Molecular Cloning and Characterization of TIEG2 Reveals a New Subfamily of Transforming Growth Factor-β-inducible Sp1-like Zinc Finger-encoding Genes Involved in the Regulation of Cell Growth*

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Sp1-like zinc finger transcription factors are involved in the regulation of cell growth and differentiation. Recent evidence demonstrating that mammalian cells express novel, yet uncharacterized, Sp1-like proteins has stimulated a search for new members of this family. We and others have recently reported that the transforming growth factor (TGF)-β-regulated gene TIEG encodes a new Sp1-like protein that inhibits cell growth in cultured cells. Here we report the identification, nuclear localization, DNA binding activity, transcriptional repression activity, and growth inhibitory effects of TIEG2, a novel TGF-β-inducible gene related to TIEG. TIEG2 is ubiquitously expressed in human tissues, with an enrichment in pancreas and muscle. TIEG2 shares 91% homology with TIEG1 within the zinc finger region and 44% homology within the N terminus. Biochemical characterization reveals that TIEG2 is a nuclear protein, which, as predicted from the primary structure, specifically binds to an Sp1-like DNA sequence in vitro and can repress a promoter containing Sp1-like binding sites in transfected Chinese hamster ovary epithelial cells. Furthermore, functional studies using [3H](thymidine uptake and MTS (3-(4,3-dimethyltiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium) assays demonstrate that the overexpression of TIEG2 in Chinese hamster ovary cells inhibits cell proliferation. Thus, TIEG2, together with TIEG1, defines a new subfamily of TGF-β-inducible Sp1-like proteins involved in the regulation of cell growth.

The transcription factor Sp1 is the founding member of a family of zinc finger proteins that regulate a variety of genes involved in cell growth and differentiation (1–22). Sp1-like binding sites, for example, are critical for the expression of a large group of genes necessary for DNA synthesis and cell cycle progression (2, 14–22). In addition, the overexpression of some members of the Sp1-like family of proteins in cultured cells has been shown to induce cell proliferation, cell cycle arrest, or apoptosis (9, 10, 13, 23). Furthermore, the disruption of Sp1 by homologous recombination demonstrates that at least some members of this subfamily of proteins are essential for normal development in vivo (24). Thus, Sp1-like proteins are emerging as critical regulators of the cellular events underlying morphogenesis.

The current members within the Sp1-like family of proteins include Sp1, Sp2, Sp3, Sp4, BTEB1, BTEB2, CPBP, BKL, EKLF, GKL, LKL, and TIEG1 (1, 3–15). These proteins are characterized by the presence of three highly conserved C-terminal zinc finger domains, which bind to GC-rich sequences. The growth regulatory effects of these proteins are believed to be mediated by the tight regulation of a hierarchical cascade of gene expression resulting from their binding to cis-regulatory GC-rich sites and subsequent interaction with the basal transcriptional machinery (25–29). In several instances, the identity of the specific Sp1-like protein that regulates distinct promoters through GC-rich sequences has been determined (5, 7, 14–22). However, emerging evidence reveals that GC-rich sequences in other promoters bind to as yet uncharacterized proteins (22, 30–36). This evidence has led many laboratories to search for novel members of the Sp1-like proteins. We and others, for example, have recently reported that the TIEG gene encodes an Sp1-like zinc finger protein that represses transcription and inhibits cell growth in epithelial and osteoblastic cell populations (13, 23, 37–39). In the current study, we describe the identification and functional characterization of TIEG2, a novel, ubiquitously expressed, Sp1-like zinc finger encoding cDNA. We have called this protein TIEG2 because, among the known members of the Sp1-like proteins, it displays the highest level of homology with TIEG. Sequence analysis demonstrates that TIEG2 contains the typical three zinc finger array at the C-terminal region of the protein and an N-terminal proline-rich domain. Biochemical studies indicate that TIEG2 encodes a nuclear protein that binds to a GC-rich consensus Sp1-like binding site and behaves as a transcriptional repressor. Moreover, functional studies demonstrate that the overexpression of TIEG2 in Chinese hamster ovary (CHO) cells inhibits cell proliferation. Thus, the identification and characterization of TIEG2 expands the repertoire of Sp1-like proteins expressed in mammalian cells and extends our understanding of the molecular machinery involved in the regulation of cell growth.

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The abbreviations used are: TIEG, transforming growth factor (TGF)-β-inducible early gene; CHO, Chinese hamster ovary; FBS, fetal bovine serum; FITC, fluorescein-5-isothiocyanate; GFP, green fluorescent protein; GST, glutathione S-transferase; IGF, insulin-like growth factor; MTS, (3-(4,3-dimethyltiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium; PKG, platelet-derived growth factor; ZF, zinc finger; DMEM, Dulbecco’s modified Eagle’s medium; CAT, chloramphenicol acetyltransferase; FACS, fluorescence-activated cell sorting.
**EXPERIMENTAL PROCEDURES**

**Cloning and Sequencing TIEG2**—We searched for TIEG-related proteins by comparing the rat TIEG sequence (GenBank accession number U88630) (13) against the Washington University Library expressed sequence tag (EST) database using the BLAST program from the National Center for Biotechnology Information (National Institutes of Health, Bethesda, MD). This comparison indicated the presence of a related EST (Accession No. T07800) (American Type Culture Collection (ATTC), Rockville, MD). For the isolation of TIEG2-encoding cDNAs, a total of 10^6 amplified recombinant plaques from a human epithelial CF Pac-1 cell line cdNA library (Stratagene, La Jolla, CA) were transferred onto nitrocellulose filters (Schleicher & Schuell, Keene, NH) and screened using the TIEG-related expressed sequence tag clone described above as a probe labeled to a specific activity of 2 x 10^9 cpmp/µg in a sodium acetate (0.15 M NaCl and 0.015 M sodium citrate) plus 0.1% SDS at 65 °C. Autoradiography was carried out at 5 h using LipofectAMINE according to the manufacturer's suggestions (GIBCO-BRL, Gaithersburg, MD). Recombinants were hybridized overnight at 68 °C in a solution containing 6 x SSPE (1 x SSPE = 0.18 NaCl, 10 mM NaHPO4, 1 mM EDTA, pH 7.7), 5 x Denhardt's solution, 1% SDS, 10% dextran sulfate, and 100 µg/ml denatured salmon sperm DNA. Filters were washed with a final stringency of 0.1 x SSPE containing 0.1% SDS at 65 °C. Positive plaques were isolated for a secondary screening, which was performed under the same conditions as the first. cDNAs were rescued using the QA-ExAssist helper phage-mediated protocol (Stratagene, San Diego, CA). Sequencing was performed using a sequencing-cycler-based double-stranded DNA sequencing system (Life Technologies, Inc., Gaithersburg, MD). The analyses of sequences and data base comparisons were performed using GCG (Genetics Computer Group, Madison, WI) and BLAST (National Library of Medicine, Bethesda, MD) DNA analysis software.

**Cell Culture and Northern blot analysis**—The human pancreatic epithelial cell line, PANC1, and the Chinese hamster ovary epithelial cell line, CHO, were obtained from ATCC. PANC1 cells were cultured in DMEM, and CHO cells were cultured in Ham's F-12 media, both supplemented with 5% FBS, 5% newborn calf serum, 100 units/ml streptomycin, and 100 units/ml penicillin (Life Technologies, Inc., Gaithersburg, MD). For experiments on the growth factor-mediated regulation of TIEG2 gene expression, PANC1 cells were cultured in 0.5% FBS-containing DMEM for 24 h and were transfected with 10 µg of pGAGC6 or pGAGC0 (American Type Culture Collection, Rockville, MD) DNA. Transfections were performed in triplicate in at least six independent experiments with similar results. To search for repression motifs within TIEG2, cdNA encoding the regions N-terminal (1-389) or C-terminal (461-495 amino acids) to the zinc finger DNA binding domain were cloned in-frame with the GAL4 DNA binding motif present in the effector vector pSG424 (kindly provided by Dr. Zeleznik-Le, University of Chicago) (45). This vector was co-transfected along with a luciferase reporter construct carrying five GAL4 binding sites upstream of the thymidine kinase basal promoter driving the expression of the chloramphenicol acetyltransferase (CAT) gene. As a control for basal transcriptional activity, the reporter construct was co-transfected with a plasmid carrying only the GAL4 DNA binding domain. Transfection of 5 x 10^5 CHO cells in 60-mm tissue culture dishes was performed in triplicate in at least three independent experiments with similar results.

**Immunofluorescence and Confocal Microscopy**—The subcellular localization of TIEG2 was determined using an epitope tag approach as described previously (13). A plasmid encoding an epitope-tagged form of TIEG2 was assembled by cloning the coding sequence of this gene in-frame with the Xpress™ epitope in the pcDNA3.1/HisA vector (Invitrogen, Carlsbad, CA). Approximately 2 x 10^6 CHO cells/100-mm dish were transfected with the Xpress-tagged TIEG2 (pcHisTIEG2) for 5 h using LipofectAMINE™ according to the manufacturer's suggestions (Life Technologies, Inc., Gaithersburg, MD).Twenty-four h after enhancement of cell transfection, cells were harvested by trypsinization and plated on poly(L-lysine)-coated coverslips. After an additional 24 h, cells were washed with phosphate-buffered saline and fixed for 10 min with −20 °C methanol. The localization of the tagged TIEG2 was performed using a monoclonal mouse anti-GFP antibody (Clontech, Palo Alto, CA). Cells were then double stained with fluorescein (FITC)-conjugated anti-mouse secondary antibody (Molecular Probes, Portland, OR) by indirect immunofluorescence as described previously (41). Cells were observed with a Zeiss LSM-310 confocal laser scanning microscope (Carl Zeiss, Inc., Oberkochen, Germany) using a 63 x oil immersion objective with a numerical aperture of 1.25. The excitation was performed using 488- and 568-nm wavelengths from an argon krypton laser. For illustration purposes, the cytoplasm was given a red pseudocolor to show the cell outline.

**Gel Shift Assays**—Gel shift assays were performed essentially as described (42, 43). Briefly, a double-stranded GC-rich Sp1 binding site, 5'-ATTCGATCGGGGCGGGGCGAGC-3', or a mutant GC box in which the two underlined G residues are replaced with two residues (Santa Cruz Biotechnology, Santa Cruz, CA) were cloned into phEGX-T4-S (Amersham Pharmacia Biotech). In order to map the consensus TIEG2-binding site, we employed the polymerase chain reaction, cloned in-frame with GST in the vector pGEX-T4-S (Amersham Pharmacia Biotech), and verified by sequencing. BL21 bacteria were induced to express the GST-ZF fusion protein with the addition of 2 µM isopropyl-1-thio-β-D-galactoside for 2 h, and the recombinant protein was purified from a GST-Sepharose column according to the manufacturer's suggestions (Amersham Pharmacia Biotech). Purified GST or GST-ZF (∼200 ng) was incubated 10 min at room temperature with or without cold competitor or anti-GST antibody (Amersham Pharmacia Biotech). Reactions were performed in a buffer containing 4% glycerol, 1 mM MgCl2, 0.5 mM EDTA, 0.5 mM dithiothreitol, 50 mM NaCl, 10 mM Tris-HCl, pH 7.5, and 50 µg/ml poly(dI-dC)poly(dI-dC) (Promega). End-labeled oligonucleotide was added to a final concentration of 20 µM, and reactions were loaded immediately onto a 4% nondenaturing polyacrylamide gel and run for 3 h at 120 V at room temperature. Autoradiography was carried out at room temperature using X-Omat film (Kodak).

**Reporter Assays**—The transcriptional regulatory activity of TIEG2 was determined in transfected CHO cells using the luciferase reporter plasmid pGAGC6, which contains six GC-rich Sp1 binding sites (kindly provided by Dr. Jeffery Kudlow, University of Alabama, Birmingham, AL) (44). The plasmid pGAGC0, with the Sp1-like binding sites deleted, was used as a control. CHO cells were transfected with the pcHisTIEG2 vector and either the pGAGC6 or pGAGC0 reporter plasmids. Control experiments were performed using the parental plasmid pCDNA3.1/His. For these experiments, 3 x 10^5 CHO cells were plated in 60-mm tissue culture dishes and transfected with 1.5 µg of reporter plasmid and 4.5 µg of pcHisTIEG2 or control pcDNA3.1/His vector. Twenty-four h after transfection, cells were lysed, and luciferase assays were performed using a Turner 20/20 luminometer and a luciferase assay system according to the manufacturer's suggestions (Promega, Madison, WI). Relative luciferase activity was determined using equal amounts of protein (20 µg) and was expressed as a ratio between the activity of pGAGC6 and pGAGC0. Studies were performed in triplicate in at least three independent experiments with similar results.

**Transfection of CHO Cells**—CHO cells were plated in triplicate in at least three independent experiments with similar results.
were plated in a 48-well microtiter dish. Twelve to 72 h after plating, CHO cells were co-transfected with GFP to measure overall cell death, and TIEG2-expressing plasmids. Annexin V analysis on CD20-positive cells was performed by FACS as described previously.

CD20 positive cells were counted using a FACScan flow cytometer and analyzed for CD20/annexin V-positive cells using WinMDI 2.5 software. All studies were performed using a Beckman liquid scintillation counter. Beckman Instruments, Palo Alto, CA. For annexin V studies, CHO cells were co-transfected with the CD20 transmembrane receptor (a gift kindly provided by Dr. David McKean, Mayo Clinic, Rochester, MN) and TIEG2-expressing plasmids. Annexin V analysis on CD20-positive cells was performed by FACS as described previously.

RESULTS AND DISCUSSION

Identification of a TIEG-related Sp1-like Zinc Finger-encoding Gene, TIEG2—The TGF-β-inducible immediate early gene, TIEG, encodes an Sp1-like transcription factor that has the ability to inhibit cell growth in cultured osteoblastic and epithelial cell populations (12, 23). Evidence for the existence of a novel TIEG-related cDNA reported in this study was gathered during the process of comparing our previously published rat aminos acid sequence against the I.M.A.G.E. Washington University Expressed Sequence Tag data base using the BLAST software system. Using this approach, we identified a human cDNA clone (I.M.A.G.E. Clone 770800) that encodes a novel peptide sequence displaying 39% identity to amino acids 1 to 79 of the TIEG protein (data not shown). This novel cDNA clone was obtained from the Washington University Consortium (deposited at ATCC, Rockville, MD) and used as a probe to screen a cDNA library originating from the human epithelial CF Pac-1 cell line under high stringency conditions. From this screening, we isolated five overlapping clones that provided the entire coding sequence for a novel protein of 55.24 kDa containing three Sp1-like zinc finger motifs at the C terminus and a proline-rich N terminus (Fig. 1, a and b). The zinc finger motifs of this protein are 91% homologous to the corresponding domains within TIEG (referred to as TIEG1 in the rest of this article). In addition, as shown in Fig. 1c, this region is remarkably similar to other members within a growing family of Sp1-like transcription factor proteins (72% to 78%), all containing three highly homologous CxHxH zinc finger motifs at the C terminus. Outside of the zinc finger domains, this protein is 44% similar to TIEG1 but does not share any significant homology with any other previously identified protein. Moreover, both of these proteins also share the presence of several proline-rich sequences within the N-terminal domain, a property commonly found in other transcription factor domains (6–8, 50).

Interestingly, recent studies from other laboratories have begun to group the Sp1-like transcription factors into distinct structural subfamilies based upon the presence of conserved regions found outside of the zinc finger motifs. The subfamilies that have been identified thus far, using this criteria, include the SP proteins (Sp1, Sp2, Sp3, and Sp4) and the KLF proteins (EKLF, LKLF, and GKLKF) (3, 4, 8). Members of the SP sub-family of proteins show significant homology throughout the N-terminal domain, whereas the KLF proteins share a single 20-amino acid motif directly upstream of the zinc finger domain. Our data reveal the existence of at least two TIEG-related proteins that share several regions of similarity both N-terminal and C-terminal to the zinc finger domain. Thus, this analysis led us to name the novel protein identified in this study as TIEG2.

Nuclear Localization, Sp1-like Binding, and Transcriptional Repressor Activity of TIEG2—Previous structural studies on zinc finger peptides bound to DNA target sequences have provided information that has allowed, in some instances, to make predictions on the putative DNA sequences that novel zinc finger proteins may recognize (51–54). One of the groups of proteins that allows us to make these predictions is the Sp1-like subfamily of transcription factors, since this supporting structural analysis already exists (51, 53). Interestingly, the amino acid residues within the first (KHA), second (RER), and third (RHK) zinc finger domains of TIEG2 that are predicted to make contact with DNA are identical to the corresponding regions within Sp1 (see Fig. 1c) that bind to the sequences GGG (ZF1), GCG (ZF2), and GGG (ZF3), respectively (51, 55). We therefore tested if TIEG2 would, like Sp1, bind to the GGG GCG GGG sequence (GC box). We performed electrophoresis on the three zinc finger motifs of TIEG2 (GST-ZF) and a probe containing the GC box. Control experiments were performed using a mutant GC box and binding sites for unrelated transcription factors. The results of these experiments, shown in Fig. 2a, demonstrate that GST-ZF (lane 3) but not GST alone (lane 2), binds to the wild-type Sp1 binding site GGG GCG GGG. No binding was observed using a mutant GC box in which the third and fourth G residues (underlined) had been replaced for T residues GGATT (lane 4). A supershift of the GST-ZF/GC box complex using an anti-GST antibody (lane 5) demonstrates that this binding activity is due to the fusion protein and not to trace amounts of any other bacterial protein that may have co-purified with GST. No supershift was detected using mouse IgG as a control (data not shown). The GST-ZF binding activity is competed by unlabeled GC box but not by the binding sites for other transcription factors such as NF1 (lane 7) or AP2 (data not shown). Therefore, these studies demonstrate that TIEG2 specifically binds to the consensus Sp1 binding site in vitro.

Subsequently, we determined the ability of TIEG2 to regulate promoter activity through Sp1-like binding sites in vivo. For this purpose, we performed reporter assays using a luciferase plasmid containing six GC-rich Sp1 consensus binding sites cloned upstream of the adenovirus major late basal promoter (pGAGC6) (44). This approach has been previously used to characterize the transcriptional regulatory activity of several members of the Sp1-like family of proteins (3–8, 11, 12, 15, 39). TIEG1, for instance, has been recently shown to behave as a transcriptional repressor in this assay (39). A TIEG2 expression vector was used to transfect CHO cells along with a reporter plasmid either containing (pGAGC6) or lacking (pGAGC0) six Sp1-like binding sites. Control assays were performed using cells transfected with the reporter plasmid and the parental expression plasmid lacking the TIEG2 cDNA. The reporter activity corresponding specifically to the Sp1-like sites was calculated from the ratio between the values obtained with the pGAGC6 and the pGAGC0 reporters (pGAGC6/pGAGC0 ratio). Fig. 2b shows that the overexpression of TIEG2 represses luciferase activity by 49% of control values (3.7 ± 3.7 versus 73.4 ± 3.1, TIEG2 versus control). Thus, these studies demonstrate that, similar to TIEG1, TIEG2 can repress pro
moter activity in vivo through Sp1-like binding sites. Subsequently, we used the well characterized GAL4-based transcriptional regulatory assay to determine whether TIEG2 contains transcriptional repressor domains that modulate gene expression. These studies were performed using chimeric constructs carrying the GAL4 DNA binding domain and either the C-terminal or N-terminal region of TIEG2. Constructs were co-transfected with an expression vector containing a CAT reporter gene carrying five GAL4 binding sites upstream of the thymidine kinase basal promoter. CAT activity was determined using a colorimetric assay. The results of these experiments, shown in Fig. 3, demonstrate that although the C-terminal region of TIEG2 did not modify CAT activity, the N-terminal portion of the molecule reduced the activity of this reporter in a dose-dependent manner. Therefore, the transcriptional regulatory function of TIEG2 can be accounted for by the presence of a potent repressor activity located within the N-terminal region of the protein.

To determine the subcellular localization of TIEG2, we transfected CHO epithelial cells with a plasmid encoding the full-length TIEG2 protein with an eight-amino acid Xpress™ epitope on the N terminus. The localization of this protein was determined by immunofluorescence using confocal microscopy and a monoclonal anti-Xpress™ antibody. Using this approach, we found that TIEG2 is localized to the cell nucleus but is excluded from the nucleolus (Fig. 4). No detectable staining was observed in cells transfected with the parental plasmid used as a control (data not shown). Together, the results of the gel shift assays, reporter studies, and the immunolocalization analysis demonstrate that TIEG2 displays biochemical features characteristic of an Sp1-like transcription factor, suggesting a role for this protein in the regulation of gene expression.

FIG. 1. Sequence analysis of TIEG2. a, physical map of the deduced TIEG2 protein. Sequence analysis indicates the presence of several proline-rich regions within the N terminus of the protein, two putative nuclear localization signals (*) and three C2-H2 zinc finger domains near the C terminus. b, alignment of the human TIEG1 and TIEG2 protein sequences (GenBank® accession numbers U21847 and AF028008, respectively). Numbers to the right refer to amino acid residues. Red denotes identical amino acids, whereas blue denotes conserved amino acids. Proline residues within the N-terminal domain of TIEG2 are bold. The putative nuclear localization signals (*) were identified using the program PSORT (www.psort.nibb.ac.jp). Individual zinc fingers are underlined. c, alignment of the zinc finger motifs of TIEG2 with the corresponding regions of previously identified Sp1-like proteins reveals high homology with TIEG1 (U88630), BTEB1 (D31780), Sp1 (U21847), Sp2 (M93506), Sp3 (M97191), Sp4 (U62522), and EKLF (U37106). Individual zinc finger motifs are underlined. Residues predicted to interact with DNA according to the Klevit model (51) are indicated by arrows.

The percentage of similarity between TIEG2 and other Sp1-like zinc finger domains is indicated on the right.
Expression Pattern and Growth Factor Regulation of TIEG2—Some previously described members of the Sp1-like family of genes are ubiquitously expressed (e.g. Sp1), whereas others display a more restricted pattern of expression (e.g. EKLF) (2, 8). We therefore performed Northern blot analysis on RNA isolated from various human tissues to gain insight into the spatial distribution of the TIEG2 mRNA. Fig. 5 shows that the TIEG2 mRNA is ubiquitously expressed in human tissues, with the highest levels found in pancreas and skeletal muscle. Note that the expression pattern for TIEG1 and TIEG2 is slightly different. TIEG2, for example, is more prominently expressed in human pancreas, whereas TIEG1 is expressed in human skeletal muscle and liver. Because some of the TIEG2 every tissue exhibit TIEG2 expression, we therefore examined the levels of TIEG2 mRNA in response to serum deprivation, which induces TIEG2 expression in many cell types. Serum deprivation of CHO cells results in increased TIEG2 mRNA levels (Fig. 6c and data not shown), which can be abolished by the addition of the RNA polymerase II transcriptional inhibitor, actinomycin D, indicating that this effect is mediated, at least in part, at the level of transcription. Neither cycloheximide nor actinomycin D treatment alone affected TIEG2 mRNA levels (Fig. 6c). The importance of the finding that TIEG2 is a TGF-β-inducible early response gene should be considered in light of the fact that TGF-β-mediated signaling pathways inhibit epithelial cell growth (57, 58). Because early response genes have been proposed to be a direct link between growth factor signaling cascades and distinct cellular functions (59), it is therefore possible that TIEG2 may be an effector of anti-proliferative signaling pathways. Interestingly, Fig. 6b shows that the levels of TIEG2 mRNA also increase in response to serum deprivation and decrease with the addition of serum. Furthermore, mitogenic growth factors such as epidermal growth factor, IGF-I, IGF-II, and PDGF, known to activate these cells (Refs. 60 and 61 and data not shown), did not modify TIEG2 expression levels (Fig. 6c). Thus, these results establish a correlation between the expression of TIEG2 and anti-proliferative treatments.

Effect of TIEG2 on Epithelial Cell Proliferation—Based upon its structural similarity to TIEG1 and its regulation by anti-proliferative agents, TIEG2 becomes a good candidate to inhibit epithelial cell growth. We therefore transiently expressed TIEG2 in the highly transfectable epithelial CHO cell line and measured proliferation both by [3H]thymidine uptake and the
The N terminus (amino acids 1–389) or C terminus (amino acids 461–495) of TIEG2 were fused to the DNA binding motif of GAL4 and co-transfected into CHO cells along with the reporter plasmid containing five GAL4 recognition sites upstream of the CAT gene. CAT activity was determined using an enzyme-linked immunosorbent assay assay (Boehringer Mannheim). Basal transcriptional activity was measured using the reporter plus the effector plasmid carrying the GAL4 DNA binding motif alone (Control). For normalization purposes, a plasmid carrying β-galactosidase was co-transfected with each experimental condition. Relative CAT activity using 1 µg of the reporter construct with the GAL4 DNA binding motif alone (Control, 3000 ng), the N terminus-GAL4 construct (N-term, 10, 100, 500, and 3000 ng), and the C terminus-GAL4 construct (C-term, 3000 ng) ±S.E. are plotted. Note that the N-terminal region of TIEG2 displays a dose-dependent transcriptional repressor activity, whereas the C-terminal region does not have any significant transcriptional regulatory activity over the control.

![Figure 3](image3.png)

**Fig. 3. Transcriptional regulatory activity of TIEG2.** The N terminus (amino acids 1–389) or C terminus (amino acids 461–495) of TIEG2 were fused to the DNA binding motif of GAL4 and co-transfected into CHO cells along with the reporter plasmid containing five GAL4 recognition sites upstream of the CAT gene. CAT activity was determined using an enzyme-linked immunosorbent assay assay (Boehringer Mannheim). Basal transcriptional activity was measured using the reporter plus the effector plasmid carrying the GAL4 DNA binding motif alone (Control). For normalization purposes, a plasmid carrying β-galactosidase was co-transfected with each experimental condition. Relative CAT activity using 1 µg of the reporter construct with the GAL4 DNA binding motif alone (Control, 3000 ng), the N terminus-GAL4 construct (N-term, 10, 100, 500, and 3000 ng), and the C terminus-GAL4 construct (C-term, 3000 ng) ±S.E. are plotted. Note that the N-terminal region of TIEG2 displays a dose-dependent transcriptional repressor activity, whereas the C-terminal region does not have any significant transcriptional regulatory activity over the control.

cho cells were transfected with an Xpress™ epitope-tagged TIEG2 vector, and the localization of the tagged protein was performed by confocal microscopy following staining with a monoclonal anti-Xpress™ antibody and an FITC-conjugated anti-mouse secondary antibody. The arrowheads show nuclear staining of the TIEG2 construct in the transfected CHO cells, whereas untransfected cells in the field show no FITC staining. For illustration purposes, the cellular cytoplasm was given a red pseudocolor.

![Figure 4](image4.png)

**Fig. 4. Nuclear localization of TIEG2.** CHO cells were transfected with an Xpress™ epitope-tagged TIEG2 vector, and the localization of the tagged protein was performed by confocal microscopy following staining with a monoclonal anti-Xpress™ antibody and an FITC-conjugated anti-mouse secondary antibody. The arrowheads show nuclear staining of the TIEG2 construct in the transfected CHO cells, whereas untransfected cells in the field show no FITC staining. For illustration purposes, the cellular cytoplasm was given a red pseudocolor.

The effect of TIEG1 and TIEG2 on cell proliferation is in agreement with an emerging concept that a major role for Sp1-like proteins is to regulate cell growth. Some members of this family of transcription factors, such as Sp1, participate in mitogenic pathways, whereas other Sp1-like proteins clearly inhibit cell proliferation (2, 10, 13, 23). It is important to note that among the Sp1-like transcription factors, the effects of both of the TIEG proteins on cell growth most resembles the function of GKL (10). The expression of GKL is also upregulated by anti-proliferative stimuli, and its overexpression in cultured cells inhibits [3H]thymidine uptake. Currently, understanding the molecular mechanisms by which TIEG1, TIEG2, and GKL inhibit cell growth remains a significant challenge. However, it is likely that these proteins regulate the expression of genes involved in cell proliferation through the use of GC-rich cis-regulatory sequences. GC-rich sequences are required for the regulation of a large number of growth-regulatory genes, including specific cell cycle regulators (p15, p21, p27), MAP kinases, regulatory GTPases (Ha-Ras), DNA synthesis proteins (histones, thymidine kinase, DHFR, POLD1), growth factors (PDGF, TGF-β), and growth factor receptors (insulin receptor, insulin-like growth factor receptor, cyes) (2, 14, 15, 17, 20–22, 65–71). Interestingly, recent evidence indicates that Sp1, Sp3, and novel, uncharacterized Sp1-like proteins bind to some of these promoters (2, 14–22, 30–36). The [M] of some of these uncharacterized proteins are similar to the predicted molecular weight of TIEG2, raising the possibility that this protein may participate in the regulation of some of these genes. Thus, future studies aimed at understanding the molecular mechanisms underlying TIEG-mediated transcriptional regulation may provide critical insight into potential target genes for these proteins. Nevertheless, the knowledge on
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Fig. 6. Expression of TIEG2 mRNA in response to growth factors and serum. Northern blot analysis of TIEG2 expression in PANC1 cells treated with TGF-β1 (a), serum addition and withdrawal (b), and epidermal growth factor (EGF), IGF-I, IGF-II, and PDGF (c) for the times indicated as described under “Experimental Procedures.” Note that TIEG2 mRNA expression is up-regulated by TGF-β1 treatment, even in the presence of the protein synthesis inhibitor cycloheximide (CHX), supporting a role for this gene product as an early response gene for this growth factor. Furthermore, up-regulation is abolished with the addition of the transcriptional regulator, actinomycin D (ActD), indicating that the increase in mRNA levels is due to a transcriptional event rather than mRNA stability. TIEG2 mRNA is also up-regulated upon serum withdrawal but down-regulated with the addition of serum. Finally, TIEG2 expression is not up-regulated by the proliferative growth factors EGF, IGF-I, IGF-II, and PDGF.

Fig. 7. Proliferation of CHO cells overexpressing TIEG2. CHO cells were co-transfected with a GFP-expressing plasmid and either a TIEG2 expression vector (pcHisTIEG2) or the control parental plasmid (pCMV). GFP-positive cells were plated, and proliferation was measured by [3H]thymidine incorporation (a) or MTS assay (b) 12, 24, 48, or 72 h after plating. Note that cells transfected with TIEG2 show a significant decrease in [3H]thymidine uptake (84.8 ± 0.01% reduction by 72 h) and MTS values (67.4 ± 2.75% reduction by 72 h) as compared with cells expressing the control vector alone. Error bars represent S.E.

Table I

Percentage of cell death in TIEG2-overexpressing cells

CHO cells were co-transfected with TIEG2 and CD20 (annexin V studies) or GFP (propidium iodide). Control cells were co-transfected using the parental plasmid. 24, 48, and 72 h later, cells were harvested and processed for annexin V-FITC staining or propidium iodide staining as described under “Experimental Procedures.” Values represent the percentage of CD20/annexin V- or GFP/propidium iodide-positive cells in TIEG2-transfected cells normalized to control cells ± S.E. Note that no significant effect on cell death is detected in TIEG2-expressing cells.

|                      | 24 h       | 48 h       | 72 h       |
|----------------------|------------|------------|------------|
| Annexin V            | 0.467 ± 0.003 | 0.546 ± 0.067 | 0.522 ± 0.044 |
| Propidium iodide     | 1.250 ± 0.117 | 1.453 ± 0.138 | 1.062 ± 0.155 |

the existence of different transcription factors that share similar DNA binding motifs poses an exciting challenge for understanding the biology of these proteins.

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