The signal transducer and activator of transcription 3 (Stat3) transcription factor is required for the antiproliferative effects induced by cytokines, such as the interleukin-6 type. In order to investigate the role of Stat3 in inhibition of cell proliferation, we have used an inducible Stat3 construct in A375 melanoma cells. We found that activation of Stat3 to moderate levels was sufficient to repress A375 proliferation, by slowing cell transit through the cell cycle. Enhanced and prolonged Stat3 activity led to cell cycle arrest and apoptosis. Genes whose expression was altered by Stat3 activation were identified by oligonucleotide microarray analysis. We found that TEL (ETV6), a novel Stat3 target identified in this study, is a negative regulator of Stat3 activity. Small interfering RNA-mediated inhibition of TEL expression resulted in increased Stat3-dependent transcriptional activity and stronger Stat3 antiproliferative activity. Confirming these results, overexpression of TEL repressed Stat3 transcriptional activity. Intriguingly, Stat3 repression did not require TEL DNA binding and appeared to proceed via recruitment of TEL to Stat3. Inhibition of Stat3 activity by TEL represents a novel mechanism regulating the Stat3 signaling pathway.

Signal transducer and activator of transcription 3 (Stat3) is a latent cytoplasmic transcription factor that can be activated by a variety of kinases, including receptor tyrosine kinases and cytoplasmic kinases associated with cytokine receptors. Stat3 appears to play a critical role in the control of many biological processes during development (1–3) and has been implicated in cell proliferation, differentiation, and survival (4). However, constitutive activation of Stat3 has been associated with oncogenesis. Indeed, there is increasing evidence that Stat3 is activated in a wide variety of human tumors, including head and neck, prostate and breast carcinomas, multiple myelomas, and lymphomas (5, 6). Moreover, Stat3 is required for transformation by oncogenic tyrosine kinases such as v-Src, TEL-ABL, and TEL-JAK (7–9), and Stat3 activation is sufficient for transformation of fibroblasts and Ba/F3 cells (9, 10). On the other hand, members of the interleukin (IL)-6 family of cytokines, including IL-6, oncostatin M (OSM), leukemia-inhibitory factor, and ciliary neurotrophic factor, have been shown to inhibit proliferation of some leukemia, melanoma, prostate, and breast cancer cells (11–14), and this inhibition was shown to be mediated by Stat3 (15–17). Moreover, recent clinical studies revealed that active nuclear Stat3 correlates with a better prognosis in node-negative breast tumors and nasopharyngeal tumors (18, 19). These results suggest that in some cellular contexts, Stat3 has antiproliferative and possibly anticancerogenic effects.

Binding of IL-6-type cytokines to their receptors leads to hetero-oligomerization with the common signal transducer gp130, resulting in activation of the receptor-associated Janus kinases (JAKs) and phosphorylation of specific tyrosine residues in the cytoplasmic tail of gp130. The phosphorylated tyrosines serve as docking sites for two major signal-transducing molecules: Src homology domain 2 protein-tyrosine phosphatase (SHP2), which interacts with adapter molecules such as Gab1/2, p85, and Grb2, leading to activation of the mitogen-activated protein kinase and the phosphatidylinositol 3-kinase intracellular signaling pathways (20); and Stat3, which upon binding is phosphorylated by JAK (21). Tyrosine phosphorylation allows Stat3 dimerization, translocation to the nucleus, binding to specific DNA sequences and activation of transcription (10, 22). Considering the critical role of Stat3 in controlling cell growth and differentiation, it is not surprising that negative regulators of Stat3, controlling its activity at multiple levels, have been identified (22): e.g. control of JAK activity by SOCS (23), dephosphorylation of Stat3 by nuclear phosphatases (24), regulation of Stat3 nuclear translocation by GRIM-19 (25), and prevention of DNA binding by PIAS3 (26).

In the work presented here, we have investigated the mechanism underlying the antiproliferative effect of Stat3 on tumor cells. By expressing a conditionally active Stat3 in A375 melanoma cells, we found that Stat3 activation is sufficient to inhibit tumor cell proliferation. Transcriptional targets of Stat3 in A375 cells were determined by oligonucleotide microarray analysis. We have identified a novel Stat3 transcriptional target, TEL/ETV6, a member of the ETS family of transcription factors. Furthermore, we show here that TEL is a novel repressor of Stat3 transcriptional and biological activity.

**EXPERIMENTAL PROCEDURES**

*Reagents and Plasmids—Recombinant human IL-6 and OSM were from PeproTechEC (London, UK). 4-Hydroxytamoxifen (4HT) was from Sigma. Human erythropoietin (EPO) was from R&D Systems (Minneapolis, MN). A plasmid, containing the mutated ligand-binding domain of the murine estrogen receptor (ER)R56), was kindly provided by T. Littlewood (Imperial Cancer Research Fund, London, UK). ERR was cloned into pcDNA3.1 as a BamHI-EcoRI fragment. The Stat3 sequence, with an additional BamHI site, was obtained by PCR amplification from pReCMVStat3 (kindly provided by J. E. Bromberg,
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Rockefeller University, New York) and cloned into pcDNA3.1-ER(TM). Chimeric receptors, consisting of the extracellular part of the murine EPO receptor fused to various regions of the gpl30 cytoplasmic domain (16), were a kind gift from M. Kortylewski and I. Behrmann (RWTH, Aachen, Germany). The pCMV-TEL, pCMV-TEL3P, pCMV-TEL Δ122–176, pCMV-TEL Δ122–217, pCMV-TEL Δ268–335, and pCMV-TEL Δ303–333 constructs were generously provided by S. W. Hiebert (Vanderbilt University School of Medicine, Nashville, TN) and have been previously described (27). The TEL DNA-binding domain mutant construct (28), kindly provided by G. Grosveld (St. Jude Children’s Research Hospital, Memphis, TN), was subcloned into pCMV.

Cell Culture and Cell Transfection—A375 melanoma cells were maintained in RPMI supplemented with 5% fetal calf serum (Invitrogen). HEK293T cells were maintained in Dulbecco’s modified Eagle medium supplemented with 10% fetal calf serum (Invitrogen). For transient expression, plasmids were introduced into cells using the Effectene reagent (Qiagen, Hilden, Germany) according to the manufacturer’s protocol. Stable ASER cells were obtained by transfection of A375 cells with pcDNA3.1-Stat-ER. Transfected cells were selected using 1 mg/ml G418 (Invitrogen), and several clones were picked, expanded, and analyzed for STAT3-ER expression. Similar results were obtained with two independent clones.

For siRNA transfection, cells were plated at a density of 5 × 10^4 cells/well in 6-well dishes 24 h before transfection. The RNA oligonucleotides were obtained from XERAGON Inc. (Huntsville, AL). siRNA duplexes (20 nM/well) were introduced into the cells using 3 μl of OligofectAMINE reagent (Invitrogen) according to the manufacturer’s protocol. The following 21-mer oligonucleotide pairs were used: for TEL (accession number NM001987), nucleotides 540–560; and for control LacZ (accession number M50568), nucleotides 427–429 (obtained from D. Cappellen, FMI, Basel; siRNA sequences were “blasted” against the GenBank@EMBL database to ensure gene specificity. Transfection with expression vectors was carried out using Effectene, 24 h after the introduction of siRNA.

Immunoprecipitation and Western Blots—Cells were harvested and lysed in Nonident P-40 extraction buffer for 5 min on ice as previously described (15). The lysates were clarified by centrifugation, and protein concentrations were determined using the Bio-Rad protein assay (Bio-Rad Laboratories). For immunoprecipitation, equal amounts of proteins were incubated with an anti-Stat3 antibody (F-2 or C-20; Santa Cruz Biotechnology, Santa Cruz, CA) for 1 h. Immune complexes were collected with protein G-Sepharose or protein A-Sepharose (Sigma) and subjected to SDS-PAGE. Proteins were analyzed by Western blotting analysis of the oligonucleotide-bound proteins, as previously described (15), with specific antibodies: c-TEL (kindly provided by G. Grosveld); TEL, Stat3, cyclin D2, cyclin D3, and cyclin E (Santa Cruz Biotechnology, Inc., Santa Cruz, CA); phospho-Stat3 (Tyr 705) and p27 (Groveld); TEL, Stat3, cyclin D2, cyclin D3, and cyclin E (Santa Cruz Biotechnology, Inc., Santa Cruz, CA); phospho-Stat3 (Tyr257) and p27 Kip1 (Cell Signaling Technology, Beverly, MA); and cyclin D1 (Novoceastra Laboratories Ltd., Newcastle upon Tyne, UK). Proteins were visualized with peroxidase-coupled secondary antibodies using the enhanced chemiluminescence detection system (Amersham Biosciences). DNA Binding Assay—Nuclear extracts from cells treated with 4HT (1 μM) or OSM (100 ng/ml) were prepared as described (29). To measure ETS DNA binding, nuclear extracts were precleared with immobilized NeutraVidin beads (Fierce). A 5′-biotinylated double-stranded oligonucleotide, corresponding to the ETS-binding sequence 5'-ATAAACAG-GAACTGG-3′ (30), was precipitated with NeutraVidin beads. The immobilized ETS oligonucleotides or the Stat3 TransCruz oligonucleotide-agarose conjugate (Santa Cruz Biotechnology) were added to 80 μg of nuclear extracts in binding buffer (20 mM HEPES, 0.5 mM EDTA, 1 mM dithiothreitol, 2 μg/ml poly(dI-dC) and rotated for 2 h at 4°C. Samples were centrifuged, and the pellets were washed three times with binding buffer. Proteins were eluted from the beads by boiling in loading buffer, and SDS-PAGE was performed as described above.

Flow Cytometric Analyses—Cells were harvested after treatment with 4HT or OSM/4HT; cell pellets were washed three times with ice-cold phosphate-buffered saline and resuspended in propidium iodide-containing buffer. After a 30-min incubation on ice, cell cycle distribution was measured with a BD Biosciences FACScan flow cytometer. Cell cycle kinetics were determined by pulse-labeling control and 4HT-treated cells with 10 μM BrdUrd for 45 min and collecting cells every 3 h for 21 h. BrdUrd-positive cells were detected using a fluorescence isothiocyanate-conjugated anti-BrdUrd antibody (BD Biosciences) according to the manufacturer’s protocol. DNA was stained with 7-aminoactinomycin D. Cell cycle position of the BrdUrd-labeled cell population was determined by two-color flow cytometric analysis. Results were expressed as the percentage of BrdUrd-positive cells that have progressed to a certain phase of the cell cycle, relative to the total number of BrdUrd-positive cells.

Luciferase Assays—Cells (2 × 10^5 cells in 6-well plates) were transfected with a luciferase reporter construct (a generous gift from Drs. M. Hibi and T. Hirano, Osaka University, Osaka, Japan), containing four copies of the acute phase response element (APRE) from the α2-macroglobulin gene in front of the minimal junB promoter (14), together with the Renilla control plasmid, pRL-SV40, from Promega. Twenty-four hours later, cells were either left unstimulated or stimulated with OSM, 4HT, or the combination of 4HT/OSM for 24 h. In some instances, 250 ng trichostatin A (Sigma) was added to the medium. Cell extracts were prepared, and luciferase activity was measured using the dual-lucerase reporter assay system (from Promega) in an Autolumat LB953 (Berthold Technologies, Wildbad, Germany). Changes in firefly luciferase activity were expressed relative to Renilla luciferase activity in the same sample.

Microarray Analysis and Quantitative Radioactive Reverse Transcription-PCR—The microarray analyses were performed using HG U95A GeneChips(TM) (Affymetrix, Santa Clara, CA). Biotin-labeled cRNA probes were generated from each sample starting from 10 μg of total cellular RNA, which was extracted using TRIzol (Invitrogen) and further purified with the RNeasy mini kit (Qiagen). The cRNA probes were hybridized on the arrays, and signals were detected, according to the manufacturer’s instructions. Chip analyses were performed using the Affymetrix Microarray Suite 5 (target intensity 500 used for chip scaling) and GeneSpring 4.2.1 (Silicon Genetics). Changes in gene expression were assessed by looking for concordant changes between replicates, using a signed Wilcoxon rank test, as recommended by Affymetrix. The “change” p value threshold was <0.003 for increase and >0.997 for decrease. After concordance analysis, these values become <9 × 10^-6 and >0.999991, respectively. Any gene whose detection p value was >0.05 was discarded from the analysis.
The effect of 4HT is due to Stat3-ER activation. These results demonstrate that transcriptional activation and biological effects of Stat3-ER are equivalent to the effects of physiological activation of Stat3 with OSM. In summary, whereas Stat3 was previously shown to be required for OSM-induced inhibition of cell proliferation (15, 16), our results show that it is also sufficient for this effect.

**OSM Enhances Stat3-ER Transcriptional Activity and DNA Binding**—The combination of 4HT and OSM had a dramatically stronger antiproliferative effect on ASER cells, compared with individual treatments (90% versus 40% fewer cells after 3 days) (Fig. 1C). This observation raised the possibility that specific signaling pathways induced by OSM enhanced Stat3 biological activity. The mechanism by which 4HT and OSM cooperate to repress cell proliferation was further investigated. The combination of 4HT and OSM dramatically increased APRE-driven reporter activity, compared with the activity induced by 4HT or OSM alone (Fig. 1B, right panel). For instance, transcription induced by the combination of 1 μM 4HT with 100 ng/ml OSM was more than 100-fold stronger than that induced by the single agents (Fig. 1B, right panel). IL-6 showed the same cooperative effect with 4HT (data not shown). Thus, in ASER cells, 4HT and gp130 signaling synergize to increase Stat3 transcriptional activity.

Treatment of A375 cells with OSM or IL-6 triggers multiple signaling pathways, including the JAKStat, the mitogen-activated protein kinase, and phosphatidylinositol 3-kinase pathways (data not shown). We have investigated which pathway is responsible for enhancing Stat3-ER transcriptional activity, by using chimeric receptors composed of the extracellular domain of the EPO receptor fused to various regions of the gp130 cytoplasmic domain (Fig. 2A), in order to specifically activate individual pathways (32). EPO was used to activate chimeric but not endogenous gp130 signaling (33). A375 cells were transfected with the EG chimera, which contains the transmembrane and the entire cytoplasmic domain of gp130 (Fig. 2A), together with the Stat3-ER construct and the APRE-driven luciferase reporter. Stat3 transcriptional activity was evaluated in EG-expressing cells after treatment with EPO and/or 4HT. Individually, EPO or 4HT induced Stat3 transcriptional activity to similar levels, whereas the combined addition of EPO and 4HT induced very strong luciferase activity (Fig. 2B). A chimera containing only the membrane-proximal box 1/box 2 region of gp130, which mediates association with the JAKs but has none of the Tyr residues required for Stat3 or SHP2 recruitment (32), failed to respond to the concomitant addition of 4HT and EPO. These results show that the chimeric receptors can be used to study the synergistic effect between 4HT- and gp130-mediated Stat3 activation. This was accomplished using cells transfected with receptor constructs containing only the SHP2 (Tyr759) tyrosine module, or one of the Stat3 (Tyr814) tyrosine modules attached to the EG receptor. In response to EPO, these cells specifically activate the mitogen-activated protein kinase/phosphatidylinositol 3-kinase pathways or Stat3, respectively (34).
OSM and 4HT synergeste to induce Stat3 activity. A, schematic representation of chimeric receptors containing the extracellular and transmembrane region of the mouse EPO receptor fused to various portions of the gp130 cytoplasmic domain. EG, chimera with the full-length gp130 cytoplasmic tail; ΔB, truncated gp130 construct containing only the membrane-proximal box 1/box 2 region; Y759 and Y914, ΔB with a fusion of "tyrosine modules" mediating SHP2 or Stat3 binding, respectively. B, A375 cells were co-transfected with constructs coding for the indicated chimeric receptor and the Stat3-ER, together with an APRE-luciferase reporter plasmid and a Renilla plasmid, before stimulation with EPO (3.5 units/ml), 1 μM 4HT, or EPO/4HT for 24 h. Luciferase activity was determined as described in the legend to Fig. 1C. ASER cells were treated with 4HT or the combination of 4HT and OSM for the indicated times. Binding of Stat3 and Stat3 transcriptional activity, compared with the response observed when the ligands were added individually (Fig. 2B). In contrast, in cells expressing the receptor with the SHP2 module (Tyr758), no synergy was observed between EPO and 4HT (Fig. 2B). These results indicate that the gp130/4HT synergy involves only Stat3 signaling and not the SHP2-dependent pathways.

Next, we examined gp130/4HT synergy by measuring specific Stat3 DNA binding. ASER cells were treated with 4HT to activate Stat3-ER, and then OSM was added for different times, and DNA binding of Stat3-ER and endogenous Stat3 was monitored. Binding of Stat3 could be detected 15 min after the addition of OSM, peaked at 30 min and returned to low levels after 1 h of treatment (Fig. 2C, upper panel). In the presence of OSM, Stat3-ER DNA binding was enhanced and, unlike the endogenous Stat3, was maintained for several hours (Fig. 2C, upper panel). Stat3 and Stat3-ER Tyr755 phosphorylation levels followed the kinetics of DNA binding (Fig. 2C, lower panel). These results indicate that OSM-induced gp130 activation leads to phosphorylation of both Stat3 and Stat3-ER; whereas Stat3 is rapidly dephosphorylated, Stat3-ER displays prolonged phosphorylation and DNA binding. This result very likely explains the synergy between 4HT and OSM on Stat3-dependent transcriptional activity and inhibition of cell proliferation.

Effect of Stat3 Activity on the A375 Cell Cycle—In order to understand the mechanism by which Stat3 activity leads to decreased cell number, cell cycle analyses were performed, and nuclear cell cycle regulators were examined after treatment of ASER cells with 4HT and/or OSM. Flow cytometry revealed that neither 4HT (Fig. 3A) nor the physiological activator OSM (data not shown) significantly affected the cell cycle profile. There was no obvious accumulation of cells in a particular phase of the cell cycle, despite the fact that both treatments led to a 40% decrease in cell number (Fig. 1C). In accordance with these results, in cells treated with 4HT or OSM there were no detectable changes in the levels of the major regulators of the G1/S transition, including cyclin D1, D2, and D3, and the cyclin-dependent kinase inhibitors p27Kip1 (Fig. 3D) and p21Cip1 (data not shown). In addition, there was no evidence of cell death (Fig. 3C). Interestingly, analysis of cell cycle kinetics indicates that 4HT-treated cells were transiting slower through the cell cycle. ASER cells were pulse-labeled with BrdUrd, and the cell cycle position of BrdUrd-positive cells was determined by flow cytometry. The percentage of BrdUrd-positive cells that progressed to the G1, the G2/M, or the next G1 phase of the cell cycle 6, 15, and 21 h, respectively, after the BrdUrd pulse, was markedly reduced upon 4HT treatment (Table I). Thus, whereas Stat3 activation does not affect the distribution of cells in the different phases of the cell cycle, it does prolong transit of cells through the cell cycle.

In contrast to treatment with 4HT or OSM, ASER cells treated for 24 h with the combination accumulated in G1 (+24%; Fig. 3B). These cells also had lower amounts of cyclin D2, increased expression of p27Kip1 (Fig. 3D), and increased levels of Cdk2-p27Kip1 complexes (data not shown), which is the likely cause of the G1 accumulation. After 3 days, a large proportion of these cells underwent apoptosis, as indicated by the appearance of a sub-G1 peak (quantified in Fig. 3C) and poly(ADP-ribose) polymerase cleavage (data not shown). Thus, the striking effect of the combined 4HT and OSM treatment is largely due to cell death.

Transcriptional Profiling of Stat3 Target Genes—We used oligonucleotide microarrays to identify Stat3 target genes that contribute to the biological effects of Stat3 on A375 cells. Experiments were carried out on two independent ASER clones expressing similar amounts of Stat3-ER and showing a similar response to 4HT, with respect to DNA binding, reporter gene assays, and inhibition of cell proliferation (data not shown). Stat3 DNA binding (Fig. 1A) and reporter gene activity (data not shown) are strongly increased following 2–4 h of 4HT treatment. Thus, RNA was collected from Stat3-ER-expressing cells 4 h and also 24 h after the addition of 4HT. RNAs from three independent experiments were pooled, and the resulting biotinylated cRNAs were hybridized to the Affymetrix U95A oligonucleotide array, containing probe sets for over 12,000 transcripts. Genes that were significantly increased or decreased in both clones, relative to untreated cells (change p <
Fig. 3. ASER cell cycle analyses. ASER cultures were treated with 4HT (A) or OSM/4HT (B) for 24 h (or for the times indicated in C). Cells were harvested, and nuclei were stained with propidium iodide, before flow cytometry was performed. Representative histograms are shown. Changes in the percentage of cells in each phase of the cell cycle and the percentage of apoptotic cells are indicated. D. Western blotting (WB) analysis of cell cycle regulators in lysates from ASER cells.

**TABLE I**

Activation of Stat3 slows down cell cycle progression

|               | Control | 4HT    |
|---------------|---------|--------|
| Percentage progressed to G₁ (6 h) | %   | %     |
| Percentage progressed to G₂ (12 h) | %   | %     |
| Percentage progressed to next G₁ (21 h) | %   | %     |

0.00009) were identified. Genes that were significantly increased or decreased in nontransfected parental A375 cells treated with 4HT were excluded from the study.

Upon 4HT treatment of ASER cells, expression of 62 genes was significantly altered after 4 h. These included several known, direct targets of Stat3, such as the JAK inhibitor SOCS3, α-antichymotrypsin (serpin A3), and the transcription factors CCAAT/enhancer-binding protein δ and JunB, results that validate our experimental approach. Some genes, which have not been previously described as Stat3 targets, were also up-regulated within 4 h; these include the transcription factor TEL (ETV6), the death-associated protein kinase 1 (DAPK1), and the serine protease inhibitor serpin B3.

After 24 h, 4HT-induced Stat3 activation resulted in increased expression of 154 genes and decreased expression of 23 genes. Genes whose expression was increased more than 1.5-fold (64 genes) or decreased more than 1.3-fold (11 genes) are listed (Fig. 4). These genes belong to various functional groups encoding transcriptional regulators that could mediate the Stat3 effect such as TEL, CCAAT/enhancer-binding protein δ, and FOS; receptors, such as OSM receptor; intracellular signaling molecules; and adhesion proteins such as osteopontin/secreted phosphoprotein 1 and genes involved in various aspects of cellular metabolism.

Most of these genes were also activated by OSM. It is noteworthy that the majority of these genes are sensitive to the level of Stat3 activity, since they are very highly induced by the combined 4HT/OSM treatment (Fig. 4). However, 4HT/OSM triggered the activation of many more genes that were not induced by 4HT alone (data not shown). These might be involved in specific programs leading to G1 arrest and cell death.

**TEL Is a Stat3-induced Repressor of Stat3**—The function of TEL, a new Stat3 target gene, identified in this screen, was investigated further. TEL, which belongs to the ETS family of transcription factors, is known to repress transcription of some genes that have an ETS consensus site in their promoter (35). TEL expression is induced by 4HT and by OSM treatment, and since it is already increased at 4 h, it might control expression of secondary target genes contributing to biological effects of Stat3.

The role of TEL in the antiproliferative effect of Stat3 was further evaluated. Most cells express two TEL isoforms, corresponding to initiation of TEL mRNA translation at ATG codon 1 and 43. After the addition of 4HT to ASER cells, protein levels of both isoforms, TEL and TEL-43, steadily increased from 8 h onward (Fig. 5A). We also examined TEL levels, in response to IL-6-type cytokine treatment, in a panel of cancer cell lines. TEL levels were significantly increased after IL-6 or OSM treatment of T47D and SKBr-3 breast carcinoma cells, DU145 prostate carcinoma cells, and HepG2 hepatoma-derived cells (Fig. 5B), suggesting that TEL is a fairly common Stat3
The role of TEL in Stat3 signaling was explored by siRNA knockdown. Transfection of TEL siRNA strongly reduced TEL expression relative to the level observed in cells transfected with the control LacZ siRNA (Fig. 6A). Intriguingly, in the presence of TEL siRNA, Stat3-mediated inhibition of ASER cell proliferation, induced by 4HT or OSM, was stronger (Fig. 6B). These results suggest that TEL might be a Stat3-induced negative regulator of Stat3 signaling.

Since TEL is a transcriptional repressor (36), we tested whether siRNA-mediated knock-down of TEL affected Stat3 transcriptional activity. Stat3-dependent transcription induced by 4HT or OSM was significantly increased when TEL expression was reduced (Fig. 6C). Conversely, overexpression of TEL in ASER cells (Fig. 7, A and C) and in HEK-293T cells (Fig. 7D) resulted in decreased activation of Stat3 by 4HT and OSM, respectively. These results demonstrate that TEL is a negative regulator of Stat3 transcriptional activity.

Mechanism of TEL-dependent Repression of Stat3 Activity—Our results show that TEL is a repressor of Stat3 transcriptional activity. A number of negative regulators acting at different levels of the Stat3 signaling cascade have been identified. The SOCS proteins inhibit JAK activity, leading to decreased Stat3 Tyr phosphorylation; GRIM-19 interferes with Stat3 nuclear shuttling, and PIAS3 prevents Stat3 from binding to DNA (25, 26, 37). Overexpression of TEL in A375 cells did not affect the Stat3 expression levels or Tyr phosphorylation in response to OSM (data not shown). Moreover, Stat3 nuclear translocation and DNA binding were not changed when TEL was overexpressed in A375 cells (data not shown). Thus, TEL appears to function differently from the previously identified negative regulators of Stat3.

TEL repressor activity is dependent on the recruitment of a co-repressor complex, including molecules such as mSin3A, NcoR, and SMRT (27, 38). Since these three proteins are known to interact with histone deacetylases (HDACs), we used trichostatin A (TSA), a general HDAC inhibitor, to test the possibility that HDAC recruitment plays a role in TEL-dependent repression of Stat3 activity. The addition of TSA to 4HT-stimulated ASER cells prevented the repression of Stat3 activity by TEL (Fig. 7A), suggesting that TEL repressor activity toward Stat3 is dependent on the recruitment of HDACs. The co-repressor, HDAC-containing complex has been shown to interact with at least two different domains of TEL: the pointed domain and a central repression domain (27, 38). To identify the domains of TEL essential for repression of Stat3 transcriptional activity, various TEL mutants (Fig. 7B) were overexpressed in ASER cells or in HEK-293T cells, and 4HT- or OSM-induced Stat3 transcriptional activity was evaluated (Fig. 7C and D). Among the TEL mutants tested, only TEL/H9004P, lacking the pointed domain, failed to repress Stat3 activity, whereas mutants with deletions in the central domain were still able to block Stat3 activity. Intriguingly, a TEL DNA-binding domain mutant retained the ability to repress Stat3 activity in ASER and HEK-293T cells, respectively (Fig. 7, C and D, right panels). Thus, repression of Stat3 activity by TEL requires an intact pointed domain, but does not require DNA binding of TEL.

The results suggest that TEL blocks Stat3 transcription by forming a complex with Stat3. This complex was identified using ASER cells transfected with intact TEL or the TEL mutants, TEL ΔP and TEL Δ122–217. Immunoprecipitates of Stat3 from cellular lysates revealed that TEL and TEL ΔP, lacking the pointed domain, failed to repress Stat3 activity, whereas mutants with deletions in the central domain were still able to block Stat3 activity. Intriguingly, a TEL DNA-binding domain mutant retained the ability to repress 4HT- or OSM-induced Stat3 activity in ASER and HEK-293T cells, respectively (Fig. 7, C and D, right panels). Thus, repression of Stat3 activity by TEL requires an intact pointed domain, but does not require DNA binding of TEL.

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ment. As expected, there was an increase in nuclear Stat3 levels following the addition of OSM, whereas nuclear TEL levels were unaffected by cytokine treatment (Fig. 8B, right panels). Confirming the results in transfected cells (Fig. 8A), endogenous TEL was detected in immunoprecipitates of Stat3, with increased levels in the OSM-treated lysates, reflecting increased nuclear Stat3 (Fig. 8B, left panel). Finally, TEL pull-downs, using an immobilized ETS consensus site-containing oligonucleotide, confirmed this association, showing that there was more of the TEL-Stat3 complex in the nuclei of OSM treated cells (Fig. 8B, middle panels). Taken together, these results lead us to suggest that TEL represses Stat3 transcriptional activity by interacting with Stat3 and recruiting HDACs to the transcriptional complex.

DISCUSSION

Stat3 mediates the effects of multiple growth factors and cytokines; as such, Stat3 is involved in many normal physiological processes but also in disease, such as cancer. The identification of Stat3 effectors and regulators should improve our understanding of the multiple and sometimes contradictory biological roles attributed to this transcription factor. Indeed, some studies describe a role for Stat3 in oncogenesis, whereas others demonstrate its ability, acting downstream of cytokine receptors, to inhibit tumor cell proliferation. We have evaluated the antiproliferative effect of Stat3 by introducing a conditionally active Stat3 into A375 melanoma cells. Our results show that depending on the extent of Stat3 activity, the cancer cells show decreased proliferation or growth arrest accompanied by apoptosis. Stat3 targets in the melanoma cells were identified using oligonucleotide microarray analyses. A functional study was carried out on TEL, a novel Stat3 target, which is a member of the ETS family of transcription factors. We report here that TEL is a novel repressor of Stat3 activity; TEL overexpression leads to decreased Stat3-driven transcription, whereas the loss of TEL results in stronger Stat3-dependent transcription and antiproliferative effects.

Stat3 activation caused a general decrease in the progression of A375 cells through all phases of the cell cycle, without affecting the levels of the major regulators of the G1-S transition, including p21, p27, D-type cyclins, or cyclin E. Our results are in contrast to previous studies showing accumulation of cells in G1, accompanied by an increase in p27 levels, upon cytokine-triggered Stat3 activation (16, 39). This discrepancy might reflect differences in levels of Stat3 activity achieved or differences in the sensitivity of the cells to active Stat3. Indeed, we observed that further enhancing Stat3 activity in A375 cells, by combining 4HT with OSM, led to an increase in p27 levels, an accumulation of cells in G1, and massive apoptosis.

Whereas a number of previously described Stat3 target genes (e.g. junB, serpin A3, fibronectin, or vascular endothelial growth factor) (5, 40, 41) were detected in our analysis, others, including pim1 or angiotensinogen (5, 42), were only detected when Stat3 activity was synergistically enhanced by 4HT and
However, some genes, such as \textit{bcl-X}_L, \textit{CCND1}, and \textit{myc}, which were strongly up-regulated by Stat3 activation in rat fibroblasts (10), were not increased even after combined treatment of A375 cells with 4HT and OSM. Thus, the ability of Stat3 to affect specific target genes is dependent upon the cellular context.

Considering the importance of Stat3 in different biological processes, tight regulation of the Stat3 signaling pathway is essential and is achieved via diverse mechanisms. We have identified TEL, a novel Stat3 target, and have shown that it

\textsuperscript{N. Schick and A. Badache, unpublished observations.}
TEL Is a Stat3-induced Repressor of Stat3

plays a role in a Stat3 negative feedback loop. In contrast to other negative regulators of Stat3 (e.g., SOCS3, GRIM-19, and Pias3) (25, 26, 43), which act at various levels of the JAK/Stat3 pathway to cause an overall reduction in DNA-bound Stat3, TEL does not affect Stat3 DNA binding but represses its transcriptional activity. Our finding that TEL levels are induced in many different cancer cell lines after IL-6 or OSM treatment, suggests that TEL might be a general Stat3-induced negative regulator.

The repressive activity of TEL has previously been shown to depend on recruitment of a co-repressor complex comprising mSin3A, NcoR, and HDACs to distinct TEL domains (27, 38, 44, 45). These include the C-terminal pointed domain, which is necessary for TEL oligomerization and association with other proteins (36, 45, 46); the N-terminal ETS domain, which interacts with specific DNA elements but also mediates protein–protein interactions (27, 46, 47); and the central TEL region, spanning amino acid residues 268–333, that associates with HDAC3 (27). We show here that TEL-mediated repression of Stat3 is dependent on HDAC activity and that the TEL pointed domain, but not the central region, is required for this repression.

Interestingly, neither point mutations in the ETS domain, which preclude TEL DNA binding, nor complete deletion of the ETS domain prevented repression of Stat3 activity. To our knowledge, this is the first study showing that TEL repressor function does not require the ETS DNA binding domain. The fact that TEL and Stat3 could be co-immunoprecipitated from nuclear extracts suggests that TEL can be recruited to the Stat3 transcriptional complex, even in the absence of DNA binding, through protein–protein interaction.

By unlocking TEL expression levels, we show here that TEL regulates the antiproliferative effects of Stat3. Recent studies suggest that TEL might also control Stat3 oncogenic activity. Indeed, it has been shown that TEL-43 inhibits transcription of NIH-3T3 fibroblasts by Src (48), which is known to depend upon Stat3 for its oncogenic activity (7, 8). Our data suggest that TEL might inhibit Src-induced transformation through its ability to repress Stat3 activity.

Recent evidence suggests that the balance between Stat3-activating signals and Stat3-repressing signals determines the biological outcome. Excessive signaling to Stat3, which is known to occur in many tumors, has been associated with abnormal activity of intracellular kinases as well as growth factor and cytokine receptors (9, 49–51). Recent studies demonstrate that persistent Stat3 signaling can also result from the silencing of negative regulators, such as SOCS1 or Pias3 (52, 53). The absence of TEL might have a similar role in some tumors, especially leukemias.

The TEL gene is often disrupted by chromosomal translocation in leukemia, most commonly with AML-1 (t12, 21), resulting in production of TEL-AML fusion proteins. This is often associated with a loss of heterozygosity (54, 55). Fusions of TEL with the ABL and JAK2 tyrosine kinases, leading to constitutively active kinases, have also been reported (55–58). Interestingly, TEL-JAK2 and TEL-ABL fusion proteins induce constitutive activation of Stat3, which was shown to cause hematopoietic precursor cell transformation (9). Our results suggest that chromosomal translocations involving TEL could promote transformation, not only through JAK- or ABL-mediated activation of Stat3 but also through attenuation of TEL-mediated negative regulation of Stat3 due to the loss of one TEL allele, especially if the nonrearranged TEL allele is also lost or mutated (55, 56, 58). Our data showing that TEL is a novel type of negative regulator of Stat3 activity call for further analysis of the impact of TEL on Stat3 biology.

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