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

Filomena de Nigris, Tiziana Mega, Nicole Berger, Maria Vittoria Barone, Massimo Santoro, Giuseppe Viglietto, Pasquale Verde, and Alfredo Fusco

Centro di Endocrinologia ed Oncologia Sperimentale del Consiglio Nazionale delle Ricerche I/Dipartimento di Biologia e Patologia Cellulare e Molecolare, Facoltà di Medicina e Chirurgia, Università degli Studi di Napoli, 80131 Naples, Italy (V. B., M.V.B., M.S.); Istituto Internazionale di Genetica e Biofisica, Consiglio Nazionale delle Ricerche, 80125 Naples, Italy (T. M., P. V.); Laboratoire d’Anatomie Pathologique, Hôpital de L’Antiquaille, Lyon, France (N. B.); Istituto dei Tumori di Napoli, Fondazione Senatore Pascale, 80131 Naples, Italy (G. V.); and Dipartimento di Medicina Sperimentale e Clinica, Facoltà di Medicina e Chirurgia di Catanzaro, Università degli Studi di Catanzaro, 88100 Catanzaro, Italy (A. F.)

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-dominant 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 polynomavirus 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 placental 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 Flt-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-dominant 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 Cl 3 and FRITL-5 cells are normal rat thyroid cells; PC MPSV cells and FRTLS-KiMSV are PC Cl 3 and FRITL-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 each of plasmids pSVEts-2, pSVEts/lacZ, pSVLacZ (25, 31), and pSVMyr, which are described elsewhere (32), in 100-mm dishes or 3 × 10⁶ units of avian myeloblastosis virus reverse transcriptase (Promega). The first two exons of the human c-myc were removed by reverse transcription using random exonucleotides (100 mM) and [α³²P]dCTP using the random oligonucleotide primer (Ready-To-Go; Pharmacia) at a specific activity ≥ 7 × 10⁶ cpm/μg. The probes were used: a) 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 gene (36). The correct DNA sequence were 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 was extracted with the guanidine thiocyanate method (34). Northern blots and hybridizations were performed 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 Hospital de L’Antiquaille (Lyon, France). Tumor samples were frozen in liquid nitrogen and stored frozen until RNA and/or proteins were extracted.

**DNA Extraction and Northern Analysis.** Total RNA was extracted with the guanidine thiocyanate method (34). Northern blots and hybridizations were performed as described previously (34, 35). Northern blots and hybridizations were carried out following a standard procedure (34). cDNA probes were labeled with [α³²P]dCTP using the random oligonucleotide primer (Ready-To-Go; Pharmacia) at a specific activity ≥ 7 × 10⁶ cpm/μg. The probes were used: a) 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 gene (36). The correct DNA sequence were 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 [α³²P]dCTP using the random oligonucleotide primer (Ready-To-Go; Pharmacia) at a specific activity ≥ 7 × 10⁶ cpm/μg. The probes were used: a) 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 gene (36). The correct DNA sequence were 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.

**Electrophoretic Mobility Shift Assay.** Nuclear extracts (2.5–5 μg) of nuclei were prepared as described above, and the green autofluorescence was analyzed on epifluorescence microscopy.

**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-HCl (pH 7.4), 10 mM NaCl, 10 mM EDTA, 1% SDS, and 0.1 mg/ml proteinase K. The samples were 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-garose 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 CELL-FIT program (Becton Dickinson) was used for cell cycle data analysis.

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

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

**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 glyc erol, 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 disso-
requirement of Ets activity in thyroid carcinogenesis

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 × 10⁵) were plated in six multwells and incubated with 1, 10, and 100 μm z-VAD-fmk, carbobenoxzy-Val-Ala-Asp-fluoromethylcheton, z-DEVD-cho, carbobenoxzy-Asp-Glu-Val-Asp-fluoromethylcheton, and Ac-YVAD-cho, carbobenoxzy-Tyr-Val-Ala-Asp-7-amino-4-trifluoromethylcoumarin, and ZFA-fmk, carbobenoxzy-Phe-Ala-fluoromethylcheton (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 7–13) 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 pSVEts-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 Myeloproliferativavasarcoma virus) cells with the same construct (Table 1). Different, 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 (pSVEts-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 antiserum 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 LacZ 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 versus the untransfected NPA cells with a perturbation in cell cycle progression with a peak accumulation in G1 phase (Fig. 5B). Finally, TUNEL assay revealed the presence of 30% of apoptotic NPA Ets-Z cell clones and only 3% of apoptotic NPA control cells (data not shown). Therefore, all of the three assays were consistent with an apoptotic cell death induced in NPA cells by the ets-dominant negative construct.

Subsequently, we investigated whether Ets-Z construct was able to induce apoptosis also in other thyroid carcinoma cell lines. Because we were not able to obtain ARO clones that stably express EtsZ, we analyzed the effects exerted by transient transfection of pSV-Ets-Z and pSV-lacZ on ARO cell line. After 48 h, transfected cells were analyzed. As shown in Fig. 6A, the ARO cells expressing the Ets-Z construct, detected by immunofluorescence, showed an apoptotic morphology after staining with fluorochrome Hoechst 3325 (i.e., the presence of picnotic cells after Hoechst staining; Fig. 6A). The observation that the Ets-Z construct induces programmed cell death was confirmed by TUNEL assay. As shown in Fig. 6C, 80% of Ets-Z-transfected ARO cells were positive for TUNEL, whereas in control cells (ARO LacZ), an average of only 2% of transfected cells were apoptotic.

**Analysis of the Apoptotic Mechanisms Induced by Ets-Z.** We assayed the effect of caspase inhibitors on cell death of NPA Ets-Z. The cells were cultured for 48 h in a medium containing a control peptide (ZFA-cho) or the caspase inhibitors, z-VAD-fmk, z-DEV-D-fmk, or Ac-YVAD-cho, at different concentrations as indicated. As shown in Fig. 7A, only the z-DEV-D-fmk conferred protection from apoptosis in a dose-dependent manner. No effect was observed with z-VAD-fmk, Ac-YVAD-cho, and ZFA-cho by a TUNEL assay (Fig. 7A). Subsequently, cell lysates from NPA and Ets-Z were analyzed by Western blot for the cleavage of the PARP. As shown in Fig. 7B, a M₁, 85,000-band corresponding to a cleaved form of PARP was detected only in NPA Ets-Z cells, whereas only the major band of M₂, 112,000 corresponding to the uncleaved PARP protein was detected in NPA and LacZ-LacZ cells.

<table>
<thead>
<tr>
<th>Table 1</th>
<th>Colony-forming assay by transfecting the Ets-Z construct and backbone vector on normal and thyroid carcinoma cell lines</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cell line</td>
<td>pSV-lacZ</td>
</tr>
<tr>
<td>PC CI 3</td>
<td>190 ± 3</td>
</tr>
<tr>
<td>PC MPSV</td>
<td>205 ± 10</td>
</tr>
<tr>
<td>NPA</td>
<td>210 ± 12</td>
</tr>
<tr>
<td>ARO</td>
<td>150 ± 9</td>
</tr>
<tr>
<td>FRO</td>
<td>260 ± 5</td>
</tr>
</tbody>
</table>
and pSVmyc. With the backbone vector were detected after cotransfection of Ets-Z alone, whereas a number of colonies comparable with those obtained no or very few colonies were obtained after transfection with Ets-Z. In fact, Table 4 shows the results of a colony assay. In fact, 30% of apoptotic cells were detected among the NPA Ets-Z cells infected with Pinco vector (Fig. 8A). 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 pSVEts/Z and pSVmyc, a myc expression vector that does not contain the gene for 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

![Graph](image-url)

**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>
<thead>
<tr>
<th>Cell clones</th>
<th>Colony-forming efficiency (%)</th>
<th>Tumor incidence^a</th>
</tr>
</thead>
<tbody>
<tr>
<td>NPA</td>
<td>23</td>
<td>8/10^4</td>
</tr>
<tr>
<td>NPA LacZ Cl 1</td>
<td>20</td>
<td>8/10^4</td>
</tr>
<tr>
<td>NPA Ets-Z Cl 1</td>
<td>22</td>
<td>4/6^a</td>
</tr>
<tr>
<td>NPA Ets-Z Cl 2</td>
<td>20</td>
<td>5/6^a</td>
</tr>
<tr>
<td>NPA Ets-Z Cl 1</td>
<td>1-2</td>
<td>5/7^d</td>
</tr>
<tr>
<td>NPA Ets-Z Cl 2</td>
<td>1-2</td>
<td>3/7^d</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.

^b 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 pSVEts/Z 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.

![Image](image-url)

**Fig. 5.** The expression of Ets-Z induces programmed cell death in the NPA carcinoma cell line. A, internucleosomal DNA cleavage. DNA was separated by agarose gel electrophoresis. DNA fragmentation was detected in samples from NPA Ets-Z cells but not from NPA cells. The sources of DNAs are indicated. B, flow-cytometric analysis of NPA cells and NPA Ets-Z. The DNA content of NPA and NPA-Ets-Z cells was analyzed by flow cytometry after propidium iodine staining. The apoptotic cell population is shown by the first peak (Apo) cells.

**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

**Table 3** Analysis of the transformed phenotype of the PC Cl 3 cells transfected with the pSVEts-2 construct

<table>
<thead>
<tr>
<th>Cell clones</th>
<th>Colony-forming efficiency (%)</th>
<th>Tumor incidence^a</th>
</tr>
</thead>
<tbody>
<tr>
<td>PC Cl 3</td>
<td>0</td>
<td>0/12</td>
</tr>
<tr>
<td>PC Cl 3 Ets-2 Cl 1</td>
<td>0</td>
<td>0/8</td>
</tr>
<tr>
<td>PC Cl 3 Ets-2 Cl 2</td>
<td>0</td>
<td>0/8</td>
</tr>
<tr>
<td>PC MPSV</td>
<td>67</td>
<td>5/5</td>
</tr>
<tr>
<td>PC MPSV Ets-2 Cl 1</td>
<td>70</td>
<td>5/5</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.
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 (Table 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-

---

**Fig. 6.** Apoptotic effect of Ets-Z construct on the ARO cell line. A (top), ARO pSVEts-LacZ-transfected cells stained by immunofluorescence with anti-β-galactosidase antibody. A (bottom), the same field nuclei cells stained for Hoechst. The arrow indicates apoptotic nucleus. B (top), ARO pSV-LacZ-transfected cell stained by immunofluorescence with anti-β-galactosidase antibody. B (bottom), the same cells stained for Hoechst. The arrows indicate nuclei with normal morphology. C, percentage of ARO-transfected cells positive for apoptosis by TUNEL assay after 24 h and 48 h from transfection with pSVEts-LacZ or pSVLacZ.

---

**Fig. 7.** Analysis of the apoptotic proteins in the NPA Ets-Z cell clones. A, inhibition of apoptotic cells by caspase proteinase inhibitors. Percentage of apoptotic cells after 48 h. B and C, Western blot analysis of total proteins extracted (50 μg/lane) from NPA, PC Cl 3, and NPA Ets-Z cells, as indicated, with antibodies specific for the PARP, c-Myc, Bax, and Bcl-2 proteins. Analysis of γ-tubulin protein was performed as a control for protein loading. The sources of proteins are indicated.
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-cho in a dose-dependent manner but not by z-VAD-fmk or Ac-YVAD-cho. The caspase inhibitor z-VAD-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.

**ACKNOWLEDGMENTS**

We thank the Associazione Partenopea Ricerche Oncologiche for its support. We also thank Jean Gilder for editing the text.

**REFERENCES**


**Table 4** Colony-forming assay by transfecting the Ets-Z-dominant negative construct on thyroid carcinoma cell lines in the presence or absence of c-myc

<table>
<thead>
<tr>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>NPA</td>
<td>50</td>
<td>3</td>
<td>15</td>
<td>45</td>
</tr>
<tr>
<td>ARO</td>
<td>44</td>
<td>0</td>
<td>15</td>
<td>42</td>
</tr>
<tr>
<td>FRO</td>
<td>60</td>
<td>0</td>
<td>12</td>
<td>48</td>
</tr>
</tbody>
</table>

*The ratio between the vectors (pSV Ets-Z and pSVMyc) was 1:1.

*The ratio between the pSV Ets/Z and pSVMyc was 1:3.*
REQUIREMENT OF ets ACTIVITY IN THYROID CARCINOGENESIS


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

Filomena de Nigris, Tiziana Mega, Nicole Berger, et al.


Updated version
Access the most recent version of this article at:
http://cancerres.aacrjournals.org/content/61/5/2267

Cited articles
This article cites 56 articles, 22 of which you can access for free at:
http://cancerres.aacrjournals.org/content/61/5/2267.full.html#ref-list-1

Citing articles
This article has been cited by 17 HighWire-hosted articles. Access the articles at:
/content/61/5/2267.full.html#related-urls

E-mail alerts
Sign up to receive free email-alerts related to this article or journal.

Reprints and Subscriptions
To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

Permissions
To request permission to re-use all or part of this article, contact the AACR Publications Department at permissions@aacr.org.