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The Tumor Suppressor p53 Down-Regulates Glucose Transporters *GLUT1* and *GLUT4* Gene Expression

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ABSTRACT

Tumorigenesis is associated with enhanced cellular glucose uptake and increased metabolism. Because the p53 tumor suppressor is mutated in a large number of cancers, we evaluated whether p53 regulates expression of the *GLUT1* and *GLUT4* glucose transporter genes. Transient cotransfection of osteosarcoma-derived SaOS-2 cells, rhabdomyosarcoma-derived RD cells, and C2C12 myotubes with GLUT1-P-Luc or GLUT4-P-Luc promoter-reporter constructs and wild-type p53 expression vectors dose dependently decreased both GLUT1 and GLUT4 promoter activity to approximately 50% of their basal levels. PG_{13}-Luc activity, which was used as a positive control for functional p53 expression, was increased up to ~250-fold by coexpression of wild-type p53. The inhibitory effect of wild-type p53 was greatly reduced or abolished when cells were transfected with p53 with mutations in amino acids 143, 248, or 273. A region spanning −66/+163 bp of the GLUT4 promoter was both necessary and sufficient to mediate the inhibitory effects of p53. Furthermore, *in vitro* translated p53 protein was found to bind directly to two sequences in that region. p53-DNA binding was completely abolished by excess unlabeled probe but not by nonspecific DNA and was super-shifted by the addition of an anti-p53 antibody. Taken together, our data strongly suggest that wild-type p53 represses *GLUT1* and *GLUT4* gene transcription in a tissue-specific manner. Mutations within the DNA-binding domain of p53, which are usually associated with malignancy, were found to impair the repressive effect of p53 on transcriptional activity of the *GLUT1* and *GLUT4* gene promoters, thereby resulting in increased glucose metabolism and cell energy supply. This, in turn, would be predicted to facilitate tumor growth.

INTRODUCTION

Tumor suppressors regulate cellular growth and differentiation by controlling DNA synthesis, gene expression, cell-to-cell communication, and various signaling processes. In the absence of tumor suppressors, uncontrolled proliferation occurs, leading to tumorigenesis (1). Tumorigenesis is associated with enhanced cellular glucose uptake and increased metabolism, which is required for rapid proliferation (2). Because the transmembrane glucose transporter (GLUT) proteins mediate glucose uptake in eukaryotic cells and are involved in the first step of the glucose utilization cascade, they represent potential regulatory targets of oncogenes or tumor suppressors (3). However, the molecular mechanisms by which tumor suppressors regulate *GLUT* gene expression at the promoter level are largely unknown.

Over the past several years, the p53 tumor suppressor protein has emerged as one of the most important cellular factors for preserving the integrity of the genome (4, 5). The p53 protein is a tetrameric phosphoprotein that mediates signal transduction from damaged DNA to genes involved in regulation of the cell cycle and apoptosis (reviewed in Ref. 6). p53 acts to reduce tumorigenic events by inducing apoptosis and eliminating relevant transformed cells from the system. Thus, it is critical that p53 protein levels, cellular localization (*i.e.*, nuclear or cytoplasmic), and activation state are tightly controlled to maintain normal cellular function. Approximately 50% of human cancers exhibit mutations within the *p53* gene (7). These mutations abrogate its tumor suppressor function and represent the most common known genetic defect associated with human cancer (8). Mutations in p53 facilitate tumorigenesis either by impairing the normal tumor suppressor function of p53 or by providing it with oncogenic potential (6).

Whereas GLUT1 is the major glucose transporter isoform found in most cells, other isoforms exhibit tissue-specific distribution patterns (9). Thus, insulin-sensitive GLUT4 is expressed in insulin-responsive tissues, such as heart muscle, skeletal muscle, and white and brown adipose tissues (10). Recently, we found that GLUT4 is also expressed in IGF-I-responsive tissues, such as the growing bone center (11). Thus, increased levels of IGF-I receptors, which are a characteristic feature of tumorigenesis (12), could lead to aberrant expression of GLUT4 in certain cancer states.

High expression of various GLUT isoforms can provide cancer cells with a metabolic advantage. Indeed, both GLUT1 and GLUT4 are aberrantly expressed in many tumors; for example, overexpression of GLUT1 is found in breast (13), thyroid (14), pancreatic, gastric (15), primary lung, and liver cancers (16). In some cases, overexpression of GLUT1 is correlated with a poorer prognosis (17). GLUT4 is present in 20% of human astrocytic tumors (18), in a subset of lung cancers (19), and in all gastric carcinomas, whereas it is expressed in only in 40% of normal gastric mucosa samples (20). We have recently shown that GLUT4 is aberrantly yet functionally expressed in alveolar rhabdomyosarcoma (21) as well as in papillary thyroid carcinoma.[1]

The correlation between oncogenes, glucose transport, and GLUT proteins was manifested in cells transfected with activated *Ras* and *Src* oncogenes (3). The cells exhibited an increase in the rate of glucose transport, which was paralleled by a marked increase in the amount of the GLUT1 glucose transporter protein and mRNA (3). The metabolic changes that occur during tumorigenesis facilitate tumor cell growth and are closely correlated with tumor aggressiveness; these alterations may therefore represent useful prognostic features (22).

Several lines of evidence suggest that the p53 and insulin signaling pathways interact to regulate glucose metabolism and other cellular functions. First, insulin receptor (23) and IGF-I receptor (24) gene expression are repressed by p53; second, glucose transporter expression is enhanced in tumors (15); and, third, p53 is mutated in a large number of cancers (7). Therefore, in the present study, we examined whether p53 regulates *GLUT1* and *GLUT4* gene expression at the molecular level.

MATERIALS AND METHODS

Cell Cultures. *In vitro* transfection studies were performed in the following cell lines: human osteosarcoma-derived SaOS-2 (ATCC no.

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[1] B. Fishman, G. Maor, M. Armoni, Z. Kraiem, B. Bishara, O. Ben-Izhak, M.J. Quon, A. Fusco, and E. Karnieli. The Ret/PTC1 fusion gene product regulates GLUT4 gene expression in human thyroid carcinoma, submitted for publication.

HTB-85), which lacks both p53 alleles (25); human embryonal rhabdomyosarcoma derived RD (ATCC no. CCL-136), which expresses one wild-type p53 allele and an Arg248Trp mutant on the other p53 allele (26); and murine skeletal muscle-derived C2C12 myoblasts (ATCC no. CRL-1772), which express GLUT4 upon differentiation into myotubes. Cells were grown in an atmosphere of 95% air/5% CO_2 in the appropriate medium, with DMEM (25 mM glucose), 10% FCS, 2 mM L-glutamine, 1 mM sodium pyruvate, 1% penicillin-streptomycin, 2.5 ng/ml amphoterycin, and 0.1% gentamicin.

GLUT1, GLUT4, and PG_{13} Luciferase Promoter Reporters. The GLUT promoter reporters were kindly provided by Dr. Michael Quon (National Center for Complementary and Alternative Medicine, NIH, Bethesda, MD; Ref. 27) and described previously by us (28). Full-length GLUT1 and GLUT4 promoter-reporter constructs (pGLUT1-P-Luc and pGLUT4-P-Luc) were generated by sub-cloning the upstream 5′ region of either the rat *GLUT1* (bp 1–2240) or *GLUT4* (bp 1–2376) gene into pGEM7-Luc, upstream of the luciferase gene. Progressive 5′ deletion mutants of the GLUT4 promoter were generated by restriction endonuclease digestion of pGLUT4-P-Luc with suitable enzymes, followed by religation. The pGEM7-Luc construct was referred to as p0-Luc, because it is devoid of any eukaryotic promoter, and was used as a negative control throughout the experiments. The PG_{13}-Luc promoter reporter was kindly provided by Dr. Lee Johnson (Ohio State University, Columbus, OH; Ref. 29). This reporter consists of an artificial promoter with 13 p53-binding sites adjacent to a TATA box and was used as positive control for p53 transcriptional activation.

p53 Expression Vectors. Expression vectors encoding wild type and various mutants of human p53 were kindly provided by Dr. Frank J. Raucher III (Wistar Institute, Philadelphia, PA). The p53 mutants used included mutant 143 (Val143Ala), mutant 248 (Arg248Trp), and mutant 273 (Arg273Trp).

Transient Transfection Assays. Regulation of *GLUT1* and *GLUT4* gene expression was studied by transiently cotransfecting the SaOS-2, RD, and C2C12 cell lines with the promoter-reporter constructs together with wild-type or mutant p53, according to our previously detailed protocols (21, 24). In brief, SaOS-2 and RD cells were plated in 100-mm dishes at a density of 650,000 cells/dish, and the cultures were transfected 24 h later. C2C12 cells were plated in 100-mm dishes at a density of 900,000 cells/dish. Differentiation of C2C12 myoblasts was induced by partial serum starvation of fully confluent C2C12 cultures in DMEM medium supplemented with 2% FCS, and the transfections were performed on the 5th day after cells reached confluency. All cells were transfected with affinity-purified cDNA (Qiagen, Inc., Hilden, Germany), using the calcium phosphate DNA precipitation method (30). Depending on the specific experimental conditions, cells were transfected with 10 μg of pGLUT1-P-Luc, pGLUT4-P-Luc, or PG_{13}-Luc promoter-reporters and increasing amounts of wild-type or mutant p53 expression vectors (0–5 μg). The DNA-containing medium of SaOS-2 and RD cells was replaced with complete medium 5 h after transfection. C2C12 myotubes were subjected to glycerol shock for 30 s 4 h after transfection. Cells were then incubated for 48 h at 37°C. Transcriptional activity of GLUT1, GLUT4, or PG_{13}-Luc promoters was determined as explained below.

Determination of Promoter Activity. Promoter activity was determined by measuring relative luciferase activity in the transfected cells using a luciferase reporter kit (Promega, Madison, WI) and a Lumat LB9501 luminometer (Berthold Systems, Inc., Nashua, NH). In preliminary experiments, for the internal control purposes, cells were cotransfected with a CMV-β-gal vector. In these experiments, no significant differences were found when the data were normalized to protein levels and when the data were normalized to β-galactosidase activity, and this promoter was hardly affected by the exogenous p53. Because it is known that the CMV promoter can be affected by p53, such data are typically normalized to total protein in sample (24). Thus, we normalized the data in all subsequent experiments to total protein levels in each sample, which was measured using a BCA protein assay (Pierce, Rockford, IL). In each set of experiments, the induced promoter activity was expressed as a percentage of its basal levels, *i.e.*, luciferase activity in cells that were transfected with the promoter-reporter in the absence of exogenous p53.

Determination of p53 Exogenous Expression. SaOS-2 cells that lack endogenous p53 were transfected with the various p53 expression vectors encoding the wild type and the mutated forms of p53, lysed with SDS-PAGE sample dissociation buffer (65 mM Tris, 2% SDS, and 8% glycerol) and electrophoretically resolved on 10% SDS-polyacrylamide gel. Proteins were transferred to nitrocellulose membranes (0.2 μm; Schleicher&Schuell, Germany) using semidry electroblotter (E&K Scientific Products, Saratoga, CA), and the membranes were saturated in blocking buffer [5% nonfat dry milk, 0.1% Tween-20, 0.5% BSA, 0.5 M NaCl, and 50 mM Tris (pH 7.5)] for 1 h at room temperature. The blots were than incubated overnight at 4°C in a solution containing anti-p53 (DO-1; Santa Cruz Biotechnology, Santa Cruz, CA) diluted in TBST [137 mM NaCl, 20 mM Tris (pH 7.6), 0.1% Tween-20]. Filters were than washed six times for 10 min in TBST and exposed to antimouse IgG-horseradish peroxidase conjugate (Pierce) diluted in TBST, followed by another series washes. After incubation with SuperSignal West Pico Chemiluminescent Substrate (Pierce), membranes were dried and exposed to X-ray films. Additionally, p53 protein levels were determined in cell lysates obtained from the SaOS-2 transfected cells. For this quantitative analysis of p53 protein, we used the p53 Pan ELISA kit (Roche, Basel, Switzerland). In brief, the transfected SaOS-2 cells were lysed with radioimmunoprecipitation assay buffer (20 mM Tris, 0.5 mM EDTA, 1% NP40, 0.5% sodium deoxycholate, 0.05% SDS, 1 mM phenylmethylsulfonyl fluoride, 1 μg/ml aprotinin, and 2 μg/ml leupeptin) and centrifuged for 10 min at 10,000 $\times$ *g*. The resulting supernatant fractions were used in ELISA assays using anti-p53-peroxidase conjugate, and the quantity of p53 was calculated using the supplied p53 standards.

***In Vitro* Translation and Electrophoretic Mobility Shift Assays (EMSAs).** The p53 cDNA was subcloned into the pCDNA3 vector to generate the pCDNA3-p53 plasmid that was used for *in vitro* translation. The TnT T7 transcription/translation system with rabbit reticulocyte lysate (Promega) was used to generate *in vitro* translated p53 protein from p53 cDNA. The resulting protein lysate was used in mobility shift assays. The production of proteins translated in the presence of [^{35}S]methionine was confirmed by SDS-PAGE, followed by phosphorimager analysis (FujiFilm, Tokyo, Japan).

For EMSAs, promoter-derived probes were prepared from the PG_{13}-Luc and GLUT4-P-Luc plasmids, respectively, by enzymatic digestion. PG_{13}-Luc was digested with *Hin*dIII to give a fragment containing 13 repeating p53-binding sites. The pGLUT4-P-Luc was digested with *Nco*I-*Hin*dIII to give a fragment of 230 bp (−66 to +163, in which nucleotide 1 corresponds to the transcription start site). The fragments were dephosphorylated with calf intestinal phosphatase (Promega) and purified from an agarose gel using the SUPREC system (Takara Shuzo Co., Shiga, Japan). Furthermore, we examined in detail the −66/+163-bp region of the GLUT4 promoter using the Motif program[2] and the FindPattern program[3] and found two sequences of 28 bp (−60/−33 bp, 5′-CGGGGCGGGAGTGAGGAGGTGGCTTCAG-3′) and 35 bp (+40/+74 bp, 5′-GGTTGTGGCAGTGAGTCCCACCAGACCCGCCCTTT-3′) within the −66/

[2] http://motif.genome.ad.jp/.

[3] http://inn.weizmann.ac.il:81/gcg-bin/seqweb.cgi.

+163-bp region that contain potential binding sites for p53 protein. Thus, GLUT4 promoter-derived −60/−33-bp sequence and +40/+74-bp sequence oligonucleotides were commercially synthesized. The double-stranded DNA probe was end-labeled with [γ-^{32}P]ATP in the presence of polynucleotide kinase (Roche). Protein-DNA-binding reactions for EMSA included *in vitro* translated p53 in the presence of 20 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (pH 7.5), 12% glycerol, 0.05% NP40, 0.1 mg/ml poly(dI-dC), 70 mM KCl, 50 mM DTT, 1 mM $MgCl_2$, 100 mM $ZnSO_4$, and 1 mg/ml BSA. Samples were incubated for 30 min at room temperature before the addition of the radiolabeled probe (~75,000 cpm). Reactions were incubated in room temperature for an additional 30 min and the DNA-protein complexes were resolved by electrophoresis on 5% nondenaturing polyacrylamide gels at 25 mA in cold 0.5× Tris-borate EDTA buffer (24). Gels were fixed, dried, and then analyzed by phosphorimaging.

***In Vitro* Mutagenesis.** Site-directed mutagenesis of the −66/+163-bp construct of GLUT4 promoter was performed with a QuickChange kit (Stratagene, La Jolla, CA) according to the manufacturer's protocol.

The two potential p53-binding sites within the −66/+163-bp promoter fragment were deleted using the following primers: Δ−50/−39, 5′-GGCGGGAGTGAAGCTCTCCGCATCTTTC-3′; and Δ+48/+67, 5′-TGGCAGTGAGTGAGTTTTGCACACCACTTCC-3′. The mutations were verified by enzymatic restriction digestion and by DNA sequencing.

RESULTS

To study the role of the p53 protein in regulation of *GLUT1* and *GLUT4* gene expression at the transcriptional level, cells were transiently transfected with either GLUT1-P-Luc or GLUT4-P-Luc promoter-reporter plasmids, together with either wild-type or mutant p53 expression vectors.

Coexpression of Wild-Type p53 Represses GLUT1 and GLUT4 Promoter Activity. Cotransfection of SaOS-2, RD, and C2C12 cells with GLUT1-P-Luc or GLUT4-P-Luc promoter-reporters and increasing amounts of pCB6+/wt p53 expression vectors dose dependently decreased both GLUT1 and GLUT4 promoter activity. The wild-type p53 expression vector repressed GLUT1 promoter activity down to 55% of basal levels in RD cells (Fig. 1*A*). Similarly, the wild-type p53 expression vector repressed GLUT4 promoter activity to 25%, 40%, and 67% of its basal levels, in SaOS-2, RD, and C2C12 cells, respectively (Fig. 1*B*). p0-Luc, which is a promoterless construct, was used as a negative control and exhibited only low levels of basal activity that were not affected by exogenous p53 (data not shown). PG_{13}-Luc, which includes an artificial promoter with 13 p53-binding sites adjacent to a TATA box, was used as a positive control for p53 expression vector activity. PG_{13}-Luc exhibited low levels of basal activity that were increased in a dose-dependent manner in response to coexpression of wild-type p53, by ~250-fold in RD cells and by ~7.5-fold in C2C12 myotubes (Fig. 1*C*).

Point Mutations Abolish p53-Mediated Repression of GLUT1 and GLUT4 Promoter Activity. Several "hot spot" elements that are mutated in various types of human cancer have been detected within the p53 protein. To determine whether any of these elements are important for the inhibitory effects of p53, various point-mutated forms of p53 were used, including those with mutations in amino acids 143, 248, or 273. SaOS-2 cells, RD cells, and C2C12 myotubes were cotransfected with either the GLUT1-P-Luc or the GLUT4-P-Luc promoter reporters and the expression vectors encoding wild-type or mutant p53. As shown in Fig. 2*A*, p53 protein was dose dependently expressed from the relevant expression vectors. The mutated forms of p53, m143 and m248, resulted in significant higher protein

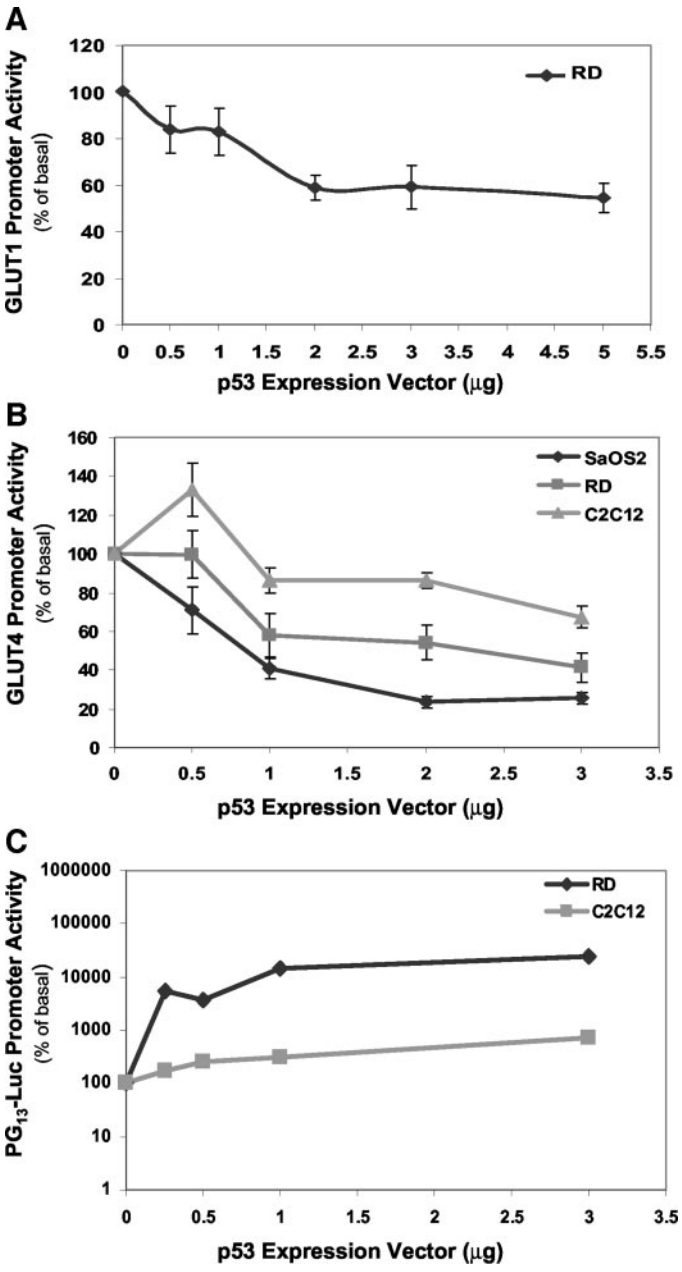

Fig. 1. Wild-type p53 represses GLUT1 and GLUT4 promoter activity in a dose-dependent manner. SaOS-2, RD, and C2C12 cells grown in 100-mm dishes were transfected with affinity-purified cDNA using the calcium phosphate DNA precipitation method. Cells were transfected with 10 μg of pGLUT1-P-Luc, pGLUT4-P-Luc, or PG_{13}-Luc promoter-reporters and 0–5 μg of wild-type p53 expression vector. Forty-eight h after transfection, activation of the GLUT1, GLUT4, and PG_{13} promoters was determined by measuring relative luciferase activity normalized to total protein levels within each sample. The induced promoter activity was expressed as a percentage of its basal levels, *i.e.*, luciferase activity in cells that were transfected with the promoter-reporter in the absence of exogenous p53. Each point in the figure represents the average of data obtained in three separate experiments performed in triplicate ± SE. *A*, RD cells were cotransfected with GLUT1-P-Luc and 0–5 μg of wild-type p53 (pCB6+/wt p53). *B*, SaOS-2, RD, and C2Cl2 cells were cotransfected with GLUT4-P-Luc and 0–3 μg of wild-type p53 expression vector (pCB6+/wt p53), as indicated. *C*, RD cells and C2C12 myotubes were cotransfected with PG_{13}-Luc and 0–3 μg of wild-type p53 expression vector (pCB6+/wt p53).

levels as compared with the wild-type form and m273 mutant. Those results were confirmed by the quantitative analysis using the p53 Pan ELISA (Roche). Transfecting SaOS-2 cells with 5 μg of wild-type p53, m143, m248, or m273 plasmids resulted in p53 protein levels of 11.5 ± 0.5, 316.4 ± 19, 44.3 ± 1.5, and 10.1 ± 0.1 μg/μl, respectively. This might be due to variation in the expression or higher

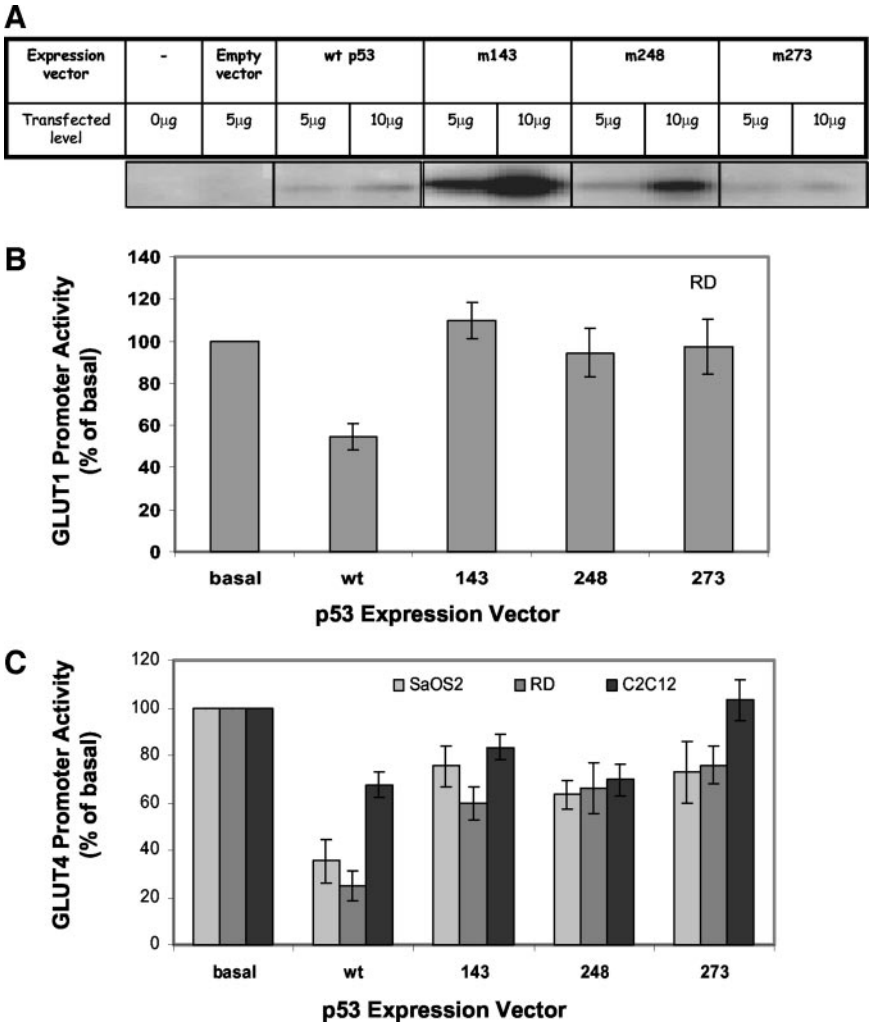

Fig. 2. Reduced repression of GLUT1 and GLUT4 promoter activity by mutant p53. SaOS-2, RD, and C2C12 cells were transfected in 100-mm dishes with affinity-purified cDNA using the calcium phosphate DNA precipitation method. Cells were transfected with 10 μg of pGLUT1-P-Luc or pGLUT4-P-Luc promoter-reporter and 3 μg of wild-type (*wt*) p53 expression vector or the various mutant forms of p53, m143 (Val143Ala), m248 (Arg248Trp), and m273 (Arg273Trp), as described in "Materials and Methods." Forty-eight h after transfection, transcriptional activity of the GLUT1 and GLUT4 promoters was determined by measuring relative luciferase activity normalized to total protein levels in each sample. The induced promoter activity was expressed as a percentage of its basal levels, *i.e.*, luciferase activity in cells that were transfected with the promoter-reporter in the absence of exogenous p53. Each point in the figure represents the average of data obtained in three separate experiments performed in triplicate ± SE. *A*, Western blot analysis of transfected and lysed SaOS-2 cells using anti-p53 (DO-1), antimouse IgG-horseradish peroxidase-conjugated secondary antibody, and SuperSignal West Pico Chemiluminescent substrate. *B*, RD cells were cotransfected with GLUT1-P-Luc and 3 μg of wild-type p53 (pCB6+/wt p53) or various mutant forms of p53, as indicated. *C*, SaOS-2 cells (*light gray*), RD cells (*gray*), and C2Cl2 myotubes (*black*) were cotransfected with GLUT4-P-Luc and 3 μg of wild-type p53 expression vector (pCB6+/p53) or various mutant forms of p53, as indicated.

protein stability of some mutated forms of p53 as previously shown (31, 32). p53 was undetected in nontransfected and empty vector transfected SaOS-2 cells. Whereas wild-type p53 repressed GLUT1 and GLUT4 transcription, none of the mutants had any significant effect on GLUT1 promoter transcriptional activity in RD cells (Fig. 2*B*) and only partially repressed GLUT4 promoter transcriptional activity in SaOS-2, RD, and C2C12 cells (Fig. 2*C*). As seen in Fig. 2, despite the elevated protein levels achieved by the mutated *p53* genes as compared with the wild type (Fig. 2*A*), their protein products did not repress the transcriptional activity of GLUT1 and GLUT4 promoters (Fig. 2, *B* and *C*). These data show that the inhibitory effect of wild-type p53 was greatly reduced or abolished when mutated forms of the p53 were used. This indicated that p53-induced inhibition of transcription from the GLUT promoter depends on the wild-type configuration of the protein.

Because the role of GLUT1 and its contribution to tumorigenesis have been described previously (15, 16, 18–20), we have focused our attention on better understanding p53-induced regulation of the GLUT4 promoter transcription activity.

The Region −66/+163 bp on GLUT4 Promoter Is Responsible for Transcriptional Repression by p53. Although consensus sequences for p53 transactivation have been repeatedly described (33), the inhibitory effect of p53 has been attributed to the presence of several specific negative elements (34–36) or to the general inhibition of basal transcription factors (37–39). Therefore, we have screened the GLUT4 promoter region (−2213/+163 bp, relative to transcription start site) for the presence of any of these inhibitory p53 response elements. Sequence analysis of the GLUT4 promoter region revealed the presence of several potential binding sites that are similar to the consensus sequence. Analyzing the effect of wild-type p53 on progressively 5′-deleted GLUT4 promoter reporter constructs identified target sequences for p53 within the GLUT4 promoter. For clarity, the effect of p53 was expressed as a percentage of the basal transcriptional activity of each promoter construct. Thus, we found that a promoter region spanning −66/+163 bp is both necessary and sufficient to mediate the inhibitory effects of p53 in both RD and C2C12 cells (Fig. 3).

p53 Protein Binds Directly to the −60/−33 bp and to the +40/+74 bp Regions on GLUT4 Promoter. EMSA analysis was used to determine whether the p53 protein and the −66/+163-bp DNA fragment of the GLUT4 promoter physically interact with each other. The *in vitro* translated p53 protein (Fig. 4*A*) was functionally viable, as confirmed by its ability to form complexes with a ^{32}P-labeled PG_{13} probe containing p53-binding sites (Fig. 4*B, Lane 2*). p53 was found to bind to the −66/+163-bp region of the rat GLUT4 promoter (Fig. 4*C, Lane 2*). This binding could be progressively competed by the addition of increasing amounts of unlabeled probe and was completely abolished by the addition of 130-fold molar excess of unlabeled probe (Fig. 4*C, Lane 3*). However, p53-DNA binding was unaffected by 130-fold molar excess of nonspecific DNA (data not shown). The p53-DNA complex was also super-shifted by the addition of an anti-p53 antibody (DO-1; Santa Cruz Biotechnol-

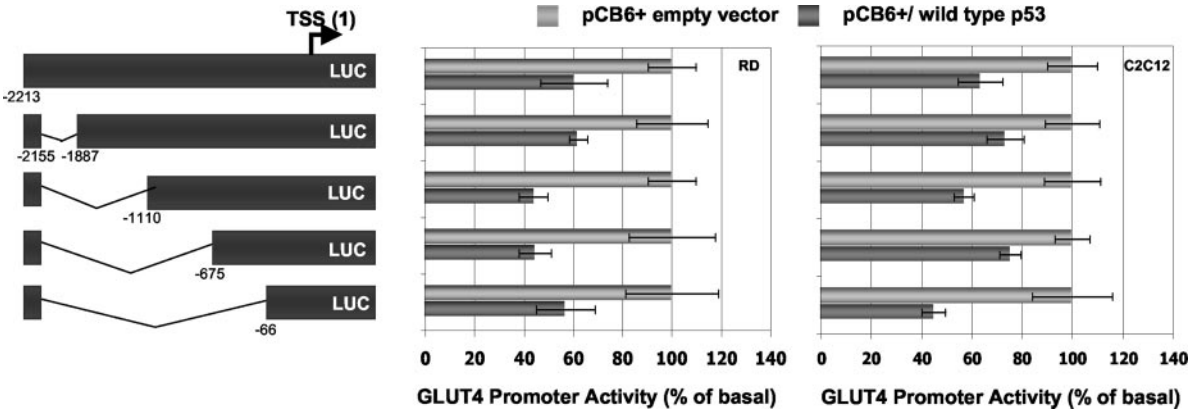

Fig. 3. Effect of p53 on progressive 5′ deletion constructs of the GLUT4 promoter. RD cells (*middle panel*) and C2C12 myotubes (*right panel*) were transfected in 100-mm dishes with affinity-purified cDNA using the calcium phosphate DNA precipitation method. Cells were transfected with 10 μg of 5′ deletion constructs of pGLUT4-P-Luc promoter-reporter (*left panel*) and 3 μg of wild-type p53 expression vector, as described in "Materials and Methods." Forty-eight h after transfection, GLUT4 promoter transcription activity was determined by measuring luciferase activity and normalizing to total protein in each sample. The induced promoter activity was expressed as a percentage of its basal levels, *i.e.*, luciferase activity in cells that were transfected with the promoter-reporter in the absence of exogenous p53. For clarity, the effect of p53 was expressed as a percentage of the basal promoter transcriptional activity within each construct. Each point in the figure represents the average of data obtained in four separate experiments performed in triplicate ± SE.

ogy; Fig. 4*C, Lane 4*). Similar results were observed when using nuclear extracts prepared from RH-30 alveolar rhabdomyosarcoma cells that express endogenous p53 (data not shown). These results suggest that wild-type p53 can bind directly and specifically to a sequence within the −66/+163-bp region of the GLUT4 promoter. To more precisely identify the bound sequences, two oligonucleotides corresponding to two shorter regions of the GLUT4-P, −60/−33 bp and +40/+74 bp, which contain potential p53-binding sites, were synthesized. The p53 protein was found to bind to each of these sequences (Fig. 4*D, Lanes 2* and *4*). The binding to both sequences could be competed by the addition of 100-fold molar excess of specific unlabeled probe (data not shown).

The −50/−39-bp and the +48/+67-bp Regions on the GLUT4 Promoter Mediate the Responsiveness to p53. Site-directed mutagenesis was used to examine the importance of the p53-binding sites on the −66/+163 bp of GLUT4 promoter. The p53-binding sites from the −66/+163-bp promoter fragment were deleted, and the mutated promoter fragments were examined in transient transfection assay as described previously. As seen in Fig. 4*F*, the double mutated construct lacking both p53-binding sites lost most of its responsiveness to p53 as compared with the nonmutated reporter construct (−66/+163 bp) or with the mutants lacking one p53-binding site. Thus, the identified two p53-binding sites are necessary for the transcriptional repression of p53 on the GLUT4 promoter.

DISCUSSION

We have examined the hypothesis that the tumor suppressor p53 regulates *GLUT1* and *GLUT4* gene expression in both normal and cancerous states. Our findings demonstrate that the GLUT1 and GLUT4 promoters are directly regulated by p53 in a dose-dependent and cell type-specific manner.

The inhibitory effect of p53 on transcriptional activity of the GLUT4 promoter was significantly greater than its effect on the GLUT1 promoter. This may be due to the fact that GLUT1 is a general "housekeeping" glucose transporter, whereas GLUT4 is a tissue-specific and insulin-sensitive glucose transporter (40). These results are in agreement with findings suggesting a possible connection between p53 and insulin signaling, such as repression of insulin receptor and insulin-like growth factor-I (IGF-I) receptor gene expression by p53 (23, 24). These findings are also consistent with activation of antiapoptotic pathways and repression of *p53* gene transcription by the serine/threonine kinase Akt/PKB, which is downstream of insulin receptor signaling (41). Additionally, we recently showed that the PPARγ nuclear receptor also inhibits the transcriptional activity of GLUT4 promoter (28).

Point mutations in p53 either partially or completely abolished its inhibitory effects on *GLUT1* and *GLUT4* gene expression. This may explain the increases in GLUT expression that are associated with certain cancerous states characterized by the loss of p53 function (15). Our study shows that the GLUT1 promoter is unaffected by mutant p53, whereas the GLUT4 promoter is slightly repressed by mutant p53. This effect of mutant p53 on the GLUT1 and GLUT4 promoters in malignant states is advantageous in a metabolic sense, because it increases GLUT protein levels and cellular glucose uptake, thereby allowing tumor cells to proliferate more rapidly.

We further focused our attention on better understanding the mechanism by which p53 regulates the transcriptional activity of the insulin-sensitive glucose transporter, GLUT4, because the role of GLUT1 and its contribution to tumorigenesis has been described previously (2, 13, 14, 16, 17). Progressive 5′ deletion analysis of the GLUT4 promoter revealed a promoter sequence that is both necessary and sufficient for p53-induced repression activity. This sequence is located between −66 and +163 bp, relative to the transcription start site. EMSA analysis detected direct binding of p53 to the −66/+163-bp region of the GLUT4 promoter, as well as to two shorter regions spanning −60/−33 bp and +40/+74 bp; therefore, it seems that p53 has a direct effect on transcriptional activity of the GLUT4 promoter.

It is widely accepted that p53 activates transcription by binding DNA in a sequence-specific manner through a highly conserved DNA-binding domain that consists of two copies of the 10-bp motif 5′-PuPuPuC(A/T)(T/A)GPyPyPy-3′ separated by 0–13 bp (33). Each half-site binds a dimer of p53, resulting in the formation of a functional p53 tetramer-DNA activator complex. In contrast to the relative wealth of information regarding p53 as a transcriptional activator, the mechanism by which p53 represses transcription is largely unknown. Relatively few promoters have been shown to be repressed by direct interaction of p53 with DNA (42–44). In these cases, p53 binds to a consensus site that overlaps the binding site of a potent activator protein. Although promoter-bound p53 still activates transcription, it also displaces the more potent activator, resulting in a net decrease in transcriptional output and therefore apparent "repression." This mech-

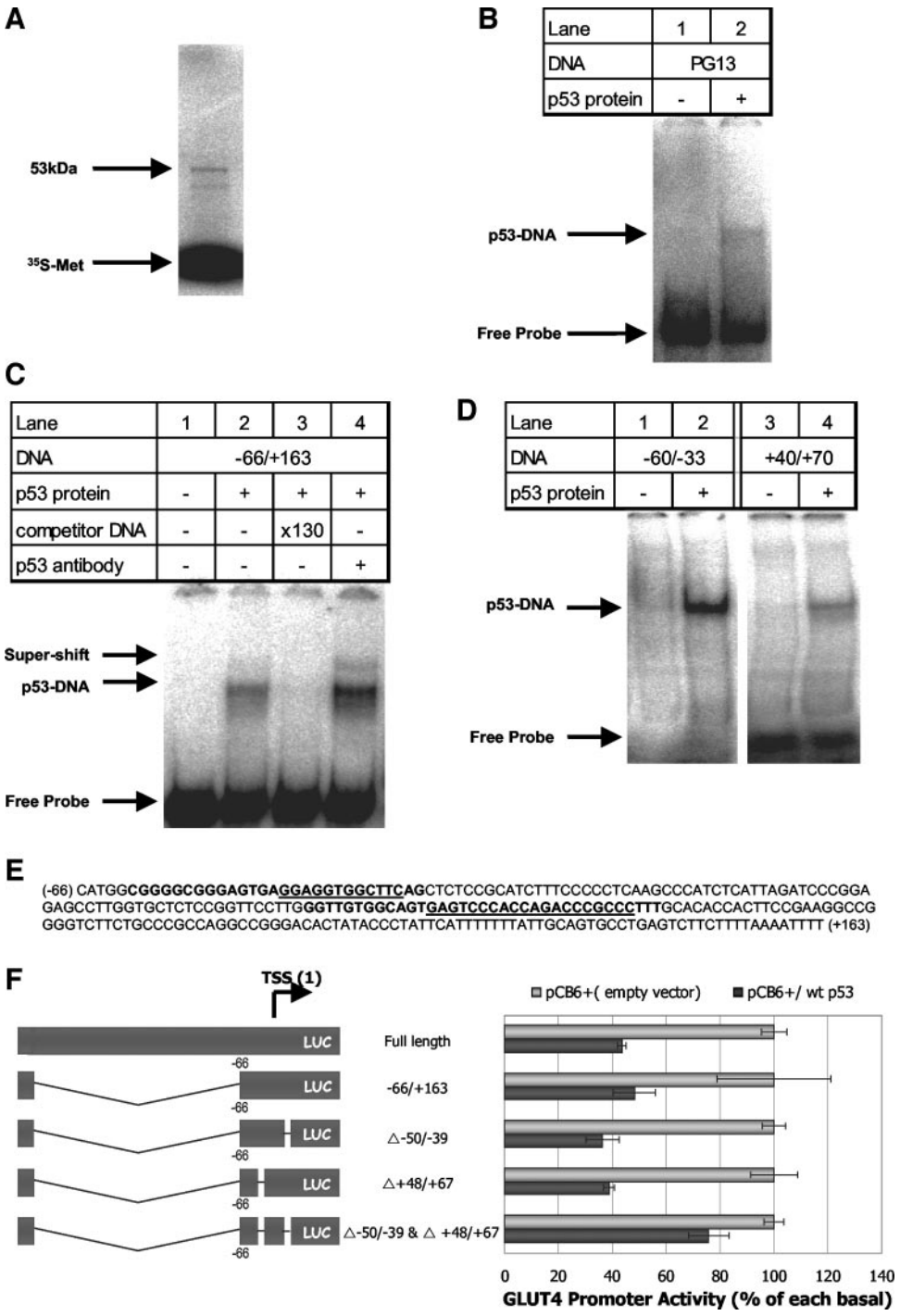

Fig. 4. *In vitro* translation and EMSAs. *A*, *in vitro* translated p53. The p53 protein was *in vitro* translated using the pCDNA3-p53 vector and TnT T7 transcription/translation system with rabbit reticulocyte lysate (Promega). Protein production was confirmed by SDS-PAGE, followed by phosphorimager analysis (PhosphorImager FLA2000) of proteins translated in the presence of [^{35}S]methionine. For EMSA studies, promoter-derived probes were prepared from the PG13-Luc and from GLUT4-P-Luc plasmids, respectively, by enzymatic digestion. Relevant oligonucleotides were commercially synthesized. The double-stranded DNA probes were end-labeled with [γ-^{32}P]ATP in the presence of polynucleotide kinase (Roche). The *in vitro* translated p53 protein was used for protein-DNA-binding reactions in the presence of radiolabeled probe (~75,000 cpm) in binding buffer, as described in "Materials and Methods." DNA-protein complexes were resolved by electrophoresis on 5% nondenaturing polyacrylamide gels at 25 mA in cold 0.5× Tris-borate EDTA buffer. Gels were fixed, dried, and then analyzed by phosphorimaging. *B*, p53 binding to the artificial p53-binding sites in the PG13 promoter. The DNA fragment of the p53-binding site sequences was digested from the PG13-Luc plasmid and was end-labeled with [γ-^{32}P]ATP. *C*, p53 binding to the −66/+163 bp region of the GLUT4 promoter. A GLUT4 promoter fragment extending from −66 to +163 bp was digested from the GLUT4-P-Luc promoter-reporter and was end-labeled with [γ-^{32}P]ATP. The corresponding unlabeled fragment of the GLUT4 promoter was used as a specific DNA competitor (*Lane 3*), whereas an OCT1-derived oligonucleotide was used for a nonspecific competition (not shown). *Lane 4*, the complex was super-shifted by incubating with anti-p53 antibody (DO-1; Santa Cruz Biotechnology) 30 min before addition of the probe. *D*, p53 binding to the GLUT4 promoter −60/−33 bp and +40/+74 bp regions. *Lanes 2* and *4*, binding reactions included the [γ-^{32}P]ATP-labeled oligonucleotides and purified p53 protein. *Arrows* indicate the relative mobilities of bound and free probes. The fold molar excess of specific competitor DNA or the addition of nonspecific DNA is indicated above the relevant lanes. *E*, the sequence of −66/+163 bp of GLUT4 promoter. Bold type indicates the sequences used in the EMSAs and underlined type indicates the sequences deleted in the mutagenesis and reporter assays. *F*, RD cells were transfected with 10 μg of mutated −66/+163 bp constructs lacking one or two p53-binding sites (*left panel*) and 3 μg of wild-type p53 expression vector, as described in "Materials and Methods." The activity of the induced promoters was expressed as a percentage of its basal levels, *i.e.*, luciferase activity in cells that were transfected with the promoter-reporter in the absence of exogenous p53 (*right panel*). For clarity, the effect of p53 was expressed as a percentage of the basal promoter transcriptional activity within each construct. TSS, transcription start site.

anism has been shown to down-regulate Bcl-2 (42), α-fetoprotein (43), and the hepatitis B virus (44). In addition, Johnson *et al.* (45) identified a novel alternative p53 DNA-binding site within the MDR1 (P-glycoprotein) promoter, in which the relative orientation of the four consensus quarter-sites defined p53 as a transcriptional repressor.

Based on these data, we have analyzed the relevant sequence, −66 to +163 bp, for potential p53-binding sites. This analysis revealed several potential binding sites for p53. When these sites were evaluated in EMSA studies, they were shown to bind the p53 protein (Fig. 4*E*). Furthermore, the importance of these binding sites was confirmed by reporter assay with mutated promoter fragments lacking these p53-binding sites (Fig. 4*F*). Interestingly, these sites overlap known binding sites for the activator p300. Taken together, these data suggest that p53 represses GLUT4 promoter transcriptional activity in a tissue-specific manner. Furthermore, this repression might be mediated, at least in part, by silencing the p300 activator through direct binding to its overlapping or nearby binding sites. This hypothesis is additionally supported by the findings that the point-mutated forms of p53 that we used, which are mutated in their DNA-binding domains, completely or partially abolished the repression of the GLUT promoter transcriptional activity.

In conclusion, our data strongly suggest that wild-type p53 represses *GLUT1* and *GLUT4* gene transcription and that several mutant forms of p53 have no effect or only slightly repress the transcription of these genes. This may have important clinical significance. In physiological situations, the negative control exerted by wild-type p53

limits the energy capacity for tumor cell proliferation. Mutation in tumor suppressors or activation of oncogenes, two events usually associated with malignancy, will up-regulate transcriptional activity of the glucose transporter gene promoters, as compared with the effect of wild-type p53. This, in turn, will result in increased glucose metabolism and cell energy supply, which would be predicted to facilitate tumor growth.

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