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ORIGINAL PAPER

Tumor suppressor p53 inhibits transcriptional activation of invasion gene thromboxane synthase mediated by the proto-oncogenic factor ets-1

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Cancer formation and progression is a complex process determined by several mechanisms that promote cell growth, invasiveness, neo-angiogenesis, and render neoplastic cells resistant to apoptosis. The tumor suppressor p53 and the proto-oncogenic factor ets-1 are important regulators of such mechanisms. While it is well established that p53 and ets-1 influence various aspects of cell behavior by regulating the transcription of specific genes, little is known about the functional relationship between these transcription factors. We found that the gene encoding thromboxane synthase (TXSA), which we recently identified as a factor promoting invasion and resistance to apoptosis in gliomas, is a novel target gene for both p53 and ets-1. We demonstrate that p53 and ets-1 coregulate TXSA in an antagonistic and interrelated manner, with ets-1 being a potent transcriptional activator and p53 inhibiting ets-1-dependent transcription. Negative interference with ets-1 transcription requires functional p53 and is lost in mutant p53 proteins. We show that ets-1 and p53 associate physically *in vitro* and *in vivo* and that their interaction, rather than a direct binding of p53 to the TXSA promoter, is required for transcriptional repression of TXSA by wild-type p53. An important implication of our findings is that the loss of p53-mediated negative control over ets-1-dependent transcription may lead to the acquisition of an invasive phenotype in tumor cells.

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Keywords: invasion genes; glioma; p53, ets-1, thromboxane synthase, transcription

Introduction

Metabolites of arachidonic acid influence various aspects of cellular behavior such as mitogenesis, cellular adhesion, invasion, and apoptosis (Bennett, 1986; Goetzl *et al*., 1995; Pica *et al*., 1996). Deregulation of the prostanoid synthesis plays an important role in pathogenesis and cancer progression (Surette *et al*., 1999). Prostanoid synthesis in neoplasms of breast, lung, colon, and also in neuroepithelial tumors of the central nervous system exceeds the levels of normal tissues (Bennett *et al*., 1977, 1987; Castelli *et al*., 1989). The initial step in the biochemical pathway of prostanoid synthesis is the release of arachidonic acid from cellular membranes mediated by phospholipase A_2. Arachidonic acid is metabolized by cyclo-oxygenases (COX) to prostaglandin H_2 (PGH_2), a precursor of various prostanoids. The inducible form of COX, COX-2, is not detectable in most normal tissues, but is found highly expressed in malignant tumors of several histotypes (Kargman *et al*., 1995; Sano *et al*., 1995; Ristimaki *et al*., 1997; Deininger *et al*., 1999). The overexpression of COX-2 inhibits intercellular adhesion, susceptibility to apoptosis (Tsujii and DuBois, 1995), and increases the angiogenic and invasive potential of neoplastic cells (Tsujii *et al*., 1997, 1998; Liu *et al*., 2000). These cellular phenotypes are associated with an increased tumorigenic potential, indicating an important role of the COX pathway in the biology of neoplasms. The role of COX-2 as a cancer-promoting gene is further supported by the fact that COX-2 expression is controlled by tumor suppressor genes, including p53, and also by oncogenes. p53 inhibits the transcription of COX-2 (Subbaramaiah *et al*., 1999), whereas viral and cellular oncogenes stimulate COX-2 expression (Kutchera *et al*., 1996; Subbaramaiah *et al*., 1996). It is thought that the build-up of arachidonic acid metabolites downstream of COX-2 may be one mechanism underlying the cancer-promoting effects of COX-2 (Daniel *et al*., 1999; Nie *et al*., 2000).

One of the downstream metabolites of COX is thromboxane A_2 (TXA-2), which is converted from PGH_2 by thromboxane synthase (TXSA). One of the physiological functions of TXA-2 is to promote platelet aggregation and vasoconstriction. It has recently emerged that TXSA and TXA-2 play an important role

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in the biology of neoplasms. The overexpression of TXSA for example is found in human astrocytic tumors, but not in quiescent human astrocytes (Giese *et al*., 1999). Furthermore, experimental overexpression of TXSA promotes tumor growth *in vivo*, which has been demonstrated in an adenocarcinoma mouse model (Pradono *et al*., 2002). Although the cancer-promoting effects of TXSA remain poorly understood, it appears that high levels of TXSA enhance angiogenesis and increase the invasive potential of neoplastic cells (McDonough *et al*., 1998; Giese *et al*., 1999; Rodrigues *et al*., 2001; Kurzel *et al*., 2002; Yoshizato *et al*., 2002). TXSA may also play a certain role in rendering tumor cells resistant to apoptosis, and we have recently reported that specific inhibitors of TXSA block motility and sensitize migration-arrested glioma cells to apoptosis (Yoshizato *et al*., 2002). The mechanisms that regulate TXSA expression are poorly defined. In this report, we demonstrate for the first time that the transcription factors p53 and ets-1 control TXSA expression in an antagonistic manner.

The regulation of transcription is a major molecular mechanism underlying the tumor-suppressing effects of the p53 protein. Genes activated by p53 include those that mediate apoptosis, DNA repair, cell cycle arrest, and control angiogenesis (el-Deiry *et al*., 1993; Van Meir *et al*., 1994; Macleod *et al*., 1995; Levine, 1997; Wang and Ohnishi, 1997; Gottlieb and Oren, 1998; Ryan *et al*., 2001; Vousden and Lu, 2002), whereas antiapoptotic and pro-proliferative genes are suppressed by p53 (Modugno *et al*., 2002; Zhou *et al*., 2002).

The proto-oncogene *ets-1* belongs to the ETS family of transcription factors (Wasylyk *et al*., 1998; Pastorcic and Das, 2000; Wasylyk *et al*., 2002), and plays an important role in cancer progression due to its ability to activate transcription of metastasis-, angiogenesis- and invasion-associated genes (Watabe *et al*., 1998; Valter *et al*., 1999; Fenrick *et al*., 2000; Behrens *et al*., 2001; Kita *et al*., 2001). Indeed, the overexpression of ets-1 found in different human cancers is associated with invasiveness and the degree of malignancy, thus defining ets-1 as a potential target in anticancer therapy (Watabe *et al*., 1998; Nakada *et al*., 1999; Kitange *et al*., 2000; Jiang *et al*., 2001).

In this study, we demonstrate that ets-1 is a potent transcriptional activator of the *TXSA* gene *in vitro* and *in vivo*. The ets-1-dependent transcriptional activation of *TXSA* is under the tight control of p53, which inhibits *TXSA* induction mediated by ets-1. The ability to interfere with ets-1-mediated transcription requires a wild-type p53 protein and is lost in mutant p53 proteins. We show here that p53 and ets-1 proteins physically associate *in vitro* and *in vivo* and that the regulatory C-terminal domain of p53 is required for the interaction between p53 and ets-1 proteins. Our findings provide new mechanistic insights into transcriptional repression mediated by p53, and further underline the importance of protein–protein interactions for this activity of p53.

Results

The TXSA gene is positively regulated by the transcription factor ets-1

To gain an insight into the regulation of *TXSA*, we analysed the $-306/+12$ region of the minimal *TXSA* promoter for the presence of putative elements that may regulate the *TXSA* transcription. The analysis showed several GGAA/T motives corresponding to ETS-binding sites (EBSs) recognized by ETS transcription factors, and several motives closely resembling the p53 consensus (Figure 1). Considering that one of the ETS family members, ets-1, regulates the transcription of a number of invasion/apoptosis-related genes (Sementchenko and Watson, 2000), we reasoned that *TXSA*, which we recently identified as an invasion-promoting and antiapoptotic gene (Yoshizato *et al*., 2002), may also be a target gene of ets-1. To test this hypothesis, we transiently transfected SaOs-2 cells with (−306)TXSA-Luc DNA expressing fire fly luciferase under the control of the minimal *TXSA* promoter, alone or cotransfected with a pcDNA/4TO/*c-ets-1* plasmid expressing recombinant human ets-1 protein (Yamamoto *et al*., 2000). The results show that ets-1 stimulated the *TXSA* promoter in a dose-dependent and specific manner (Figure 2a), whereas neither the *p21* promoter activity (Figure, p21-Luc) nor the background activity of the $pGL3_{basic}$ vector (data not shown) was affected by ets-1. We constructed a set of unidirectional deletion mutants of the *TXSA* promoter (Figure 2b) to identify regions essential for transactivation mediated by ets-1. Reporter assays showed that the deletion of the $-306/-200$ region reduced the responsiveness of the *TXSA* promoter to ets-1 by 25% in (−200)TXSA-Luc DNA compared to the parental (−306)TXSA-Luc DNA (Figure 2c). Further deletion of the $-200/-128$ region did not alter the responsiveness of the *TXSA* promoter to ets-1, whereas the deletion of additional 49 bp upstream from the −79 position completely abrogated the ets-1 transactivation (constructs (−128)TXSA-Luc

Figure 1 Schematic presentation of the $-306/+1$ region of the human TXSA promoter. The sequences in lower-case letters correspond to eight putative EBSs. The putative p53 cognate motifs are boxed. The major transcription initiation site identified by Miyata *et al*. (1994) is designated as '+1'

and (−79)TXSA-Luc, respectively). These data indicate that two regions, −306/−200 and −128/−79 of the *TXSA* promoter, contain regulatory sequence elements essential for the activation of the *TXSA* promoter by ets-1.

To substantiate the results of our *in vitro* studies, we examined the effects of ets-1 on *TXSA* expression *in vivo*. We analysed the steady-state levels of the *TXSA* mRNA by an RNAse protection assay (RPA) in SNB19/B3 cells (a clonal derivative of the glioblastoma cell line SNB19), which express recombinant ets-1 under the control of a tetracycline-inducible promoter (Yamamoto *et al*., 2000 and Figure 3a). Antisense RNAs were prepared and used as specific probes, which hybridize to *TXSA* or β-actin (internal control) mRNAs and protect dsRNA fragments of 238 and 127 bp, respectively, from RNase digestion (Figure 3b). The RPA experiments showed that induction of ets-1 upon tetracycline treatment led to a significant increase of the *TXSA* mRNA levels over baseline in SNB19/B3 cells (compare lanes 3 and 4 and Figure 3d). The effects cannot be attributed to nonspecific effects of the drug, because tetracycline treatment did not affect the *TXSA* mRNA levels in the parental SNB19 cells (lanes 1 and 2). Three independent experiments showed that induction of *TXSA* mRNA ranged from 1.5- to 2.8-fold in SNB19/B3 cells (not shown). It is possible that this experimental system underestimated the stimulation of the *TXSA* mRNA by ets-1, because we reproducibly observed that the basal level of the *TXSA* mRNA was significantly higher in SNB19/B3 cells compared to levels in parental SNB19 cells (compare lanes 1 and 3 in Figure 3c). One explanation could be that basal *TXSA* expression may be elevated in noninduced SNB19/B3 cells due to 'leaky' expression of ets-1 under tetracycline-free conditions (Figure 3a, compare lanes 2 and 4).

ets-1 is a DNA-binding protein that regulates transcription by specific binding to sequences containing a GGAA/T core usually found in ets-1-regulated promoters (Sementchenko and Watson, 2000). Both the −306/−200 and −128/−79 promoter regions contain putative ets-1 cognate motives (Figure 1, EBS-1, -2 and -5, respectively), suggesting that ets-1 may activate the *TXSA* promoter by binding to EBSs. To test this hypothesis, we examined by electrophoretic mobility shift assay (EMSA) whether ets-1 sequence-specifically binds to the *TXSA* promoter. Nuclear extracts prepared from SNB19/B3 cells, treated with tetracycline (high ets-1 amounts) or nontreated (low ets-1 amounts), were used as a source for ets-1 protein (Figure 4a). First we examined DNA binding with a PCR-derived and radioactively labeled DNA fragment, which represented

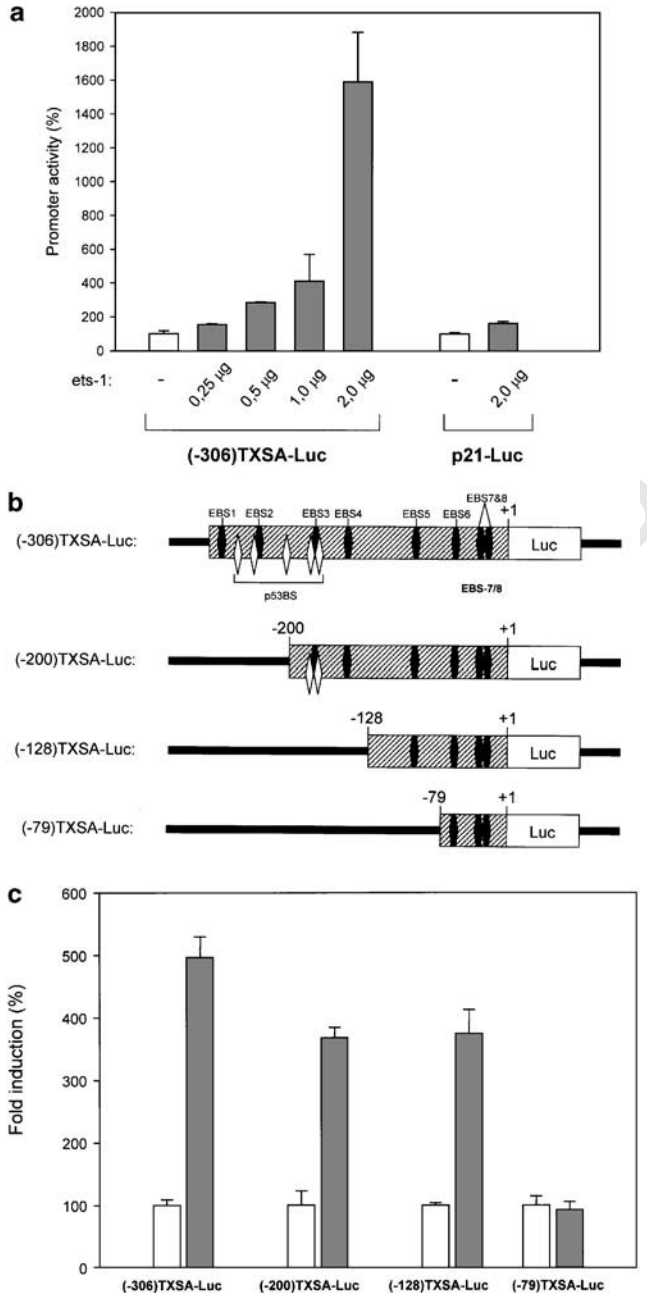

Figure 2 ets-1 activates the *TXSA* promoter *in vitro*. (**a**) SaOs-2 cells were transiently transfected with (−306)TXSA-Luc or p21-Luc reporter plasmids, alone or cotransfected with increasing amounts of pcDNA4/TO/c-*ets-1*. pUC18 DNA was used to maintain the total amounts of transfected DNA constant. The promoter activity is expressed relative to the activity of the TXSA-Luc DNA in the absence of ectopically expressed ets-1 taken as 100% (white bars). Values are the mean of at least three independent experiments, each performed in triplicate. The amounts of pcDNA4/TO/c-*ets-1* used per well of a six-well plate are indicated, the amount of reporter DNA was constant at 0.5 µg per well. (**b**) Reporter constructs used in the promoter studies. Different regions of the *TXSA* promoter (diagonally striped box) were cloned upstream from the luciferase coding sequence (Luc) of the promoterless vector pGL3basic. The black hexagons indicate the positions of eight putative ets-1-binding sites numbered arbitrarily (EBS 1–8). The white diamonds correspond to p53 consensus-like decamers. (**c**) Identification of the essential *TXSA* promoter regions for ets-1 transactivation. SaOs-2 cells were transiently transfected with reporter constructs of different *TXSA* promoter deletion mutants alone (white bars) or with pcDNA4/TO/c-*ets-1* DNA (gray bars). Transfections were performed as in Figure 2a, except that a constant amount of pcDNA4/TO/c-*ets-1* DNA (1.0 µg per well) was used. The promoter activity is expressed relative to the activity of the TXSA promoter constructs in the absence of ets-1

the −128/−79 region of the *TXSA* promoter and contained EBS 5–8 (Figure 1). Incubation of DNA with nuclear extracts produced very similar patterns of protein : DNA complexes with both, low ets-1 and ets-1-enriched nuclear extracts (Figure 4b, compare lanes 1 and 3, respectively). One complex that appeared to correlate with the amounts of the ets-1 protein in nuclear extracts (indicated by the arrowhead in lane 3) was very weak, precluding further analysis of its specificity for ets-1. Since the intrinsic DNA binding of ets-1 is weak (Li *et al*., 2000) and can be enhanced *in vitro* by ets-1-specific antibodies (Reisdorff *et al*., 2002), we also examined DNA binding in the presence of the ets-1-specific antibody C-20. In fact, the presence of the C-20Ab antibody resulted in the formation of a new complex (lanes 2 and 4, complex designated C-20). Significantly higher amounts of the C-20 complex were formed with ets-1-enriched nuclear extracts compared to those containing low amounts of ets-1 (compare lanes 4 and 2, respectively), indicating that the complex was formed by the ets-1 protein. We also analysed DNA binding with a larger fragment of the *TXSA* promoter containing the −306/−79 region of the promoter, which includes EBS 1–8 (Figure 1). Two complexes were formed with this DNA fragment after incubation with ets-1 enriched nuclear extracts in the presence of C-20Ab (Figure 4c, lanes 2–7). To test whether the complexes formed were specific for ets-1, we performed competition experiments with oligonucleotide DNAs that contained either the ets-1 consensus (ets-1_{CON}) or its mutated version (ets-1_{MUT}). The results showed that the presence of ets-1_{CON} (lanes 3 and 4), but not ets-1_{MUT} (lanes 5 and 6) competitor, led to a notable reduction of binding, indicating its specificity for ets-1. These results strongly support the hypothesis that ets-1 activates the *TXSA* promoter via EBSs.

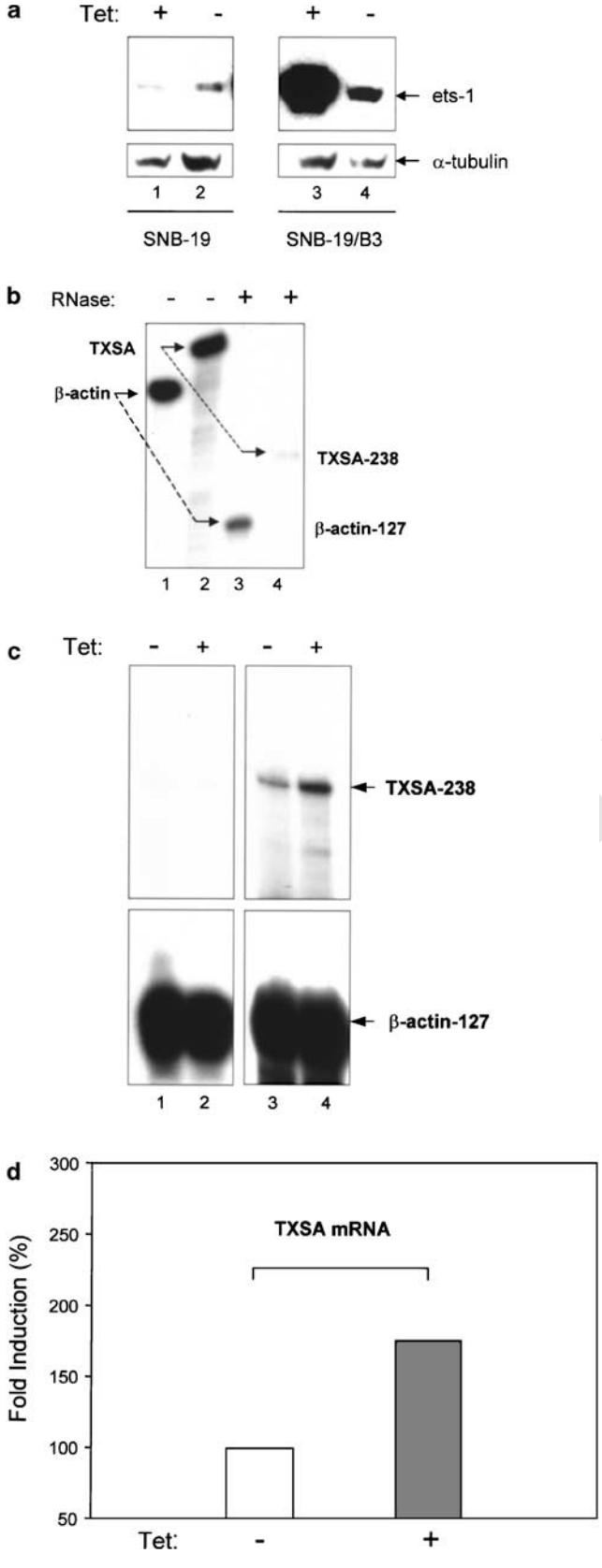

Figure 3 ets-1 stimulates *TXSA* expression *in vivo*. (**a**) Conditional expression of ets-1 in SNB19/B3 cells assessed by Western blot. The parental cell line SNB19 was used as a control. A 24 h treatment with tetracycline (1 μg/ml) induces ets-1 expression in SNB19/B3 cells (lanes 3 and 4), but not in parental SNB19 cells (lanes 1 and 2). (**b**) RNA probes used in RNAse protection experiments (RPA). Radioactively labeled β-actin- and TXSA antisense RNA probes (lanes 1 and 2) were prepared by *in vitro* transcription and hybridized with human RNA (20 μg, Ambion). The samples in lanes 3 and 4 (but not in lanes 1 and 2) were digested with RNAse after hybridization. RNAse digestion results in shortening of both RNA probes due to the presence of nonhybridized unspecific sequences. TXSA-238 and β-actin-127 represent specific regions in RNA probes that hybridize with target RNA and therefore become protected from digestion. (**c**, **d**) Analysis of the *TXSA* mRNA in SNB19 and SNB19/B3 cells by RPA. Radioactively labeled TXSA and β-actin antisense RNA probes were simultaneously hybridized with cell lysates from SNB 19 (lanes 1 and 2) and SNB19/B3 (lanes 3 and 4) cells. β-actin RNA levels served as an internal control to normalize the data. The upper panels show TXSA RNA at a longer exposure of the same gel showing β-actin RNA (lower panels). (**d**) densitometry quantification using TINA 2.0™ of the data shown in (**c**)

Wild-type p53 abrogates ets-1-dependent activation of the TXSA promoter

Sequence analysis of the *TXSA* promoter demonstrated the presence of several motives with a high degree of homology to the p53 consensus-binding site, suggesting

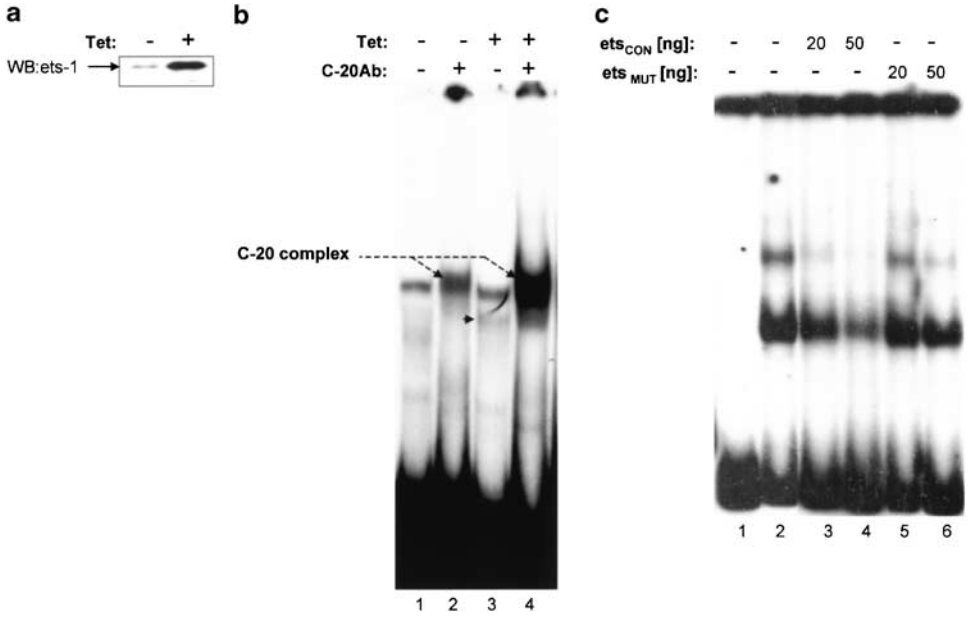

Figure 4 Analysis of ets-1 DNA binding by EMSA. DNA probes were prepared by PCR as described in Materials and methods using specific primers flanking the or −306/−79 regions of the *TXSA* promoter. (**a**) Western blot analysis of nuclear extracts prepared from SNB19/B3 cells grown in the absence or the presence of tetracycline with the ets-1-specific antibody C-20. A measure of 5.0 μg of total protein was loaded per lane. (**b**) EMSA with the radioactively labeled PCR fragment spanning the −128/−79 region of the *TXSA* promoter and nuclear extracts from SNB19/B3 cells. DNA (20 000 c.p.m.) was incubated with nuclear extracts (5.0 μg total protein per sample) with or without the addition of 2.0 μg of the C-20 antibody as indicated. The arrowhead (lane 3) indicates the complex formed with ets-1-enriched nuclear extracts in the absence of C-20Ab. The dotted arrows show the complex formed in the presence of C-20Ab. (**c**) EMSA using radioactively labeled PCR fragment spanning the −306/−79 region of the *TXSA* promoter and ets-1-enriched nuclear extracts from SNB19/B3 cells. DNA (20 000 c.p.m.) was incubated with nuclear extracts (5.0 μg total protein per sample) in the presence or absence of competitor DNA as indicated. The C-20 antibody (2.0 μg) was added to all samples

that p53 may be involved in transcriptional regulation of the *TXSA* promoter (Figure 1). We examined the effects of wild-type p53 on *TXSA* promoter activity by reporter assays in SaOs-2 cells (p53 null) transiently transfected with (−306)TXSA-Luc DNA alone or with ets-1 and/or p53 expression vectors. Figure 5a shows that the basal activity of the *TXSA* promoter was strongly inhibited by wild-type p53, but not by R175Y mutant p53. The *p21* promoter, a known target of p53, was strongly activated by p53, as expected. The *TXSA* promoter activity was also repressed by p53 in H1299 lung carcinoma cells and in G-130 glioblastoma cells (Anker *et al*., 1993), indicating that transcriptional regulation of the *TXSA* promoter by p53 is not restricted to a specific cell type (data not shown). Remarkably, wild-type p53 inhibited not only the basal but also the ets-1-stimulated activity of the *TXSA* promoter in a dose-dependent manner (Figure 5b), suggesting that p53 represses *TXSA* by interfering with the positive regulator ets-1. Again, mutant R175Y p53 did not inhibit ets-1-stimulated activity of the *TXSA* promoter, indicating that the inhibition requires wild-type p53 protein (Figure 5c).

To gain further insight into the mechanisms of transcriptional regulation of *TXSA*, we analysed the influence of p53 on ets-1-stimulated transcription in LNZ-2024 cells, a clone derived from LNZ-308 human glioma cells that conditionally expresses wild-type p53 in the presence of doxycycline (Albertoni *et al*., 1998, 2002). LNZ-2024 cells represent a suitable experimental system for studies of p53 transcriptional activities because treatment with doxycycline leads to p53 induction and activation of p53 transcriptional targets (Figure 6a, shown for MDM2 and Albertoni *et al*., 2002). We transfected LNZ-2024 with *c-ets-1* cDNA. One fraction of the sample was incubated with doxycycline to induce p53 and one fraction was kept under doxycycline-free conditions (no p53). The *TXSA* mRNA was analysed in transfected cells by RPA. We found that ets-1 expression in LNZ-2024 cells led to an increase of the *TXSA* mRNA levels by about 1.74-fold in the absence of p53 (Figure 6b, compare lanes 1 and 2 in the upper panel). It should be noted that this increase of endogenous *TXSA* mRNA was detectable under experimental conditions allowing transfection rates of approximately 20%. However, the induction of p53 in transfected cells prevented ets-1-dependent activation of endogenous *TXSA* (lane 3). These results add strong support to our promoter studies, and demonstrate that p53 blocks the transcriptional activation of *TXSA* mediated by ets-1.

Direct binding of p53 to the TXSA promoter DNA is dispensable for transcriptional inhibition of the TXSA gene

Direct binding to regulated promoters is indispensable for transcriptional activation mediated by p53. However, it is still unclear whether p53-mediated transcriptional repression requires the p53 DNA-binding activity. The presence of p53 consensus-like elements

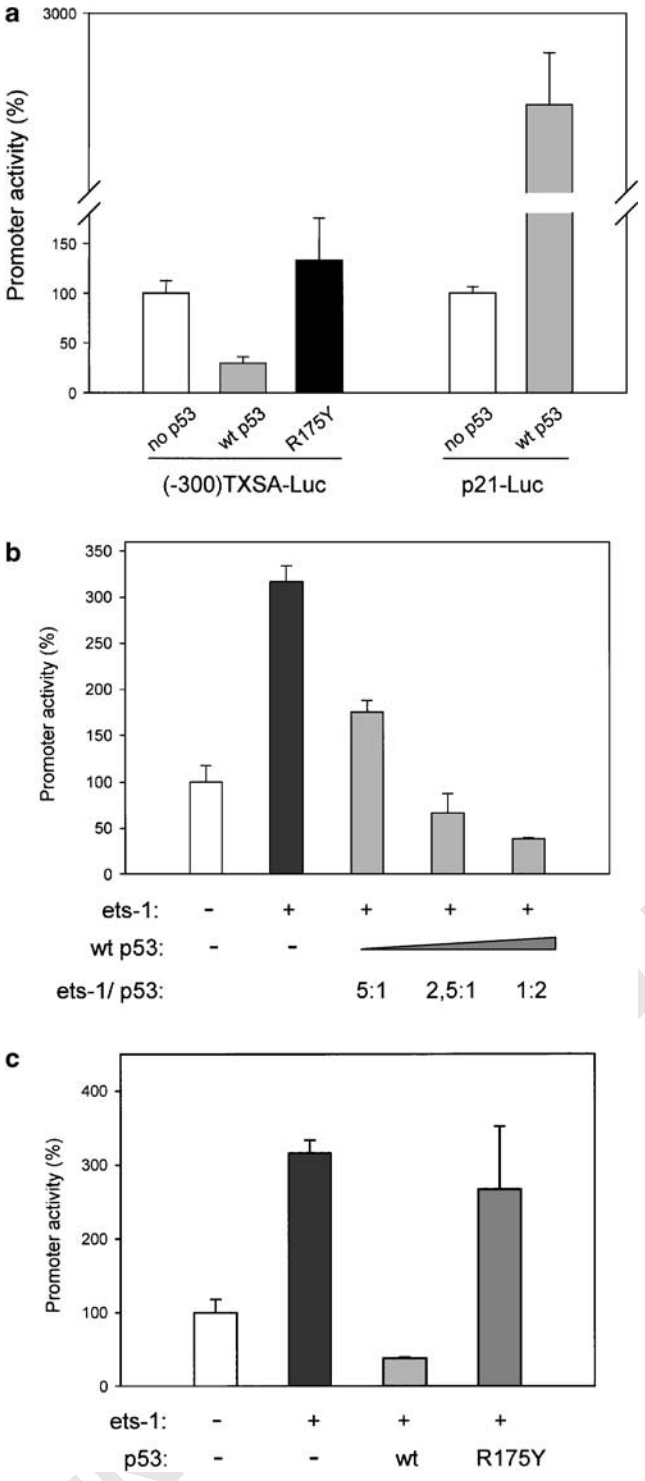

Figure 5 Wild-type p53 inhibits the basal and ets-1-stimulated activity of the *TXSA* promoter. (**a**) (−300) TXSA-Luc DNA was transiently transfected into SaOs-2 cells as described in Materials and methods alone or with expression vectors for wild-type or R175Y p53. p21-Luc DNA (Kim *et al*., 1999) expressing a luciferase gene under the control of the human p21 promoter was used as a positive control for p53-specific transcriptional activity. (**b**) SaOs-2 cells were cotransfected with (−300) TXSA-Luc DNA and pcDNA4/TO/c-*ets-1* DNA and increasing amounts of a wild-type p53 expressing vector. The total amounts of DNA transfected were kept constant using pUC18 DNA. (**c**) The transcriptionally impaired mutant R175Y does not inhibit the ets-1-mediated transactivation of the *TXSA* promoter

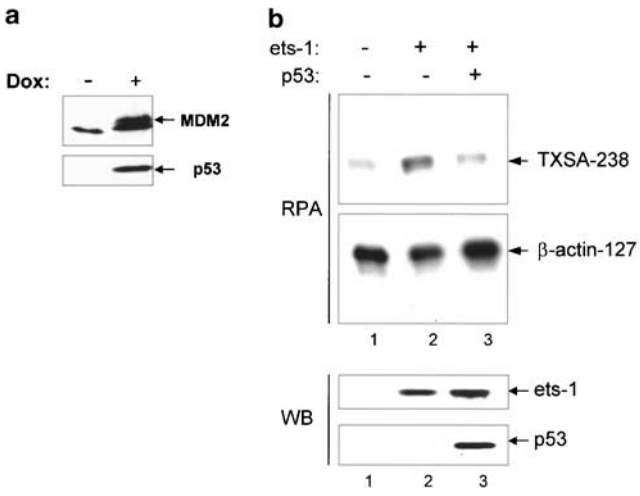

Figure 6 Wild-type p53 protein inhibits ets-1-dependent stimulation of *TXSA in vivo*. (**a**) Conditional expression of transcriptionally active p53 in glioma cell line LNZ-2024. Cell lysates were prepared from LNZ-2024 cells (Albertoni *et al*., 2002) grown in the absence or presence of doxycycline (1 μg/ml for 24 h). Doxycycline induces the expression of p53 and its transcription target MDM2. (**b**) RPA analysis of the *TXSA* mRNA from LNZ-2024 cells transiently transfected with pcDNA4/TO/c-*ets-1* DNA (lanes 2 and 3 in the upper panels) or mock-transfected DNA (lane 1). Transfected cells were incubated in the absence (lanes 1 and 2) or presence of doxycycline (lane 3). The lower panel designated 'WB' shows the analysis of cell lysates (50 μg protein) from transfected cells by Western blot with anti-ets-1 and anti-p53 antibodies

in the *TXSA* promoter suggested that transcriptional inhibition of *TXSA* may be mediated by direct binding of p53 to the *TXSA* promoter. We tested whether putative p53-binding sites in the *TXSA* promoter could bind p53 protein *in vitro*. Radiolabeled DNA fragments derived from the *TXSA* promoter containing the putative p53 binding sites were incubated with purified recombinant human p53 protein in the presence or the absence of p53-specific antibodies PAb421 or DO-1 (Figure 7, lanes 1–4). As a positive control, a DNA oligonucleotide containing the well-characterized p53-binding site from the *p21* promoter (p53BS-p21) was used (lanes 5–8). The results showed that recombinant p53 protein bound both the *TXSA* promoter fragment and p53BS-p21 in the presence of the C-terminal antibody PAb421 (Figure 7, compare lanes 2 and 6 with lanes 4 and 8, respectively). The effect of PAb421 was specific, because the N-terminal antibody DO-1 did not significantly stimulate the formation of a specific complex (lanes 3 and 7). Since the enhancement of DNA binding by PAb421 is a characteristic feature of p53 sequence-specific DNA binding under EMSA conditions (Hupp and Lane, 1994), these results seemed to support the idea that p53 consensus-like sequences may mediate transcriptional inhibition of the *TXSA* promoter by p53. To test whether the observed DNA binding of p53 correlates with the inhibition of the *TXSA* promoter by p53, we obtained several deletion mutants of the *TXSA* promoter that either lacked or contained p53 consensus-like sequences, and compared their responsiveness to p53 inhibition in reporter assay. Table 1 shows that the

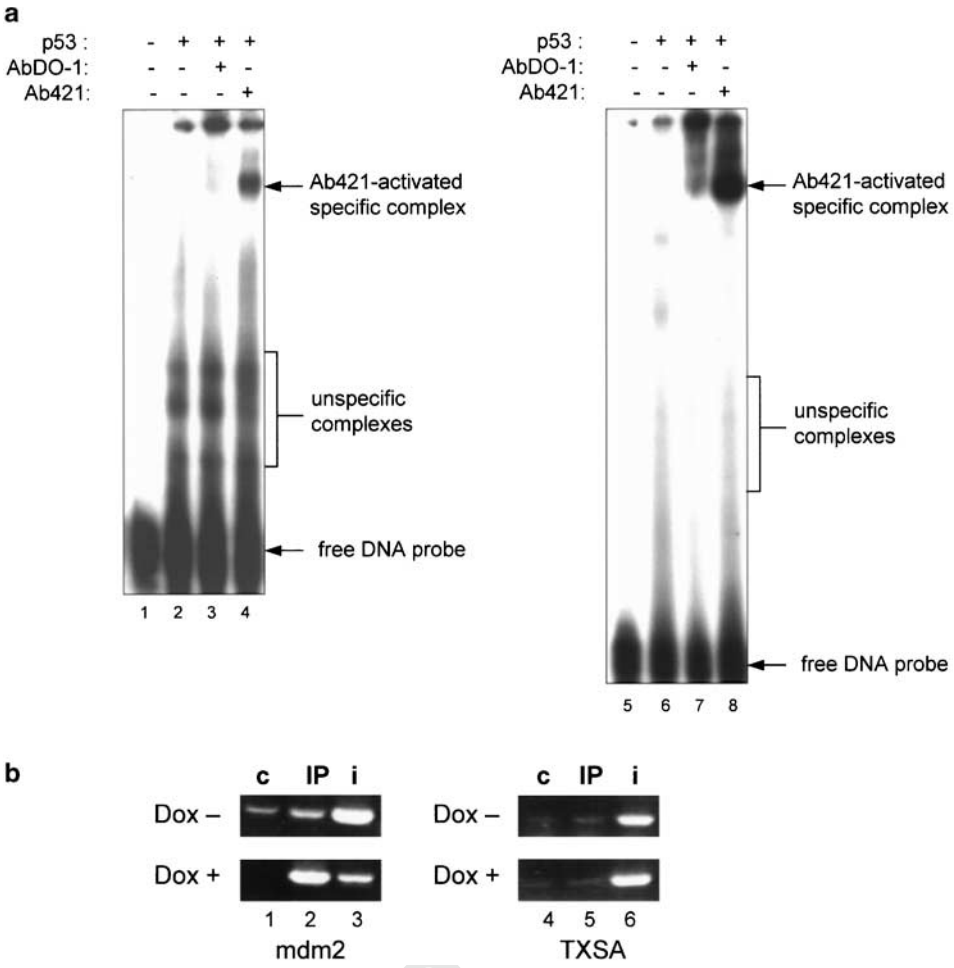

Figure 7 Wild-type p53 protein binds to the *TXSA* promoter *in vitro*. (**a**) EMSA experiments using recombinant p53 protein and a radioactively labeled 157 bp fragments spanning the −255/−99 region of the *TXSA* promoter containing a putative p53-binding site (lanes 1–4). Radiolabeled double-stranded oligonucleotide DNA containing a p53-binding site from the human p21 promoter was used as a control (lanes 5–8). Lanes 1 and 5: probe alone; other lanes probe and p53 protein alone or combined with antibodies AbD01 or Ab421 as indicated. (**b**) *In vivo* analysis of p53 DNA binding in LNZ 2024 cells by ChIP. Occupation of the mdm2 or the *TXSA* promoter by p53 was analysed in LNZ2024 cells that were treated or not treated with doxycycline (Dox+ and Dox−, respectively). Control lane (**c**) corresponds to the IP control samples processed in the absence of antibody. Immunoprecipitated genomic DNA and input DNA are shown in lanes IP and i, respectively

Table 1 The presence of p53-binding sites is not essential for the inhibition of ets-1-stimulated transcription by wild-type p53

TXSA construct	*Number of p53 CON-like decamers*	*P53-dependent inhibition (fold)*
(−306/+1)TXSA-Luc	4	8.58×
(−200/+1)TXSA-Luc	2	8.10×
(−128/+1)TXSA-L	None	9.60×

A reporter assay using promoter deletion mutants with different numbers of consensus-like p53 binding decamers was used to analyse the significance of p53 DNA-binding sites for repression of ets-1-dependent transcription of the *TXSA* promoter. Inhibition of ets-1-dependent stimulation of the promoter activity by p53 does not correlate with the number and presence of p53-binding sites. Results of a representative experiment in SaOs-2 are shown. Similar results were obtained in p53 null G-130 glioma cells and p53 null H-1299 lung carcinoma cells

derivatives of the *TXSA* promoter lacking putative p53 binding motives were inhibited by p53 as efficiently as those that contained four putative p53-binding sites.

It has recently been established that p53 binds to DNA in chromatin in a mode different from the DNA binding under *in vitro* conditions (reviewed in Kim and Deppert, 2003), and that natural p53 response elements do not always match the established p53 consensus. Therefore, the possibility remained that p53 may still bind some noncanonical sequences in the *TXSA* promoter. We analysed the DNA binding of p53 *in vivo* by chromatin immunoprecipitation (ChIP) in LNZ-2024 cells. The experiments showed that p53 bound to the *mdm2* promoter (Figure 7b lane 2 in the lower panel) as expected, but not to the *TXSA* promoter (lower panel, lane 5) when presented in the context of chromatin. We

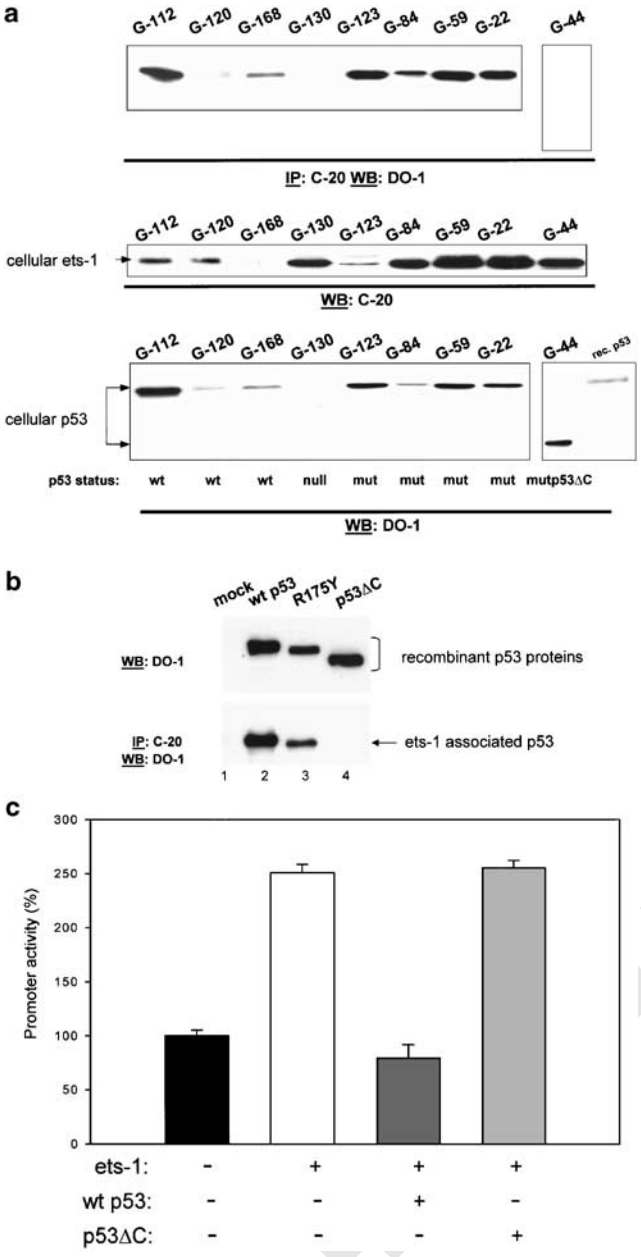

Figure 8 P53 and ets-1 proteins physically associate *in vivo* and *in vitro*. (**a**) Cell lysates were prepared from a panel of glioma cell lines and subjected to IP using the ets-1-specific antibody C-20. The presence of p53 in immunoprecipitates was analysed by Western blot using the p53-specific antibody DO-1 (upper panel). The p53 null cell line G-130 was used as a negative control. G-44 cells express a C-terminally truncated p53 protein due to a homozygous 'amber' mutation at the codon 224 (Kim *et al*., unpublished data). The middle and the lower panels show Western blot analysis of ets-1 and p53 in cells lines used in IP experiments. (**b**) Recombinant wild-type p53, R175Y, or p53ΔC-331 proteins were expressed in SaOs-2 cells by transient transfection and the expression of p53 proteins was verified by Western blot using AbDO-1 (upper panel). Cell lysates from the transfected cells were subjected to immunoprecipitation with the ets-1-specific antibody C-20 and analysed for coprecipitated p53 by Western blot with AbDO-1 (lower panel). (**c**) p53ΔC-331 protein does not inhibit ets-1 transactivation. The influence of the p53ΔC-331 protein on ets-1 transactivation was assessed by a reporter assay in transiently transfected SaOs-2 cells

conclude from these results that although p53 protein can bind to the *TXSA* promoter *in vitro*, such binding is unlikely to reflect the mechanism for transcriptional repression of *TXSA in vivo*.

p53 and ets-1 proteins associate physically in vitro *and* in vivo

A characteristic feature of the ETS family members is that their transcriptional activities are modulated by interactions with other proteins (Sato *et al*., 2001). This raised the possibility that p53 may influence the transcriptional activity of ets-1 by physically associating with the ets-1 protein. We tested this possibility using immunoprecipitation (IP). Cell lysates were prepared from a panel of glioma cell lines with known p53 status, and were subjected to IP with the ets-1-specific antibody C-20. The presence of p53 in the immunoprecipitates was assessed by Western blot with the p53-specific antibody DO-1. p53 was found to coprecipitate with ets-1 in all cell lines except G-130, which is null for p53 and G-44 expressing a C-terminally truncated p53 due to a homozygous mutation (E224 $\Rightarrow$ stop) in exon 6 (Figure 8a, upper panel). The amounts of the coprecipitated p53 directly correlated with the levels of p53 present in the corresponding cellular lysates (Figure 8a, lower panel). The data demonstrate that both wild-type and mutant p53 proteins physically associate with ets-1 in cells. The fact that the C-terminally truncated p53 protein of G-44 cells did not coprecipitate with ets-1 indicated that the C-terminus of p53 may be essential for ets-1 binding. To analyse the role of the p53 C-terminus in ets-1 binding, we further studied the interaction between ets-1 and p53 proteins in Saos-2 cells transiently transfected with expression vectors for different p53 proteins (wild-type, R175Y and a C-terminally truncated p53). The results showed that only full-length wild-type and R175Y p53 proteins, but not the p53ΔC-331 protein lacking 62 C-terminal amino-acid residues, coprecipitated with the endogenous ets-1 protein (Figure 8b) supporting the idea that the C-terminus of p53 is essential for the interaction with ets-1.

Having established that p53 and ets-1 proteins form a complex *in vitro* and *in vivo*, we next asked whether such an interaction may be important for the inhibition of ets-1 transcription by p53. We addressed this question by analysing the ability of the p53ΔC-331 protein that does not complex with ets-1 to influence ets-1-mediated transactivation of the *TXSA* promoter using reporter assays. SaOs-2 cells were transiently cotransfected with (−306)TXSA-Luc DNA either alone or together with expression vectors encoding ets-1, wild-type p53, or the C-terminally truncated p53ΔC-331 protein. The results showed that p53ΔC-331 no longer inhibited ets-1-mediated stimulation of the *TXSA* promoter, in contrast to wild-type p53, which inhibited ets-1 transcription as expected (Figure 8c). The results thus demonstrate that the ability of p53 to physically associate with ets-1 is essential for the inhibition of ets-1 transcription by p53.

Discussion

Increased invasiveness, neo-angiogenesis, and resistance to apoptosis are distinct features of neoplastic cells and are associated with the progression of malignant tumors. TXSA, an enzyme operating downstream of the COX, has recently been identified as an antiapoptotic and invasion-associated factor in several human cancers (McDonough *et al*., 1998; Daniel *et al*., 1999; Giese *et al*., 1999; Rodrigues *et al*., 2001; Kurzel *et al*., 2002; Yoshizato *et al*., 2002). In this study, we demonstrate for the first time that p53 and ets-1 regulate transcription of the *TXSA* gene in an antagonistic manner. Our results show that ets-1 is a potent transcriptional activator of *TXSA*, whereas p53 negatively interferes with ets-1 and thereby inhibits *TXSA* transcription stimulated by ets-1. Negative interference with ets-1 requires the wild-type p53 protein and is abrogated by hot-spot p53 mutations.

Our promoter studies and the analysis of p53 DNA binding *in vivo* indicate that direct binding of p53 to the *TXSA* promoter is an unlikely mechanism for the p53-dependent repression of *TXSA*, although p53 can bind to the *TXSA* promoter specifically *in vitro* according to our EMSA experiments. The physical interaction between p53 and ets-1 proteins, which we demonstrate *in vitro* and *in vivo*, is a more likely mechanism. We found that ets-1 physically associates with wild-type p53 and that the C-terminal part of p53 is essential for both ets-1 binding and inhibition of ets-1 transcriptional activity. Our finding that wild-type p53 interacts with ets-1 *in vitro* and *in vivo* is in contrast to recently reported data reporting that wild-type p53 does not associate with ets-1 (Sampath *et al*., 2001). A possible explanation for these contrasting results may be that in our experiments, 0.5–1.0 mg of a total protein input was used for IP, whereas a much lower amount of protein (0.05–0.1 mg of total protein from transiently transfected cells) was used in the study of Sampath *et al*. (2001). In agreement with previously reported findings of Sampath *et al*. (2001), we also find that mutant p53 proteins are associated with ets-1. Sampath *et al*. (2001) showed in their study that mutant p53 enhances ets-1-mediated transcription of the multidrug resistance-associated gene MDR1. However, in our experiments shown in Figure 5, mutant R175Y p53 had no significant effect on ets-1-mediated transcription of the *TXSA* promoter. Again, this discrepancy might depend on different cellular systems and different promoters analysed and deserves further investigation. It remains unknown whether ets-1 directly binds to the C-terminus of p53 or whether this interaction may be mediated by other proteins bridging the interaction between p53 and ets-1. However, our findings generally support the idea that the p53 C-terminus is crucial for the transcriptional repression mediated by wild-type p53 (Fritsche *et al*., 1998; Murphy *et al*., 1999; Hong *et al*., 2001).

The precise mechanism by which binding of p53 to the ets-1 protein influences ets-1-mediated transactivation remains to be elucidated. One possible scenario would be that a physical association between the two factors may affect ets-1 interaction with other transcriptional coactivators or basal transcription factors, which then may preclude the assembly of an active transcription complex. Such a mechanism has been described for the inhibition of HIF-activated transcription by wild-type p53. p53 forms a trimeric complex with the transcriptional HIF/p300 complex and thereby inhibits transcriptional activity of HIF (Blagosklonny *et al*., 1998). In this respect, it is worth noting that an interaction with the CBP/p300 coactivators is crucial for the transcriptional activities of both ets-1 and p53 (Avantaggiati *et al*., 1997; Lill *et al*., 1997; Jayaraman *et al*., 1999).

Considerable evidence indicates that the functional interaction between p53 and ets-1 occurs at several levels and may lead to diverse outcomes depending on the cellular context and experimental conditions. It has recently been reported that ets-1 modulates p53 transcriptional activity and UV-induced apoptosis in embryonic stem cells (Xu *et al*., 2002). On the other hand, our study demonstrates that ets-1 activities are under the tight control of p53, disruption of which may lead to the acquisition of an increased invasive potential and resistance to apoptosis in neoplastic cells. *TXSA* is not the only gene-coregulated by p53 and ets-1. The antiapoptotic gene *presenilin-1* is also coregulated by these two factors (Pastorcic and Das, 2000). Considering that ets-1 stimulates the transcription of many invasion-, metastasis- and antiapoptotic genes and that p53 repression of ets-1 activity is mainly mediated via a physical association between the two proteins, it is likely that the inhibition of ets-1 may be a more general mechanism by which p53 can suppress cancer progression. The identification of target genes common for p53 and ets-1 may help in elucidating the generality and functional consequences of p53 – ets-1 interaction in the biology of neoplasms.

Materials and methods

Cell culture

The human glioblastoma-derived cell lines (NCE-G22, G44, G59, G84, G112, G120, G123, G130, G168) (Anker *et al*., 1993) were propagated in MEM with 10% FCS (Biochem, Berlin, Germany), the glioma cell line SNB19 and the SNB19-derived clone SNB19/B3 (SNB19 TR/TO/ets-1-B3, Yamamoto *et al*., 2000), the human glioma cell line LN-Z308 (Albertoni *et al*., 1998), and its derived clone 2024 (Albertoni *et al*., 2002); osteosarcoma cell line SaOs-2 and H1299 lung carcinoma cells (ATCC) were cultured in DMEM, with 10% FCS in humidified incubators containing 5% CO_2. ets-1 or p53 expression was induced by incubating SNB19/B3 or LNZ2024 cells in the presence of 1 μg/ml of tetracycline or doxycycline, respectively.

Western blot analysis

Subconfluent cultures were washed with cold PBS twice, then scraped in cold PBS, and pelleted by centrifugation at 3000 *g* at 4°C for 5 min. The cells were lysed in lysis buffer (50 mM Tris-HCl (pH 8.0), 120 mM NaCl, 1% NP40) containing inhibitors of proteases. After 30 min incubation on ice, the samples were

centrifuged for 25 min at 13 000 *g*, and protein concentrations were determined in the supernatants using a BCA protein detection kit (Pierce, Rockford, IL, USA). A measure of 50–100 μg of cell lysate was separated on SDS–PAGE (7.5–15%), and transferred onto a polyvinylidene fluoride membrane (Millipore Corporation, Bedford, MA, USA). Anti-p53 antibodies PAb421 and DO-1, anti-p21 antibody (Santa Cruz Biotechnology, Santa Cruz, USA), and the anti-MDM2 antibody 2A10 (Oncogene) were used for the detection of the respective proteins with an ECL detection system (Amersham, Freiburg, Germany).

Immunoprecipitation

Cells were harvested from a subconfluent monolayer culture, washed in PBS, and resuspended in IP-buffer (20 mM Tris-HCl (pH 8.0), 1 mM EDTA, 0.5% NP-40, 150 mM NaCl, 1 mM DTT, 10% glycerol) containing proteases inhibitors. After 30 min incubation on ice, lysates were cleared by centrifugation and the protein concentration was determined using a BCA protein detection kit (Pierce). After preclearing with protein-A–sepharose (PAS), 0.5–1.0 mg of lysate was incubated with 10 μg of anti-ets-1antibody C-20 (Santa Cruz Biotechnology) or 10 μg of DO-1 antibody (Oncogene) at $+4^{\circ}$C overnight on the rotating platform. A measure of 70 μl of PAS was added and incubated for 60 min at 4°C with the rotation. PAS-bound proteins were collected by centrifugation, washed three times with the IP-buffer, and analysed by Western blot.

Transient transfection and luciferase reporter gene assay

For transient transfection, 2.0×10^5 cells were plated into six-well tissue culture plates (Nunc, Roskilde, Denmark) and transiently transfected using Effectene® transfection reagent (Qiagen, Hilden, Germany) according to the manufacturer's instructions. A measure of 0.5 μg per well of reporter plasmids containing a luciferase gene under the control of the respective promoter was used. The p21-LUC reporter plasmid containing human p21 promoter (el-Deiry *et al*., 1993) was used as a control. For transactivation assays, 0.1 μg per well of pCMV-wtp53 DNA (Rowan *et al*., 1996) and/or indicated amounts of pcDNA4/TO/c-*ets-1* (Yamamoto *et al*., 2000) were transfected together with reporter DNA. Transfections were performed in triplicate. At 30 h after transfection, cells were washed with PBS twice, scraped in PBS, pelleted by centrifugation, and lysed in cell culture lysis buffer (Promega, Madison, USA). Cell lysates were cleared by centrifugation at 13 000 rpm for 5 min at 4°C and transferred to new tubes. The protein concentration was measured in the cell lysates and adjusted with the lysis buffer. The luciferase activity was measured using a luciferase assay system (Promega, Madison, USA) according to the manufacturer's instruction.

RNAse protection assay

Radioactively labeled RNA probes were prepared from linearized plasmids containing human β-actin or TXSA cDNA by *in vitro* transcription (Ambion). RNAse protection experiments were performed with cell lysates prepared from 0.5–2×10^6 cells using a Direct Protect Kit (Ambion) or with 30 μg total RNA (Multi-NPA Kit, Ambion).

Electrophoretic mobility shift assay

Recombinant wild-type p53 protein was expressed in insect cells and purified by ion-exchange chromatography (FPLC, Amersham Pharmacia Biotech) as described in Gohler *et al*. (2002). Radiolabeled DNA probes were prepared by PCR using (−306) TXSA-Luc DNA as a template. PCR was performed under standard conditions, except that α[^{32}P-dATP] was used instead of dATP. To amplify the region containing p53 consensus-like sites, we used primers 5′-atatagatagacattttg-gagaa-3′ (fw) and 5′-tgactgcactgtcaataacattta-3′ (rev). −306/−128 and −128/−79 regions of the TXSA promoter were amplified using pairs of primers RV3 (Promega) with 5′-tgactgcactgtcaataaacattta-3′ and 5′-taaatgtttattgacagtgcagtca-3′ (fw) with 5′-gggcacaacaaaggg-3′. Radioactively labeled PCR fragments were purified from polyacrylamide gels and used as DNA probes in EMSA experiments. For DNA binding, purified p53 protein (50 ng) or nuclear extracts (5.0–10.0 μg total protein) was incubated with the labeled DNA probe in a reaction mixture containing 25 ng poly (dI : dC)(dI : dC) (Amersham Biosciences) and 2 μg BSA in 50 mM Tris-HCl (pH 7.5), 0.1 mM EDTA, 1 mM DTT, 20% glycerol, 50 mM NaCl. After 20 min preincubation at room temperature, 30 000 c.p.m. of the labeled DNA probe was added for another 25 min. The samples were loaded onto a 4% native polyacrylamide gel and separated by electrophoresis in 10 mM Tris-HCl (pH 7.8), 0.2 mM EDTA, 1.25 mM NaOAc, and 8 mM acetic acid at 200 V at room temperature.

Chromatin Immunoprecipitation

LNZ 2024 cells (0.5×10^7 cells per condition) were incubated in the presence or absence of doxycycline (1 μg/ml) for 24 h and cross-linked with 1% formaldehyde for 10 min at 37°C. Formaldehyde was neutralized by the addition of glycine to 0.125 M for 10 min at room temperature. Cells were washed twice with cold PBS, scraped, and collected by centrifugation. IP of the chromatin cross-linked p53 was performed with the DO-1 antibody using a ChIP kit (Upstate Biotechnology) according to the recommendations of the supplier.

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