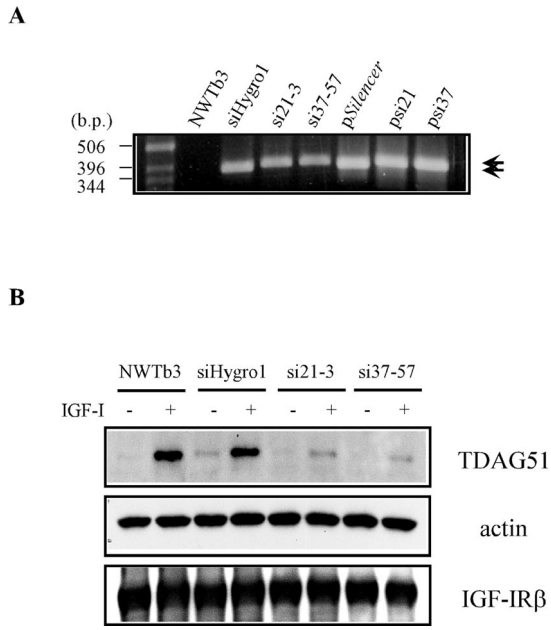

FIG. 4. **Knock down of TDAG51 expression by siRNA in NWTb3 cells.** NWTb3 cells were stably cotransfected with *TDAG51* siRNA expression vectors (psi21 or psi37), p*Silencer*, or pcDNA3.1-hygro, as described under "Experimental Procedures." *A*, PCR analysis to confirm the expression of siRNA-expressing vectors. Genomic DNA was isolated and analyzed by PCR, as described under "Experimental Procedures." psi21, psi37, and p*Silencer* served as positive controls. As a negative control, we used genomic DNA derived from NWTb3 cells. *B*, TDAG51 expression in parental NWTb3 cells and cells expressing siHydro1 or *TDAG51* siRNA. Each cell line was subjected to serum starvation and then incubated in the presence or absence of IGF-I (50 nM) for 6 h and homogenized in lysis buffer. The resulting cell lysates were subjected to SDS-PAGE and immunoblotting with an anti-TDAG51 antibody or an antibody directed against the IGF-IR β-subunit. After autoradiography and quantification, membranes were stripped and reprobed with an antibody to actin as the internal control.

total forms of Akt, p38 MAPK, ERK1/2, and JNK, as described previously (7, 24). Each inhibitor effectively prevented the IGF-I-induced phosphorylation of each of these protein kinases (data not shown). We next analyzed the effects of these inhibitors on IGF-I-induced expression of TDAG51. NWTb3 cells were pretreated with these protein kinase inhibitors for 1 h and prior to stimulation with IGF-I (50 nM for 1.5 h). Total RNA was then isolated and subjected to Northern blot analysis, as described under "Experimental Procedures." As shown in Fig. 3, pretreatment with LY294002, U0126, and SP600125 did not significantly alter the effects of IGF-I on *TDAG51* expression. However, pretreatment with SB202190 blocked IGF-I-induced *TDAG51* expression by 80%. These results suggest that the effects of IGF-I on *TDAG51* gene expression are mediated primarily by the p38 MAPK pathway.

Knock Down of TDAG51 Expression in NWTb3 Cells—To examine the role of TDAG51 in the functional effects of IGF-I, we generated clones expressing *TDAG51* siRNA to knock down TDAG51 expression in NWTb3 cells. The two different *TDAG51* siRNA expression vectors, psi21 and psi37, were transfected into NWTb3 cells as described under "Experimental Procedures". Two clones were selected and designated as si21-3 and si37-57. In addition, one of the control clones was cotransfected with p*Silencer* vector and the hygromycin-resistant plasmid (pcDNA3.1-hygro), which was designated as siHygro1. These hygromicin-resistant clones were analyzed by PCR to confirm that the siRNA expression vectors were expressed in these clones. As shown in Fig. 4*A*, the selected clones expressed the siRNA construct. We then performed Western blot analysis using an anti-TDAG51 antibody to evaluate protein expression levels in these clones. Because TDAG51 protein is expressed at very low levels in the basal state (in complete culture medium), the cells were stimulated with 50 nM IGF-I for 6 h to induce TDAG51 expression. It can be seen in Fig. 4*B* that IGF-I robustly induced TDAG51 expression in the parental NWTb3 cells and siHygro1 cells. However, TDAG51 expression was 75–80% lower in si21-3 and si37-57 cells (Fig. 4*B*), indicating that the siRNA constructs effectively reduced TDAG51 levels. Expression of si21-3 and si37-57 had no effect on actin or IGF-IR expression levels (Fig. 4*B*).

TDAG51 Regulates the Inhibitory Effects of IGF-I on Apoptosis Induced by Serum Starvation in NWTb3 Cells—Previous studies have shown that TDAG51 is associated with enhanced apoptosis in several cell lines (8–10, 25). However, in NWTb3 cells, IGF-I (which has an anti-apoptotic function) induced TDAG51 expression. To determine how TDAG51 affects IGF-I function, control cell lines (parental NWTb3 and siHygro1) and clones expressing *TDAG51* siRNA (si21-3 and si37-57) were incubated in serum-free medium in the presence or absence of 50 nM IGF-I for 48 h. The cells were then evaluated for apoptosis by Annexin V and 7-AAD staining (Fig. 5*A*). In control cell lines, the number of apoptotic cells (Annexin V-positive and 7-AAD-negative) represented less than 3% of the population in the basal state (in complete culture medium containing 10% FBS). Apoptotic cells made up about 40% of the population in cells that were subjected to serum starvation for 48 h. In the presence of IGF-I, the population of apoptotic cells was about 15% in control cell lines, reflecting a 60% reduction as com-

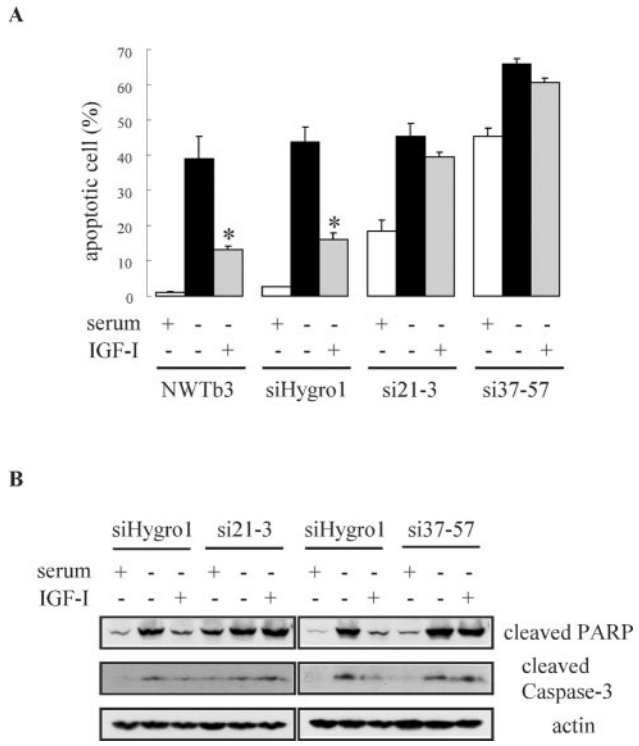

FIG. 5. **Knock down of TDAG51 expression attenuates the anti-apoptotic action of IGF-I in NWTb3 cells.** Each cell line was plated on 100-mm dishes in complete culture medium. After 18 h of incubation, medium was changed to serum-free medium with or without IGF-I (50 nM) and incubated for 48 h. *A*, analysis of flow cytometry. The cells were collected, stained with Annexin V and 7-AAD, and analyzed by flow cytometry as described under "Experimental Procedures." Values shown are the means ± S.E. for three separate experiments. *, $p < 0.05$ *versus* the serum-starved groups in each cell lines. *B*, analysis of the level of cleaved caspase-3 and PARP. The cells were collected and homogenized in lysis buffer, and then the extracted proteins were resolved by SDS-PAGE and immnoblotted with antibodies to the cleaved caspase-3 and the cleaved PARP. Immunoreactivity was detected by ECL and quantified by densitometric scanning. The membranes were then stripped and reprobed with an anti-actin antibody, as an internal control. The results shown are representative of those obtained in two independent experiments.

pared with cells subjected to serum starvation in the absence of IGF-I. On the other hand, si21-3 and si37-57 cells exhibited significantly more apoptotic cells under even basal conditions (18% in si21-3 cells and 45% in si37-57 cells), and serum starvation induced 45% of si21-3 cells and 65% of si37-57 cells to become apoptotic. Furthermore, IGF-I exhibited very little ability to rescue si21-3 and si37-57 cells from serum withdrawal-induced apoptosis, in that 40 and 60% of these cells, respectively, were apoptotic even in the presence of IGF-I. The changes in the relative percentage of necrotic cells (Annexin V-positive and 7-AAD-positive) were similar to the changes in the percentage of apoptotic cells.

Since the apoptotic cells were increased in clones expressing *TDAG51* siRNA even in the presence of serum, we further examined whether this increased apoptosis was merely because of inhibition of the effect of IGF-I in serum. siHygro1 cells and si21-3 cells were preincubated in serum-free medium with mouse IgG or αIR-3 antibody (1.0 μg/ml) to block the IGF-IR function for 2 h and then FBS (10%) and IGF-I (10 nM) were added or not to the medium for an additional 48 h. In siHygro1 cells, αIR-3 antibody blocked the IGF-I effect in preventing apoptosis induced by serum starvation but had no effect on the population of apoptotic cells in the presence of FBS (about 3% in both treatment with mouse IgG and aIR3 antibody). In si21-3 cells, αIR-3 antibody also had no effect on the apoptosis under the basal conditions (data not shown). These results demonstrate that the prevention of basal apoptosis by TDAG51 was not only contributed by IGF-I, suggesting that TDAG51 has an IGF-I-independent anti-apoptotic effect in mouse fibroblast.

We also examined caspase-3 and PARP, downstream effectors of the apoptotic pathway that may mediate the anti-apoptotic actions of IGF-I. Caspase-3 and PARP play key roles in regulating apoptotic DNA fragmentation. Caspase-3, a member of the family of aspartate-specific cysteine protease, is comprised of two subunits, 17 kDa and 12 kDa in size, which are derived from a common proenzyme. Caspase-3 plays a central role in the execution of apoptosis and is responsible for the cleavage of PARP during cell death (26, 27). Using antibodies that recognized the large fragment of activated caspase-3 (17 kDa) and the large fragment of activated PARP (89 kDa), we examined the level of cleaved caspase-3 and PARP. As shown in Fig. 5*B*, in the siHygro1 control cell line, cleaved caspase-3 and PARP levels were very low in cells cultured in FBS and increased in cells subjected to serum starvation for 48 h. IGF-I prevented the cleavage of caspase-3 and PARP by 50–60% in siHygro1 cells. On the other hand, si21-3 and si37-57 cells, even under basal conditions, exhibited levels of cleaved PARP that were 3-fold higher than those in siHygro1 cells. The cleavage of caspase-3 and PARP was further induced by serum starvation, and IGF-I had no effect on the cleavage of caspase-3 and PARP in si21-3 and si37-57 cells. These results are entirely consistent with the fluorescence-activated cell sorter analysis described above.

DISCUSSION

IGF-I and insulin share a number of common signaling pathways that are activated by their receptors. However, IGF-I and insulin have distinct biological functions. Whereas insulin is implicated in metabolic actions, IGF-I is associated with cell proliferation, cell growth, and protection against apoptosis (1–3). In a previous study, we used cDNA microarray analysis to compare the effects of IGF-I and insulin on regulation of gene expression patterns in NIH-3T3 cells overexpressing either the human IR or the human IGF-IR. We identified 30 genes that were specifically induced by IGF-I but not insulin (6). We went on to show that one of these IGF-I-induced genes, *Twist*, mediates the anti-apoptotic effects of IGF-I (7).

In the present study, we used NIH-3T3 fibroblasts overexpressing the human IGF-IR (NWTb3 cells) and studied the molecular mechanisms whereby IGF-I enhances the expression of *TDAG51*, one of the IGF-I-specific genes identified in our microarray study (6). We demonstrated that *TDAG51* mRNA and protein levels were dramatically increased after IGF-I stimulation. In addition, we confirmed that the kinase activity of IGF-IR is essential for the induction of *TDAG51* gene expression in response to IGF-I, in that this effect was absent in NIH-3T3 cells overexpressing a dominant-negative form of the IGF-IR.

To study the role of TDAG51 in mediating the actions of IGF-I, we generated *TDAG51* siRNA-expressing clones to reduce endogenous TDAG51 levels in NWTb3 cells. Through the use of Annexin-V staining and analysis of the level of caspase-3 and PARP cleavage, we measured the level of apoptosis induced by serum withdrawal. Expression of *TDAG51* siRNA dramatically reduced TDAG51 levels in NWTb3 cells (by 75–80%). Furthermore, *TDAG51* siRNA expression abolished the ability of IGF-I to prevent cells from undergoing apoptosis in response to serum starvation. Interestingly, *TDAG51* siRNA expression increased apoptosis in the presence of serum, which was independent of the IGF-I in serum, suggesting that TDAG51 might have the ability of cell survival by itself.

These findings suggest that TDAG51 is a critical regulator of the anti-apoptotic effects of IGF-I. Interestingly, previous studies have reported that TDAG51 is a pro-apoptotic molecule. In mice T cell hybridomas, TDAG51 plays an important role in regulating Fas expression and activation-induced apoptosis of T cells (8). In rat neuronal cells, the rat TDAG51 homologue was induced in response to fibroblast growth factor and promoted apoptosis without inducing Fas expression (9). Decreased expression of TDAG51 in metastatic human melanoma correlated closely with the increased resistance of these cells to apoptosis; conversely, overexpression of TDAG51 enhanced the sensitivity of these cells to apoptosis (10). Recently, it has been shown that TDAG51 is induced in response to homocystein, which causes endoplasmic reticulum stress and programmed cell death, and that overexpression of TDAG51 decreased cell adhesion and promoted detachment-mediated apoptosis in human vascular endothelial cells (25). In addition, TDAG51 has been shown to inhibit protein synthesis (28). However, the molecular mechanism by which TDAG51 mediates apoptosis remains uncertain. In addition, the *in vivo* study of TDAG51 is limited, and there is no evidence to suggest that TDAG51 enhances apoptosis *in vivo*. Rho *et al.* (29) generated *TDAG51* knock-out mice. These mice exhibited no embryonic abnormalities and the overall morphological phenotype of the adult mice was indistinguishable from that of wild-type mice, with no differences in Fas expression or T cell apoptosis (29). Since there is no obvious phenotype of *TDAG51* knock-out mice, it is possible that TDAG51 may have specific functions in apoptosis, depending on the cell type involved.

It is well known that IGF-I is a powerful inhibitor of apoptosis (4, 5). IGF-I protects cells from apoptosis induced by withdrawal of interleukin-3 (13, 14), c-*myc* overexpression (12), anti-cancer drugs (16), transforming growth factor-β1 (30), UV-B irradiation (17), osmotic stress (31), serum withdrawal (18, 32, 33), and rapamycin (34). It has also been demonstrated that overexpression of the activated IGF-IR inhibits apoptosis induced by etoposide (15), osmotic shock (35), tumor necrosis factor-α (36), p53 (37), and okadaic acid (38). The mechanism by which the IGF-IR protects cells from apoptosis induced in response to such a wide array of apoptotic stimuli has been the subject of many investigations.

The best defined pathways known to regulate the anti-apoptotic effects of IGF-I are those mediated by the PI3K/Akt pathway. Using pharmacological kinase inhibitors, it has been shown that the PI3K pathway mediates IGF-I-induced protection against apoptosis in several cell lines (17, 31–33). In addition, the expression of constitutively active Akt prevents COS-7 cells from undergoing from apoptosis, whereas expression of a kinase-dead Akt inhibits the anti-apoptotic effect of IGF-I in these cells (17). One of the downstream molecules in this IGF-I/PI3K/Akt cell survival signaling pathway is BAD, which interacts with Bcl-2 and Bcl-X_L and prevents them from performing their anti-apoptotic functions in the non-phosphorylated state (39). While BAD is well known to be directly phosphorylated and inactivated by Akt (40), the MAPK/ERK1 kinase pathway can also phosphorylate BAD (41, 42). Several studies have shown that IGF-I is capable of preventing apoptosis by activating multiple signaling pathways, especially the MAPK pathways (18, 43, 44).

We have previously demonstrated that *Twist* expression, which is involved in the cell survival-promoting effects of IGF-I, was induced by IGF-I via the ERK1/2 kinase pathway (7). In this study, we found that the ability of IGF-I to induce TDAG51 expression, which is also an important molecule for the anti-apoptotic effect of IGF-I in our cell system, is almost completely blocked by SB202190, a specific inhibitor of p38 MAPK. In contrast, LY294002 (a PI3K inhibitor), U0126 (a MEK1/2 inhibitor), and SP600215 (a JNK inhibitor) did not affect IGF-I induced *TDAG51* expression. Thus, the p38 MAPK pathway appears to be the primary pathway by which IGF-I induces *TDAG51* expression. Taken together, these findings suggest that the MAPK pathways are important for the induction of specific components involved in IGF-I-mediated cell survival signaling. Although ERK is generally considered to promote cell survival and to be involved in the protective effects of growth factors against apoptosis (45), p38 MAPK has been frequently implicated in the induction of apoptosis and inflammation in response to exposure to a variety of agents (46). Thus, p38 is designated as a "stress-kinase pathway." However, we reported in a previous study that p38 MAPK mediates the anti-apoptotic effects of IGF-I in PC-12 cells (47). The present study also suggests that p38 MAPK is involved in the IGF-I-induced cell survival signaling pathway.

In summary, our findings indicate that IGF-I induces TDAG51 expression through activation of the IGF-IR and the downstream p38 MAPK pathway in NWTb3 cells. We also demonstrate that knocking down TDAG51 expression abolished the protective effect of IGF-I against apoptosis induced by serum withdrawal in NWTb3 cells. The results show that TDAG51, like *Twist*, plays a role in the anti-apoptotic effects of IGF-I. Moreover, TDAG51 itself might have anti-apoptotic effects in NWTb3 cells. Further studies will be needed to advance our understanding of the pro- and anti-apoptotic effects of TDAG51 under various conditions and in different cell types.

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TDAG51 Mediates the Effects of Insulin-like Growth Factor I (IGF-I) on Cell Survival

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J. Biol. Chem. 2004, 279:25898-25904.
doi: 10.1074/jbc.M400661200 originally published online March 22, 2004
