Insulin-like Growth Factor 1 (IGF-1)-induced Twist Expression Is Involved in the Anti-apoptotic Effects of the IGF-1 Receptor*

Joëlle Dupont‡, Ana M. Fernandez‡, Charlotta A. Glackin§, Lee Helman¶, and Derek LeRoith‡§

From the ‡Section on Molecular and Cellular Physiology, Clinical Endocrinology Branch, NIDDK, and the ¶Section on Molecular Medicine, Beckman Research Institute of the City of Hope, Duarte, California 91010

In this study we investigated the molecular mechanisms whereby insulin-like growth factor 1 (IGF-1) induced Twist gene expression and the role of Twist in the anti-apoptotic actions of the IGF-1 receptor. In NIH-3T3 fibroblasts overexpressing the human IGF-1 receptor (NWTb3), treatment with IGF-1 (10−8 m) for 1 and 4 h increased the level of Twist mRNA as well as protein by 3-fold. In contrast, insulin at physiological concentrations did not stimulate Twist expression in NIH-3T3 fibroblasts overexpressing the human insulin receptor. The IGF-1 effect was specific for the IGF-1 receptor since, in cells overexpressing a dominant negative IGF-1 receptor, IGF-1 failed to increase Twist expression. Pre-incubation with the ERK1/2 inhibitor U0126 or expression of a dominant negative MEK-1 abolished the effect of IGF-1 on Twist mRNA expression in NWTb3 cells, suggesting that Twist induction by IGF-1 occurs via the mitogen-activated protein kinase signaling pathway. In vivo, IGF-1 injection increased the mRNA level of Twist in mouse skeletal muscle, the major site of Twist expression. Finally, using an antisense strategy, we demonstrated that a reduction of 40% in Twist expression decreased significantly the ability of IGF-1 to rescue NWTb3 cells from etoposide-induced apoptosis. Taken together, these results define Twist as an important factor involved in the anti-apoptotic actions of the IGF-1 receptor.

Insulin and the insulin-like growth factors (IGFs) exert their biological effects by binding to their respective transmembrane receptors. Insulin and IGF-1 receptors (IR and IGF-1R, respectively) share the same overall structure. They are composed of two extracellular α subunits containing the ligand binding domain and two transmembrane β subunits possessing tyrosine kinase activity (1, 2). Both receptors are capable of binding insulin and IGF-1, but each receptor binds its own ligand with a 100–1000 fold higher affinity than that of the heterologous peptide. IR and IGF-1R have intracellular downstream events that are highly similar. Both receptors phosphorylate on tyrosine residues the same substrates such as the insulin receptor substrate proteins (1 through 4 (Refs. 3–6)), Gab-1 (7), and Shc (8), leading to activation of the mitogen-activated protein kinase (MAPK) also defined as extracellular signal regulated kinase (ERK) (9) and the phosphoinositide 3-kinase (PI3K/Akt (10) pathway. Despite close similarities in their receptor structure and signaling pathways, insulin and IGF-1 have different physiological functions (11–14). Insulin acts primarily as a regulator of metabolism, whereas IGF-1 functions predominantly in the control of cell growth, cell survival and differentiation. In order to understand the molecular basis for the different functions of the IGF-1 and insulin receptors, we previously used cDNA microarray expression profiling to identify genes that are differentially regulated by IGF-1 and insulin in mouse fibroblast NIH-3T3 cells.2 The mRNA level of Twist was increased more than 2-fold following 90 min of IGF-1 stimulation. However, no increase in Twist expression was observed following insulin stimulation.

Twist belongs to the basic helix-loop-helix family of transcription factors, which play a central role in cell type determination and differentiation in both vertebrates and invertebrates (15). The basic helix-loop-helix proteins are defined by the basic domain which mediates specific DNA binding and the helix-loop-helix domain that acts as a protein-protein interaction domain (16). Twist is expressed in mesodermal and cranial neural crest cells during embryogenesis (17). It has been implicated in the inhibition of differentiation of multiple cell lineages including muscle (18, 19) and bone (20, 21). For example, in C2C12 myoblasts (22) as well as in ES cell-derived embryoid bodies (23), Twist expression inhibits muscle differentiation. In C2C12 cells, this inhibition was reversible by blocking Twist expression using an antisense Twist oligonucleotides. In chicken skeletal muscle, the induction of Twist by hepatocyte growth factor (HGF) enables the satellite cells to proliferate and prevents differentiation (24). Although the inhibitory mechanisms are as yet unclear, some hypotheses have been proposed. In vitro, some studies show that both murine and Drosophila Twist protein can inhibit the expression and activity of myogenic genes. The suggested mechanisms involve the inactivation of the myogenic factors such as MyoD through heterodimerization with Twist or through inhibition by Twist of myogenic factors binding to DNA (18, 22). Twist has also been demonstrated to inhibit apoptosis in muscle embryonic...
fibroblasts that express both E1A and Ha-Ras (C8 MEF), a cell line in which apoptosis has a demonstrated dependence on p53 function (25). It is well known that IGF-1 is a powerful inhibitor of apoptosis caused by a variety of injuries in various cells. For example, addition of IGF-1 inhibits apoptosis induced by serum withdrawal in PC12 cells (26), IL-3 withdrawal in hematopoietic cells (27), as well as by c-Myc overexpression (28), anticancer drugs (29), and transforming growth factor β1 in fibroblasts (30). However, in different cell types and depending on the apoptotic stimulus, the mechanisms by which the IGF-1R protects cells from apoptosis vary considerably.

In this study, we investigated the mode of IGF-1 action on Twist gene expression and the role of Twist in the anti-apoptotic effects of IGF-1. We show that IGF-1 increases mRNA as well as protein levels of Twist in vitro in mouse fibroblasts overexpressing the human IGF-1R (NWTb3), whereas insulin fails to affect Twist expression in mouse fibroblasts overexpressing the human IR (IR cells). Moreover, in vivo, IGF-1 injections increased Twist expression in mouse skeletal muscle where Twist is predominantly expressed. We also investigated which IGF-1R signaling pathway was involved in the induction of Twist expression using different pharmacological inhibitors and a dominant negative MEK-1 construct. The results indicate that the MAPK pathway is primarily involved in IGF-1-induced Twist expression. Finally, using an antisense strategy in NWTb3 cells, we show that Twist is positively involved in the IGF-1 inhibition of apoptosis induced by etoposide treatment.

**EXPERIMENTAL PROCEDURES**

**Chemicals and Antibodies**—The radionucleotide α-[32P]dCTP (6000 Ci/mmol) was purchased from PerkinElmer Life Sciences. Recombinant human IGF-1 (rhIGF-1) was a gift of Genentech (South San Francisco, CA). FSK-specific inhibitor LY 294002 and etoposide were purchased from Sigma. Both MEK1/2-specific inhibitor U 0126 and p85 MAPK-specific inhibitor SB 202190 were from Calbiochem (La Jolla, CA). The stock solutions of pharmacological inhibitors such as LY 294002, U 0126, and SB 202190 were all prepared in Me2SO at a concentration of 1000-fold, so that when they were added to the culture medium, the concentrations of Me2SO was below 0.1%. Monoclonal anti-actin (clone AC) was obtained from Sigma. Rabbit polyclonal antibodies to phospho-Akt (Ser473), Akt, phospho-Erk1/2 (Thr202/Tyr204), and cleaved caspase-3 were purchased from New England Biolabs Inc. (Beverly, MA). Rabbit polyclonal antibody to PARP, which reacts with the PARP full-length and the 24/31-kDa, cleaved PARP product, was from Oncogene (Cambridge, MA). Rabbit polyclonal antibody to Erk1 (C16) and to Erk2 (C16) were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). All antibodies were used at a 1:1000 dilution in Western blotting. The dominant negative construct of MEK-1 (pCDNAIII-MEKA) was kindly provided by Dr. J. S. Gutkind (NICHD, National Institutes of Health, Bethesda, MA). Rabbit polyclonal antibodies to Twist and the Twist antisense pcDNA3 construct were kindly donated by Dr. C. A. Glackin (Division of Molecular Medicine, Loma Linda University, Loma Linda, CA).

**Cell Culture and Stable Transfection**—MCF-7, COS-7, C2C12, and PC3 cells were obtained from ATCC (Rockville, MD). The parental mouse embryonic fibroblast NIH-3T3 cell line expresses 16 × 10^3 IGF-IR/cell (31). The NWTb3 cell line established in our laboratory expresses the normal human IGF-IR at a level of about 3 × 10^5 receptors/cell (33). The parental NIH-3T3, MCF-7, COS-7, C2C12, and PC3 cells were routinely cultured in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% heat-inactivated fetal bovine serum (FBS), 100 units/ml penicillin, 100 μg/ml streptomycin, and 300 μg/ml l-glutamine in a humidified atmosphere of 95% air and 5% CO2 (37 °C). NWTb3, IR, and NKR1 cells were cultured in the same medium with the addition of Geneticin (0.5 g/liter, Life Technologies, Inc.). For Twist antisense, the transfection method using Effectene (Qiagen, Valencia, CA) was carried out according to the manufacturer’s instructions. Subconfluent NWTb3 cells were transfected with either hygromycin pcDNA3 (Hygro, negative control) or Twist antisense hygromycin pcDNA3 construct (TWIAs). After 48 h, the cells were cultured in the same medium supplemented with Genetnicin (0.5 g/liter) and hygromycin (0.2 μg/liter, CLONTECH, Palo Alto, CA). IGF-1 Stimulation in Vivo—C57BL/6 mice of weights 15–20 g were obtained from the Jackson Laboratory (Bar Harbor, ME) at 3 weeks of age. They were maintained on a 12-h light/12-h darkness cycle in a temperature-controlled room and given free access to commercial mouse chow and water. Mice were fasted overnight, anesthetized with avertin (15 ml/kg body weight) intraperitoneally, and abdominal cavi- ties were opened and the inferior vena cava exposed. A bolus of IGF-1 was then injected. For dose responses we utilized 0.5–10 mg/kg IGF-1, and for the time course we utilized 2.0 mg/kg IGF-1.

**Western Blot Analysis**—Cell lysates were prepared in lysis buffer A (10 mm Tris (pH 7.4), 150 mm NaCl, 1 mm EDTA, 1 mm EGTA, 0.5% Nonidet P-40) containing various protease inhibitors (2 mm phenyl- methylsulfonyl fluoride, 10 mg/ml leupeptin, 10 mg/ml aprotinin) and phosphatase inhibitors (100 mm sodium fluoride, 10 mM sodium pyrophosphate, 2 mM sodium orthovanadate). Lysates were centrifuged at 12,000 × g for 20 min at 4 °C, and then the protein concentration in the supernatants was determined using the BCA protein assay. 50 μg of total protein samples were electrophoresed in a SDS-PAGE gel and transferred to nitrocellulose membranes. The membranes were blocked with 5% nonfat milk in Tris-buffered saline for 1 h, then washed for 1 h at room temperature. Blots were probed with the various antibodies as indicated in the figure legends. After extensive washing, bands were detected with the appropriate horseradish peroxi- dase-conjugated secondary antibodies followed by enhanced chemilumi- nescence. Densitometry was performed by scanning the radiographs and then analyzing the bands with the MacBas version 2.52 software (Fuji Photo Film U.S.A., Inc.).

**Clonage of Poly(ADP-ribose) Polymerase (PARP) and Caspase-3**—PARP catalyzes the ADP-ribosylation of nuclear proteins at the sites where DNA strands break spontaneously and thereby facilitates the repair of this DNA damage (34). A critical step in the regulation of apoptosis is the proteolytic inactivation of PARP by caspases (34). Hygro and TWIAs cells were plated in 100-mm dishes in DMEM sup- plplemented with 10% FBS. After 16 h of incubation, medium was changed to DMEM with 1% FBS and cells were treated or not with 25 μM etoposide. Etoposide is a topoisomerase II inhibitor that induces cell cycle arrest or apoptosis when applied in high concentrations (35). After 2 days of treatment, IGF-1 (10^{-7} M) was added or not for 24 h and cell lysates were prepared in the lysis buffer containing 50 mM Pipes (pH 6.8), 2 mM EGTA, 0.1% Chaps, 5 mM dithiothreitol, 10 μg/ml leupeptin, 10 μg/ml aprotinin, and 1 mM phenylmethylsulfonyl fluoride. The cleavage of PARP and caspase-3 in Hygro and TWIAs cells was analyzed by immunoblotting with anti-PARP and anti-cleaved caspase-3 antibodies.

**Analysis of Apoptotic and Necrotic Cell Populations—**Cells (Hygro and TWIAs) were washed twice in Hepes buffer (10 mM HEPES, 140 mM NaCl, 2.5 mM CaCl2, 1 mM MgCl2, pH 7.4) and incubated at 37 °C in a temperature-controlled room for 1 h (36). Cells were resuspended in 0.2 ml of Hepes buffer supplemented with 2 μl of annexin-V-FLUOS (Roche Molecular Biochemicals) and 5 μl of 7-amino-actinomycin solution (Via-Probe, Pharmingen) and were incubated for 15 min at room temperature in the dark. The staining was analyzed by flow cytometry using a FACSCalibur (Becton Dickinson, Mountain View, CA).

**Northern Blot**—At the indicated times after treatment with IGF-1, mouse tissues were excised and frozen immediately. Total RNA from tissue was extracted using TRIzol reagent according to the manufacturer’s instructions. The same technique was used to isolate total RNA from cells stimulated or unstimulated. 20 μg of total RNA from tissue or cells was separated by denaturing formaldehyde electrophoresis, then transferred to nitrocellulose membranes. The membranes were blocked with 5% nonfat milk in Tris-buffered saline for 1 h, then washed for 1 h at room temperature. Blots were probed with the various antibodies as indicated in the figure legends. After extensive washing, bands were detected with the appropriate horseradish peroxi- dase-conjugated secondary antibodies followed by enhanced chemilumi- nescence. Densitometry was performed by scanning the radiographs and then analyzing the bands with the MacBas version 2.52 software (Fuji Photo Film U.S.A., Inc.).
were assessed using the human RNA 18 S probe from Ambion, Inc. (Austin, TX).

**Transient Transfection of MEK-1 Dominant Negative Construct—**
NTb3 cells were seeded the day before the transfection. Cells were then transiently transfected using the Effectene transfection reagent (Qiagen, CA) according to the manufacturer's instructions with 1 μg of a MEK-1 dominant negative construct (MEK-1 \((-/-)\)) or an empty vector (empty vector). 24 h after transfection, cells were switched to serum-free medium (DMEM) for 16 h, followed by the addition of IGF-1 (10^{-9} M, 90 min), and then total RNA was extracted. In order to check ERK1/2 phosphorylation in NTb3 cells transfected with the MEK-1 \((-/-)\) construct and compare with cells transfected with vector alone, cells were starved overnight 24 h after transfection and stimulated with IGF-I (10^{-8} M, 10 min at 37 °C). Protein extracts were prepared as described previously, and Western blot analyses were performed with an anti-phospho Erk-1/2 antibody to detect Erk-1/2 phosphorylation induced by IGF-I stimulation as well as with an Erk-1 antibody to detect Erk-1/2 protein levels.

**RESULTS**

**Differential Effect of IGF-1 and Insulin on Twist mRNA Expression in Mouse Fibroblasts—** To characterize the differential effects of insulin and IGF-1 on Twist expression in NIH-3T3 fibroblasts, cells overexpressing either the human IR (33) or IGF-1 receptor (NTb3, Ref. 31) were treated with various concentrations of insulin or IGF-1, respectively. Expression of Twist was then determined by Northern blot analysis. As shown in Fig. 1A, incubation for 90 min with IGF-1 (10^{-9} M) or (10^{-8} M) increased the abundance of Twist mRNA in NTb3 cells by ~2- and ~3-fold, respectively. Interestingly, in IR cells, treatment with insulin (10^{-9} M) or (10^{-8} M) for 90 min failed to increase Twist mRNA levels (Fig. 1B). However, a very high concentration of insulin (10^{-6} M) increased Twist mRNA. Thus, physiological concentrations of IGF-1 and non-physiological concentrations of insulin stimulate Twist mRNA expression in NTb3 or IR fibroblasts, respectively.

In order to demonstrate that IGF-1 increases Twist mRNA expression through the IGF-1R, NIH-3T3 parental cells and a cell line overexpressing the dominant negative human IGF-1R, NKR1 cells (32) were stimulated with IGF-1 (10^{-8} M) for 90 min. Total RNA was extracted and analyzed by Northern blotting for the expression of Twist. After quantification by densitometry and autoradiography, membranes were stripped and rehybridized with the 18 S ribosomal RNA as a loading control. Results shown are representative of three experiments.

**Fig. 1.** Dose-dependent effects of IGF-1 (A) and insulin (B) on Twist mRNA expression in NIH-3T3 fibroblasts. Serum-starved NTb3 (A) and IR (B) cells were stimulated with the indicated concentrations of IGF-1 and insulin, respectively, for 90 min. Total RNA was isolated, resolved by agarose gel electrophoresis, and transferred to a nylon membrane. Membranes were hybridized to a cDNA probe for Twist. Signal intensities, determined with a Phospholmog apparatus (FujiFilm), were corrected for differences in loading by dividing the signal intensity for Twist by the signal intensity for 18 S ribosomal RNA. The graphs represent the corrected intensities for the Twist signal. The error bars represent the mean ± S.D. from three separate experiments.

**Fig. 2.** Effect of IGF-1R activation on Twist mRNA expression in NIH-3T3 fibroblasts. Serum-starved NIH-3T3 parental, NTb3, and NKR-1 cells were exposed to IGF-1 (10^{-8} M) for 90 min. Total RNA was extracted and analyzed by Northern blotting for the expression of Twist. After quantification by densitometry and autoradiography, membranes were stripped and rehybridized with the 18 S ribosomal RNA as a loading control. Results shown are representative of three experiments.

IGF-IR/cell). As shown in Fig. 2, the increase in Twist expression in response to IGF-1 was observed only in NTb3 cells overexpressing normal IGF-1R as compared with parental NIH-3T3 and NKR1 cell lines. The lack of identifiable response in the parental NIH-3T3 cells is mostly due to the fewer number of IGF-1 receptors. Taken together, these data demonstrate that IGF-1R activation is necessary to increase Twist expression.

We also studied the level of Twist mRNA and protein in NTb3 cells treated with IGF-1 for various times. Using Northern blot analysis, we show that IGF-1 (10^{-8} M) increased Twist mRNA level maximally after 4 h of stimulation. This effect is reduced after 8 h and abolished after 16 h of stimula-
tation (Fig. 3A). We also show that the amount of Twist protein (26 kDa) increased 3-fold after 4 h of IGF-1 treatment (Fig. 3B). This increase was observed up to 12 h of IGF-1 treatment. However, after 24 h of stimulation, the effect of IGF-1 was abolished. Thus, the time course for Twist protein expression correlates well with the expression of Twist mRNA induced by IGF-1. These observations demonstrate that the stimulatory effect of IGF-1 on Twist gene expression in NWTb3 cells leads to the production of Twist protein under these conditions.

**MAP Kinase Is Involved in IGF-1-induced Twist Expression**—In order to study the signaling pathways involved in IGF-1’s induction of Twist expression, inhibitors specific for p38, ERK1/2, and PI3K were used. First, we determined the active concentration of each inhibitor. NWTb3 cells were treated for 1 h with the inhibitors prior to the IGF-1 stimulation (10^{-8} M, 10 min). Western blot analysis (Fig. 4A) revealed the dose-dependent inhibition of SB 202190 (p38 pathway), U 0126 (ERK1/2 pathway), and LY 294002 (PI3K pathway), respectively, on the IGF-1-induced p38, ERK1/2, and Akt phosphorylation. As shown in Fig. 4A, 50 \mu M LY 294002 was able to inhibit Akt phosphorylation induced by IGF-1 and in the same manner, 0.5 \mu M U 0126 and 50 \mu M SB 202190 inhibited ERK1/2 and p38 phosphorylation, respectively (Fig. 4A). Western blot analyses, performed to detect all forms of Akt, ERK1/2 and p38 kinases, revealed that protein levels were similar among the samples. Moreover, we found that the same dose of each inhibitor (50 \mu M LY 294002, 0.5 \mu M U0126; and 50 \mu M SB 202190) also inhibited Akt, MAPK, and p38 in vitro activities respectively (data not shown). Fig. 4B indicates that neither PI3K pathway inhibitor LY 294002 nor p38 inhibitor SB 202190 affected IGF-1-induced Twist expression. However, the addition of U0126 abrogated the increase of Twist expression induced by IGF-1 stimulation. Thus, in NWTb3 cells, the expression of Twist induced by IGF-1R activation is apparently mediated through the MAPK pathway.

In order to confirm this result, NWTb3 cells were transiently transfected with a dominant negative MEK-1 construct (MEK-1 (−/−)). Upon IGF-1 stimulation, there was an 80% reduction in ERK1/2 phosphorylation after transfection of the dominant negative construct (Fig. 5A). Northern blot analysis was performed on NWTb3 transfected with MEK-1 dominant negative (MEK-1 (−/−)) or empty vector (empty vector) as a control (Fig. 5B). Upon IGF-1 stimulation, in cells transfected with empty vector, Twist mRNA expression was increased by 2.5-fold as compared with unstimulated cells. However, when NWTb3 cells were transfected with a dominant negative MEK-1, the IGF-1-induced Twist expression was not observed. Thus, the inhibition of the MAPK pathway, either by a specific ERK1/2 inhibitor (Fig. 4B) or by the expression of MEK-1 dominant negative in NWTb3 (Fig. 5B), results in inhibition of the IGF-1-induced Twist mRNA expression.

**Twist Is Involved in the IGF-1 Inhibition of Apoptosis Induced by Etoposide Treatment in NWTb3 Cells**—Twist has been shown to be implicated in the control of diverse developmental processes in different tissues as well as inhibition of apoptosis (25). In order to determine the role of Twist in the anti-apoptotic action of IGF-1, we generated Twist antisense clones (TWIAS). The Twist antisense clones were developed by stable transfection of NWTb3 cells with pcDNA3-hygromycin-TWIAS expression vector as described under “Experimental Procedures.” Several hygromycin resistant clones were analyzed by immunoblotting using an anti-twist antibody. Three clones were selected, designated as AS-1 to AS-3. In parallel, NWTb3 cells were stably transfected with the pcDNA3 hygromycin empty vector and two control clones (hygro1 and 2) were generated. Twist (26 kDa) immunoreactivity was reduced by 40–45% in the AS-1, AS-2, and AS-3 compared with the hygro1 and hygro2 clones (Fig. 6A). Control and TWIAS cells were preincubated or not with 25 \mu M etoposide for 48 h (a dose that induces apoptosis (35, 38)), and then IGF-1 (10^{-8} M) was added or not to the medium for an additional 24 h. Apoptosis was determined by annexin-V staining, which measures the trans-
location of phosphatidylserine to the outer plasma membrane, one of the earliest detectable events in the induction of apoptosis (39). In both control (hygro1) and Twist antisense (TWIAS-2) NWTb3 cells, the percentage of apoptotic cells represented less than 5% in basal state (DMEM with 1% FBS) and about 30% after etoposide treatment for 48 h. IGF-1 treatment represented less than 5% in basal state (DMEM with 1% FBS) and about 30% after etoposide treatment for 48 h. IGF-1 treatment.

We also investigated the effect of IGF-1 (10−8 m) for 90 min, Twist expression in various cell lines (Fig. 7A) and in vivo in different mouse tissues (Fig. 7B). In vitro, using Northern blot analysis, we detected Twist as a transcript of about 3.5 kb in human cell lines (MCF-7, PC-3, and HEK-293 cells) and as a transcript of about 1.6 kb in mouse fibroblast NWTb3 cell line, respectively. As expected, mouse myoblast C2C12 cells and COS-7 cells do not express Twist (Fig. 7A; Refs. 19 and 22). When all the cell lines studied were stimulated with IGF-1 (10−8 m) for 90 min, Twist expression was increased only in NWTb3 cells suggesting that IGF-1-induced Twist expression is specific to cultured fibroblasts. In vivo, in mouse tissues, we detected Twist as three transcripts (about 1.6, 4, and 4.9 kb). Twist 1.6-kb transcript is mainly expressed in skeletal muscle and in testis (Fig. 7B).

IGF-1 Increases Twist mRNA Expression in Vivo in Mouse Muscle—We also investigated the effect of IGF-1 injections on mouse skeletal muscle Twist expression. Three-week-old mice (C57bl/6j) were fasted overnight and infused with either saline (0) or IGF-1 via the inferior vena cava at the concentrations and times indicated in Fig. 8 (A and B), respectively. Initially, mice were infused with saline (0) or varying concentrations of IGF-1 (0.5–10 mg/kg) for 20 min (Fig. 8A). IGF-1 (1.5 mg/kg) increased the level of Twist mRNA by 1.4-fold as compared with saline infusion. This increase was maximal after IGF-1 (2 mg/kg) infusion. The time course of IGF-1’s effect at 2 mg/kg indicated that 20 min is the optimal time to obtain the maximal response of Twist expression to IGF-1 (Fig. 8B).

Thus, IGF-1 increases Twist expression in vitro in mouse
fibroblasts NIH-3T3 cells but also in vivo in mouse skeletal muscle.

DISCUSSION

The biological effects of IGF-1 and insulin are mediated by distinct but highly homologous cell surface receptors. Despite close similarities in the structure and signaling pathways of their receptors, insulin and IGF-1 have different biological actions. Whereas IR is mainly implicated in metabolic responses, the IGF-1R is primarily involved in mitogenesis, cell differentiation, and other important functions such as anti-apoptosis. The activation of distinct cellular responses by IR and IGF-1R might result in association with different intracellular signaling proteins or induction of different signaling mediators. We were interested in studying the molecular mechanism of IGF-1 action on Twist gene expression. We show that, in mouse fibroblast NIH 3T3 cells, the level of Twist mRNA is increased by IGF-1 at physiological concentrations and by insulin at only pharmacological doses. Twist has been demonstrated to inhibit muscle differentiation (18) but also to be an anti-apoptotic factor in a mouse embryo fibroblast-derived cell line (25). Thus, Twist could represent an important molecule involved in mediating IGF-1R-specific cellular responses.

In this study, we used NWTb3 cells, a NIH-3T3-derived cell line overexpressing the normal human IGF-1R (since parental NIH-3T3 cells have far fewer IGF-I receptors and do not show a response of Twist expression to IGF-I stimulation). As control cells, we used a cell line overexpressing tyrosine kinase-deficient human IGF-1R, where the lysine 1003 residue at ATP binding site was replaced by arginine (KR mutant; Ref. 32). This cell line (NKR) was characterized as an IGF-1R dominant negative NIH-3T3-derived cell line. Indeed, it has been reported previously that a human IGF-1R cDNA with a point mutation at the ATP binding site is an inactive receptor, unable to transmit a mitogenic signal, to transform (40), or to protect cells from apoptosis (41). By Northern blot analysis, we showed that IGF-1 was able to induce Twist expression in NWTb3 cells but not in NKR cells. We also showed that, in vivo, IGF-1 injections increased Twist expression in mouse skeletal muscle. Moreover, this effect was abolished in MKR mice that overexpress the negative dominant KR of IGF-1R specifically in

![Fig. 5. Dominant negative MEK-1 abolishes IGF-1-induced Twist mRNA expression in NIH-3T3 fibroblasts. NWTb3 cells were transiently transfected with pCDNAIII-MEKA, a dominant negative MEK-1 construct (MEK−/−), or vector alone (empty vector) as a control experiment. ERK1/2 phosphorylation and protein levels were determined in response to IGF-1 (10−8 M, 10 min) in MEK−/− and empty vector cells as described under “Experimental Procedures” (A). After 90 min of IGF-1 stimulation, total RNA was also extracted and analyzed by Northern blotting for the expression of Twist (B). 18 S rRNA is shown as loading control. The error bars represent the mean ± S.D. from three separate experiments.](http://www.jbc.org/)

![A.](http://www.jbc.org/)

![B.](http://www.jbc.org/)
Role of Twist in the Anti-apoptotic Actions of IGF-1R

A. Blot : aTwist

B. Hygro 1 TWIAS-2

C. Hygro 1 TWIAS-2

FIG. 6. Twist antisense expression interferes with IGF-1 inhibition of apoptosis induced by the etoposide treatment in NWTb3 cells. A. Twist protein expression in NWTb3 cells stably transfected with a Twist antisense construct (TWIAS). NWTb3 cells were stably transfected with pcdNA3 (hygro) or TWIAS constructs, as described under “Experimental Procedures.” 50 µg of protein from whole cell lysates was subjected to SDS-PAGE, followed by immunoblotting with a rabbit Twist polyclonal antibody. Control cells are designated as hygro 1 and hygro 2. As a negative control, we used C2C12 cell lysates. As control for the protein loading, the blot was stripped and reprobed with a mouse actin monoclonal antibody. B. IGF-1 treatment fails to rescue the apoptosis induced by etoposide in NWTb3 cells overexpressing Twist antisense cDNA. Control (hygro 1) and Twist antisense (TWIAS-2) cells were plated on 100-mm dishes in DMEM supplemented with 10% FBS. After 16 h of incubation, medium was changed to DMEM with 1% FBS, and cells were treated with etoposide (25 µM) as indicated. After 48 h of treatment, IGF-1 (10^{-8} M) was added or not in the medium for 24 h. The cells were collected and analyzed by flow cytometry as described under “Experimental Procedures.” The error bars represent the mean ± S.D. of percentage of apoptotic cells from three separate experiments. C. IGF-1 effects on the PARP cleavage in control and Twist antisense cells. Cells (hygro1 and TWIAS-2) were incubated in DMEM with 1% FBS in the presence of etoposide (25 µM) for 48 h, and IGF-1 (10^{-8} M) was added or not in the medium for 24 h. Cell lysates were subjected to Western blot analysis using an anti-PARP antibody as described under “Experimental Procedures.” These results are representative of two independent experiments.

The muscle (data not shown). These results indicate that the kinase activity of IGF-1R is essential for the induction of Twist expression in response to IGF-1. In chicken muscle satellite cells, another growth factor, HGF inhibits cell differentiation and stimulates cell proliferation by increasing the expression of Twist and reducing the level of cyclin-dependent kinase inhibitor p27 (24). However, the signaling pathway of HGF on Twist gene expression was not fully characterized. In the present study, we found that the ability of IGF-1 to induce Twist expression was blocked by U0126, a specific inhibitor of ERK1/2 kinase, whereas LY294002 (PI3K inhibitor) and SB202190 (p38 MAPK inhibitor) did not block the IGF-1 action on Twist expression. Thus, the pathway used by the IGF-1 system to induce Twist expression acts through the ERK1/2 kinase cascade.

Twist has been described mainly as an anti-myogenic and anti-apoptotic factor. In vitro, C2C12 myoblasts do not normally express Twist (19, 22). However, in Twist-transfected C2C12 myoblast cells, Twist inhibits cellular differentiation but does not affect cellular proliferation (42). In vivo, Twist is expressed in human and mouse muscle but its role has not been fully elucidated (43). Recently, we have shown that the hypoplasia found in MKR mice muscle at 3 weeks of age was correlated with an up-regulation of Twist expression and a down-regulation of MyoD as compared with the wild-type animal, suggesting that Twist could also be involved in vivo in mouse muscle differentiation. Twist-null mice die at day 11.5 post coitum (44). Just prior to death, Twist null mice show a massive wave of apoptosis in the developing somites, a site in which Twist is normally expressed (44). Studies in Drosophila also indicated that Twist might affect the transcription of fibroblast growth factor receptor, which is implicated in craniosynostosis (45). Many syndromes of craniosynostosis result from local perturbation of the apoptotic program (46). In C8 mouse embryo fibroblasts (cells expressing both E1A and Ha-RasV12), ectopic expression of Twist has been shown to inhibit the apoptosis induced by serum starvation and contact inhibition (25). In the present work, we generated Twist antisense clones and studied the action of IGF-1 on apoptosis of NWTb3 cells induced by etoposide treatment. Etoposide is a topoisomerase II inhibitor inducing cell cycle arrest (usually G2-M, although some reports also show G1 arrest) or apoptosis when applied at high concentration (35). Some studies demonstrated that the addition of IGF-1 markedly inhibits etoposide-induced apoptosis in p6 cells (BALB/c 3T3 cells that constitutively overexpress the human IGF-1 receptor; Ref. 38) as well as in glo- merular mesangial cells (47). It also been demonstrated that overexpression and activation of IGF-1R protects cells from apoptosis induced by a variety of stimuli and conditions including IL-3 withdrawal (48, 49), Fas activation (50), osmotic shock (51, 52), tumor necrosis factor-α (53), p53 (54), ionizing and nonionizing radiation (41), and okadaic treatment (55). However, the mechanisms by which the IGF-1R protects cells from apoptosis have been the object of many investigations. Inhibitors of both PI3K and MAPK pathways have been shown to block the anti-apoptotic effects of IGF-1 (26, 41, 55). In Cos-7 cells, expression of activated Akt protects cells from apoptosis, whereas a kinase-dead Akt does not, suggesting that Akt is a target of PI3K in anti-apoptotic signaling of the IGF-1R (41). One mechanism by which the activated Akt in response to IGF-1 may promote cell survival is through the inhibition of BAD, one component of the cell death machinery (56). However, some studies show that the MAPK pathway can also phosphorylate BAD (57). The IGF-1R may also protect cells from apoptosis independently of BAD phosphorylation (55). In the present study, we show that a decrease of Twist expression by 40% in NWTb3 reduces significantly IGF-1's ability to inhibit apoptosis induced by etoposide. These data indicate that Twist could be a regulator in the anti-apoptotic actions of IGF-1. Maestro et al. (25) showed that, in C8 mouse embryo fibroblasts, Twist inhibits apoptosis by antagonizing the p53 pathway that plays a critical role in regulating cell death in response to a variety of stimuli. It has been shown that Twist can directly bind two independent histone acetyltransferase

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domains of acetyltransferases, p300 and p300/CBP-associated factor, and regulate their histone acetyltransferase activities (19). One hypothesis is that Twist may modulate p53 activity through effects on p300/CBP and p300/CBP-associated factor (25).

In summary, our results indicate that in NIH-3T3 fibroblasts that overexpress human IGF-1 receptor (NWTb3 cells), IGF-1 induces Twist expression through activation of its receptor via the MEK/MAPK signaling pathway. We additionally demonstrate that a reduction in Twist expression impairs significantly the inhibition of IGF-1 on the apoptosis induced by...
etoposide. Thus, we identified a new component involved in the anti-apoptotic action of IGF-1 receptor. Finally, we show that IGF-1 also increases Twist expression in vivo in mouse skeletal muscle. Further investigation will enable us to discover the implications of these effects.

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