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TReP-132 Controls Cell Proliferation by Regulating the Expression of the Cyclin-Dependent Kinase Inhibitors $p21^{WAF1/Cip1}$ and $p27^{Kip1}$

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The transcriptional regulating protein of 132 kDa (TReP-132) has been identified in steroidogenic tissues, where it acts as a coactivator of steroidogenic factor 1 (SF-1). We show here that TReP-132 plays a role in the control of cell proliferation. In human HeLa cells, TReP-132 knockdown by using small interfering RNA resulted in increased $G_1 \rightarrow S$ cell cycle progression. The growth-inhibitory effects of TReP-132 was further shown to be mediated by induction of G_1 cyclin-dependent kinase inhibitors $p21^{WAF1}$ (p21) and $p27^{KIP1}$ (p27) expression levels. As a consequence, G_1 cyclin/cyclin-dependent kinase activities and pRB phosphorylation were markedly reduced, and cell cycle progression was blocked in the G_1 phase. The stimulatory effect of TReP-132 on p21 and p27 gene transcription involved interaction of TReP-132 with the transcription factor Sp1 at proximal Sp1-binding sites in their promoters. Moreover, in different breast tumor cell lines, endogenous TReP-132 expression was positively related with a lower proliferation rate. In addition, TReP-132 knockdown resulted in enhanced cell proliferation and lowered p21 and p27 mRNA levels in the steroid-responsive and nonresponsive T-47D and MDA-MB-231 cell lines, respectively. Finally, a statistic profiling of human breast tumor samples highlighted that expression of TReP-132 is correlated with p21 and p27 levels and is associated with lower tumor incidence and aggressiveness. Together, these results identify TReP-132 as a basal cell cycle regulatory protein acting, at least in part, by interacting with Sp1 to activate the p21 and p27 gene promoters.

Cell proliferation is regulated by a balance between cell division, growth arrest, differentiation, and programmed cell death. A network of genes, including cell cycle regulatory genes (30, 37), protooncogenes (33), and tumor suppressor genes (49), play major roles in normal physiological processes, such as development and aging, as well as in various pathological states, such as neurodegenerative disorders, immunodeficiency syndromes, and cancer (49). Recently, several genes encoding transcription regulating proteins, including retinoblastoma (RB), Wilms' tumor, p53, and BRCA have been characterized as tumor suppressor genes (52).

Cell cycle progression in eukaryotic cells is regulated by general mechanisms that involve phosphorylation of specific proteins through each stage of the cell cycle. Notably, phosphorylation of the retinoblastoma gene product pRB (and the related protein p107) represents a critical checkpoint of the $G_1 \rightarrow S$ transition (32). When underphosphorylated, pRB sequesters the E2F family transcription factors, which regulate genes encoding proteins required for S-phase DNA synthesis (58). Phosphorylation of pRB releases E2F that permits the induction of E2F-dependent genes and therefore the irreversible induction of the mitosis process, after which cells are refractory to extracellular growth inhibition signals. Thus, many cell cycle regulatory pathways, including response to growth factors and hormones (16, 39), act through modulation of mechanisms controlling pRB phosphorylation.

Phosphorylation of cell cycle proteins, including pRB, is performed by cyclin-dependent kinases (CDKs), whose activity depends on interactions formed with the timely expressed cyclins and cyclin-dependent kinase inhibitors (CDKIs) that activate or inhibit their activity, respectively (51, 83). Notably, whereas the D-type cyclins activate CDK4/6 to phosphorylate pRB, cyclin E and cyclin A mediate CDK2 kinase activity to phosphorylate histone H1. Among the CDKIs, $p16^{INK4A}$ (p16), a member of the INK4 protein family, is specifically induced at the end of the G_1 phase in response to pRB phosphorylation as a retrocontrol mechanism to inhibit CDK4/6. In addition, $p21^{Cip1/WAF1}$ (p21) and $p27^{Kip1}$ (p27), members of the Cip/Kip family, inhibit a broad range of CDKs, including CDK4/6 and CDK2. Since p21 and p27 are expressed in the G_1 phase to control pRB phosphorylation (83), their transcriptional regulation is a primary target for growth signaling factors such as steroid hormones (83). Moreover, decreased expression of both CDKIs is associated with the promotion of tumor formation and a poor prognosis in many types of cancer (81, 85). Therefore, characterization of mechanisms underlying the transcriptional regulation of p21 and/or p27 genes is important in our understanding of the genesis of cancers and in the search of novel therapies, notably for breast cancer (47, 78, 85).

The 132-kDa transcriptional regulating protein (TReP-132) was recently cloned based on its ability to activate P450scc

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gene expression (26). TReP-132, which contains two coactivator LXXLL nuclear receptor recognition motifs (26), was shown to act as a coactivator of the nuclear receptor steroidogenic factor 1 (SF-1), thus enhancing the expression of various steroidogenic genes (27, 28). Although steroid receptors control cell growth in steroidogenic tissues (12, 22, 77), several steroid receptor coregulators, including CBP/p300 and Wilms' tumor suppressor protein 1 (WT-1) (both cofactors of SF-1), have recently been shown to also influence cell proliferation and cancer development in both nonsteroidogenic and steroidogenic tissues (29, 49, 70, 71). Concurring with this, during our subsequent studies to further establish its biological functions, it became apparent that TReP-132 is involved in the control of cell proliferation. To characterize the role of TReP-132 in cell growth, the effects of TReP-132 overexpression or silencing by siRNA were studied by using HeLa cells and several breast cancer cell lines as models. Our results show that TReP-132 acts as a cofactor for Sp1 to increase expression of p21 and p27. As a consequence, TReP-132 lowers kinase activities of G_1 cyclin/CDK complexes, decreases pRB phosphorylation levels, and halts $G_1 \rightarrow S$ progression. Finally, TReP-132 expression was negatively associated with breast tumor formation and development in humans.

MATERIALS AND METHODS

Plasmids. The vectors encoding TReP-132, as well as the glutathione *S*-transferase (GST)–TReP-132, were constructed as previously described (26, 27). The pcDNA3-Sp1 was kindly provided by D. Monté (IBL, Lille, France) (4), the p21Luc plasmids were from A. Kraft and J. Biggs (Division of Oncology, University of Colorado, Health Sciences Center, Denver) (3), and the p27 promoter reporter constructs from T. Sakai (Kyoto Prefectural University of Medicine) (38). The nucleotide locations are indicated relative to the ATG.

Cell culture assays. Human cervical HeLa and breast cancer cells were obtained from American Type Culture Collection (ATCC; Rockville, MD) and cultured as recommended. Medium was supplemented with 10% fetal calf serum (HyClone, Logan, UT), L-glutamine (2 mM), and antibiotics gentamicin (1%) (Gibco, Burlington, Ontario, Canada).

For transient-transfection assays, HeLa cells were incubated for 24 h in 24-well plates at an initial density of 1.5×10^4 and 1×10^5 cells per well. Transfections were carried out with ExGen 500 (MBI Fermentas, Flamborough, Ontario, Canada) at a ratio of 4 μl of ExGen 500 per 0.5 μg of DNA. The indicated reporter constructs (100 ng) were cotransfected with the indicated amounts of expression vectors and the internal control *Renilla* luciferase reporter plasmid pRL-Null (10 ng) for 12 h. After 10 h of incubation in a fresh medium, cells were harvested and cell lysates (20 μl) were assayed for luciferase activity as previously described (28). Representative results of the mean ± the standard deviation (SD) of values obtained in triplicate from a single experiment ($n = 3$/time point) repeated at least three times are shown.

For colony formation assays, HeLa cells were seeded in a 100-mm-diameter plate at an initial density of 10^6 cells/well. After 24 h, cells were transfected by using the ExGen 500 with 1 μg of the indicated plasmids containing the neomycin resistance gene. After 48 h, medium was supplemented with 400 mg of G418 (Sigma-Aldrich CA, Ltd.)/ml. After 2 weeks, cells were washed with phosphate-buffered saline (PBS) and fixed with 10% acetic acid–10% methanol for 15 min and then colored with crystal violet (Sigma-Aldrich CA, Ltd.).

For assays using cell cycle synchronization, HeLa cells were synchronized before entering the S phase by a double thymidine block as previously described (6, 80). Cells were incubated for 14 h with thymidine (5 mM), then for 9.5 h without thymidine, and then for 5 h with thymidine. The time of end of the second incubation with thymidine corresponds to time point zero.

Establishment of inducible HeLa tet-off cell lines. HeLa tet-off (HTO) cells expressing the tetracycline-controlled transactivator, obtained from Clontech Laboratories (Palo Alto, CA), were grown in HeLa cell medium supplemented with 200 μg of G418 (Sigma-Aldrich CA, Ltd.)/ml. HTO cells, expressing both green fluorescent protein (GFP) and Flag-tagged TReP-132 (HTO-GFP/TReP cells) or GFP alone (HTO-GFP cells) upon doxycycline (Dox) removal, were, respectively, obtained by stable cotransfection of the pcDNA6 plasmid containing the blasticidin resistance gene (Invitrogen, Ontario, CA) and either the bidirectional pBI-EGFP or pBI-EGFP-TReP vectors at a ratio of 1:50. Cell colonies were then obtained after 2 weeks of selection with blasticidin (2 μg/ml; Sigma-Aldrich, CA, Ltd.) and were screened for inducible expression of GFP and/or TReP-132. Two cell lines of either HTO-GFP or HTO-GFP/TReP clones were analyzed with similar results. Cell clones were maintained in the presence of Dox (2 μg/ml), and the medium was changed every 2 days. The zero time point corresponds to Dox removal, and induction of TReP-132 was fully achieved at 48 h (data not shown).

siRNA analyses. Oligonucleotides were used in a T7 polymerase-based in vitro transcription reaction (Silencer small interfering RNA [siRNA] construction kit; Ambion) to generate double-stranded RNA with dideoxythymidine overhangs complementary to targeted mRNA regions. Sequences of siRNA tested in the present study were not present in any other known mRNA as determined by BLAST analysis. Oligonucleotides used for generation of p21 and p27 siRNAs were from Ambion (1436 and 14072, respectively). Oligonucleotides used for the generation of two TReP-132 siRNAs were synthesized by Eurogentec (siRNA-1, nucleotides 298 to 318 [sense, 5′-AACATGTTTGAGTTGGCCAGG-3′; antisense, 5′-AACCTGGCCAACTCAAACATG-3′; siRNA-2, nucleotides 362 to 382 [sense, 5′-AATGGCCTGGGAGTAGGTGTA-3′; antisense 5′-AATACAC CTACTCCCAGGCCA-3′). The nonsilencing siRNA oligonucleotide from Qiagen, which does not target any known mammalian gene, was used as a negative control. Transfection of siRNA duplex was performed by using the GeneSilencer (Gene Therapy Systems, San Diego, CA) reagent for the T-47D cells and the jetSI (Polyplus Transfection, Illkirch, France) reagent for the HeLa and MDA-MB-231 cells. siRNA transfection was performed at 0 and 3 days. The efficiency of the transfection was ascertained by fluorescence-activated cell sorting (FACS) analysis (see below) after control transfection of a fluorescein-coupled nonsilencing siRNA. The loss of endogenous TReP-132 protein expression 48 h after transfection with TReP-132 siRNA was verified in each studied cell line by immunofluorescence with an anti-TReP-132 antibody as described previously (26) (data not shown). The nonsilencing siRNA control did not influence TReP-132 expression. The experiments shown here were performed with the TReP-132 siRNA-1. TReP-132 expression knockdown using the siRNA-2 led to an ~65% decrease in TReP-132 mRNA levels and comparable effects on cell proliferation, as well as p21 and p27 gene expression as obtained with siRNA-1.

Cell proliferation and cell cycle analyses. The cellular DNA content was determined with fluorochrome 3,5-diaminobenzoic acid free acid (DABA) as described previously (21, 74). To analyze growth curves of HTO cell clones, cells were plated in 24-well plates at an initial density of 3×10^3 cells per well and 24 h later were grown in the presence or absence of Dox (2 μg/ml). Flow cytometry profiles were determined by propidium iodide staining of nuclear DNA as described in reference 40.

RNase protection assays. RNase protection assays were carried out as described previously (26). Analysis of transcripts encoding the cell cycle regulating proteins was performed by using the hCC-2 Multi-Probe template set according to the manufacturer's protocol (Pharmingen, Woodbridge, Ontario, Canada). Radioactivity was visualized and quantified by using a PhosphorImager (Molecular Dynamics).

RT and quantitative PCR. Total RNA was reverse transcribed, and the levels of TReP-132 transcripts were then assessed by quantitative reverse transcription-PCR (RT-PCR) as described previously (28) by using oligonucleotide primers specific for TReP-132 (5′-GTCAACAATATGGCCCAGGTG-3′ and 5′-GCCA GAGGCTGCTGGTCGTC-3′; GenBank no. NM_033502), for p21 (5′-TAATG GCGGGCTGCATCCAG-3′ and 5′-GCGAGGCACAAGGGTACAAGACA G-3′), and for p27 (5′-AGCCGGAGCCCCAATTAAAGG-3′ and 5′-AGTGT CTAACGGGAGCCCTAGCCTG-3′ for T-47D cells or 5′-CAAGTACGAGT GGCAAGAGGTGGAG-3′ for MDA-MD-231 cells).

Protein assays. Protein assays were performed as described previously (27) according to the Santa Cruz protocol. Immunoprecipitations were performed for 1 h at 4°C with one of the following antibodies: anti-CDK2 (sc-163) (Santa Cruz), anti-CDK6 (RB-017), anti-cyclin A (RB-010), or anti-cyclin D1 (MS-395) (Microm France, Francheville, France). Western blot analyses were performed by incubation with the following primary antibodies: anti-p21 (OP64; Merck Eurolab, Fontenay-sous-Bois, France), anti-p27 (K25020; Transduction Laboratories, Lexington, KY), anti-pRB (554136; Pharmingen [recognizing all phosphorylation states of pRB]), anti-ppRB-Ser780 (sc-12901) raised against a pRB peptide phosphorylated on Ser780, and anti-PR (MS-298-P) and anti-β-actin (sc-7210) according to the manufacturer's protocols. Histone H1 or pRB kinase activity of immunoprecipitates was measured as described by Draetta et al. (18). Relative abundances were quantified by using SCANWISE and Perfect-IMAGE V-5.3 (Clara Vision) software.

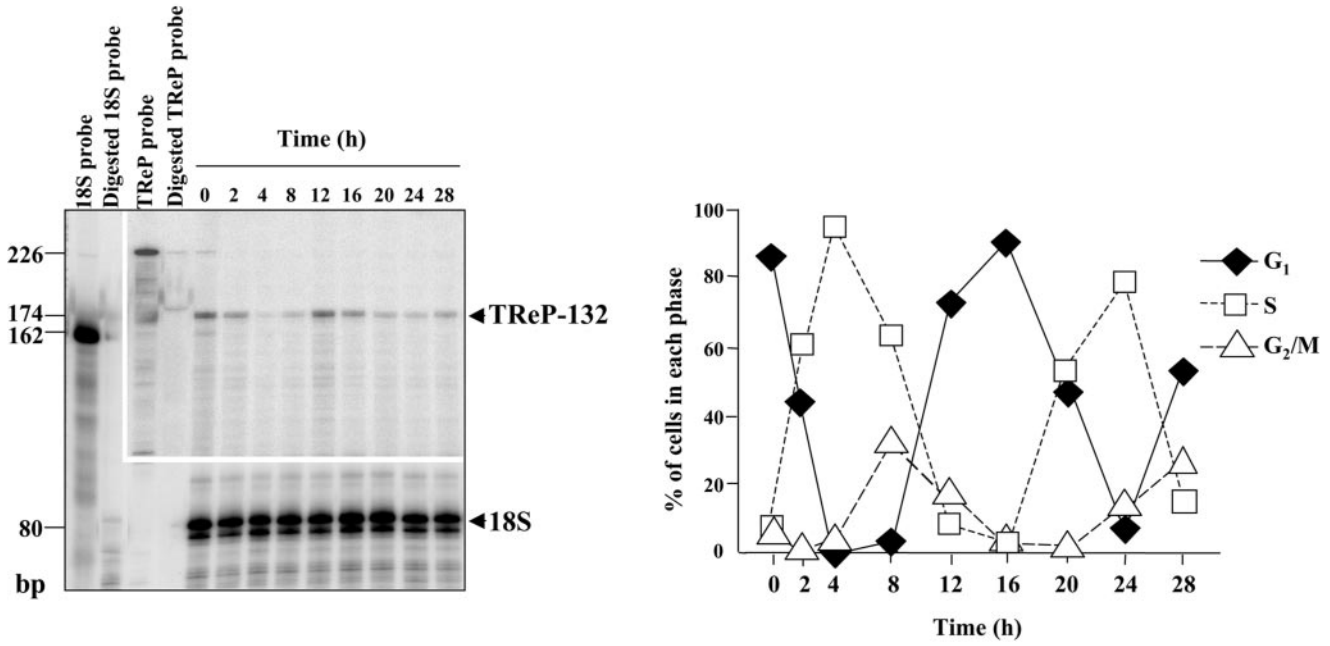

FIG. 1. TReP-132 transcript levels fluctuate during the cell cycle in HeLa cells. HeLa cells were synchronized before entering the S phase by a double thymidine block. RNase protection analysis of TReP-132 transcript (left panel) and flow cytometry profiles (right panel) were performed on cells harvested at the indicated time points over a 28-h period. One representative result from two independent experiments is shown.

In vitro protein binding assay. GST pull-down analyses were performed as described by Frangioni and Neel (23) with slight modifications (55). The gels were stained with Coomassie blue to ascertain that equal amounts of GST proteins were loaded, after which the gels were dried and visualized by autoradiography.

ChIP assays. Chromatin immunoprecipitation (ChIP) experiments were performed according to the method of Shang et al. (73), as modified by Barbier et al. (2). For the HTO cells, Dox was removed from half of the dishes, and the cells were incubated for 48 h before lysis. Cell lysates were sonicated and then immunoprecipitated by using the indicated antibodies and an anti-HA antibody as a negative control. Extracted DNA was PCR amplified by using primers for p21 (5′-GGCACTCTTGTTCCCCCAGGC-3′ and ACCATCCCCTTCCTCAC CTG-3′ [p21 Sp1 response element] or 5′-GCACACTGACGCAGCACACA G-3′ and 5′-CAGTTTGAGAAGCAGCCACCT-3′ [distal p21 element]), for p27 (5′-AGGCCAGCCAGAGCAGGTTTGT-3′ and 5′-GGAGGAGATCCAT TGGTTGCGG-3′ [p27 Sp1 response element] and 5′-GACTTGCATCTAGTC CTGACTCCGG-3′ and 5′-GCCTACCTCATCTCATACGCTCCAG-3′ [distal p27 element]), and for β-actin (5′-AAACTCTCCCTCCTCCTCTTCCT-3′ and 5′-CGAGCCATAAAAGGCAACTTTCG-3′).

Studies on human breast cancer biopsies. A total of 88 primary breast tumor samples were obtained from female patients undergoing surgery for locoregional disease in the Centre Oscar Lambret (the Anticancer Center of the North of France) between November 1989 and December 1990. The mean age of the patients was 60.6 years (range, 26 to 90 years). The median duration for follow-up of living patients was 83.4 months. The number of deaths was 27, and the number of relapses was 34. Estrogen receptor (ER) and progesterone receptor (PR) levels in each sample were determined by the dextran-coated charcoal method as described previously (63) according to the assay quality control levels of the European Organization For Research and Treatment of Cancer Receptor and Biomarker Study Group (43). Total RNA was isolated from 40 mg of individual tumor samples by using the RNeasy Minikit (Qiagen) as described earlier (61). All of the statistical analyses were done by using the SPSS software

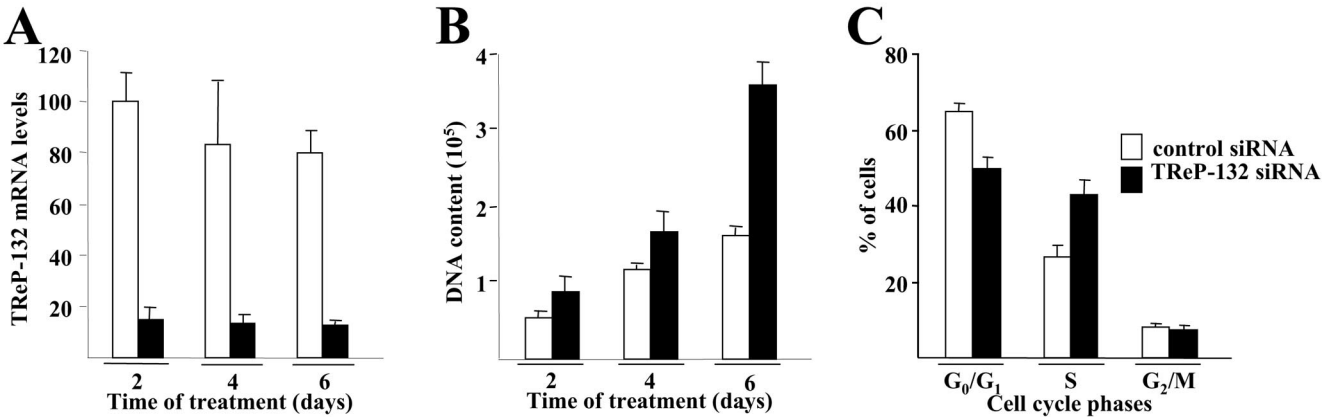

FIG. 2. TReP-132 inhibits HeLa cell proliferation. Cells, transfected with control or TReP-132 siRNAs (400 ng) at 0 and 3 days, were harvested at 2, 4, or 6 days. TReP-132 mRNA levels (expressed relative to amounts in control siRNA-transfected cells harvested at 2 days) (A) and DNA content (expressed as arbitrary units) (B) were measured. (C) Cell cycle phase distribution was determined at 4 days. Experiments were performed three times in triplicate, and values represent the means ± SD of a representative experiment.

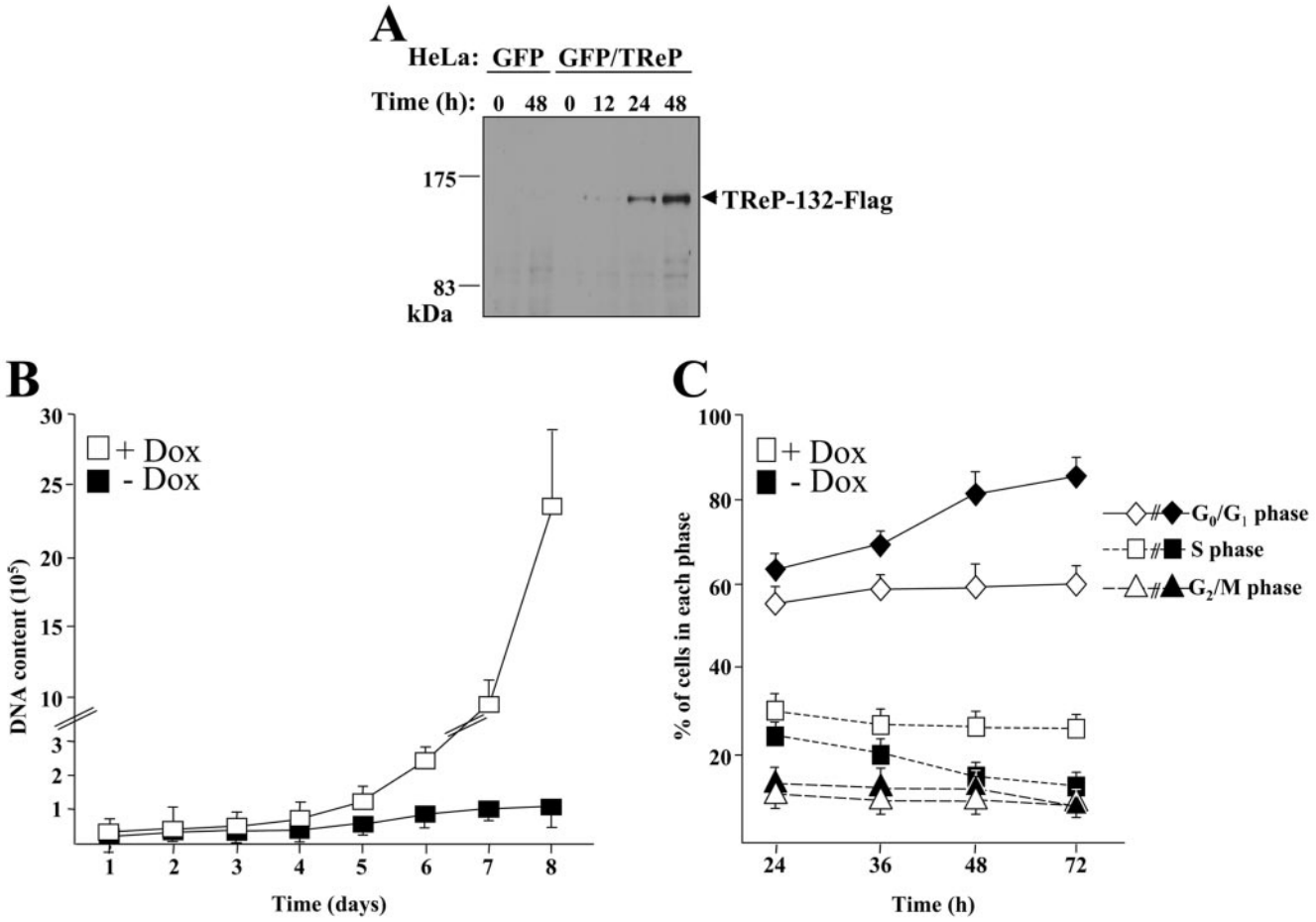

FIG. 3. Inducible overexpression of TReP-132 arrests the proliferation of HeLa cells in the G_1 phase of the cell cycle. HTO cells expressing GFP and Flag-tagged TReP-132 or GFP alone were harvested for protein analysis (A), cell proliferation assays (B), and cell cycle analysis (C) at the indicated times after Dox removal as described in Materials and Methods. (A) Immunoblot analysis of TReP-132-Flag expression. Protein extracts (30 μg) were resolved by sodium dodecyl sulfate-8% polyacrylamide gel electrophoresis and then analyzed by Western blotting with an anti-Flag antibody. (B) Growth curve. The data are plotted as DNA content relative to day 0. The figure represents the mean ± SD of values obtained in triplicate from a single experiment (n = 3/time point), which was repeated four times with similar results. (C) Cell cycle phase distribution. The percentages of cells in each phase of the cell cycle in a representative experiment out of four performed in triplicate are presented.

(version 8.0.1F). Relationships between qualitative variables were determined by using the χ^2 test (with Yates' correction when necessary). Correlations between parameters were assessed according to the Spearman nonparametric test. Comparison between curves was carried out by the log rank test. RNA from normal human epithelium mammary gland samples (n = 5) were obtained from BD Biosciences (catalog no. 636691).

RESULTS

TReP-132 gene expression is decreased during the S phase and TReP-132 negatively controls G_1→S transition. TReP-132 was isolated as a factor that regulates expression of the P450scc gene involved in the steroid synthesis pathway. To further analyze its biological function, we established stable clones overexpressing TReP-132 constitutively in HeLa cells, which express relatively low endogenous levels of the gene (25, 26). Interestingly, selection of cells transfected with the TReP-132 expression vector led to significantly fewer cell colonies of smaller size than when transfected with the empty pcDNA3 expression vector or with a construct encoding the firefly luciferase gene (data not shown). This suggested that TReP-132 overexpression results in altered cell proliferation.

To assess a putative role of TReP-132 on cell proliferation, we first evaluated whether TReP-132 gene expression is cell cycle dependent. To this end, mRNA levels were measured by RNase protection analysis in HeLa cells resuming proliferation after synchronization at the end of the G_1 phase (Fig. 1). Interestingly, TReP-132 expression displayed a cyclical pattern with lowest levels when the majority of cells are in the S phase (4, 20 and 24 h) compared to the G_1 and G_2 phases. Furthermore, silencing of TReP-132 by using a siRNA approach resulted in increased DNA content over a period of 6 days, indicating that endogenous TReP-132 inhibits cell proliferation (Fig. 2B). TReP-132 deficiency was associated with a lower percentage of cells in G_0/G_1 and a concomitant increase in the percentage of cells in the S phase (Fig. 2C), thus showing that TReP-132 may regulate cellular growth by controlling molecular events occurring at the G_1 phase. At present, a specific role for TReP-132 during the G_2 phase cannot be excluded and will require further work.

TReP-132 overexpression arrests cells in the G_1 phase. To determine the molecular mechanisms involved in the cell

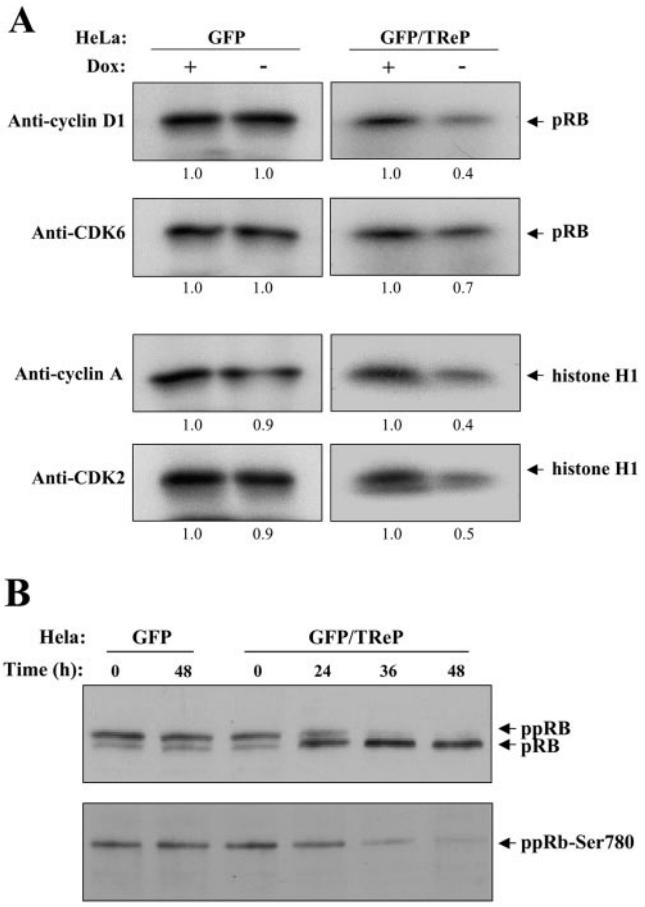

FIG. 4. TReP-132 overexpression decreases G_1 CDK activities and phosphorylated pRB levels. (A) In vitro kinase assays. HTO-GFP or HTO-GFP/TReP-132 cells were incubated 48 h without Dox and CDKs or cyclins were immunoprecipitated with the indicated specific antibodies. Immunoprecipitates were assayed for kinase activities using as canonical substrates pRB to measure cyclin D1 and CDK6 associated activities (86) or histone H1 to measure cyclin A and CDK2 associated activities (44). Amounts of phosphorylated histone H1 or pRB in induced HTO cells are expressed relative to amounts in uninduced HTO cells, arbitrarily set to 1.0. One representative result from three independent experiments is shown. (B) In-cell analysis of pRB phosphorylation status. Whole-cell protein extracts (20 μg) from HTO cells incubated with or without Dox during the indicated periods were analyzed by Western blotting with anti-pRB antibodies raised against total RB protein (upper panel) or only against the phosphorylated RB at Ser780, a residue preferentially phosphorylated by cyclin D1/CDK4 (41) (see lower panel). pRB, hypophosphorylated RB; ppRB, phosphorylated and highly phosphorylated forms of RB.

growth regulation by TReP-132, inducible Dox-regulated HTO cell lines expressing GFP alone (HTO-GFP) or both GFP and Flag-tagged TReP-132 (HTO-GFP/TReP) were generated. The TReP-132-Flag protein was detectable with an anti-Flag antibody as early as 24 h after the removal of Dox, whereas it was barely detectable in cells cultured in the presence of Dox (Fig. 3A). In addition, no signal was found in protein extracts from HTO-GFP cells expressing only GFP. Thus, these stable cell lines constitute a suitable model to study the effects of inducible TReP-132 expression on cell proliferation.

The growth curve of HTO-GFP/TReP cells cultured in the presence of Dox (repressing exogenous TReP-132 expression) showed that, after an initial lag phase of approximately 4 days, cells resumed a normal growth rate comparable to that of nontransfected HeLa cells. In contrast, the proliferation of cells cultured in the absence of Dox was significantly decreased (Fig. 3B). Since no differences in cell proliferation were observed when either HTO-GFP cells or parent HTO cells were incubated with or without Dox (data not shown), it can be concluded that TReP-132 overexpression results in an inhibitory effect on HeLa cell proliferation.

To determine which stage(s) of the cell cycle is affected by TReP-132, the cell cycle phase distribution was analyzed by flow cytometry. Induction of TReP-132 expression by Dox removal increased the proportion of cells in the G_0/G_1 phases and decreased the proportion of cells in the S phase (Fig. 3C), whereas the proportion of cells in G_2/M was not affected. Thus, in good agreement with Fig. 2C, overexpression of TReP-132 inhibits cell cycle progression by arresting the cells in the G_1 phase.

TReP-132 inhibits G_1 CDK activities by upregulating the expression of the CDKIs p21 and p27. Phosphorylation of pRB and of histone H1 by the G_1 cyclin/CDKs represents major events in the $G_1 \rightarrow S$ progression (62, 86). To determine whether TReP-132 influences G_1 cyclin/CDK complex activities, we examined in vitro the kinase activities associated with two cyclins (i.e., cyclin D1 and A) and two CDKs (i.e., CDK6 and -2) that preferentially phosphorylate either pRB or histone H1 (41, 44, 68). Induction of TReP-132 expression markedly reduced the kinase activities associated with both cyclins, as well as both CDKs immunoprecipitated from HTO-GFP/TReP cells (Fig. 4A).

The in-cell phosphorylation state of pRB after TReP-132 overexpression was also determined. Whereas the RB protein was predominantly present in its inactive hyperphosphorylated form in noninduced HTO-GFP/TReP cells, increased TReP-132 expression caused a significant increase in the amount of hypophosphorylated pRB concomitantly with a marked reduction of cellular levels of phosphorylated pRB (Fig. 4B). The levels of phosphorylated RB protein decreased as early as 24 h after Dox removal and was almost undetectable at 48 h. In contrast, no change in phosphorylation of pRB was observed during the same time period in HTO-GFP cells. These results are consistent with a role for TReP-132 in cell cycle arrest at the G_1 phase, since the hypophosphorylation of the RB protein reached its maximum at 36 h, a time point preceding the maximum accumulation of cells in the G_1 phase observed at 48 h (see Fig. 3C).

Considering that TReP-132 can act as a transcriptional regulating protein (26, 27), it was next investigated whether the TReP-132-dependent inhibition of cell proliferation is associated with changes in gene expression of certain cell cycle regulating factors by using a "multiprobe" RNase protection analysis. At 48 h after Dox removal, the induction of TReP-132 expression was associated with an increase in mRNA levels of the CDKIs p27, p21, and p16, as well as a slight decrease in pRB mRNA levels (Fig. 5A, middle panel). The induction of p21 and p27 mRNA levels after TReP-132 expression was further confirmed by quantitative RT-PCR analyses (Fig. 5A, right panel). In correlation, p21 and p27 protein levels were increased between 24 and 48 h, respectively, after TReP-132

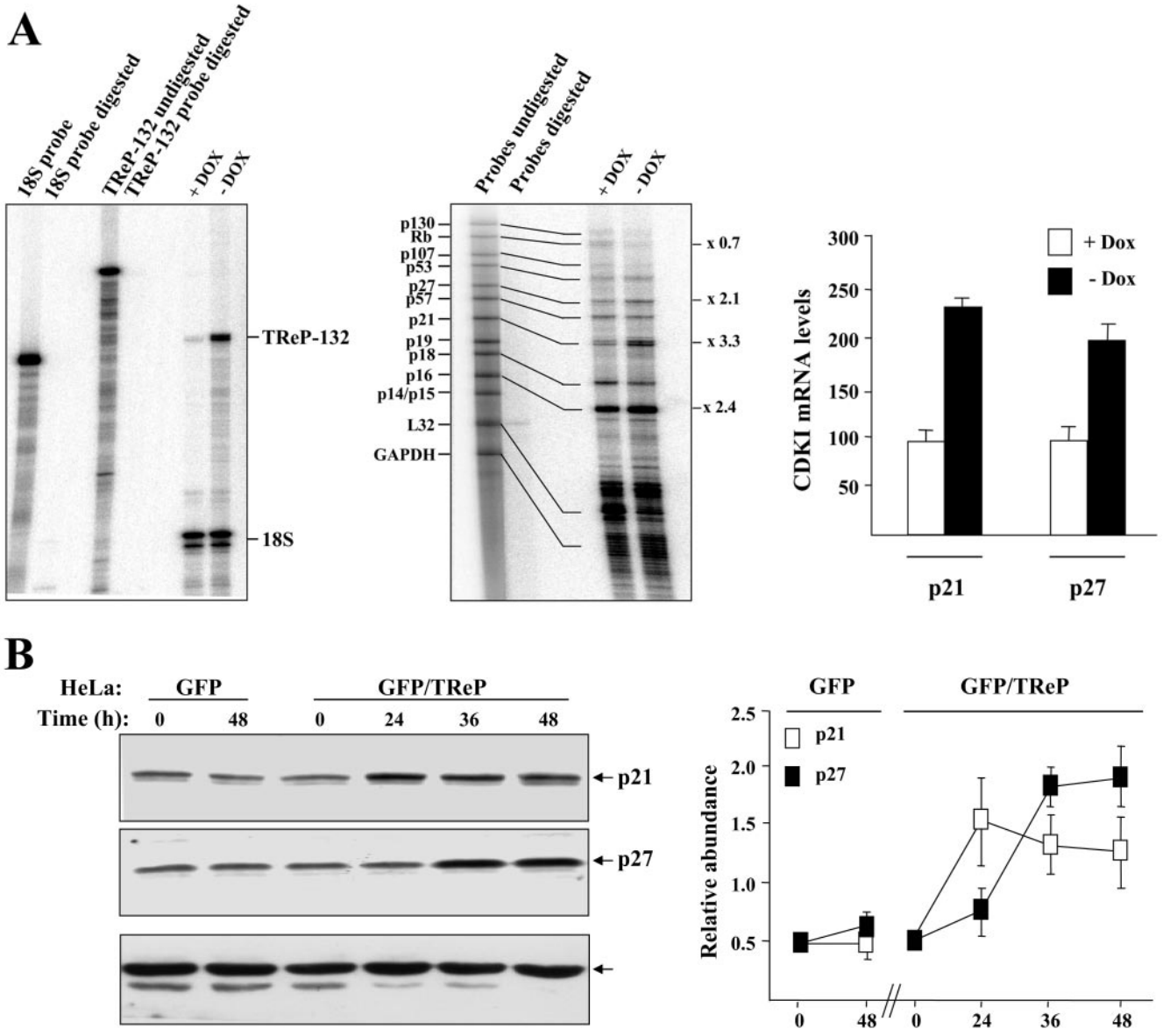

FIG. 5. TReP-132 inhibits cell proliferation by inducing the expression of the CDKIs p21 and p27. (A) mRNA levels. RNA from HTO-GFP/TReP cells incubated for 48 h with or without Dox were analyzed by RNase protection for TReP-132 transcript expression (left panel) and transcript levels of the indicated cell cycle-regulatory genes (middle panel, the fold variations of specific transcript levels normalized to L32 and GAPDH are indicated on the right), as well as by quantitative RT-PCR for p21 and p27 transcript levels (right panel). The results from one representative experiment out of three are shown. (B) Protein levels. Protein extracts (30 μg) from HTO-GFP and HTO-GFP/TReP cells incubated with or without Dox during the indicated periods of time (h) were subjected to Western blot analysis with antibodies to p21, p27, and β-actin as a control for protein loading. The blot represents a typical result of three independent experiments (left panel). Protein levels were quantified (means ± standard errors of the mean from three independent experiments) and expressed relative to the level in uninduced HTO cells, arbitrarily set as 1.0 (right panel). (C) Cell proliferation after p21 and p27 siRNA transfection. HTO-GFP/TReP cells incubated with or without Dox for 24 h were transfected with p21 and p27 siRNAs (10 nM). DNA content (means ± SD) was determined 4 days after transfection and expressed relative to cells incubated with Dox. One representative experiment of three performed in triplicate is shown.

expression, whereas the protein levels of these CDKIs remained unchanged in cells overexpressing only GFP (Fig. 5B).

To assess the role of p21 and p27 in mediating the effect of TReP-132 on cell growth, it was determined whether silencing of these CDKIs using a siRNA approach affects the effects of TReP-132 overexpression on HTO cell proliferation (Fig. 5C). As observed previously (Fig. 3B), the induction of TReP-132 in HTO-GFP/TReP cells by Dox removal led to a decrease of cell proliferation. Strikingly, this effect was nearly abolished in cells in which p21 and p27 expression levels were decreased by 86% ± 12% and 64% ± 5%, respectively, by transfection of both p21 and p27 siRNAs. These data thus demonstrate that expression of p21 and p27 is required for the negative control of TReP-132 on cell growth.

TReP-132 regulates the p21 and p27 gene promoters through interaction with Sp1. The transcriptional mechanisms

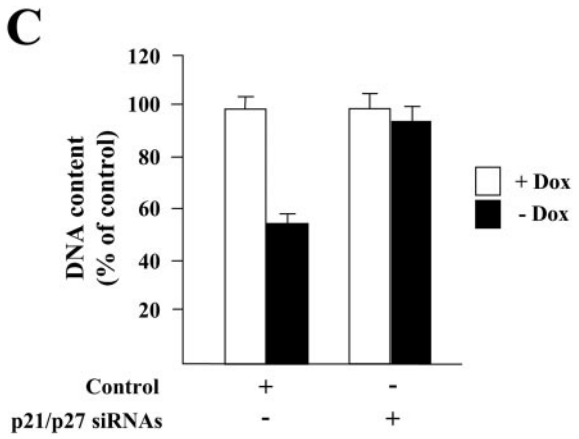

FIG. 5—*Continued*.

involved in the induction of both CDKIs by TReP-132 were further investigated. Luciferase reporter constructs controlled by the promoter regions of p21 (p21Luc, from bp −2320 to +16) and p27 (p27Luc, from bp −3568 to +12) were transfected in HeLa cells in the presence of increasing amounts of the pcDNA3-TReP-132 expression vector (Fig. 6A). Transfection of TReP-132 resulted in a dose-dependent stimulatory effect on both promoters, demonstrating that TReP-132 regulates gene expression of these CDKIs at the transcriptional level.

Previous reports have identified six proximal Sp1-binding sites in the −117/−51 region of the p21 promoter and two sites at positions −544 and −536 of the p27 promoter, which constitutively bind Sp1 (3, 38) and play major roles in the regulation of these genes (see, for example, references 14, 50, and 59 for p21 and references 38, 48, and 66 for p27). To investigate whether TReP-132-mediated activation of the p21 and p27 gene promoters occurs via these Sp1 sites, the p21 and p27 reporter constructs containing wild-type, mutant, or deleted Sp1 response elements were transiently cotransfected with Sp1 and/or TReP-132 expression vectors in HeLa cells (Fig. 6B and C). Interestingly, whereas the activities of the −2320p21Luc and −3568p27Luc constructs were induced in the presence of Sp1 and TReP-132 alone, the greatest increases in promoter activities were obtained when both transcription factors were cotransfected. The shorter −154p21Luc construct, in which the Sp1-binding element is still present, was also cooperatively activated by Sp1 and TReP-132, but the response to Sp1 and/or TReP-132 was abolished when the Sp1-binding elements were deleted (Fig. 6B). Importantly, whereas the −93p21Luc construct was activated by Sp1 and/or TReP-132 comparable to the −2320p21Luc construct, mutations of Sp1-3 or Sp1-4 sites, respectively, abolished or markedly reduced induction of the promoter by both factors (Fig. 6B, lower panel). Similarly, whereas the construct driven by the first −549 bp of the p27 promoter was still activated by Sp1 and/or TReP-132, further 5′ deletion of the promoter that removed the Sp1 sites resulted in a lack of activation by either transcription factor alone or together (Fig. 6C). Sp1 and TReP-132 also failed to activate the −3568p27 promoter in which either the Sp1-1 or the Sp1-2 site was mutated (Fig. 6C). Overall, these results indicate a cooperative interaction between TReP-132 and Sp1 at proximal Sp1-binding sites of both the p21 and the p27 gene promoters.

To determine whether TReP-132 interacts with the proximal Sp1-binding regions in intact cells, ChIP experiments were performed in HTO-GFP/TReP cells with an anti-Flag antibody to immunoprecipitate the Flag-TReP-132 fusion protein (Fig. 6D). The genomic DNA regions encompassing the Sp1 elements of both the p21 and the p27 genes were immunoprecipitated in HTO-GFP/TReP cells induced for TReP-132 expression but not in HTO-GFP cells. PCR amplification with primers covering either a region ~1-kb upstream of the proximal Sp1-binding regions in the p21 and p27 genes or primers specific for the β-actin gene did not result in any significant signal, thus demonstrating the specificity of immunoprecipitation and PCR amplification reactions. Thus, TReP-132 is part of complexes bound to the proximal Sp1-binding regions in the p21 and p27 gene promoters.

To determine whether TReP-132 directly binds to these Sp1 elements, electrophoretic mobility shift assays were performed. However, the formation of a protein-DNA complex was not detected (data not shown). To further test the ability of TReP-132 to bind to Sp1, a pull-down assay was performed with the GST or GST-TReP-132 fusion proteins and in vitro produced radiolabeled Sp1 protein. Whereas the GST protein did not interact with Sp1, a strong protein-protein interaction was observed between Sp1 and the GST–TReP-132 fusion protein (Fig. 6E). Altogether, these results indicate that TReP-132 activates the promoter through direct interaction with the DNA-binding protein Sp1.

TReP-132 gene expression is a growth-inhibitory factor of mammary cancer cells. Next, we addressed the putative role of TReP-132 in the breast cancer, taken as an example of steroid-dependent cancer. As a first approach, relative expression levels of TReP-132 were examined in several types of human epithelial breast cancer cell lines, which have been proposed to appropriately reflect the characteristic features of the human tumors from which they are derived (46). Both the primary and the metastatic cancer cell lines express TReP-132, but lower levels are found in the three highest proliferating cell lines (Fig. 7). Interestingly, TReP-132 expression is very low in the SK-BR-3 and MDA-MB-453 cells, which produce high levels of c-erb-2 (36, 45), a factor positively correlated with tumor aggressiveness (24, 69). In addition, significant levels of TReP-132 are found in all cell lines that express ER and PR, which are currently used as predictive markers of favorable disease prognosis (10, 67) and response to hormonal therapy (34, 35).

To assess the influence of TReP-132 on mammary cell growth, endogenous TReP-132 was decreased by using the siRNA approach in MDA-MB-231 and T-47D cells, which are negative and positive, respectively, for expression of the ER and the PR (46). Similarly, as in HeLa cells, silencing of TReP-132 expression by siRNA provoked an increase of cell proliferation in both cell lines (Fig. 8). Since the regulation of p21 and p27 has been reported to play a key role in the steroid regulation of T-47D cell growth (31, 56, 57) and since TReP-132 regulates the expression of these two genes in HeLa cells, the effects of TReP-132 knockdown on the expression of both genes were also analyzed. The decrease in TReP-132 expression resulted in a significant downregulation of both p21 and

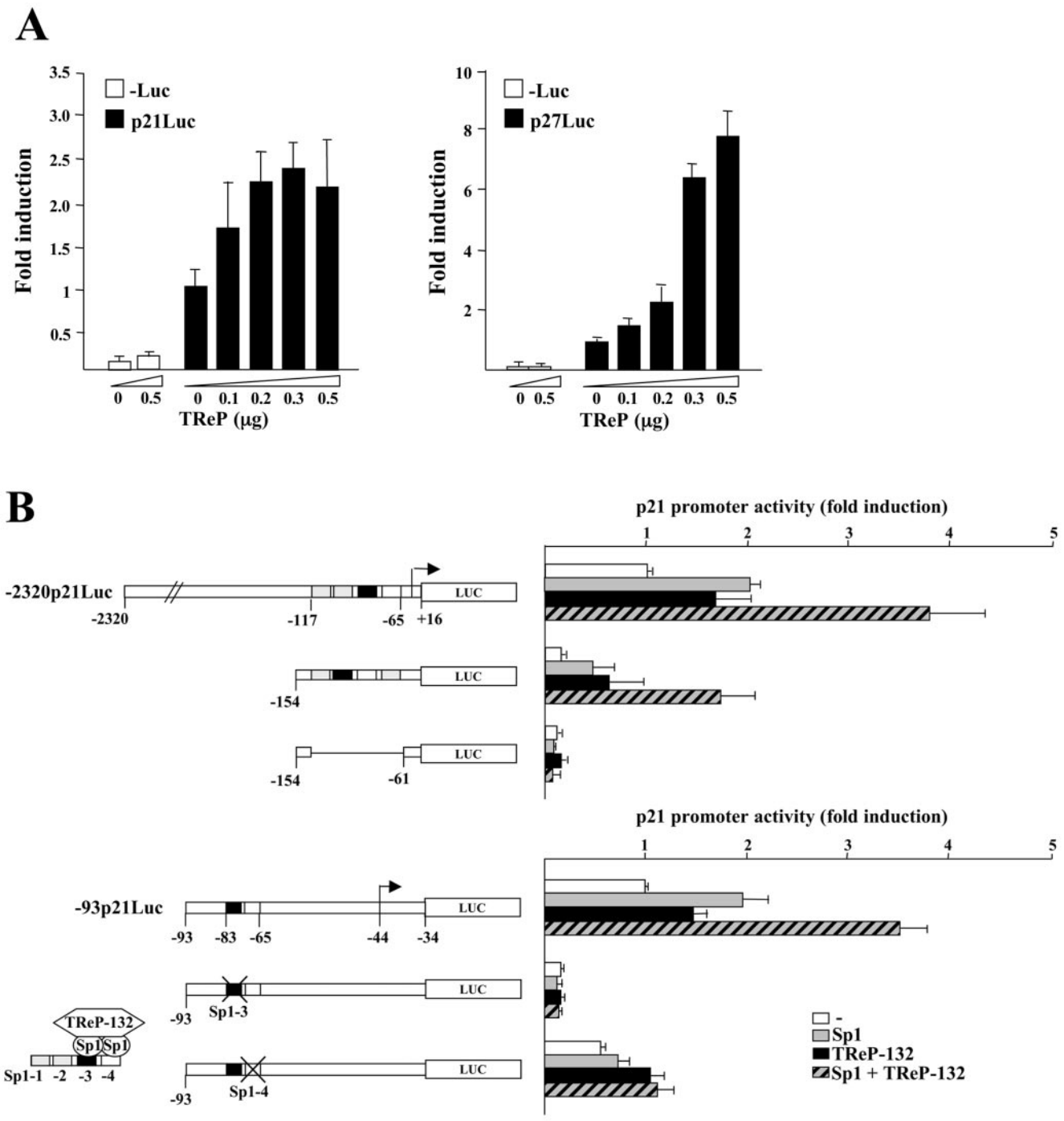

FIG. 6. TReP-132 enhances p21 and p27 promoter activities through interaction with Sp1 in HeLa cells. (A) Regulation of the p21 and p27 gene promoters by TReP-132. HeLa cells were transiently cotransfected with the p21Luc and p27Luc reporter constructs and increasing amounts of the pcDNA3-TReP-132 expression plasmid. Basal reporter activities were arbitrarily set to 1. (B and C) Cooperation between TReP-132 and Sp1. Luciferase reporter constructs driven by the indicated p21 (B) or p27 promoter (C) deletion or mutation fragments were each cotransfected in HeLa cells with Sp1 (0.1 μg) and/or TReP-132 (0.3 μg) expression plasmids. The results represent the fold increase (mean ± SD) with the luciferase activity levels of the −2320p21Luc, −93p21Luc, or −3568p27Luc promoter constructs arbitrarily set as 1. The arrows indicate the transcription start sites. (D) ChIP assay. Soluble chromatin was prepared from HTO-GFP or HTO-GFP/TReP cells incubated with or without Dox during 48 h. Immunoprecipitations were performed with an anti-Flag antibody. Control PCRs were done without DNA (H_2O) or with nonimmunoprecipitated genomic DNA (input). DNA extractions were amplified by using primer pairs covering either the −117/−65 p21 gene promoter (top panel), a distal region of the p21 gene located ~1 kb from this element, the −549/−511 p27 gene promoter, a distal region of the p27 gene located ~1 kb from this element, or a β-actin gene region (*lower panel*). (E) Pull-down assay. GST-TReP-132 was incubated with ^{35}S-labeled Sp1 and immobilized on glutathione-coupled Sepharose. The specificity of the interaction was assessed by comparison with background levels obtained by incubation of GST alone with ^{35}S-labeled Sp1. The lane denoted Sp1 input contains one-tenth the amount of radioactive Sp1 protein used in all incubations.

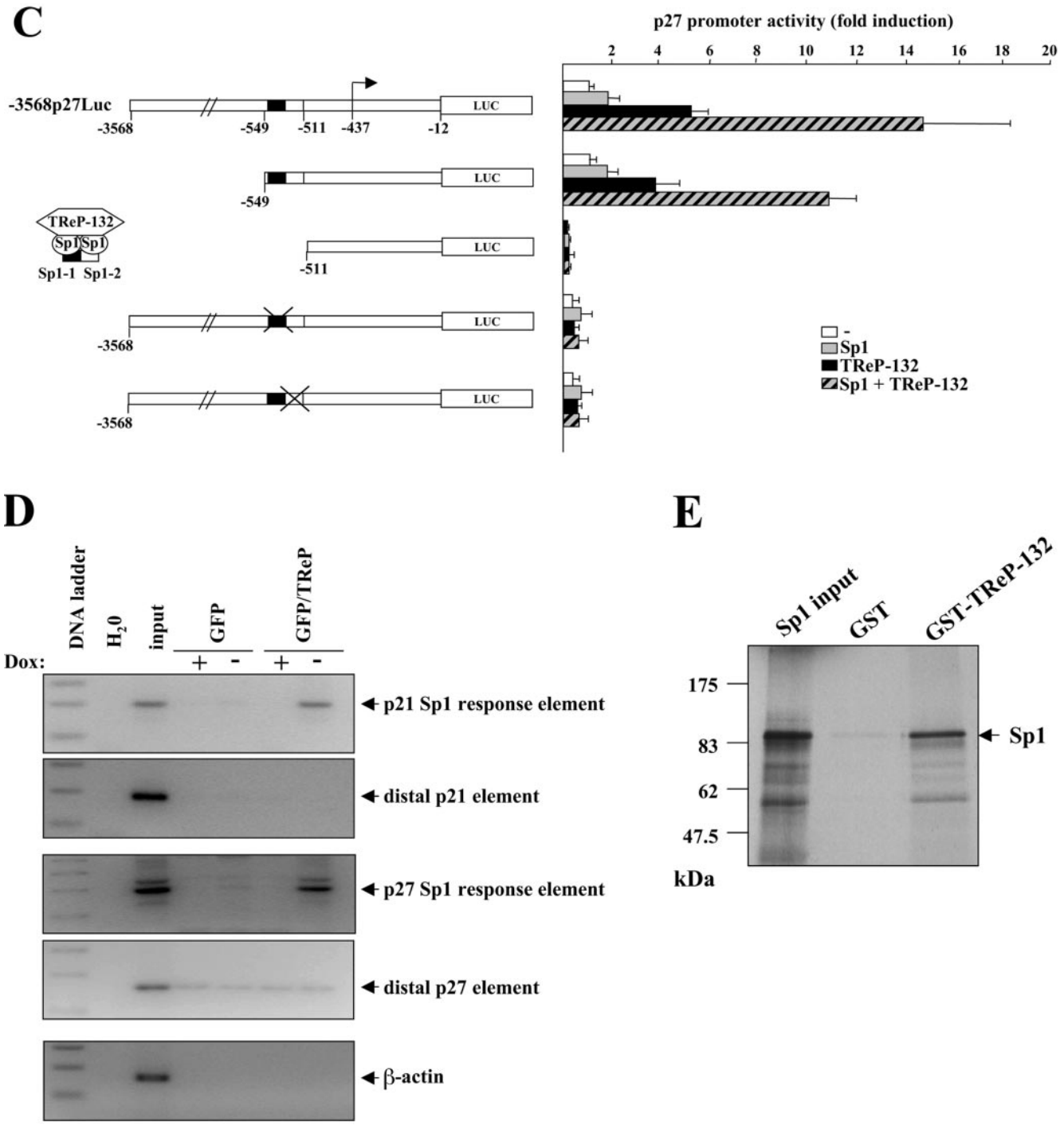

FIG. 6—*Continued*.

p27 mRNA. Together, these data indicate that TReP-132 controls positively p21 and p27 gene expression and influences negatively breast cancer cell proliferation.

TReP-132 expression is associated with lower breast tumor incidence and aggressiveness in humans. To further ascertain whether TReP-132 expression is associated with clinical prognostic factors of mammary tumors, TReP-132 mRNA levels were measured and correlated with their clinical, histological, and biological parameters in a pilot study with 88 unselected primary human breast tumor samples. Interestingly, TReP-132 mRNA levels were markedly lower in tumor tissues compared to levels in normal epithelium tissues. Moreover, TReP-132 mRNA levels in the tumor tissues ranged from 10 to 859% of the mRNA levels found in T-47D cells (Fig. 9). The expression level of TReP-132 in the different tumor samples did not follow a Gaussian curve, since the majority of tumors expressed very low TReP-132 mRNA levels (Fig. 9). In fact, whereas the mean of TReP-132 expression in the samples was 173% of mRNA level in

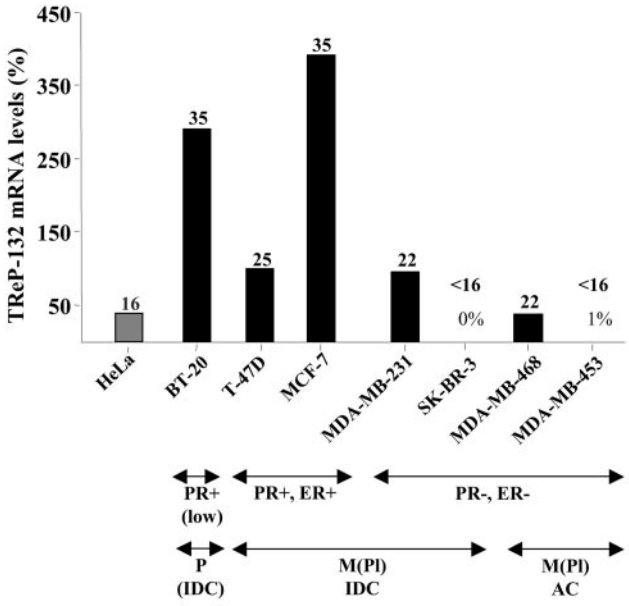

FIG. 7. TReP-132 mRNA levels in human breast cancer cell lines. TReP-132 mRNA levels normalized to 28S rRNA were measured by quantitative PCR in human mammary epithelial cancer cell lines and expressed as percentage relative to the levels obtained in T-47D cells. The approximate doubling times (hours) and a few main characteristics (46) of the studied cell lines are indicated on the bar and below the graph, respectively. The relative expression level of TReP-132 in SK-BR-3 and MDA-MB-453 cells is indicated as a percentage. P, primary; M(Pl), metastasis from pleural effusion; IDC, invasive ductal carcinoma; AC, adenocarcinoma.

T-47D cells, the median value of TReP-132 mRNA levels was pronouncedly lower at 117%. This indicates that tumor incidence is inversely related to *TReP-132* expression. Among the tumor samples, 75 and 73% were ER and PR positive, respectively, and the classical correlation between ER and PR expression ($P < 0.001$, $r = 0.598$) was observed (63). Interestingly, TReP-132 transcript levels were positively correlated to ER ($P < 0.001$, $r = 0.454$) and to PR ($P = 0.011$, $r = 0.274$) levels (Table 1). In contrast, TReP-132 mRNA levels were negatively correlated with the histoprognostic grade ($P = 0.003$, $r = -0.332$), a parameter based on histological criteria such as cell differentiation, mitotic activity, and degree of nuclear polymorphism, allowing classification of tumors from best to worse prognosis (5). Moreover, TReP-132 gene expression correlated also with p21 and p27 levels (Table 1). Together, these observations suggest that TReP-132 expression may negatively influence tumor formation and development in humans.

DISCUSSION

Whereas TReP-132 was previously reported to act as a coactivator of SF-1 to modulate steroid synthesis in adrenal cells (27, 28), we now identify a function for TReP-132 in the control of cell growth. This novel function for TReP-132 was characterized by using as models HeLa cells, which do not express steroid receptors (data not shown), have a high proliferation rate (with a doubling time of ~16 h), and express relatively low levels of TReP-132 (25, 26), as well as several breast cancer cell types. Interestingly, our results show that in both steroid-responsive and unresponsive human cancer cell lines, TReP-132 expression negatively controls cell proliferation and positively regulates levels of the CDKIs p21 and p27, indicating that TReP-132 acts as a basal cell growth inhibitory protein. The antiproliferative activity of TReP-132 thus occurs at least in part via an increased expression of p21 and p27 and consequent inhibition of certain CDK activities involved in phosphorylation of pRB and histone H1 in the G_1 phase. Consistent with the growth-inhibitory function of TReP-132 in vitro, a study of TReP-132 expression levels in human breast cancer biopsies provided supportive data for a potential negative role of this factor on mammary tumor formation and development in humans. TReP-132 expression was negatively correlated with histoprognostic grading, an indicator of unfavorable prognosis (11), whereas it was positively correlated with the expression status of PR, ER, and p27, which are predictive markers of good disease prognosis (8, 10, 64, 67). Interestingly, TReP-132 gene expression was also correlated with key markers of tumor aggressiveness. Indeed, whereas TReP-132 gene expression in human tumors was positively associated with c-erb-3 ($P < 0.001$, $r = 0.349$) and c-erb-4 ($P < 0.001$, $r = 0.456$) levels (data not shown), which are recently identified markers for good recovery (42, 61), TReP-132 expression was negatively related with the level of EGFR ($P < 0.001$, $r = 0.420$) (data not shown), which is a marker of poor recovery (42, 61). Additional studies are thus warranted to ascertain the prognostic value of TReP-132 expression in primary breast cancer, in terms of either patient survival or relapse free survival.

Interestingly, the expression of ER, PR, and c-erb-4 is positively correlated with the level of differentiation of normal and carcinogenic tissues (53, 75, 76), and high histoprognostic grading is associated with tumor cell dedifferentiation (11). This suggests that TReP-132 expression is also associated with cell differentiation. In line with this, previous studies suggested that TReP-132 activity may be involved in adrenal differentiation of the zonae fasciculata and reticularis in the adrenal cortex (28). It is noteworthy that upregulation of p21 and p27 have been reported to act as molecular switches to facilitate hormone-induced cell differentiation (38). In breast tissue, induction of p21 and p27 levels is thought to be the molecular basis for the blockage in the alveolar differentiated state (54, 68). Thus, since TReP-132 upregulates p21 and p27 expression, it is tempting to speculate that TReP-132 may act as a differentiation factor, notably in breast epithelial cells.

Based on the location of the human *TReP-132* on chromosome 6p21.1-p12.1 (17, 25, 72), it is interesting to hypothesize that mutations leading to decreased expression or inactivation of TReP-132 could be associated with tumor development. Chromosome 6 is the fourth most frequently rearranged chromosome in human tumors (79), and the 6p21 locus is a hot spot for mutations associated with immortalizing events in several cancers. Notably, genetic alterations in the 6p21.1 locus have been reported in breast carcinomas (9, 13), and allelic loss at 6p21.1 was shown to accompany the acquisition of tamoxifen resistance by MCF-7 cells (1). Moreover, it has been suggested that the gene encoding the putative glycosylphosphatidylinositol (GPIM) protein, which colocalizes with TReP-132 in the p21.1 region, might be involved in tumor suppression (15). Thus, considering the mapping of *TReP-132* in a region frequently rearranged in human tumors and its role in the regu-

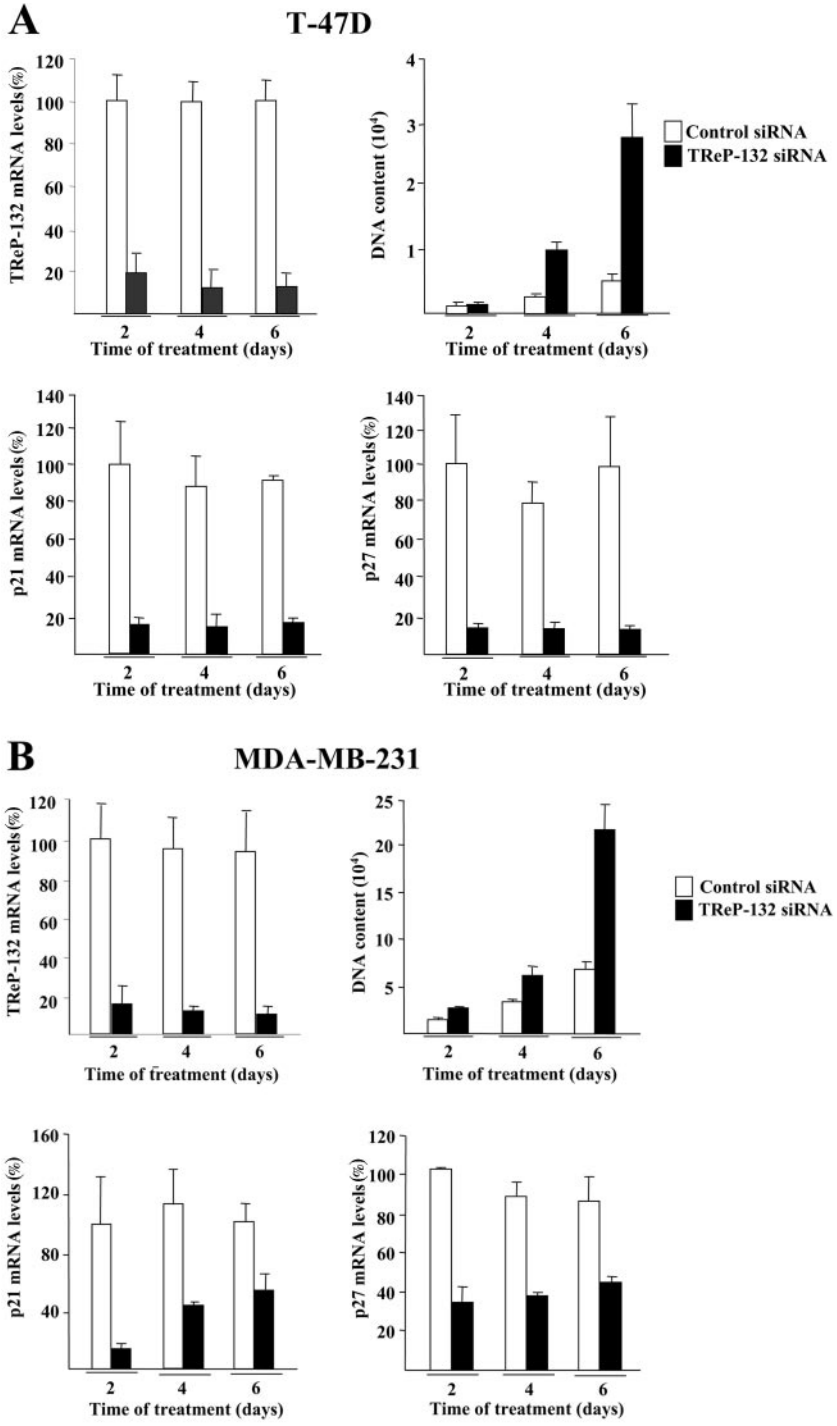

FIG. 8. siRNA silencing of TReP-132 increases breast cancer cell proliferation and decreases p21 and p27 mRNA levels. (A and B) siRNA (700 ng) transfection was performed as described in Fig. 2 in T-47D (A) and MDA-MB-231 (B) cells. DNA content as well as TReP-132, p21, and p27 mRNA levels were determined at the indicated times. DNA content values are expressed as means ± SD (arbitrary units) of one representative experiment performed three times in triplicate. The mRNA levels values are expressed relative to the amounts in control siRNA-transfected cells harvested at 2 days.

lation of cell proliferation, it is possible that *TReP-132* is a tumor suppressor gene candidate. Analyses of potential correlations between mutations of *TReP-132* and familial predisposition to cancer and tumoral development, notably in breast tissue, will be decisive to clarify this point.

In agreement with the previous demonstration that TReP-132 functions as a transcriptional regulating protein (26), TReP-132 positively regulates the expression of both CDKIs p21 and p27 at the transcriptional level in HeLa, as well as in breast cancer T-47-D and MDA-MB-231 cells. However, it

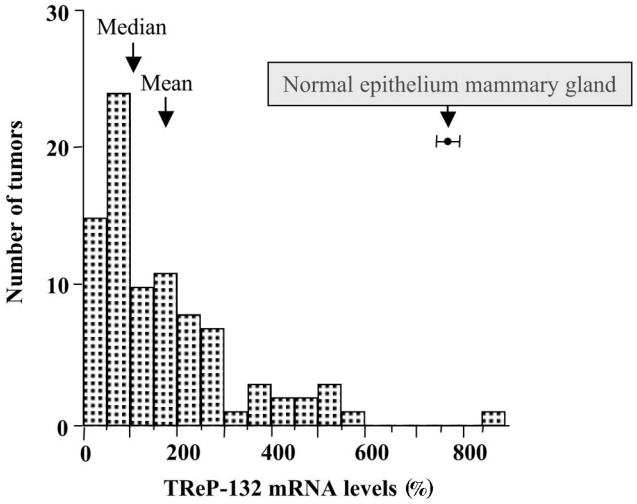

FIG. 9. Analysis of TReP-132 mRNA expression in human breast cancer biopsies. TReP-132 mRNA levels normalized to 28S rRNA were measured by quantitative PCR in 88 breast tumor biopsies and are expressed as percentages relative to the levels obtained in T-47D cells. The number of tumor samples with a given level of TReP-132 mRNA is shown in the *y* axis. The mean value of TReP-132 RNA levels in normal epithelium mammary gland ($n = 5$ individuals) is indicated.

cannot be excluded that TReP-132 could also regulate CDKI expression through indirect mechanisms. Notably, since p27 degradation through the ubiquitin-mediated proteasome pathway constitutes a key mechanism in the regulation of p27 protein levels (60), it is possible that TReP-132 also controls the expression of factors involved in this pathway. Nonetheless, from our data it is clear that TReP-132 controls *p21* and *p27* gene expression by activating their promoters through specific Sp1 DNA-binding regions, most likely by forming protein complexes with Sp1. In agreement with this observation, in gel shift analyses TReP-132 alone was unable to bind the double-stranded oligonucleotides encompassing the proximal Sp1-binding regions of the p21 and p27 promoters, but its coincubation with Sp1 had a positive effect on the binding of Sp1 (data not shown). Therefore, the physical interaction of TReP-132 with Sp1 may stabilize the tethering of Sp1 to promoters, which constitutes a mechanism often reported for signaling pathways involving Sp1 (84). Furthermore, the primary structure of TReP-132 contains putative domains for binding to the RNA polymerase II transcriptional initiation complex (26), notably a glutamine-rich domain via which it may interact with the TATA-binding protein-associated factor $dTAF_{110}$ (20). Interestingly, $dTAF_{110}$ has been suggested to mediate Sp1 transcriptional activation of TATA-less genes (such as p27 [84]) or genes that do not require a functional TATA box for promoter activity (such as p21 [59]). Therefore, in agreement with the finding that the TReP-132-responsive elements are localized in the vicinity of the transcription start sites of the p21 and p27 genes, it is possible that TReP-132 acts as a cointegrator in the regulation of the p21 and p27 promoters by interacting with components of the basal transcription machinery acting in concert with Sp1. Further studies are necessary to determine whether TReP-132 controls, in addition to p21 and p27, the expression of other cell cycle proteins containing also functional proximal Sp1 sites in their promoters, such as cyclin D1 (7).

It is noteworthy that p21 and p27 play major roles in the regulation of mammary cell proliferation by steroids or steroid antagonists (e.g., (12, 65, 77, 82). Notably, the proximal Sp1-binding regions of p21 are involved in mediating signals from progesterone (38). On the other hand, estrogens regulate cell cycle regulatory genes other than p21 and p27 through Sp1 sites, such as the cyclin D1 (7) and the c-*fos* (19) genes. Since TReP-132 can act as a nuclear receptor cofactor (26, 27), it will be interesting to determine whether it can also act as a coregulator for other nuclear receptors, such as PR and ER, in the control of cell cycle regulatory genes. In such a case, TReP-132 would act also as an integrator of the steroid effects on cell proliferation of steroid-responsive tissues, such as the breast, in addition to control basal cell cycle progression.

Taken together, the results of the present study identify a role for TReP-132 in the control of cell proliferation. Concurring with this, elevated levels of TReP-132 are correlated with lower aggressiveness of human mammary tumors. The growth-inhibitory function of TReP-132 finds its molecular basis in its ability to interact with general transcription regulators (e.g., Sp1, CBP/p300) (26) on specific cell cycle regulating gene promoters (e.g., p21 and p27). The ability of TReP-132 to influence steroid hormone production and to regulate the expression of genes involved in cell cycle progression makes it a potential key factor in different disorders related to cell proliferation. Further analysis of the role of TReP-132 to regulate the formation of multiprotein complexes formed with the PR or ER at Sp1-binding sites on certain key cell cycle gene promoters could be a promising approach to understand the molecular mechanisms controlling cell growth in steroid-responsive tissues.

TABLE 1. Relation (χ^2 test) between normalized expression of TReP-132 and clinical, histological, and biological parameters, as well as p21 and p27 mRNA levels[a]

Parameter	*P*	*r*
Age	0.748	0.035
Histoprognostic grading	**0.003**	**−0.332**
Node involvement	0.703	0.41
Tumor size	0.820	−0.37
ER	**<0.001**	**0.454**
PR	**0.011**	**0.274**
p21	**0.016**	**0.259**
p27	**0.004**	**0.31**

[a] Values in boldface are significant.

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