Induction of ETS-1 and ETS-2 Transcription Factors Is Required for Thyroid Cell Transformation

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ABSTRACT

The proteins of the Ets family are transcription factors involved in signal transduction, cell cycle progression, and differentiation. In this study, we report that thyroid cell neoplastic transformation is associated with a dramatic increase in ETS transcriptional activity, which is dependent on the accumulation of Ets-1, Ets-2, and other Ets-related proteins. Inhibition of ETS transactivation activity by the Ets-dominated negative construct (Ets-Z) induced programmed cell death in human thyroid carcinoma cell lines but not in normal thyroid cells. Apoptotic cell death induced by Ets-Z was dependent on the reduction of c-MYC protein levels, because it was prevented by overexpression of c-myc. Taken together, these data indicate that the induction of Ets-1 and Ets-2 transcription factors plays a pivotal role in thyroid cell neoplastic transformation.

INTRODUCTION

The Ets family encompasses a large number of genes (ets-1, ets-2, elf-1, elk-1, erg-1, fil-1, PU-1, etc.) that code for transcription factors related to the transforming v-Ets oncogene transduced by the E26 avian retrovirus (1, 2). The proteins of the Ets family are involved in cell proliferation, differentiation, and oncogenic transformation. They share a common DNA-binding domain (Ets domain) that recognizes a GGAA/T purine-rich core sequence, found in the promoter or enhancer region of a large variety of genes. In most cases, the Ras-responsive activity of Ets-family members is mediated by functional interaction with other transcription factors on composite DNA-binding sites. The best characterized example is represented by the Ets/AP-1 cooperation, originally discovered in the polyomavirus enhancer and subsequently described in the promoters of many genes, including those encoding extracellular matrix-degrading proteases, such as collagenase (3), stromelysin (4), and urokinase (5). In addition, the complex regulation of the c-fos serum response element requires the activity of the ternary complex factors subfamily of Ets proteins interacting with the serum response factor (6). Moreover, Ets-family components are also involved in the control of tissue-specific genes, as in the case of the Ets-1/pit-1 cooperation, involved in the hormonal regulation of the prolactin gene expression (7).

ets-1 and ets-2 genes are expressed in several tissues during mouse development (8). In the adult tissues, ets-1 gene expression is restricted to lymphoid cells (9), whereas the ets-2 gene is present, although at low levels, in a variety of adult tissues (10). By targeted deletion of the conserved DNA-binding domain, ets-2 has been shown to be essential for placentation function, normal mouse development, and the tissue-specific expression of the extracellular matrix-degrading metalloproteases MMP-3, MMP-9, and MMP-13 (11). Differently, the role of ets-1 appears to be restricted to the development of specific subpopulations of T lymphocytes (12).

The regulatory role of the Ras-Raf-mitogen-activated protein kinase-dependent phosphorylation in the control of Ets transcriptional activity has been shown for at least six subfamilies of Ets proteins (Ets, YAN, ELG, PEA3, ERF, and TCF; reviewed in Wasylyk et al., Ref. 13).

Several lines of evidence have established the correlation between Ets protein activity and neoplastic transformation. First, overexpression of c-ets-1 and c-ets-2 abolishes the serum requirements of fibroblast cells in culture and leads them to the neoplastic phenotype (14, 15). Second, Ets proteins are overexpressed in several experimental and human neoplasias such as breast (16, 17), lung (18), gastric (19), and prostatic carcinomas (20). In most of these tumors, ets gene expression levels correlate with tumor progression. Third, rearrangement of ets-family genes has been detected in human tumors. In particular, molecular analysis of the Ewing family of tumors revealed fusion of the EWS gene on chromosome 22 with either the FLI-1 or erg genes, members of the ets family, located on chromosomes 11 and 21, respectively (21, 22). Finally, the causal role of Ets-dependent activity in transformation has been established by functional inhibition, mediated by the expression of Ets transdominant mutants (23–25).

Thyroid neoplasias comprise a broad spectrum of diseases ranging from benign adenoma to the very aggressive undifferentiated carcinoma that it is lethal in a few months (26). They represent an excellent model system with which to study the role of transcription factors in the process of carcinogenesis. The aim of our study was to define the role of Ets-1 and Ets-2 transcription factors in thyroid carcinogenesis.

In this study, we report that ETS transcriptional activity and Ets-1 and Ets-2 proteins are increased in human thyroid carcinoma tissues and cell lines. The Ets-dominated negative construct (Ets-Z) suppressed the ETS-transcriptional activity and induced programmed cell death, mediated by decreased c-myc expression, specifically in thyroid carcinoma cell lines. Therefore, the activity of the Ets-1 and Ets-2 proteins is necessary for the survival of carcinoma but not of normal thyroid cell lines.

MATERIALS AND METHODS

Cell Lines and Transfection Analysis. The human thyroid carcinoma cell lines TPC-1, WRO, FRO, NPA, and ARO have been described previously (27). TPC-1 and NPA cells derive from a papillary thyroid carcinoma, and WRO cells derive from a follicular carcinoma, whereas FRO and ARO cells were established from anaplastic thyroid carcinoma. They were grown in Ham’s F-12 medium, Coon’s modification (Sigma Chemical Co.) supplemented with 5% calf serum (Life Technologies, Inc.). The PC 3 and FRTL-5 cells are normal rat thyroid cells; PC MPSV cells and FRTL-5/KMSV are PC 3 and FRTL-5 cells transformed by the myeloproliferative sarcoma virus and Kirsten murine sarcoma virus, respectively. They were grown as...
already described (28). HTC-2 cells are normal human thyroid cells and have been described elsewhere (29).

Transfections were performed with the calcium phosphate procedure as described previously (30). For stable transfections and colony assays, we used 10 μg of each plasmid pSVEts-2, pSVEts/ lacZ, pSVElacZ (25, 31), and pSVMy, which are described elsewhere (32), in 100-mm dishes or 3 μg of each plasmid in 3-mm dishes. After transfection (48 h), cells were split and selected for 15 days in G418 400 μg/ml (Life Technologies, Inc., Gaithersburg, MD). After 15 days, colonies were stained with 500 μg/ml crystal violet in 20% methanol and counted.

To evaluate ETS activity in normal and transformed thyroid cells, we transfected 5 μg of plasmid pEE18, which contains two inverted repeat ets-binding sequences fused to the luciferase reporter gene (33). As an internal control of transfection efficiency, we cotransfected 2 μg of the plasmid carrying the β-galactosidase reporter gene under the control of the CMV promoter (30). The luciferase activity and β-galactosidase were determined as described previously (34, 35).

Human Thyroid Tissues. Thyroid specimens were from the Istituto Nazionale dei Tumori di Napoli (Naples, Italy); Laboratoire d’Histologie et de Cytologie, Center Hospitalier Lyon Sud (Lyon, France); and Laboratoire d’Anatomie Pathologique and Hopital de L’Antiquaille (Lyon, France). Tumor samples were frozen in liquid nitrogen and stored frozen until RNA and/or proteins were extracted.

RNA Extraction and Northern Analysis. Total RNA was extracted with the guanidine thiocyanate method (34). Northern blots and hybridizations were carried out following a standard procedure (34). cDNAs were labeled with [α-32P]dCTP using the random oligonucleotide primer (Ready-To-Go; Pharmacia) at a specific activity of 7 × 108 cpm/mg. The probes used were: a 1.2-kb EcoRI fragment corresponding to the cDNA of the human c-ets-2 gene (31); b) a 1.2-kb EcoRI-EcoRI fragment corresponding to the cDNA of the human c-ets-1 gene (31); c) a 1.0-kb PstI-PstI fragment corresponding to the cDNA of the human c-myc gene (35); d) a 0.4-kb EcoRI-HindIII fragment corresponding to the cDNA of the human GAPDH (36). The correct DNA sequence was confirmed by automated DNA sequencing. The human erg-1 and elf-1 probe cDNAs were obtained by RT-PCR. Correct DNA sequences were confirmed by automated DNA sequencing.

RT-PCR Analysis. Total RNA (5 μg), digested with DNase free-RNase, was reverse transcribed using random exonucleotides as primers (100 mM) and 12 units of avian myeloblastosis virus reverse transcriptase (Promega). The cDNA was amplified in a 25-μl reaction mixture containing 0.2 mM deoxytriphosphate triphosphate, 1.5 mM MgCl2, 0.4 mM of each primer, and 1 μl of Taq DNA polymerase (Perkin-Elmer). The PCR were performed after a denaturing step (95°C for 2 min) for 20 cycles (95°C for 1 min, 55°C for 30 s, 72°C for 30 s). The sequences of oligonucleotide primers used for amplification of ets-1 cDNAs were: forward, 5′-ACCCAGATGGAGGTGGCCAGG-3′; reverse, 5′-TCTGAGGTTGATCCACCC-3′ (nucleotides 1376 to 1396 and 1575 to 1555, respectively; Ref. 37). For ets-2, they were: forward, 5′-GATCATCCCAAGAGAGGA-3′; and reverse, 5′-GGCTCCCTGTGGTGGAGCCC-3′ (corresponding to nucleotides 1285–1304 and 1673–1695, respectively; Ref. 37). For human MMP-1, they were: forward, 5′-GACAGATTCTATCATGCGAC-3′; and reverse, 5′-TGTCGCCAAATCAGG-3′ (corresponding to nucleotides 108–128 and 545–525, respectively; Ref. 38). For the human uPA, they were: forward, 5′-TCCGG-GACTATACAGACAT-3′; and reverse, 5′-ACTTTCTTTTGTGTTGA-3′. The erg-1 and elf-1 cDNAs were obtained by RT-PCR on total RNA using the following primers: forward, 5′-GATGACCACTGATCTCAGA-3′; and reverse, 5′-GCTGTCCTTTCCTCTGCCCT-3′ (corresponding to nucleotides 1001–1021 and 1231–1221 of elf-1 sequence; Ref. 39); and forward, 5′-GCAGGAGCAGCTGACAC-3′; and reverse, 5′-CCTCCCTGGGGCC-3′ (corresponding to nucleotides 2941–2961 and 3831–3811 of elf-1 sequence; Ref. 40). Expression of the GAPDH gene was used as an internal control. The specific primers were: forward, 5′-AATGTTTCCATATCATTGTTC-3′; and reverse, 5′-TGGACTCTACAGCTATCTAC-3′ (corresponding to the nucleotides 195–215 and 355–335, respectively; Ref. 36).

Nuclear Protein Extraction and Electrophoretic Mobility Shift Assay. Nuclear protein extraction was performed as already described (37). For the Electrophoretic Mobility Shift Assay, nuclear extracts (2.5–5 μg of proteins) were incubated for 10 min at room temperature in 20 μl of a solution containing 20 mM HEPEs (pH 7.5), 40 mM KCl, 5 mM glycerol, 5 mM spermidine, and 1 μg of poly(dI-dC). Probe and competitor were added as indicated, and the incubation was continued for another 10 min. For analysis of dissociation of the 100-fold excess of unlabeled competitor oligonucleotide was added after 10 min of incubation with probe. Aliquots from the same binding mixture were taken at different times and immediately loaded on the gel. For supershift analysis, the nuclear extracts were preincubated with Ets-1- and Ets-2-specific antibodies at room temperature for 1 h before adding the probe. The sequences of the oligonucleotide probes were: Ets-consensus binding site for Ets-1 and PEAI (sc-2555; Santa Cruz Biotechnology, Santa Cruz, CA), 5′-GATTCGCCAGGAAATGCTGA-3′; and Sp1 consensus-binding sequence, 5′-ATTCGATTGCGGCGGCGGACG-3′. Samples were then separated on 6% native polyacrylamide gels (acrylamide:bis 29:1 in 0.5 × Tris-borate EDTA).

Immunoblotting Analysis. Total proteins were prepared as already described (41). To ascertain that equal amounts of protein were loaded, the Western blots were incubated with antibodies against the γ-tubulin protein (Sigma Chemical Co.). Anti-Ets-1 (N-276), anti-Ets-2 (C-20 and SC-351X), Bcl-2 (N-19), Bax (N-20), Bcl-xL (H5), Myc (C-20), anti-Ets-1 (C-20), anti-Erg-1 (C-17), and PARP (H250) antibodies were purchased from Santa Cruz Biotechnology. β-Galactosidase monoclonal antibody was purchased from Promega.

Immunofluorescence Analysis. Cells were fixed in 4% paraformaldehyde, permeabilized with 0.1% Triton, and stained with a primary anti-β-galactosidase mouse monoclonal antibody (Promega). After several washings, rhodamine-conjugated antitoxin or FITC-conjugated antitoxin IgG was added. Fluorescence was visualized with Zeiss 140 epifluorescent microscope equipped with filters that discriminated between rhodamine and green fluorescein. For the detection of the Pinco-Myc vector, the cells were fixed as described above, and the green autofluorescence was analyzed on epifluorescent microscope.

Assay of the Transformed State. Soft agar assays were performed according to a technique described previously (42). The tumorigenicity of the cell lines was tested by s.c. injections of 2 × 106 cells into athymic mice. The animals were monitored at regular intervals for the appearance of tumors.

Laddering Assay. To analyze DNA for nucleosomal size fragmentation, adherent and nonadherent cells were collected, washed twice with ice-cold PBS, and resuspended in lysis buffer containing 10 mM Tris-Cl (pH 7.4), 10 mM NaCl, 10 mM EDTA, 1% SDS, and 0.1 mg/ml proteinase K. They were then incubated at 4°C for 30 min and centrifuged at 13,000 rpm for 10 min. The supernatants were extracted with phenol/chloroform and precipitated with ethanol. The DNA pellets were resuspended in Tris-EDTA buffer and separated on a 1.2% Tris-borate EDTA-agarose gel.

TUNEL Assay. We used the In situ cell death detection kit (Boehringer Mannheim) and the manufacturer’s instructions for the TUNEL assay. Briefly, the air-dried cells were fixed with a freshly prepared paraformaldehyde solution [%4% in PBS (pH 7.4)] for 30 min at room temperature. The slides were rinsed with PBS and incubated in permeabilization solution (0.1% Triton X-100; 0.1% sodium citrate) for 2 min on ice. Then the slides were rinsed twice with PBS, incubated for 60 min at 37°C with 50 μl of TUNEL reaction mixture containing terminal deoxynucleotidyl transferase DNA polymerase terminal deoxynucleotidyltransferase and modified nucleotides, rinsed three times with PBS, and supplemented with converted AP (anti-Fab antibody) substrate solution. After 30 min at 37°C, the slides were incubated with Fast red for 10 min at room temperature, mounted under glass coverslips, and analyzed under light microscope.

Flow-Cytometric Analysis. Cells were collected and washed in PBS. DNA was stained with propidium iodide (50 μg/ml) for 30 min at room temperature and analyzed with a FACScan flow cytometer (Becton Dickinson, San Jose, CA) interfaced with a Hewlett Packard computer (Palo Alto, CA). The CELLFIT program (Becton Dickinson) was used for cell cycle data analysis.

Generation of a Retroviral Vector Carrying the C-myc Gene (Pinco-Myc Vector). The first two exons of the human c-myc gene (43) were inserted in the BamHI and EcoRI sites of the Pinco retroviral vector (44). The amphi-

3 The abbreviations used are: CMV, cytomegalovirus; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; RT-PCR, reverse transcription-PCR; MPM, matrix metalloprotease; uPA, urokinase plasminogen activator; TUNEL, terminal deoxynucleotidyl transferase-mediated nick end labeling; PARP, poly(ADP-ribose) polymerase.

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tropic packaging cell line Phoenix was transfected with the calcium-phosphate/chloroquine method (44, 45). Culture supernatants containing viral particles were collected at 48 h after transfection. For the selection of the transfected cells, 1 μg/ml puromycin was added to the cell growth medium. Infection was performed by culturing target cells in 0.45-μm filtered viral supernatant for 3 h. Two infection cycles were run to infect the NPA Ets-Z cells.

**Inhibition of Cell Death.** Cells (6 × 10^5) were plated in six multwells and incubated with 1, 10, and 100 μm z-DEVD-cho, carboxobenzoxy-Val-Ala-Asp-fluoromethylketon, z-DEVD-cho, carboxobenzoxy-Asp-Glu-Val-Asp-fluoromethylketon, and Ac-YVAD-cho, carboxobenzoxy-Tyr-Val-Ala-Asp-7-amino-4-trifluoromethylcoumarin, and ZFA-fmk, carbobenzoxy-Phe-Ala-fluoromethylketon (Calbiochem) for 48 h. Death was assessed by measuring the percentage of fragmented DNA by TUNEL as described previously.

**RESULTS**

**Expression of Ets Proteins in Human Thyroid Carcinoma Cell Lines and Tumor Samples.** We first analyzed the expression of Ets-1 and Ets-2 in a panel of human thyroid cell lines including normal (HTC-2) and neoplastic cells from different carcinoma histotypes (TPC-1, WRO, NPA, ARO, and FRO). The results (Fig. 1A) showed that the level of both nuclear proteins was increased in the neoplastic compared with the normal cell lines. However, whereas the fold increase of Ets-1 was dramatic in all of the five transformed cell lines, Ets-2 exhibited a significantly smaller increase, which varied between the different cell lines. The semiquantitative RT-PCR analysis of the ets-1 and ets-2 transcripts (Fig. 1B) revealed increased ets-1 and ets-2 mRNA levels in the carcinoma cell lines, suggesting that the oncogene-dependent accumulation of both transcription factors is, at least partially, consequent to the increased gene expression.

We further extended our analysis by investigating the expression of the Ets gene products in surgically removed human thyroid tumors. High levels of Ets-1 and Ets-2 proteins were detected in all of the carcinoma tissues (Fig. 1C, Lanes 1–5) but not in normal thyroid tissue (Lanes 1–5). No significant changes were observed in adenoma samples (data not shown).

We have also analyzed two other ets-related transcription factors, such as erg and elf-1. Their expression in carcinoma cells (Fig. 1, A–C) and tissues is essentially quite similar to that of Ets-1 and Ets-2.

**In Vitro DNA-binding and ETS-dependent Transcriptional Activity in Human Thyroid Carcinoma Cell Lines.** To establish a correlation between the expression of Ets-1 and Ets-2 with their transcriptional activity, we first analyzed the in vitro binding to the Ets consensus oligonucleotide in the normal and thyroid carcinoma cell lines. The amount of gel-retarded complex was dramatically increased in all of the carcinoma cell lines (Fig. 2A) compared with the HTC-2 normal thyroid cells. The binding activity of the different nuclear extracts was normalized by use of an oligonucleotide probe binding the ubiquitous Sp1 transcription factor (Fig. 2A). Supershift analysis using antibodies versus the Ets-1 and Ets-2 proteins showed the presence of Ets-1 and Ets-2 in the complexes binding the Ets consensus sequence (Fig. 3A). However, the binding activity was only partially reduced, indicating that other members of the Ets family are present in these complexes.

To determine the functional consequences of the increased ETS DNA-binding activity, we analyzed by transient transfection the activity of a reporter construct (pE18-luciferase) containing an inverted and repeated ETS-2 binding site fused to the c-fos minimal promoter (46). The reporter activity was strongly increased (about 10-fold) in the transformed cell lines, compared with normal cells (Fig. 2C).

We also analyzed the expression of two known Ets transcriptional targets: the uPA and the MMP-1, both coding for proteases involved in the degradation of extracellular matrix. The results of the semiquantitative RT-PCR analysis showed that both uPA and MMP-1, essentially undetectable in the normal thyroid cell line, were induced in all of the five carcinoma cell lines (Fig. 2, D and E), suggesting a causal relationship with the increased Ets-1 and Ets-2 expression.

**Suppression of the ETS Transcriptional Activity by the Ets-Z-dominant Negative Construct Blocks the Growth of Thyroid Carcinoma Cells.** To understand the functional relevance of ETS-1 and ETS-2 activity in the maintenance of the malignant phenotype of thyroid cell lines, we transfected the NPA, ARO, and FRO cell lines with the pSVsEts-LacZ (Ets-Z) construct, expressing the transdomi-
nant negative derivative of Ets-2, containing the Ets DNA-binding domain fused to the Escherichia coli lacZ coding sequence (31). The transfected cells were selected for resistance to G418, and colonies were counted after 14 days. Few colonies were obtained when NPA cells were transfected with Ets-Z, whereas no colonies at all were obtained after transfection of the ARO, FRO, and PC MPSV (PC Cl 3 transformed by the Myeloproliferativasarcoma virus) cells with the same construct (Table 1). Differentially, all of the carcinoma cell lines gave rise to a significant number of colonies when transfected with the backbone vector (pSVlacZ, Table 1) or with the construct expressing the wild-type protein (pSV-Ets-2; data not shown). No significant differences in the number of G418-resistant colonies were observed when the normal rat thyroid cells were transfected with the pSVEts-Z or the pSVlacZ constructs (Table 1). These results suggest the possibility that the Ets-β-galactosidase chimeric protein strongly interferes with normal growth and/or survival of thyroid carcinoma cell lines, without a significant effect on the normal thyroid cells.

Therefore, we further characterized the transfected cells, by determining the activity of the dominant negative Ets-Z protein, and the expression of the two Ets target genes. To this purpose, we isolated two Ets-Z-transfected NPA cell clones (NPA Ets-Z Cl1 and Cl2). First, we showed that they stably expressed the pSVEts-Z construct by Western blot analysis using monoclonal antibodies against the β-galactosidase protein (Fig. 3A). A polypeptide recognized by the anti-β-galactosidase antisemur with a size (M, 143,000) in agreement with the predicted mass of the Ets-lacZ fusion protein was detected in the two NPA Ets-Z clones but not in the parental NPA cell line. We then examined the Ets/PEA3 DNA-binding activity of the NPA Ets-Z cell lines. Mobility shift analysis revealed two gel-retarded products with a comparable intensity. In addition to the Ets complex described previously (Fig. 2), a slower migrating band was detectable in nuclear extract from NPA Ets-Z cells but not in the NPA cell line (Fig. 3B). For further characterization, the two Ets oligonucleotide/protein complexes were subjected to dissociation analysis (off rate) to determine their relative in vitro stability. To this purpose, the complexes were diluted with unlabeled oligonucleotide and allowed to dissociate before electrophoretic separation. The upper complex (Ets-Z) exhibited a slower dissociation with a significant amount of complex still bound after 10 min, whereas the lower complex (Ets) was almost completely dissociated within 2 min, with comparable kinetics in the NPA and the NPA Ets-Z cell line (Fig. 3B). This result suggests that the Ets-Z chimeric protein might compete very effectively with the endogenous Ets proteins because of the increased binding affinity with respect to the Ets regulatory elements on DNA.

We then analyzed the activity of the Ets reporter construct (pE18-luciferase) in the NPA Ets-Z cell clones. The results (Fig. 3C) showed a significant (4–5-fold) decrease of the reporter activity in the two NPA Ets-Z cell clones compared with the NPA and the NPA LacZ cell lines, in which the activity of the transfected reporter was about 10-fold compared with a normal thyroid cell line (PC Cl 3). The expression of the collagenase (MMP-1) and urokinase (uPA) was determined by RT-PCR in the two NPA Ets-Z cell clones. Both the MMP-1 and uPA mRNAs were strongly decreased in both the NPA Ets-Z cell clones with respect to the NPA cells, although their level was clearly detectable if compared with the virtual absence of expression in the PC Cl 3 normal cell line (Fig. 3D).

Finally, we evaluated the effect of Ets-Z protein on the transformed phenotype by analyzing the growth rate, anchorage-independent growth, and tumorigenicity in athymic mice of the NPA Ets-Z cell clones. The growth rate of the NPA Ets-Z cell clones was lower when compared with untransfected or backbone vector-transfected cells (Fig. 4). The ability to form colonies in soft agar and tumors in
athymic mice was drastically reduced by Ets-Z (Table 2), whereas it was not affected by the expression of the c-ets-2 gene. Interestingly, overexpression of the normal ets-2 gene in normal thyroid cells was not able to induce the acquisition of the malignant phenotype (Table 3).

**Suppression of the ETS Activity Induces Programmed Cell Death in Thyroid Carcinoma Cell Lines.** To determine whether the suppression of the ETS transcriptional activity in thyroid carcinoma cells induced growth arrest or programmed cell death, we analyzed different stably Ets-Z-transfected NPA cell clones.

The presence of apoptotic cells was investigated by three apoptotic assays: DNA laddering, flow-cytometric analysis, and TUNEL. DNA extracted from NPA and NPAEts-Z cells did not show any laddering (Fig. 5A), whereas significant internucleosomal cleavage of DNA resulting in typical DNA fragmentation was observed in all of the NPA Ets-Z cell clones. Consistently, flow-cytometric analysis revealed a shift of the DNA profile to a sub-G1 position in NPA Ets-Z cell clones. Consistently, flow-cytometric analysis revealed a shift of the DNA profile to a sub-G1 position in NPA Ets-Z versus the untransfected NPA cells with a perturbation in cell cycle progression with a 100-fold excess of the unlabeled competitor oligonucleotide for indicated times. The arrows indicate the position of Ets (lower) and Ets-Z complexes (upper). C, analysis of reporter construct activity in NPA Ets-Z cell clones. PC CI 3, NPA cells, and NPA Ets-Z cells (clone 1 and clone 2) were transfected with the plasmid E18. Luciferase activity was assayed 48 h after transfection. All of the transfections were performed using a CMV-β-galactosidase internal control vector to normalize transfection efficiency. The diagram shows the relative luciferase activity, determined by assigning the basal level to the PC CI 3. The results, reported here, represent the average of three independent experiments. D, RT/PCR analysis of type-1 metalloproteinase and uPA receptor expression in NPA Ets-Z cell clones. All of the cDNAs were coamplified with GAPDH as an internal control. Bands of comparable intensity, obtained by the rat GAPDH sequence-specific primers, suggest comparable amplification of all of the samples. No bands are seen in nonreverse-transcribed RNAs, excluding DNA contamination (data not shown). The sources of RNAs are indicated.

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The results presented here represent the average of three independent experiments.

<table>
<thead>
<tr>
<th>Cell line</th>
<th>No. of colonies</th>
<th>pSVlacZ</th>
<th>pSVEts-Z</th>
</tr>
</thead>
<tbody>
<tr>
<td>PC CI 3</td>
<td>210 ± 12</td>
<td>190 ± 3</td>
<td></td>
</tr>
<tr>
<td>PC MPSV</td>
<td>205 ± 10</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>NPA</td>
<td>210 ± 12</td>
<td>4</td>
<td></td>
</tr>
<tr>
<td>ARO</td>
<td>150 ± 9</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>FRO</td>
<td>260 ± 5</td>
<td>0</td>
<td></td>
</tr>
</tbody>
</table>
Fig. 4. Effect of Ets-2 or Ets-Z genes on growth of the NPA cell clones. The cells were plated at 10^4 cells/dish at time 0 and allowed to grow under standard culture conditions for 7 days.

Table 2 Analysis of the transformed phenotype of the NPA cells transfected with the Ets-Z construct

<table>
<thead>
<tr>
<th>Cell clones</th>
<th>Colony-forming efficiency (%)</th>
<th>Tumor incidencea</th>
</tr>
</thead>
<tbody>
<tr>
<td>NPA</td>
<td>23</td>
<td>8/10c</td>
</tr>
<tr>
<td>NPA Ets-Z Cl 1</td>
<td>20</td>
<td>8/10c</td>
</tr>
<tr>
<td>NPA Ets-Z Cl 2</td>
<td>20</td>
<td>5/6c</td>
</tr>
<tr>
<td>NPA Ets-Z Cl 1</td>
<td>1-2c</td>
<td>3/7c</td>
</tr>
<tr>
<td>NPA Ets-Z Cl 2</td>
<td>1-2c</td>
<td>3/7c</td>
</tr>
</tbody>
</table>

*a Assayed by injecting 2 x 10^6 into athymic mice (4 to 6 weeks of age). The animals were monitored for the appearance of tumors at the inoculation site for 6 weeks.
a Tumors appeared after 4 weeks and showed a diameter of 2 cm.
c These colonies were smaller in comparison with those induced by the NPA cells.
d Tumors appeared after 7 weeks and showed a diameter of 0.2 cm.

We next analyzed the expression of the bcl-2, Bcl-xL, bax, and c-myc genes, which are known to be involved in the apoptotic process (47). As shown in Fig. 7C, Bcl-xL, Bcl-2, and c-MYC proteins drastically reduced in NPA Ets-Z clones with respect to the untransfected and NPA-LaCZ cells. Northern blot analysis showed also a down-regulation of bcl-2 and c-myc genes (data not shown).

Because the c-myc proto-oncogene could play a central role in the induction of apoptosis (48), we tested the hypothesis that reduced c-myc expression might be involved in the apoptotic process induced by Ets-Z. To this purpose, we infected NPA Ets-Z clones with Pinco-Myc, a retrovirus generated by inserting the human c-myc cDNA in the Pinco vector (44). As shown in Fig. 8A, the cells infected with Pinco vector showed apoptotic morphology (bottom panel), whereas the cells infected with Pinco-Myc (Fig. 8B) showed a normal morphology (bottom panel). Only 7% of the NPA Ets-Z cells infected with Pinco-Myc underwent apoptosis 72 h after infection. Conversely, 30% of apoptotic cells were detected among the NPA Ets-Z cells infected with Pinco vector (Fig. 8C). These results were confirmed by a colony assay. In fact, Table 4 shows the results of a colony assay performed by cotransfecting NPA and ARO cells with pSVetsZ and pSVMyc, a myc expression vector that does not contain the gene for the resistance to G418. Myc expression rescued the ability to form colonies suppressed by the Ets-dominant negative construct. In fact, no or very few colonies were obtained after transfection with Ets-Z alone, whereas a number of colonies comparable with those obtained with the backbone vector were detected after cotransfection of Ets-Z and pSVMyc.

DISCUSSION

The process of carcinogenesis occurs via multiple steps that correspond to different genetic alterations. Several genetic lesions have been described in human thyroid carcinomas. The RET/PTC and TRK oncogenes are activated in 50% and 10%, respectively, of the human papillary carcinomas, whereas mutations of the ras gene family have been found in 50% of adenomas and follicular and anaplastic carcinomas. Loss of p53 function, determined by different molecular mechanisms, is a constant feature of anaplastic carcinomas (49). All of the genetic alterations seem to influence the activity and the composition of several transcriptional factors such as c-myc (27), NFkB (29), and AP-1 (50). The aim of the present study was to evaluate the role of the Ets transcription factor family in human thyroid cell transformation in vivo.

In this study, we show that ETS activity is increased in human thyroid carcinoma cell lines and demonstrate that such increase is
required for the maintenance of the neoplastic phenotype of human thyroid carcinoma cell lines. In fact, using an Ets-dominant negative construct (Ets-Z), we were able to impair the growth of human neoplastic thyroid cell lines. The increase in ETS activity depended, at least partially, on accumulation of Ets-1 and Ets-2 proteins. The increased ETS activity does not appear to depend on specific oncogene activation because it was observed in all of the carcinoma samples. Neither does it seem to depend on HMGI protein expression because, at variance with AP-1 induction in thyroid malignant cells (41, 50), it is not modified by suppression of HMGI protein synthesis.

The expression of the ets gene family does not differ between thyroid benign adenomas and normal thyroid (data not shown). This finding is consistent with the results obtained in precancerous bronchial lesions (51) suggesting that activation of ETS transcriptional activity is an event of progression of the carcinogenesis process.

The functional role of Ets-1 and Ets-2 proteins in thyroid cell transformation was investigated by transfecting the thyroid carcinoma cell lines with an ets-dominant negative construct, which has been shown previously (31, 52) to inhibit several ras-responsive enhancers. The block of ETS activity was able to suppress the growth of human thyroid cell lines and to cause a programmed cell death in various thyroid carcinoma cell lines. In contrast, overexpression of wild-type ets gene in normal thyroid cells did not significantly modify growth conditions (see Table 3), although the cells did show a slight induction of c-myc and uPA (data not shown) and increased resistance to apoptosis, indicating that ets induction is necessary but not sufficient for the acquisition of the malignant phenotype.

Our data confirm and extend the results obtained by use of various Ets-2 derivatives in Ras-transformed (23, 24, 31) or Neu-transfected mouse 3T3 fibroblasts (46) and in breast carcinoma cells (25). However, differently from Ras-transformed cells, in which both the transcriptionally active full-length ets-2 and the transcriptionally inhibitory Ets-2DBD (DNA-binding domain alone) were able to revert the neoplastic phenotype, in the thyroid cell lines the Ets-Z inhibited both in vitro colony formation and in vivo tumor incidence, whereas the overexpressed full-length ets-2 did not interfere with the transformed phenotype (Tables 1–3). It can be speculated that distinct targets are differentially recognized by the full-length ets-2 in the different cell lines, whereas the Ets-Z fusion construct might exert a more generalized inhibitory effect.

Our results showing that suppression of ETS activity results in the induction of programmed cell death suggest that the oncogenic activation of some Ets component(s) might allow thyroid carcinoma cells to elude apoptosis and raise the question of which Ets-family component and the type of mechanism is involved. Several reports have recently addressed the functional role of Ets proteins in the control of apoptosis. Disruption of the ets-1 gene showed the essential role played by the Ets-1 protein in T-cell survival (53). In addition, the p42 splice variant of Ets-1 can induce apoptosis in human colon cancer cells (54) and is capable of rescuing the block of Fas-induced apo-

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ptosis in colon carcinoma cells by an ICE/caspase-1-dependent mechanism (55). An antiapoptotic role has been demonstrated for Ets2 in a different cellular system. In particular, it has been shown that the Ets2 gene product can protect macrophages from CSF-1 deprivation-induced apoptosis, very likely through a Bcl-XL-dependent survival pathway (56).

The analysis of the mechanisms of apoptosis of the NPA Ets-Z cells showed that it was associated with PARP cleavage and inhibited by the cysteine protease inhibitor z-DEVd-fmk and Ac-YVAD-cho. The caspase inhibitor z-DEVd-fmk was demonstrated to be an ineffective inhibitor of caspase-9, whereas it is very effective in inhibiting caspase-3 activity (57). Conversely, the z-YVAD-cho inhibitor is almost specific in suppressing caspase-1 activity. Therefore, we might exclude the involvement of caspase-1 and -3 and take in consideration the caspase-9 pathway in the programmed cell death caused by the block of the ETS transcriptional activity in thyroid carcinoma cell lines. However, the interpretation of the caspase pathway is unfortunately incomplete, because the selectivity of the caspase inhibitors still needs to be completely understood.

Moreover, apoptosis of the NPA-Ets-Z cell clones is associated with a reduced expression of the Bcl-xL, Bcl-2, and c-Myc proteins confirming previous data showing that c-myc is a direct target of Ets-1 and Ets-2 transcriptional activity (31). We show that ectopic expression of Myc protein, by the use of a retroviral construct, determined a partial rescue of apoptotic process suggesting that c-myc gene is an important gene target of Ets-Z action. Interestingly, it has been proposed that c-myc might represent a direct target of ETS transcriptional activity by means of a single binding site in its promoter targeted by ets family proteins and E2F-1 (58). The data that c-myc overexpression prevents apoptosis are in apparent contrast with other data showing that myc induces programmed cell death when expressed without growth factor stimulation or in cells arrested by other means. This apparent discrepancy could be explained by the hypothesis that the cellular context plays an important role in determining the effect of myc on cell proliferation or cell death apoptosis (48). In the presence of genetic lesions that block the cell death pathway, myc overexpression may protect the cells from apoptosis. Consistently, it has been reported recently (57) that myc-expressing p53−/−, casp9−/−, and Apaf-1−/− cells were resistant to apoptosis after growth factor depletion.

In conclusion, we show an increased ETS activity in human thyroid carcinoma tissues and cell lines that is required for the survival of carcinoma but not normal thyroid cell lines. These results suggest the Ets proteins as a target for thyroid cancer gene therapy.

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REQUIREMENT OF ets ACTIVITY IN THYROID CARCINOGENESIS


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