Three Conserved Transcriptional Repressor Domains Are a Defining Feature of the TIEG Subfamily of Sp1-like Zinc Finger Proteins*

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Sp1-like transcription factors are characterized by three highly homologous C-terminal zinc finger motifs that bind GC-rich sequences. These proteins behave as either activators or repressors and have begun to be classified into different subfamilies based upon the presence of conserved motifs outside the zinc finger domain. This classification predicts that different Sp1-like subfamilies share certain functional properties. TIEG1 and TIEG2 constitute a new subfamily of transforming growth factor-β-inducible Sp1-like proteins whose zinc finger motifs also bind GC-rich sequences. However, regions outside of the DNA-binding domain that differ in structure from other Sp1-like family members remain poorly characterized. Here, we have used extensive mutagenesis and GAL4-based transcriptional assays to identify three repression domains within TIEG1 and TIEG2 that we call R1, R2, and R3. R1 is 10 amino acids, R2 is 12 amino acids, and R3 is approximately 80 amino acids long. None of these domains share homology with previously described transcriptional regulatory motifs, but they share strong sequence homology and are functionally conserved between TIEG1 and TIEG2. Together, these data demonstrate that TIEG proteins are capable of repressing transcription, define domains critical for this function, and further support the idea that different subfamilies of Sp1-like proteins have evolved to mediate distinct transcriptional functions.

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TIEG1 also mediates repression, revealing that this region has a conserved transcriptional function in both TIEG proteins. More importantly, using deletion and site-directed mutagenesis together with transcriptional regulatory assays, we have defined three independent transcriptional repression domains conserved within TIEG proteins that we call R1, R2, and R3. R1 and R2 are 10- and 12-amino acids long, respectively, while R3 is a longer proline-rich sequence of approximately 80 aa. Interestingly, in contrast to several other members of the Sp1-like family, no transcriptional activation domain was found to be present in these transcription factors (7, 8, 14, 18, 19, 23–26, 28, 29). Together, these data strongly support the biochemical similarity between TIEG1 and TIEG2 and indicate that they primarily function to silence gene expression. Furthermore, these results expand our understanding of the repertoire of functional domains present in Sp1-like transcription factors for the regulation of gene expression in mammalian cells. The importance of these results for extending the classification of Sp1-like proteins is discussed.

EXPERIMENTAL PROCEDURES

Generation of GAL4 Effector Plasmids—TIEG2-GAL4 constructs were generated by amplifying various regions of TIEG2 by polymerase chain reaction and cloning these in frame with the pSG424 GAL4 DBD\(^\text{a}\) effector plasmid (kindly provided by Dr. N. J. Zeleznik-Le, University of Chicago, IL). TIEG1 constructs were generated in the same manner. All TIEG2 constructs were numbered by amino acid using the second in-frame methionine of the originally published sequence (the codon that best fits the Kozak consensus translational start site) as amino acid residue 1 (17). Site-directed proline mutagenesis of the R1 and R2 domains was performed by annealing overlapping mutant primers that span the region of interest. Mutagenesis of the R3 domain was performed using standard overlapping polymerase chain reaction mutagenesis. All constructs were verified by sequencing.

GAL4-based Transcriptional Regulatory Assays—GAL4-based transcriptional regulatory assays were performed as described previously (17). Briefly, 3 \(\times\) 10\(^5\) CHO cells were plated in 60-mm tissue culture dishes and transfected 48 h later with 3 \(\mu\)g of GAL4 effector plasmid DNA. 1 \(\mu\)g of GAL4, tkCAT reporter (generously provided by Dr. N. J. Zeleznik-Le), and 1 \(\mu\)g of pHook-LacZ (InVitrogen, Carlsbad, CA) using LipofectAMINE\(^\text{b}\) (Life Technologies, Inc.). As a control for basal transcriptional activity, the reporter construct was co-transfected with an effector plasmid carrying the GAL4 DNA-binding domain alone. Co-transfection with a \(\beta\)-galactosidase expression plasmid, pHook-LacZ (Promega), was performed to control for transfection efficiency. Relative CAT activity was assayed using an enzyme-linked immunosorbent assay method from Roche Molecular Biochemicals, and \(\beta\)-galactosidase activity was measured using the \(\beta\)-galactosidase enzyme assay system (Promega). In all experiments, CAT activity was determined using the same amount of protein (50 \(\mu\)g), and the values were normalized to \(\beta\)-galactosidase activity. Studies were performed in triplicate in at least three independent experiments with similar results.

Gel Shift Assays—To ascertain expression levels of each GAL4 chimeric transcription factor used in these studies, electromobility shift assays were performed essentially as described previously (17, 32, 33). Briefly, concomitant with the transfection for the GAL4-based transcriptional regulatory assay, an additional 80-mm dish of CHO cells was co-transfected with 3 \(\mu\)g (or as indicated) of each effector plasmid and 2 \(\mu\)g of filler DNA (pcDNA3.1; InVitrogen). 48 h post-transfection, cells were washed twice with ice-cold PBS, harvested by scraping, and pelleted at 3000 \(\times\) \(g\) for 5 min. Cells were then lysed in 35 \(\mu\)l of whole cell extract buffer (20 mM Tris, pH 7.5, 20% glycerol, 0.5 mM KCl, 1 mM phenylmethylsulfonyl fluoride, 5 \(\mu\)g/ml aprotinin, 1 mM dithiobistrithiol), frozen at \(-80^\circ\ C\), and thawed at 4 \(^\circ\ C\). Cellular debris and unlysed cells were pelleted at 10,000 \(\times\) \(g\) for 10 min at 4 \(^\circ\ C\), and lysates were transferred to fresh tubes. Protein concentrations were assayed using the BCA method (Pierce). 10 \(\mu\)g of whole cell extracts was incubated 10 min at room temperature in a buffer containing 4% glycerol, 1 mM MgCl\(_2\), 0.5 mM EDTA, 0.5 mM dithiobistrithiol, 50 mM NaCl, 10 mM Tris-HCl, pH 7.5, and 50 \(\mu\)g/ml poly(dI-dC)poly(dI-dC) (Promega, Madison, WI). A double-stranded GAL4 DNA-binding site, 5'-GGAGTACT-GTCCCCGGAG-3' was end-labeled with \([\gamma^32P]\)ATP with T4 polynucleotide kinase according to the manufacturer's suggestions (Promega, Madison, WI). 0.35 pmol of end-labeled GAL4 probe was then added to each reaction for an additional 20 min, and the reactions were loaded immediately onto a 4% non-denaturing polyacrylamide gel. Samples were run for 3 h at 120 V at room temperature, vacuum-dried, and exposed to X-Omat film (Eastman Kodak Co.) at room temperature.

RESULTS

TIEG1 and TIEG2 Act as Potent Transcriptional Repressors—Several studies have recently revealed the existence of a novel subfamily of Sp1-like transcription factors, TIEG1 and TIEG2, that inhibit growth of epithelial cell populations (15, 17). These proteins share 91% similarity within their C-terminal zinc finger DNA-binding domain and 44% similarity within a proline-rich N terminus. Previous biochemical studies have demonstrated that both of these proteins bind to GC-rich sequences and repress transcription of promoters containing these sites (16, 17). In addition, the N-terminal region of TIEG2, when tethered to DNA through a heterologous DBD, behaves as a potent transcriptional repression domain (17) (Fig. 1). To test whether the homologous region of TIEG1 has a similar function, in the current study, we fused the N and C terminus of TIEG1 to the GAL4 DBD and tested its ability to regulate a promoter carrying five GAL4 DNA-binding sites upstream of a CAT reporter gene. Indeed, Fig. 1b demonstrates that the N-terminal regions of both TIEG1 and TIEG2 are able to repress transcription by over 95%, whereas the C-terminal regions display no transcriptional regulatory activity. Together, these results demonstrate that the N-terminal regions of the TIEG proteins display functionally conserved transcriptional repression activity.

Deletion Mutagenesis of the N Terminus of TIEG2 Reveals the Presence of Three Transcriptional Repressor Domains—To identify domains utilized by TIEG proteins to regulate transcription, we generated a number of deletion and site-directed mutants as GAL4-TIEG chimeric constructs. The expression and the ability of each of these proteins to bind GAL4 DNA-binding sites was confirmed using gel shift assays as described under “Experimental Procedures” (data not shown). The results shown in Fig. 2 delineate three distinct domains within

\(^{a}\) The abbreviations used are: DBD, DNA-binding domain; CAT, chloramphenicol acetyltransferase; CHO, Chinese hamster ovary; aa, amino acid(s).
TIEG2, R1 (aa 24–41), R2 (aa 151–162), and R3 (aa 273–351), that repress reporter activity by at least 75%. R1 and R2 are small peptides (17 and 12 aa, respectively), whereas R3 is a larger domain composed of approximately 80 aa (residues 273–351). Regions outside of the R1, R2, and R3 domains display no transcriptional regulatory activity (Fig. 2b). Furthermore, smaller deletion constructs within R1 (27–34), R2 (158–160), and R3 (273–303 and 304–351) display a loss of repression activity, indicating that the domains defined using this analysis represent the minimal functional units necessary for transcriptional repression.

Identification of Residues Critical for R1, R2, and R3 to Repress Transcription Using Site-directed Mutagenesis—To more precisely define the residues responsible for the transcriptional regulatory activity of R1, R2, and R3, we used extensive site-directed mutagenesis. Computer-based modeling studies of R1 domain using SOPMA software (34) (available on the Internet) predict that this sequence is capable of forming an α-helical structure. Therefore, we generated sequential mutations along the R1 domain of TIEG2 using the helix-breaking proline residue as a mutagen (Fig. 3a). We then tested each of these mutants for their ability to maintain transcriptional repression. As shown in Fig. 3, mutations within the central core of the R1 domain, corresponding to amino acid residues 30–39 (AVEALCVMSS), resulted in a complete loss of transcriptional regulatory activity, while mutations outside of this core did not abolish repression. Additional results demonstrate that this decapeptide is also sufficient to repress transcription. Therefore, these results define a 10-aa core sequence that is required for the R1 domain to function.

Using a similar approach, we characterized the R2 and R3 domains of TIEG2. Mutation of nine of the 12 residues that compose R2 did not significantly influence repression (Fig. 4). In contrast, mutations in three consecutive residues, Val, Ile, and Arg (aa 158–160), resulted in a complete loss of repression activity by R2. However, these three residues alone (GAL4-VIR) were not sufficient to repress reporter activity. This result suggests that while the VIR peptide is essential for R2-mediated repression, the remainder of the R2 domain also contributes to its function.

The R3 domain of TIEG2 contains two interesting features. First, R3 is relatively proline-rich (20%), a feature that is commonly found in activation and repression domains (35, 36). Second, the R3 domain contains a core sequence of approximately 20 aa (aa 311–328) that is highly conserved (67% similar) between TIEG1 and TIEG2 (see Fig. 6). Because of the high proline content of R3, we used serine mutants to characterize this domain (Fig. 5a). Three of these mutants involved changing clusters of proline residues near the beginning (aa 288–290), middle (aa 306–310), or end (aa 332–336) of this domain; an additional mutant changed seven of the central conserved residues (aa 317–323). As shown in Fig. 5b, all of the proline-to-serine mutants were still able to repress transcription, indicating that these proline residues are not essential for the ability of R3 to repress transcription. In contrast, mutation of the conserved core abolishes the ability of R3 to repress transcription. Interestingly, one of the constructs used to define the R3 domain (304–351, Fig. 2) contains this conserved sequence but is only able to repress transcription by −42% versus 77% for the full R3 domain. This result suggests that the central core sequence is necessary but not sufficient for full R3-mediated repression activity.

R1, R2, and R3 Are Conserved Repression Domains within the TIEG Family of Transcription Factors—An important feature of the repression domains described above is that they share strong sequence homology between both TIEG proteins (65, 75, and 29% similar for R1, R2, and R3, respectively) (Fig. 6a). In addition, the central core of R3, a region that is required for repression, is 67% similar between TIEG1 and TIEG2. To determine whether these three domains are functionally conserved between the TIEG proteins, we tested the ability of the corresponding regions of TIEG1 to regulate transcription (Fig.
S.D. Note that in addition to being homologous, each of these domains bars other homology was detected with other proteins using existing data constructs. Nevertheless, a GAL4 DBD-VIR (VIR) construct is not sufficient to repress transcription. However, a GAL4 DBD-VIR (VIR) construct is not sufficient to repress transcription.

Fig. 4. Mapping of the residues involved in R2-mediated repression. Scanning proline mutations were generated to span the entire R2 domain of TIEG2 (a). These mutant GAL4 DBD chimeric effector plasmids were then tested for their ability to regulate transcription (b). Note that only mutations in residues VIR (constructs 8–10) lose the ability to repress transcription. However, a GAL4 DBD-VIR (VIR) construct is not sufficient to repress transcription.

Fig. 5. Mapping of the residues involved in R3-mediated repression. (a) Four serine-based mutants were generated in the R3 domain of TIEG2. Mutants 1, 2, and 4 mutate prolines to serines, while mutant 3 changes a core of residues present within a conserved region between TIEG1 and TIEG2 (shaded). These mutant GAL4 DBD chimeric effector plasmids were tested for their ability to regulate transcription (b). Note that mutations in the proline residues do not affect the ability of the R3 domain to repress transcription (1, 2, 4), while a mutation of the conserved region alleviates transcriptional repression (3).

Fig. 6. R1, R2, and R3 are conserved in TIEG1 and TIEG2. a, sequence comparisons of the R1, R2, and R3 domains of TIEG2 (from Fig. 2) compared with TIEG1 show a remarkable conservation between these regions. The R1 domain is 85% similar, the R2 domain is 75% similar, and the R3 domain is 29% similar. Dark gray indicates identical residues, and light gray indicates similar residues. Proline residues in the R3 domain are boxed, and a core of 20 aa in the R3 domain that is 67% similar between TIEG2 and TIEG1 is marked with asterisks. No other homology was detected with other proteins using existing databases. b, histogram representing relative CAT expression from GAL4-based transcriptional regulatory assay using increasing amounts of effector plasmids (DBD and R1: 0.25, 0.5, 1, and 2 μg; R2 and R3: 0.0375, 0.075, 0.15, and 0.3 μg). Effector and reporter plasmids were transfected in CHO cells and analyzed for relative CAP expression as described under “Experimental Procedures.” Bars, S.D. Note that in all cases, a dose-dependent repression is observed, and under all conditions, the R1 domain most effectively represses transcription. b, CHO cells were transfected with 2 μg of the GAL4-DBD, 2 μg of R1, 0.3 μg of R2, or 0.3 μg of R3 effector plasmid. pcDNA3.1+ plasmid was added to each transfection to reach a total of 5 μg, and transfections were performed concomitantly with the experiment in a as described under "Experimental Procedures." 10 μg of whole cell lysates from each sample were then used in a gel shift assay using the GAL4 DNA-binding site as a probe. The asterisk indicates a degradation product. This experiment was performed three times with identical results.

6b). Indeed, the R1, R2, and R3 regions of TIEG1 also repress transcription. Furthermore, the residues that are critical for TIEG2 repression share remarkable similarity (≥80%) between both TIEG proteins (Figs. 9–6). Interestingly, the data presented in Figs. 2 and 6 also suggested that the R1, R2, and R3 domains exhibit different levels of repressor activity. To assess their differential strengths, we compared dose–response curves for R1, R2, and R3 from TIEG2. From these experiments, we find that all three domains display repression activity in a dose-dependent manner. However, the R1, R2, and R3 domains exhibit different relative strengths: 92, 84, and 51% repression, respectively, at the highest dose tested. For this comparison, gel shift analysis of each GAL4-TIEG2 chimeric protein was performed to determine equal DNA binding activity (Fig. 7b). Together, these results reveal that the three distinct transcriptional repressor domains are not only structurally conserved but are also functionally conserved within the TIEG subfamily of Sp1-like proteins.

DISCUSSION

Continuing with our studies on the functional characterization of novel members of the Sp1-like family of proteins, in this report we demonstrate that the presence of three conserved transcriptional repression domains is a defining biochemical property of TIEG proteins. Sp1-like proteins are characterized by the presence of three highly related C_2H_2 zinc finger DNA-binding motifs (1). Currently, at least 14 different members of the Sp1-like family of transcription factors have been shown to regulate promoters containing GC-rich sequences (1). Interestingly, these sequences are present in the promoters of more than 1000 different gene products (2, 22, 37, 38). Therefore, a detailed analysis of the transcriptional regulatory properties of Sp1-like proteins will be critical for beginning to understand the complexity of gene expression in mammalian cells.

Several laboratories are attempting to define distinct subgroups within the growing family of Sp1-like proteins based upon the presence of conserved regions located outside of the zinc finger motif. We and others have recently reported that this classification can help to predict biochemical and functional properties of novel proteins that are being added to this family of transcription factors (reviewed in Ref. 1). In addition, these studies are providing useful information for understanding whether different members of the Sp1-like family of proteins display distinct or redundant functions. The Sp1-like subgroups that have been identified thus far include the SP (Sp1, Sp2, Sp3, and Sp4), BTEB (BTEB1 and BTEB2), KLF (KLF, GKL, and LKLF), CPBP (CPBP, Zf9, and UKLF), and TIEG (TIEG1 and TIEG2) proteins. The SP proteins contain glutamine-rich N-terminal transcriptional activation domains, the BTEB proteins activate transcription through a hydrophobic-rich domain, and the CPBP proteins encode an acidic activation domain (14, 18, 26, 28, 39). The KLF proteins are defined by the presence of two different types of conserved sequences that act as a nuclear localization signal and a puta-
Transcriptional Repression by TIEG Proteins

Three novel transcriptional repressor domains are a defining feature of the TIEG subfamily of Sp1-like proteins. The presence of these domains suggest that, in contrast to other Sp1-like proteins, the TIEG subfamily has evolved to silence gene expression in mammalian cells. These results, together with previous studies from other laboratories, are being used to generate a structural and functional classification of the growing family of Sp1-like zinc finger proteins. This classification will be useful for predicting common as well as distinct biochemical and functional properties of members of this family.

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