The p14ARF tumor suppressor is a key regulator of cellular proliferation, frequently inactivated in human cancer, whose mode of action is currently not completely understood. We report here that the so-called human immunodeficiency virus Tat-binding protein 1 (TBP-1), a component of the 19 S regulatory subunit of the proteasome 26 S, also involved in transcriptional regulation and with a supposed role in the control of cell proliferation, specifically interacts with ARF, both in yeast and mammalian cells. We present evidence that the overexpression of TBP-1 in various cell lines results in a sharp increase of both transfected and endogenous ARF protein levels. Moreover, this effect depends on the binding between the two proteins and, at least in part, is exerted at the post-translational level. We also show that the ARF increase following TBP-1 overexpression results in an increase in p53 protein levels and activity. Finally, our data underline a clear involvement of TBP-1 in the control of cell proliferation.

The p14ARF protein is encoded by the ARF-INK4a locus of human chromosome 9p21 that also encodes p16INK4a, an inhibitor of the CDK4/6. The genetic alteration at the ARF-INK4 locus occurs in a wide spectrum of human cancers, both familial and sporadic (1). The ARF tumor suppressor acts as a sensor of hyperproliferative signals emanating from oncogenes and inducers of S phase entry, such as Myc, E1A, Ras, and E2F-1 (2–6, 7). ARF, in turn, triggers p53-dependent growth arrest in the G1 and G2 phases of the cell cycle or, in the presence of appropriate collateral signals, sensitizes cells to apoptosis (8, 9). ARF binds directly to MDM2, enabling transcriptionally active p53 to accumulate in the nucleoplasm (2, 10–12). Emerging evidence suggests that ARF can, although less efficiently, suppress the proliferation of cells that express mutant p53 or lack both MDM2 and p53, implying interactions with other regulators (13, 14). It has been demonstrated that ARF can associate with certain members of the E2F family of transcription factors and induce their degradation through the 26 S proteasome pathway (15). In addition, ARF is also able to associate with DP1, a functional, regulatory partner of the E2F family of factors, but, surprisingly, the E2F1-DP1 complex appears to be refractory to ARF regulation (16). Very recently, it has been demonstrated that ARF acts as an inhibitor of RNA processing (17), highlighting a p53-independent role for p19ARF that couples the antiproliferative function of ARF to ribosome biogenesis (17).

Other proteins that complex with ARF include spinophilin (18), topoisomerase I, MdmX, the hypoxia-inducible transcription factor HIF-1α, Pex19, p120E4F, and CARF (19). Although the extent to which all of these ARF-complexes modulate the cell cycle-inhibitory action of ARF is not well established, spinophilin, CARF, and p120E4F appear to cooperate with ARF in promoting growth arrest.

In this paper, we report an interaction between the human p14ARF and the HIV-1 Tat-binding protein-1 (TBP-1) (20, 21). TBP-1 is a member of a large highly conserved gene family (AAA protein family) that contains two conserved domains, one resembling a nucleotide-binding motif (ATP-binding site) and the other resembling a motif common to proteins with helicase activity. Members of this protein family are the proteins MSS1 (or Rpt1), S4 (or Rpt2), TBP-7 (or Rpt3), SUG2 (or Rpt4), SUG1 (or Rpt6), and Rpt5 (TBP-1 itself), all components of the 19 S regulatory subunit of the proteasome 26 S (22). The proteasome 26 S is the chief site of regulatory protein turnover in eukaryotic cells. It is also a component of the quality control machinery that selectively degrades proteins with abnormal structures or allows their refolding (23). It thus participates in nearly all of the multiple roles played by intracellular protein degradation in cellular function. The proteasome 26 S comprises one 20 S catalytic complex and two axially positioned 19 S regulatory complexes that control substrate access to the catalytic chamber. Most known protein substrates of the proteasome are covalently modified with a polyubiquitin chain as a prerequisite for their proteolysis. Recognition of this signal is followed by substrate unfolding and translocation that are presumably catalyzed by one or more of the six nonredundant AAA ATPases located at the base of the 19 S regulatory subunit. Actually, TBP-1 is a member of this protein family and is likely to be involved in this process.

An interesting aspect of the 19 S regulatory subunits is their apparent involvement in cellular events that do not require proteolysis. In fact, 19 S protein members have been shown to be involved in transcription regulation (24, 25). Interestingly, TBP-1 itself was originally described as a factor that binds to HIV-Tat protein and suppresses Tat-mediated transactivation.
of HIV gene expression (20). Furthermore, TBP-1 binds to the HBx protein of HBV virus, stimulating HBx-specific transcription of the HBV genomic DNA (26), and also potentiates the ligand-dependent transactivation by TRp1 and TRα1 via thyroid hormone response elements (27). It has also been shown that, although unable to bind DNA, TBP-1 is a strong transcriptional activator when brought into proximity of several promoter elements; it has been demonstrated that transcriptional activity depends upon the integrity of the nucleotide binding domain and the helicase domain (21, 26).

Protein members of the 19S subunit have also been shown to play a role in nucleotide excision repair in the absence of proteasome activity (28), further supporting the notion that roles other than proteolysis could be features of 19S AAA ATPase family members.

An interesting aspect of TBP-1 regards its supposed role as a tumor suppressor, since TBP-1 mRNA levels were found to be elevated upon inhibition of the oncogenic phenotype of transformed cells expressing ErbB family receptors. Furthermore, TBP-1 overexpression diminished cell proliferation and strongly inhibited transforming efficiency in athymic mice when stably expressed in human tumor cells containing ErbB family receptors (29). In addition, TBP-1 also possesses 46% identity to KAI1, a metastasis suppressor gene for human prostate cancer (30), and maps to chromosome 11p12–13, a region frequently deleted in cancers (31).

Here we show that the overexpression of TBP-1 in various cell lines resulted in a dramatic increase in the ARF protein levels and, consequently, in an increase in p53 protein levels and activity, uncovering a functional role of TBP-1 through ARF stabilization.

Interestingly, TBP-1 appears to inhibit proliferation with an efficiency similar to that exhibited by ARF, in different cell lines. Although our observations need further elucidations, they underline a clear involvement of TBP-1 in the control of cell proliferation.

EXPERIMENTAL PROCEDURES

Plasmids

For Yeast Two-hybrid Screening—ARF constructs were as already described (18).

TBPI Constructs—pGAD10-TBP-1 (1–31), pGAD10-TBP-1 (1–61), and pGAD10-TBP-1 (1–61−404) were obtained by PCR amplification using the primers TBP-22F and TBP-31R, TBP-22F and TBP-61R, and TBP-62F and TBP-404R, respectively, and subsequent cloning in pGAD10-cut XhoI/BamHI.

PCR Primers—Primers were as follows: TBP-22F, CGCTCGAGCGCAGCCGTCGCACCGCGACTGTT; TBP-31R, AACCTCGAGATGTTGACCTCATTATAGTCAAGG; TBP-62F, AACCTCGAGATGTTGACCTCATTATAGTCAAGG; TBP-404R, ACGGGGATCCTGCCTAGGCGTAGTGGTGAGACAGAAG; TBP-61R, ACGGGGATCCTGCCTAGGCGTAGTGGTGAGACAGAAG; TBP-22F, CGCTCGAGCGCAGCCGTCGCACCGCGACTGTT; TBP-31R, AACCTCGAGATGTTGACCTCATTATAGTCAAGG; TBP-62F, AACCTCGAGATGTTGACCTCATTATAGTCAAGG; TBP-404R, ACGGGGATCCTGCCTAGGCGTAGTGGTGAGACAGAAG; TBP-61R, ACGGGGATCCTGCCTAGGCGTAGTGGTGAGACAGAAG.

Mammalian Expression Vectors—The 1.341-kg fragment containing the TBP-1 cDNA was obtained from EcoRI digestion from pGADTBP-1 and ligated into the pcDNA1.His-Invitrogen (pcDNA1.BP-1). The pGAD10-TBP-1 plasmid was cut with Smal and religated in order to eliminate 372 bp (aa 202–238); the resulting plasmid was cut with EcoRI in order to excise the deleted TBP-1 fragment that was cloned in the EcoRI site of pcDNA3.1.Hiss to give pcDNA1.BP-1 (aa 320–326). The EcoRI fragment from pGAD10-TBP-1 (61–404) was cloned in the EcoRI site of pcDNA3.1.Hiss (pcDNA1.BP-1). The PG13 artificial promoter, containing 13 repeats of a specific p53 binding site, was provided by C. A. Lane and was used as described (22).

Yeast Two-hybrid Screen

The procedure was as already described (18). Briefly, pBTM-ARF construct was used to screen a human muscle cDNA library cloned into the pGAD10 vector (Clontech). The yeast strain L40 (34) was sequentially transformed with the pBTM-ARF vector and the library. An estimated 107 transformants were screened. Yeasts containing inter-acting proteins were identified by growth on selective media lacking leucine, tryptophan, and histidine and confirmed by β-galactosidase activity. Isolated plasmids were retransformed into L40 with the negative control plasmid pBTM-galactin (a gift of L. Chiariotti) and with pBTM-ARF and tested again for growing on the selection media. Those that were negative for interaction with galactin were sequenced, and DNA sequences were used to search the nonredundant GenBankTM data base using the BLAST search algorithm available on the World Wide Web at www.ncbi.nlm.nih.gov.

Cell Culture, Transfection, and Coimmunoprecipitation

U2OS, Saso2, H1299, HeLa, and COS-7 cell lines were maintained in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal calf serum. The NIH3T3 cell line was maintained in Dulbecco’s modified Eagle’s medium supplemented with 10% calf serum. U2OS cells were transiently transfected using Superfect (Qiagen); H1299 and HeLa cells were transiently transfected using LipofectAMINE (Invitrogen); NIH3T3 cells were transiently transfected using LipofectAMINE Plus; and COS-7 cells were transiently transfected using LipofectAMINE 2000, following the manufacturer’s instructions. The total amount of DNA was brought up to 2 μg using the “empty” expression vector when necessary.

For coimmunoprecipitation, U2OS cells (two 60-mm dishes) were transfected with 1 μg of pcDNAARF or 1 μg of pcDNA1.BP-1 and 1 μg of pcDNA1.BP-2. Cells were harvested 24 h after transfection and lysed in IBP buffer (150 mM NaCl, 50 mM Tris-HCl, 0.5% Nonidet P-40, 1 mM phenylmethylsulfonyl fluoride, leupeptin (10 μg/ml), aprotinin (10 μg/ml), and trypsin inhibitor (10 μg/ml)). Debris was removed by centrifugation. Equal amounts of lysates (0.8 μg of protein) were precleared with protein A-agarose beads (Santa Cruz Biotechnology, Inc., Santa Cruz, CA; 8 μg at 4 °C) and subsequently incubated with anti-ARF antibody (sc-8613; Santa Cruz Biotechnology; dilution 1:40) or monoclonal anti-1.BP-1 (clone 1B9; Affinity Research; dilution 1:200) for 4 h at 4 °C followed by the addition of the protein A-agarose beads (25 μl) and left for 4 h at 4 °C. Beads were collected by centrifugation, and the immunoprecipitates were washed three times with IBP buffer (4 °C), solubilized in SDS-PAGE sample buffer, loaded on 8 or 12% SDS-PAGE, and analyzed by immunoblotting with anti-1.BP-1 antibody (1:4000, 2 h at room temperature) or anti-ARF (1:500, 2 h at room temperature), followed by incubation with the horseradish peroxidase anti-mouse antibody (Amersham Biosciences; 1:5000, 1 h at room temperature) or with horseradish peroxidase-anti-goat (Santa Cruz Biotechnology; 1:5000). Immunoreactive bands were visualized by enhanced chemiluminescence (ECL Plus; Amersham Biosciences).

Coimmunoprecipitation of ARF and the TBP-1 mutants were carried out under exactly the same experimental conditions except for the Western blot analysis that was carried out with monoclonal anti-Xpress antibody (Invitrogen 46-0528; 1:4000 dilution) to detect transfected wild type or mutant versions of TBP-1.

For coimmunoprecipitation of endogenous proteins, H1299 cells (one 100-mm plate) were harvested and lysed as described above. Equal amounts of lysates (2 mg) were incubated with anti-ARF antibody (1:40) or polyclonal anti-actin (Santa Cruz Biotechnology 1:19; 1:40 dilution) for 4 h at 4 °C.

RT-PCR

24 h after transfection, cells were collected, and total RNA was isolated using the Trizol LS reagent (Invitrogen), reverse transcribed (Superscript II; Invitrogen) and PCR-amplified using the forward and reverse primers for ARF and hypoxanthine phosphoribosyltransferase (HPRT) genes at 63 °C for 28 cycles: ARF forward, ATGGTGCGCGAGCCTGTTGCTG; ARF reverse, GGTTGCGCGAGCCTGTTGCTG; HPRT forward, CCTTGGGATTACCTAATAAGCGAGCAG; HPRT reverse, CCTTGGGATTACCTAATAAGCGAGCAG.
Gal4 DNA-binding domain-p14ARF fusion protein was used as a bait to screen a human muscle cDNA library cloned into a Gal4 activation domain yeast two-hybrid plasmid. From a screen of 10^7 yeast transformants, 30 colonies were scored as positive in secondary screening assays, a 1341-nucleotide clone was identified in a database search as corresponding to a transcript differentially regulated among different species from archaebacteria to humans. This domain contains a site of binding to ATP and a helix domain and is typically present in proteins of the 19 S regulatory subunit of the proteosome (21, 26) (Fig. 1). The N-terminal portion of TBP-1 is characterized by the presence of a dimerization or heterodimerization domain. Inspection of the amino acid sequence by the program “COILS” (available on the World Wide Web at www.embnet.org/software/COILS_form.html) identified a putative coiled-coil region of 29 aa between aa 11 and 40 (Fig. 1). This domain, rich in leucines, resembles features typical of leucine-zipper domains, commonly involved in protein-protein interactions.

To define which parts of the ARF protein are required for the physical interaction with TBP-1, we assayed, by yeast two-hybrid assay, an ARF mutant expressing only exon 1β (aa 1–65) and two exon 1β deletion mutants (Fig. 1). We were able to show that the region 1–65 of ARF is sufficient for the interaction with TBP-1 (Fig. 1, lanes a and b). Actually, the interaction with TBP-1 appears also stronger when the exon 1β alone is present. Deletion of either aa 27–65 or aa 1–38 of the exon 1β-encoded region (Fig. 1, lanes c and d) impairs the interaction with TBP-1, suggesting that the entire region encompassing amino acids 1–65 is essential for the binding to TBP-1.

To identify the minimal region of TBP-1 necessary for the TBP-1-ARF interaction, we constructed various mutants (details are given under “Experimental Procedures”) that were tested in yeast by two-hybrid assay. TBP-1-(1–31) and TBP-1-(1–61) are deletion mutants that maintain different portions of the N terminus of the protein, whereas TBP-1-(61–404) completely lacks all of the N terminus (see Fig. 1, lanes a, c, f, and g). As shown in Fig. 1, lane e, TBP-1-(1–61), bearing all of the putative coiled-coil region, is the only mutant capable of interacting with exon 1β of p14ARF, whereas neither TBP-1-(1–31), containing only part of the coiled-coil region, nor TBP-1-(61–404), which completely lacks it, are able to bind to p14ARF.

Analysis of TBP-1 Expression and Interaction with ARF in Mammalian Cells—TBP-1 is a widely expressed protein, conserved among different species from archaeabacteria to humans. Before proceeding to further analysis in mammalian cells, we evaluated the level of expression of TBP-1 in different cell lines, using a commercially available antibody raised against TBP-1. As a control of the antibody specificity, full-length TBP-1 was cloned in pcDNA3.1His mammalian expression vector and transfected in U2OS cells; the cellular extracts were probed with anti-Xpress or anti-TBP-1 antibodies (Fig. 2A). Both antibodies reveal a band of roughly 50 kDa, representing the transfected TBP-1, whereas the anti-TBP-1 antibody recognizes also the endogenous TBP-1, as expected. Next, cell lysates...
were prepared from U2OS, COS-7, Saos2, and H1299 cell lines, and the level of expression of endogenous TBP-1 was assayed by using anti-TBP-1 antibodies. As it is possible to see in Fig. 2B, TBP-1 is abundantly expressed in all of the cell lines tested.

By RT-PCR we could also observe comparable levels of TBP-1 mRNA among the different cell lines tested (data not shown). Finally, by indirect immunofluorescence, we verified that TBP-1 is localized both in the nucleus and cytoplasm, with a preferential distribution at the nuclear periphery (data not shown), according to Refs. 21, 26, and 33.

To confirm the ARF/TBP-1 interaction in intact cells, we decided to perform co-immunoprecipitation experiments in various cell lines. For this purpose, U2OS cells (ARF−) were transfected with mammalian expression plasmids encoding TBP-1 and/or ARF. The cellular lysates were immunoprecipitated with anti-TBP-1 antibodies, and the immune complexes were blotted and probed with anti-ARF antibodies. Coimmunoprecipitation of TBP-1 (Fig. 2C, lane 3) occurred only when this protein was coexpressed with ARF, since the complexes were not found when the protein alone was expressed into the cells (Fig. 2C, compare lane 3 with lanes 1 and 2). Similar results were obtained when the cellular lysates were immunoprecipitated with anti-ARF antibodies (Fig. 2D). The interaction was observed also in COS-7 cells using similar experimental procedures (data not shown).

Moreover, to confirm the physiological relevance of the TBP-1-ARF complexes containing ectopic proteins, lysates of H1299, in which ARF is abundantly expressed, were analyzed for endogenous TBP-1-ARF complexes. 2 mg of total cellular extract of untreated H1299 cells were used for coimmunoprecipitation with anti-ARF antibodies; the resulting immune complexes were analyzed by Western blot for their content of both TBP-1 and ARF. Both proteins were present in an anti-ARF immunoprecipitate (Fig. 2E, lane 2) but not in that of an irrelevant control immunoprecipitate of H1299 lysate (Fig. 2E, lane 1), suggesting an endogenous ARF-TBP-1 interaction.

Our yeast two-hybrid data suggested that the first 61 amino acids of TBP-1 are sufficient for the interaction with ARF. To obtain an independent evaluation of the protein-protein interaction, we wanted to verify whether the deletion of the first 61 aa at the N terminus of TBP-1 could abrogate its capacity to bind to ARF also in human cells. We thus constructed the pcDNA-TBP-1-(61–404) expression vector (Fig. 2F) and used it to transfect the U2OS cell line in combination with pcDNAARF (Fig. 2G, lane 3) or alone (Fig. 2G, lane 5). Wild type TBP-1 and a mutant TBP-1 lacking the helicase domain but retaining all
of the N-terminal region (pcDNA TBP-1; 202–326) (Fig. 2F) were used, as controls, under the same experimental conditions (Fig. 2G, lanes 1 and 7, and lanes 2 and 6). The expression level of the various proteins was assayed in Western blot analysis with anti-Xpress and anti-ARF antibodies prior to subjecting them to immunoprecipitation (Fig. 2G, upper panel). Cell lysates were immunoprecipitated with anti-ARF antibodies and probed with anti-Xpress antibody (that reveal both the full-length and the TBP-1 mutants) and with anti-ARF antibody. Lysates obtained from cells transfected only with pcDNAARF was also included as control (lane 4). Fig. 2G clearly shows that the mutant TBP-1 (61–404) does not interact with ARF, thus confirming the data obtained in yeast (compare lanes 1–3).

**p14ARF Protein Levels Increase following TBP-1 Overexpression**—TBP-1 is a member of the 19 S regulatory subunit of the 26 S proteasome, the essential multiprotein complex involved in the degradation of ubiquitinated and nonnative proteins in an ATP-dependent fashion (23). Furthermore, TBP-1 has been recently identified as one of the major ATPase subunits involved in the recognition of ubiquitinated proteins (34). ARF protein levels do not vary during the cell cycle but increase after an oncogenic stimulus. The precise mechanism through which the level of ARF protein is regulated into the cell is actually still obscure. Transcriptional regulation seems to be partially responsible for its accumulation, whereas evidence of post-translational mechanisms of regulation has been reported (35–37).

The evidence of the interaction between ARF and a component of the regulatory subunit of the proteasome raises the question of whether the ARF turnover depends on proteasome function. To elucidate this point, various cell lines expressing a transfected ARF were treated with the proteasome inhibitors ALLnL or MG132. The relative abundance of ARF was analyzed by Western blot, with gel loading of lysate protein normalized for the abundance of endogenous actin (Fig. 3A, lower panels). As shown in Fig. 3A (central panels), the use of ALLnL or MG132 drugs does not cause any accumulation of both transfected and endogenous ARF, as would have been expected for a protein that is degraded by the proteasome. As a control of the drugs’ effect, the same cell lysates were probed with an anti-MDM2 antibody, since HDM2 turnover is regulated by proteasome degradation (Fig. 3A, upper panels). These results suggest that mechanisms other than proteasome degradation are responsible for the regulation of ARF protein levels.

Nevertheless, we explored the possibility that TBP-1 could modulate the abundance of p14ARF. 0.5 μg of pcDNAARF and increasing amounts of pcDNA TBP-1 were cotransfected into H1299 cells. Lysates were analyzed by Western blot with anti-Xpress, anti-actin, and anti-ARF antibodies (Fig. 3). Interestingly, synthesis of TBP-1 resulted in a sharp, linear increase in the abundance of ectopically expressed ARF. Remarkably, also the endogenous ARF level increased relative to control, underlying the physiological relevance of the described phenomenon.

**TBP-1-induced ARF Increase Occurs at the Post-translational Level**—To gain insights into the mechanisms responsible for the accumulation of p14ARF following TBP-1 overexpression, we followed various experimental approaches. To determine whether the elevation in the ARF protein level was due to an increase in transcription or stability of ARF mRNA, we performed RT-PCR experiments. Fig. 4A shows that the relative level of ARF mRNA was similar in the presence or in the absence of ectopic expression of TBP1.

The possibility that the TBP-1-induced increase in ARF abundance was caused by a decrease of ARF protein turnover was directly investigated, comparing the half-life of ARF in the presence or absence of TBP-1 overexpression. For this purpose, U2OS cells were transfected with pcDNAARF alone or together with pcDNA TBP-1 (Fig. 4B). 24 h after transfection, cells were treated with cycloheximide to block protein synthesis. At the indicated times after exposure to the drug, cells were harvested, and the extracts were analyzed by Western blot and probed with anti-ARF antibody. As is shown, ARF is quite stable, with a half-life of about 6 h as already described (35). In the presence of ectopically expressed TBP-1, ARF decayed at lower rates; the half-life of ARF was increased from about 6 h to more than 10 h. These observations strongly suggest that the ARF stabilization, following TBP-1 synthesis, occurs at the post-translational level.

**ARF Stabilization by TBP-1 Requires the Physical Interaction between the Two Proteins**—The effect of TBP-1 on ARF protein turnover could, in principle, be associated with the formation of complexes containing TBP-1 and ARF. Hence, we wanted to verify if the TBP-1 (61–404) mutant, which is unable to interact with ARF, still exerts an effect on ARF accumulation. For this purpose, we transfected U2OS cells with ARF and increasing amounts of wild type TBP-1 (Fig. 5, lanes 2 and 3) or TBP-1 (61–404) (lanes 7–9) or TBP-1 (202–326) mutant (lanes 4–6), as control. The TBP-1 (61–404) mutant that does...
not bind to ARF does not cause any effect on ARF protein levels, suggesting that physical interaction between ARF and TBP-1 is absolutely necessary to get the ARF stabilization.

Interestingly, the mutant TBP-1-(Δ202–326), which lacks the helicase domain necessary for the transcriptional functions of TBP-1 (21, 26), is still able to stabilize the ARF protein at levels similar to that obtained with wild type TBP-1, suggesting that the stabilization of ARF does not require the transcriptional domain of TBP-1.

**TBP-1-induced ARF Stabilization Results in an Increase in the p53 Protein Levels and Activity**—An increase in the ARF cellular levels following oncogenic stimuli leads to the stabilization and stimulation of the activity of p53. However, other pathways in which ARF is involved have been described recently, suggesting alternatives to the already known mechanisms of functioning of ARF. As a first attempt, we investigated whether the ARF stabilization due to TBP-1 overexpression culminates in an increase of p53 protein levels and activity. For this purpose, we chose to test whether the transcriptional activity of endogenous p53 is affected by TBP-1 overexpression. U2OS cells were transfected with pcDNAARF, FIG. 4.

**Fig. 4. Effect of TBP-1 overexpression on ARF.** A, H1299 cells were either untransfected (lane 3) or transfected with 1 or 2 µg of pcDNA TBP-1 (lanes 4 and 5). 24 h post-transfection, cells were harvested, and total RNA was prepared. The relative amount of endogenous ARF mRNA was analyzed by RT-PCR using ARF forward and reverse primers. HPRT RT-PCR products were obtained using oligonucleotides of the HPRT gene. Controls without template (lane 1) and with RNA as template (lane 2) were also included. B, three plates (60-mm dishes) of U2OS cells were transfected with pcDNAARF (1 µg) in the presence or absence of 1 µg of pcDNA TBP-1. 12 h after transfection, the cells were trypsinized, and the cells from the same set were pooled and replated into six plates. 24 h from transfection, cycloheximide was added to the medium at a final concentration of 80 µg/ml, and the cells were harvested at the indicated time points. Total cell extract was prepared, and 30 µg of cell extract was probed, in Western blot, with anti-ARF antibody and, as control, with anti-actin. C, TBP-1 half-life. Untreated U2OS cells were trypsinized, pooled, and split into six plates. After 24 h, cycloheximide was added to the medium at a final concentration of 80 µg/ml, and the cells were harvested at the indicated time points. Total cell extract was prepared, and 30 µg of cell extract was probed, in Western blot, with anti TBP-1 and, as control, with anti-actin antibodies.

**Fig. 5. ARF stabilization by TBP-1 requires the physical interaction between the two proteins.** U2OS cells were transfected with 0.5 µg of ARF and 1 or 2 µg of pcDNA TBP-1 (lanes 2 and 3); 0.5, 1, or 2 µg of TBP-1-(Δ202–326) (lanes 4–6); or 0.5, 1, or 2 µg of TBP-1-(61–404) (lanes 7–9). Approximately 24 h after transfection, total cell extract was prepared. 30 µg of cell extract was probed, in Western blot, with anti-Xpress and anti-ARF antibodies.
pcDNATBP-1, or both. 24 h post-transfection, cells were collected, and cell lysates were analyzed for chloramphenicol acetyltransferase activity and p53 protein levels. As expected, ARF ectopic expression causes an increase in endogenous p53 protein levels and activity (Fig. 6, A and B). Coexpression of ARF and TBP-1 results in a consistently higher increase in both p53 protein level (Fig. 6B) and transcriptional activity (Fig. 6A). Moreover, a parallel increase in the level of endogenous p21WAF, a well-known target of p53, occurred (Fig. 6B).

Interestingly, we also noted an effect of TBP-1 overexpression on p53 in the absence of ARF. In fact, Fig. 6, A and B, shows a slight increment of p53 level and activity following transfection of pcDNA-TBP-1 alone. Furthermore, TBP-1 ectopic expression results in a slight decrease of HDM2, a key negative regulator of p53, as shown in Fig. 6C.

Interestingly, we also noted an effect of TBP-1 overexpression on p53 in the absence of ARF. In fact, Fig. 6, A and B, shows a slight increment of p53 level and activity following transfection of pcDNA-TBP-1 alone. Furthermore, TBP-1 ectopic expression results in a slight decrease of HDM2, a key negative regulator of p53, as shown in Fig. 6C.

**DISCUSSION**

Herein, we identify TBP-1 as a new partner of p14ARF and demonstrate its effect on the abundance of ARF.

By yeast two-hybrid assay and coimmunoprecipitation experiments, we show that the binding between these two proteins requires an intact ARF N-terminal region and the first 61 aa of TBP-1. Interestingly, between aa 11 and 40 of TBP-1 is a coiled-coil region sharing structural similarities to that present in the spinophilin/neurabin II, a cellular partner of ARF already identified (18), suggesting the existence of a common motif involved in the ARF interaction.

TBP-1 is a multifunctional protein, component of the 19 S regulatory subunit of the proteasome. The proteasome is a fundamental cellular structure that catalyzes the degradation of many rate-limiting enzymes, transcriptional regulators, and regulatory proteins; it is also critically involved in the rapid elimination of abnormal proteins and, in higher eukaryotes, in antigen processing. Interestingly, the six nonredundant
ATPases of the 19 S regulator have been shown to interact with many cellular and viral proteins. Presumably, these interactions act to modulate the recognition/degradation of either the binding proteins themselves or other cellular and viral proteins. For example, the HEC (highly expressed in cancer) protein specifically interacts with the S7 ATPase and modulates the degradation of a mitotic cyclin (38), whereas the papilloma virus E7 protein specifically interacts with the S4 ATPase and controls the degradation of the retinoblastoma protein (39, 40). Recently, it has been shown that the S6 subunit of the 19 S proteasome corresponds to gankyrin, discovered to be an oncoprotein that increases the degradation of the retinoblastoma protein (41). The “proteasome pathway” thus often appears to be the target of cancer-related deregulation and is involved in processes such as oncogenic transformation, tumor progression, and escape from immune surveillance and drug resistance.

The physiological significance of the interaction between TBP-1 and ARF could be that ARF turnover depends on the proteasome function. ARF expression has been known to be regulated at the transcriptional level, and its expression is induced by several oncogenic signals such as Myc, E1A, E2F, mutated Ras and v-Abl (reviewed in Ref. 42). ARF expression is inhibited by Twist and Tbx-2 and is actively repressed during development by the Bmi repressor. Additionally, the human ARF promoter can be silenced by methylation or expression of wild-type p53 (12). However, ARF expression appears to be regulated also at the level of translation and/or protein stability (35–37).

We tested whether ARF protein levels could depend on the proteasome function and found that, blocking the proteolytic activity of the proteasome through the use of ALLnL and MG132 drugs, no variation in the amount of ARF protein, both endogenous and transfected, occurred, as would have been expected for a protein that is degraded by the proteasome. Furthermore, ARF primary sequence does not contain any lysine residue that can allow ubiquitination in a canonical way.

Nevertheless, the results herein suggest a role for TBP-1 in post-translational regulation of ARF. In fact, we surprisingly found that synthesis of TBP-1 causes a sharp increase in the abundance of ARF. The increase in ARF protein level is unlikely to be due to a transcriptional effect of TBP-1 for various reasons: 1) the relative level of ARF mRNA was similar in the presence or in the absence of TBP-1; 2) the observed effect occurs both on endogenous and transfected ARF; and 3) the effect strictly requires binding between the two proteins; 4) the mutant TBP-1 that lacks the helicase domain, reported to be essential for the transcriptional activity of TBP-1, is still able to increase ARF levels; and 4) the ARF half-life appears to increase following TBP-1 synthesis.

Our data are not the first outcome of a physical or functional link between ARF and proteasomal components. Recently, it has been reported that colocalization between p53, HDM2, and p14ARF, once overexpressed, occurs at extranucleolar sites. Accumulation of PML bodies and proteasomes at these sites suggests that the components of the nuclear inclusions are targeted for proteasome-mediated degradation (43). These observations suggest that ARF could exert its functions in the cells in these cellular compartments, presumably bringing in contact proteins that have to be degraded by the proteasome. It has also been reported that ARF can target E2F transcription factors for degradation by the proteasome, by a not yet defined mechanism (15). Whether ARF is an adaptor that brings E2F or eventually other unknown proteins and the proteasome apparatus together remains to be determined. In this scenario, one could argue that ARF-TBP-1 interaction could exert a role in this unknown mechanism. On the other hand, 19 S proteins and TBP-1 itself appear to be also involved in cellular events that do not require the activity of the proteasome (24, 25, 28). Hence, the possibility that the ARF-TBP-1 interaction does not involve the proteasome in its whole is still open.

It is well established that ARF acts upstream of p53 and activates its transcriptional activation functions. The increase in the ARF protein levels following TBP-1 overexpression corresponds to an increase in its functions in the HDM2/p53 pathway. Fig. 6 shows that co-expression of ARF and TBP-1 results in a consistently higher increase in both p53 protein level and transcriptional activity as judged by chloramphenicol
acetyltransferase analysis and Western blot, highlighting a physiological role for the ARF/TBP-1 interaction. Although our data clearly point out for a role of TBP-1 in the regulation of ARF protein levels, we have found that TBP-1 can exert a negative control on cell proliferation in two different cell lines, NIH3T3 and U2OS, both lacking ARF expression and retaining a functional p53 gene. Therefore, in our colony formation efficiency assays, TBP-1 appears to act as growth suppressor only in cellular contexts in which p53 is present. On the other hand, monitoring the effects of TBP-1 overexpression in the absence of ARF as early as at 48 h post-transfection (see Fig. 6), we have noticed a slight increase of p53 and a decrease of HDM2 protein levels; also unknown is the precise role of TBP-1 in the context of the proteasome or in roles unrelated to the proteasome. In principle, one could speculate that the role of TBP-1 is to substract ARF from other unknown mechanisms providing some of the plasmids used in this study, Dr. C. Passananti for generous technical help. We are grateful to Drs. B. Vogelstein and L. Chiarotti for generous providing some of the plasmids used in this study, Dr. C. Passananti for the human muscle cDNA library, and Dr. G. Mansuetu for critical reading of the manuscript.

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Functional and Physical Interaction of the Human ARF Tumor Suppressor with Tat-binding Protein-1

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