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Induction of the Tat-binding protein 1 gene accompanies the disabling of oncogenic erbB receptor tyrosine kinases

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ABSTRACT **Conversion of a malignant phenotype into a more normal one can be accomplished either by down-regulation of erbB family surface receptors or by creating inactive erbB heterodimers on the cell surface. In this report, we report the identification and cloning of differentially expressed genes from antibody-treated vs. untreated fibroblasts transformed by oncogenic $p185^{neu}$. We repeatedly isolated a 325-bp cDNA fragment that, as determined by Northern analysis, was expressed at higher levels in anti-$p185^{neu}$-treated tumor cells but not in cells expressing internalization defective $p185^{neu}$ receptors. This cDNA fragment was identical in amino acid sequence to the recently cloned mouse Tat binding protein-1 (mTBP1), which has 98.4% homology to the HIV tat-binding protein-1 (TBP1). TBP1 mRNA levels were found to be elevated on inhibition of the oncogenic phenotype of transformed cells expressing erbB family receptors. TBP1 overexpression diminished cell proliferation, reduced the ability of the parental cells to form colonies *in vitro*, and almost completely inhibited transforming efficiency in athymic mice when stably expressed in human tumor cells containing erbB family receptors. Collectively, these results suggest that the attenuation of erbB receptor signaling seems to be associated with activation/induction or recovery of a functional tumor suppressor-like gene, *TBP1*. Disabling erbB tyrosine kinases by antibodies or by trans-inhibition represents an initial step in triggering a TBP1 pathway.**

ErbB family receptor kinases mediate oncogenic transformation by mutation, overexpression, or coexpression leading to homodimeric or heterodimeric complexes that mediate synergistic signaling (1–5). Continual expression of $p185^{neu}$ is necessary for the maintenance of the neoplastic phenotype of neu-transformed cells (1). Incubation of oncogenic $p185^{neu}$-expressing tumor cells with the anti-neu mAb 7.16.4 causes phenotypic reversal *in vitro* and *in vivo* (6, 7). The mechanism of phenotypic reversal of tumor cells expressing the $p185^{neu/c\text{-}erbB2}$ oncogene occurring with anti-neu-specific mAb treatment has not been defined completely, although this mechanism has been characterized as arising as a consequence of disabling the kinase complex, a process in which a fraction of the receptors becomes down-modulated (6–8).

The binding of antibody to the extracellular domain of the $p185^{neu}$ receptor mediates down-regulation and increases $p185^{neu}$ oncoprotein degradation by causing the $p185^{neu}$ complex to enter a degradation pathway. Endosomes were found to carry $p185^{neu}$-containing receptor aggregates to lysosomes where the complex was degraded (9).

Here, we used mRNA differential display (10, 11) to isolate genes that are specifically expressed in cells treated with the anti-$p185^{neu}$ mAb 7.16.4. We repeatedly identified a 325-bp cDNA fragment called 3C bearing significant homology to the HIV tat-binding protein-1 (TBP1) on antibody-mediated down-regulation of the oncogenic $p185^{neu}$ receptor associated with inhibition of transformation. The 3C fragment is completely identical to the recently cloned mouse tat binding protein-1 (mTBP1), which itself is 98% homologous to the human TBP1 over 439 aa (12). Northern blot analysis confirmed that this fragment and the TBP1 cDNA hybridized to mRNA isolated from cells undergoing phenotypic reversal by antibody treatment.

TBP1 has been reported to suppress tat-mediated transactivation of HIV replication (13). Nakamura *et al.* (12) established that full-length murine TBP1 also suppresses Tat-mediated transactivation. A TBP1-interacting protein (TBPIP), which colocalizes *in vivo* and synergistically enhances the inhibitory action of TBP1 on Tat activity *in vitro*, also has been cloned recently (14).

To investigate the biological effects of TBP1, we transfected the full length of TBP1 cDNA into a variety of human cell lines, U87MG, SK-BR-3, and MCF-7, which express erbB family genes. Ectopically expressed TBP1 was able to cause a reversion of the transformed phenotype. Additionally, basal TBP1 mRNA levels were found to be higher in phenotypically inhibited cells. These studies suggest that induction of TBP1 mediates inhibition of cell growth and transformation of erbB-inhibited cells.

MATERIALS AND METHODS

Cell Lines. B104-1-1 cells were derived from NIH 3T3 cells transfected with $p185^{neu}$ and have been described (6). U87MG cells are human brain tumor cells; SK-BR-3 cells and MCF-7 cells are primary human breast cancer cells obtained from the American Type Culture Collection. These cells were cultured in DMEM containing 10% (vol/vol) FBS, 1% L-glutamine, and 1% penicillin/streptomycin at 37°C, 95% humidity, and 5% CO_2. The NR6TintΔ cells containing internalization-defective $p185^{neu}$ receptors have been described (15).

Incubation of Cell Lines with mAb 7.16.4. The mAb 7.16.4 has been described (6, 7). B104-1-1 cells were grown overnight in 6-well dishes and treated with mAb 7.16.4 (10 μg/ml) for 0–4 h at 37°C. Cells were harvested, washed, stained with saturating amounts of mAb 7.16.4, and restained with anti-mouse IgG FITC. Cells were then processed for flow cytometric analysis as described (15).

RNA Isolation and mRNA Differential Display. Total RNA was purified from cell lysates by using the RNeasy Mini Kit (Qiagen, Valencia, CA) and the protocol supplied with the kit.

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Abbreviations: RT-PCR, reverse transcriptase–PCR; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide; EGFR, epidermal growth factor receptor.

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An mRNA Map Kit from GenHunter (Nashville, TN) was used, and the manufacturer's protocol was followed. DNase-treated total RNA (2 μg) was reverse-transcribed by using Super-Script II (United States Biochemical) with oligo(dT) primers T12MG, T12MC, or T12MA and amplified with the library 10-mers AP-3 (5′-AGGTGACCGT-3′) or AP-6 (5′-GCAATCGATG-3′) as described in the kit. The PCR products were run on a 6% sequence gel with the cDNAs that were to be compared run side-by-side. Bands representing differentially expressed genes were eluted from the gel, reamplified, subcloned into the pCRII vector as described in the TA cloning kit (Invitrogen), and sequenced on a 6% denaturing gel.

Generation of Stable TBP1 Transfectants and Confirmation of Transgene Expression by Reverse Transcriptase–PCR (RT-PCR). The TBP1 cDNA was inserted into the *Eco*RI site of the pBK-CMV (Stratagene) vector. Stable TBP1 transfectants were generated by transfecting the pBK-CMV–TBP1 plasmid into a panel of human cell lines (U87MG, SK-BR-3, and MCF-7) by using Lipofectamine (GIBCO). G418 (0.8 μg/ml; GIBCO) was used to select for the transfected cell populations, and Northern blot analysis was used to identify clones that expressed TBP1.

First-strand cDNA was prepared from 3 μg of total RNA by using the Superscript Preamplification System for first-strand cDNA Synthesis Kit (GIBCO/BRL). To confirm the integrity of the first-strand cDNAs, we amplified β-actin sequences by using the rat β-actin control amplifier set (CLONTECH), which yielded a 764-bp product. Exogenous/transfected TBP1-derived transcripts were amplified by using the pBK-CMV vector T7 primer (5′-GTAATACGCTCACTATAGGGC-3′) and a TBP1-specific primer designated C2 (5′-AGAAGAAAGCCAACCTAC-3′), which yielded a 216-bp product. After RT-PCR, the products were run on 1.8% agarose gels to evaluate the presence or absence of the amplified product.

Cell Proliferation Assay. The proliferation assay, as measured by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) incorporation, has been described (16).

***In Vitro* and *in Vivo* Tumorigenesis Assays.** Anchorage-independent growth was determined by assessing the colony-forming efficiency of cells suspended in soft agar (6, 17). For *in vivo* experiments, NCr homozygous nude mice (6–8 weeks old) were purchased from the National Cancer Institute (Bethesda, MD). Cells (1×10^6) were suspended in 0.1 ml of PBS and injected intradermally into the mid dorsum of each animal. Parental U87MG cells were injected on one side of individual animals and stably TBP1–cDNA-transfected U87/TBP1 cells were injected on the contralateral side to make direct comparisons of growth within each animal. Animals were maintained in accordance with guidelines of the Committee on Animals of the University of Pennsylvania and those of the Committee on Care and Use of Laboratory Animals of the Institute of Laboratory Animal Resources. Tumor growth was monitored twice weekly for 6–10 weeks. Tumor size was calculated by measuring tumor volume (length $\times$ width $\times$ thickness).

RESULTS

Down-Regulation of p185^{neu} Surface Receptors and Differentially Expressed Genes. Differential display provided a convenient way for us to study altered gene expression in p185^{neu}-expressing B104-1-1 murine cells treated with anti-p185^{neu}. One drawback of the differential display is its susceptibility to generating false–positive clones. To compensate, we ran two differential display trials on each of the total RNAs so that we could select bands that were present in both trials. There were nine differentially expressed bands chosen for further characterization; these bands ranged in size from 230 bp to 1,000 bp, were observed in both trials, and were not differentially expressed in NIH 3T3 cells. One of these bands represented a 325-bp cDNA termed 3C, which had 100% homology with mTBP1 (Fig. 1), which is the murine homologue of the human gene tat-binding protein 1. Northern blot analysis of 7.16.4-treated B104-1-1 cells showed increased TBP1 mRNA levels of ≈1.5 kb in size when probed by both human TBP1 cDNA (Fig. 2*A*) and 3C DNA (data not shown).

Confirmation of differential expression was achieved by comparing Northern blots of antibody-treated B104-1-1 cells and untreated cells (Fig. 2 *A* and *B*). Antibody treatment of B104-1-1 cells containing elevated levels of oncogenic p185^{neu} resulted in increased expression of the mTBP1 mRNA transcript (Fig. 2 *A* and *B*). We have shown that receptor kinase activity alone is not sufficient for the endocytic process (15). A structural element, namely an internalization sequence, is also required for both mAb- and ligand-induced receptor down-regulation (15). Importantly, NR6TintΔ cells (15), which contain an internalization-defective p185^{neu} mutant protein, TintΔ (15), and lack the ability to undergo p185^{neu} internalization, did not have an increase in mTBP1 mRNA level after 7.16.4 treatment, although the basal level of mTBP1 transcript was higher in NR6TintΔ cells (Fig. 2 *A* and *B*). Notably, B104-1-1 cells contain higher levels of transforming p185^{neu} than NR6TintΔ cells and are more oncogenic (15). The basal level of the mTBP1 transcript was, as expected, greater in the less oncogenic NR6TintΔ cell.

Because disabling erbB receptor ensembles may enable a common inhibitory pathway, we also examined cells in which EGFR was inactivated but not down-modulated by a trans-inhibitory ectodomain form of p185^{neu} (T691stop neu; refs. 17 and 18). We found that the endogenous expression of TBP1 mRNA was also higher in phenotypically inhibited U87MG-derived cells expressing the kinase-deficient T691stop neu ectodomain form compared with U87MG parental cells (Fig. 2*C*). Because T691stop inhibition of erbB kinase activity does not require erbB receptor down-modulation (17, 18), these data suggest that induction of TBP1 expression after anti-p185^{neu} mAb treatment occurs by regulation of a kinase signaling pathway (Fig. 2 *A* and *C*) and is not simply a result of receptor down-modulation and degradation (Fig. 2*C*).

Inhibition of Cell Growth and Transformation by Human TBP1. To investigate the biological effects of TBP1, we transfected, by using the pBK-CMV vector, full-length TBP1 cDNA into several different cell lines, SK-BR-3, MCF-7, and U87MG, all of which express erbB family receptors. We screened the transfected subclones by Northern blot analysis, and the expression of ectopic TBP1 in subclones was also confirmed by RT-PCR analysis (Fig. 3 *A* and *B*). We amplified a 216-bp product from all of the TBP1-cDNA-transfected clones and the pBK-CMV–TBP1 plasmid construct but not in the corresponding nontransfected parental cells (Fig. 3*A*). RT-PCR with a β-actin control amplifier set produced a 764-bp amplified product from all the cell lines except for the pBK-CMV–TBP1 plasmid construct (Fig. 3*B*).

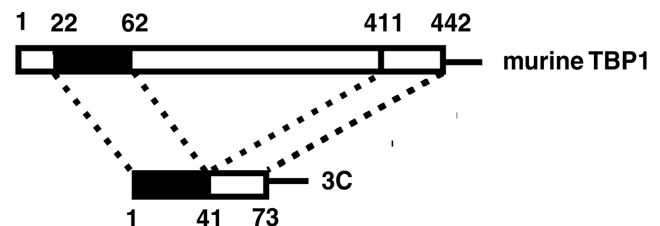

FIG. 1. Regions of homology of 3C to murine TBP1. Amino acids 1–41 of 3C correspond to amino acids 22–62 of mTBP1, and amino acids 42–73 of 3C correspond to amino acids 411–442 of mTBP1 (100% identical). The 3C C-terminal noncoding-region nucleotide sequence is 98% homologous to that of mTBP1.

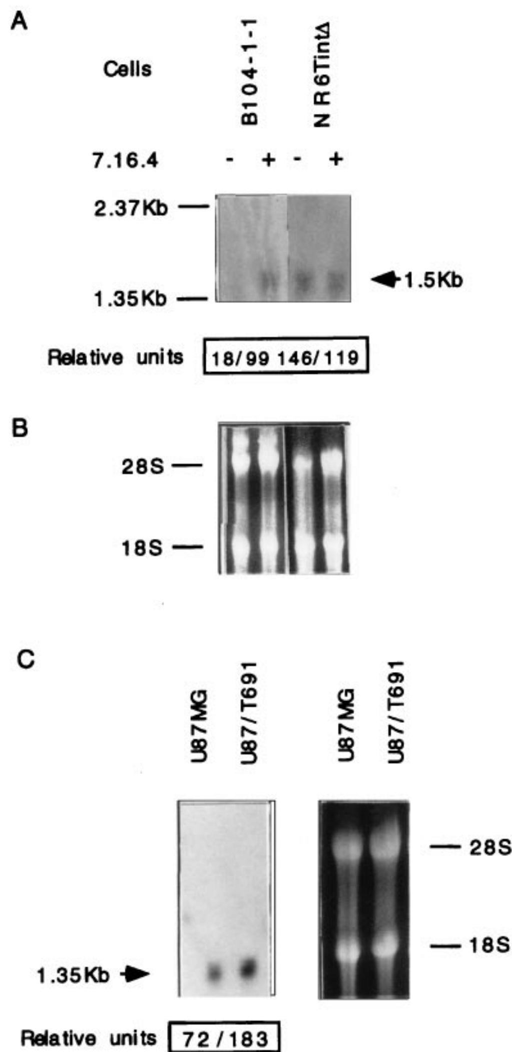

FIG. 2. Comparison and confirmation of the differentially expressed gene by Northern blot analysis and endogenous expression of TBP1 in U87MG vs. U87/T691. (*A*) B104-1-1 and NR6TintΔ cells were incubated with or without 7.16.4 (10 μg/ml) for 24 h before RNA isolation. Total RNA (10 μg) was loaded in each lane and was probed with human TBP1 cDNA probe. TBP1 expression was up-regulated with anti-p185^{neu} mAb (7.16.4) treatment in B104-1-1 cells (lanes 1 and 2) but was not changed in internalization defective NR6TintΔ cells (lanes 3 and 4). (*B*) The corresponding formaldehyde gel electrophoresis of total RNA is shown. (*C*) The U87/T691 subclone, an epidermal growth factor receptor (EGFR)-positive cell line phenotypically inhibited by the expression of a trans-inhibitory ectodomain form of p185^{neu} (T691 stop neu), showed increased endogenous levels of TBP1. Relative units are derived from scanning densitometry (Molecular Dynamics).

Cell growth of TBP1-transfected cells was evaluated by using the MTT assay (16). Transfected clones had 34–57% of proliferation inhibition compared with the corresponding parental cell lines (Fig. 4 *A–C*). Cell growth was therefore greatly diminished in TBP1-transfected erbB transformed cells. The level of TBP1 mRNA in the inhibited subclones was equal to, or exceeded, the amount of mRNA detected in parental cells inhibited by antibody treatment or T691stop expression. Thus, the mRNA level was sufficiently high to play a causal role in phenotypic reversion. Transformation efficiency was assessed by using an anchorage-independent growth assay (6, 17). The ability of TBP1 transfectants to form colonies was consistently and dramatically reduced (Fig. 5*A*). Transforming efficiency of SK-BR-3/TBP1, MCF-7/TBP1, and U87MG/TBP1 subclones was inhibited 79.2 ± 3.7% (mean ± SEM), 94.2 ± 4.5%, and

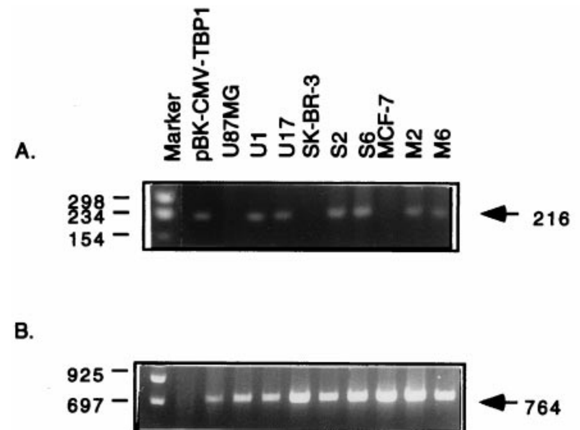

FIG. 3. Confirmation of TBP1-transfected clones by RT-PCR analysis. We made first-strand cDNA from 3 μg of each total RNA by using the Superscript Preamplification System for first-strand cDNA Synthesis Kit (GIBCO/BRL). (*A*) Amplification of the transfected TBP1 cDNA by using the pBK-CMV vector-oriented T7 primer (5′-GTAATACGCTCACTATAGGGC-3′) and the TBP1 specifically designed primer C2 (5′-AGAAGAAAGCCAACCTAC-3′) shows 216-bp product bands in only the transfected clones and the pBK-CMV–TBP1 plasmid construct and not in parental cell lines of U87MG, SK-BR-3, and MCF-7. (*B*) Amplification of actin cDNA by using the rat β-actin control amplifier set (CLONTECH) shows a 764-bp amplified product from all the cell lines except the pBK-CMV–TBP1 plasmid construct. After RT-PCR, the products were run on 1.8% agarose gel to confirm the amplified product.

65.5 ± 1.7%, respectively, as determined in three independent experiments. Transient transfection of U87MG cells with empty vector did not inhibit cell growth and transformation as determined by the MTT assay and by an anchorage-independent growth assay (data not shown).

U87/TBP1 subclones showed a profound degree of inhibition relative to U87MG parental cells after implantation into athymic mice (Fig. 5*B*). U87/TBP1 transfectants did not form appreciable tumors until 8 weeks (Fig. 5*B*), after the period of time when some animals injected with parental U87MG cells had to be killed because of excessive tumor burden. Additionally, more than 50% of the subcutaneous injections with the U87/TBP1 cell line failed to produce any palpable tumors.

DISCUSSION

Our findings indicate that disabling erbB oncoproteins with anti-erbB receptor antibodies leads to inhibition of the transformed phenotype, a feature associated with induction or recovery of TBP1 expression. Previous studies showed that kinase-deficient forms of p185^{neu} derived from the receptor ectodomain that form heterodimers with EGFR in rodent (19) and human glioblastoma cells can inhibit EGFR-dependent phenotypes contributing to transformation (17, 18). Endogenous expression of TBP1 mRNA is higher in U87MG human glioblastoma cells containing p185^{neu} kinase-deficient forms that have an inhibited phenotype. Although multiple TBP1 transfectants of three human cancer cell lines had an inhibited phenotype, all cell lines examined expressed erbB receptors. It remains possible that TBP1 induction accompanies reversion of transformation in non-erbB-containing cells. The degree to which TBP1 induction contributes to phenotypic reversion in erbB-inhibited cells is also unknown. Collectively, these observations suggest that expression of TBP1 is related to the tumorigenesis of certain malignant cells and that induction/recovery of TBP1 expression may be part of a general attenuating pathway or a specific consequence of down-regulation or attenuation of signaling from erbB family receptor tyrosine kinases.

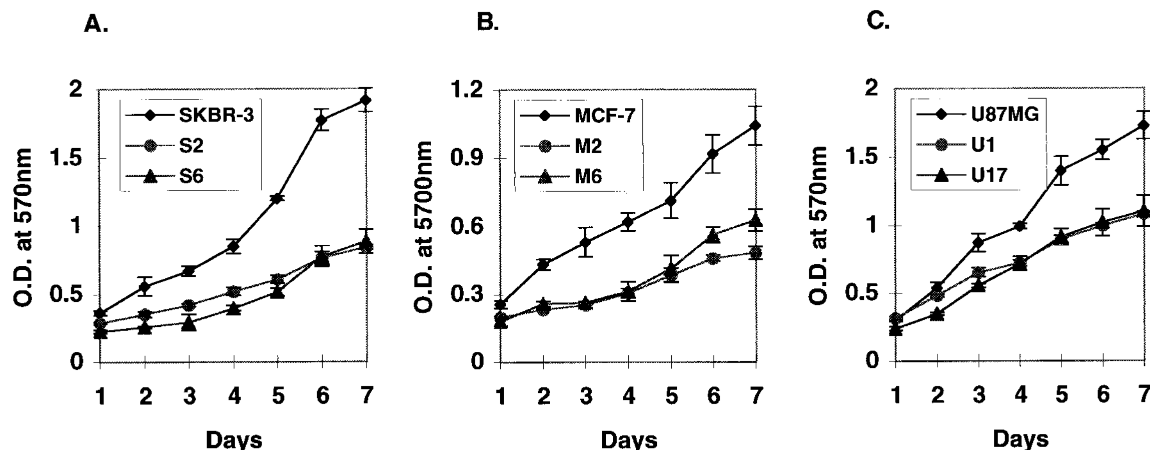

FIG. 4. Reduction of cell proliferation by expression of human TBP1 in human cancer cell lines. Cell lines were plated in 96-well plates at 4,000 cells per well in 10% DMEM and allowed to attach overnight. MTT was given to the cells for 4 h. Cells were then lysed in 50% (vol/vol) SDS/20% (vol/vol) dimethyl sulfoxide and kept at 37°C overnight. Proliferation was assessed by reading OD at 570 nm by using an ELISA reader. The number of cells used in this assay was determined to be within the linear range for this cell type. (*A*) TBP1 cDNA-transfected SK-BR-3 subclones S2 and S6 had 50% and 57% proliferation inhibition, respectively, compared with parental cells. (*B*) TBP1 cDNA-transfected MCF-7 subclones M2 and M6 had 40% and 54% proliferation inhibition, respectively, compared with parental cells. (*C*) U87MG subclones expressing elevated TBP1, U1, and U17 had 34% and 38% inhibition of proliferation, respectively, compared with parental cells.

The HIV tat protein, encoded by one of the viral regulatory genes, *tat*, is considered a powerful transactivator of viral gene expression (20–22). Human TBP1 is encoded by a 1,341-nt cDNA containing an ORF of 439 aa (23). TBP1 was originally described as a transcriptional factor of the HIV 1 by interaction with the tat protein (13, 23). TBP1 binds the HIV tat transactivator, suppressing its activity in cotransfection experiments (13). In some cases, TBP1 may also be involved in transcriptional activation (23). Nakamura *et al.* (12) isolated a full-length murine form of TBP1 that suppresses the Tat-mediated transactivation. Tanaka *et al.* (14) cloned a TBP1 interacting protein, TBPIP, that interacts with mTBP1. TBPIP colocalizes with TBP1 *in vivo* and synergistically enhances the inhibitory action of TBP1 on Tat activity *in vitro*, supporting the general concept of TBP1 ensembles that inhibit cellular functions and transcription.

We noted that TBP1 amino acids 59–63 bear 75% similarity to the motif HFRIG, and amino acids 185–189 bear 60% similarity to the motif HSRIG. The HIV gene *Vpr* contains a domain that contains two H(S/F)RIG motifs that may cause cell growth arrest and structural defects (24). *TBP1* also possesses 46% identity to *KAI1*, a metastasis suppressor gene for human prostate cancer (25). Hoyle *et al.* (26) recently localized the human *TBP1* to chromosome 11p12–13, and it has been noted that frequent loss of chromosome 11p13 occurs in a variety of cancers (27, 28). Tsuchiya *et al.* (29) reported that the tumor suppressor VHL gene product binds to TBP1. These observations suggest that the *TBP1* gene may be grouped with other possible functional tumor suppressor genes, and TBP1 may act as a negative regulator of the transcriptional elongation process by binding to tumor suppressor gene products such as pVHL.

All members of the TBP family, including TBP1, contain two highly conserved domains. One domain resembles a nucleotide-binding motif (ATP-binding site), and the other resembles a motif common to proteins with helicase activity. TBP1, TBP7, MSS1, and SUG1 are thus considered members of a large ATPase family rather than representing discrete transcriptional factors (30–33).

TBP1 has also been reported to be a component of the 26S proteasome (34), which is an essential multiprotein complex

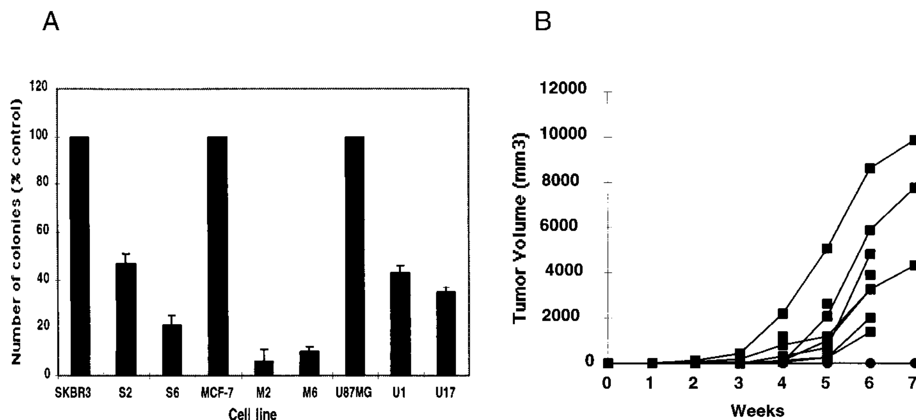

FIG. 5. Inhibition of cell growth and transformation by human TBP1. (*A*) Anchorage-independent growth. Cells of each clone ($n = 1{,}000$) were suspended in a 1-ml top layer [0.18% agarose/10% (vol/vol) FBS/10% (vol/vol) DMEM] in 6-cm culture dishes containing a 3-ml cell-free feeder layer consisting of 0.25% agarose in DMEM supplemented with 10% FBS and 20 mM Hepes (pH 7.5). Colonies (>0.3 mm) were visualized and counted on day 28 for all cell lines after staining with *p*-iodonitrotetrazolium violet (1 mg/ml). Each cell line was examined in triplicate in three separate experiments. The numbers of colonies reported represent the mean of triplicate samples. (*B*) Tumor growth in athymic mice: comparison of parental U87MG cells and U87/TBP1 transfectants. Cells of each cell line ($n = 1 \times 10^6$) were injected subcutaneously on day 0, and tumor volume was recorded weekly. These data represent individual tumor growth curves for U87MG parental cells (■) and mean tumor volumes for the U87/TBP1 subclone (●). (U87MG, $n = 7$; U87/TBP1, $n = 8$.)

that degrades ubiquitinated proteins in an ATP-dependent fashion and provides the main route for selective turnover of intracellular proteins involved with the regulation of cell growth and metabolism (35). TBP1 functions as a subunit of PA700, a nonproteasomal component of the 26S proteasome (34, 36), and a subunit of a proteasome modulator complex (34). Schnall *et al.* (37) isolated a set of 12 yeast genes, all belonging to the AAA family. Among them, the closest equivalents of the human genes *TBP1*, *TBP7*, and *MSS1* are named, respectively, *YTA1*, *YTA2*, and *YTA3*. These genes are identical or closely related to either cell cycle genes or to subunits of the 26S proteasome. We identified three proteins interacting with TBP1: p27 (34), p40 (38), and p42(SUG-2) (34, 39) in a yeast two-hybrid system by using a HeLa cell cDNA library (data not shown). Interestingly, p27 and p42 are subunits of the human proteasome 26S modulator complex, and both p40 and p42 are subunits of the regulatory proteasome PA 700. TBP1 itself is also a subunit of both the PA700 and modulator complex that enhances 26S proteasome activity (34). Moreover, DeMartino *et al.* (34) reported the purification and characterization of a proteasomal modulator complex—a trimer of TBP1, p42, and p27—which enhances proteasomal activity by as much as 8-fold. Recently, Watanabe *et al.* (40) established the association of p27, p42, and TBP1 with not only the modulator complex but also with the 26S proteasome complex. High sequence similarities of TBP1 homologues over widely different species substantiate that TBP1 function is essential *in vivo* (12). These results suggest a relationship between TBP1 expression and a protein degradation pathway.

Cell surface levels of $p185^{neu}$ and EGFR were independently down-regulated on expression of TBP1 in all subclones examined by flow cytometric analysis (B.-W.P. and M.I.G., unpublished results). These results suggest that TBP1 activity is related to inactivation of surface $p185^{neu}$ and EGFR. TBP1, as a human 26S proteasome modulator (34), may increase proteasomal activity by facilitating degradation of sequestered cell-surface proteins in addition to its role in transcriptional regulation.

This study shows that TBP1 expression was up-regulated with anti-$p185^{neu}$ mAb (7.16.4) treatment in B104-1-1 cells but not in NR6TintΔ cells expressing internalization-defective $p185^{neu}$ proteins (Fig. 2*A*). U87/T691 cells, an EGFR-disabled cell line containing a trans-inhibitory ectodomain form of $p185^{neu}$ (T691stop neu), also showed an increased endogenous level of TBP1 over that observed in parental U87MG cells (Fig. 2*C*). Our findings suggest that TBP1 expression is inversely related to the "activity" of the kinase signaling pathway (Fig. 2 *A* and *C*) and is not necessarily a direct result of the receptor degradation alone (Fig. 2*C*). Attenuation of erbB receptor signaling seems to involve the activation of a functional tumor suppressor-like gene, *TBP1*, which itself is associated with a proteasomal protein degradation pathway.

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