Journal of Cellular Biochemistry 101:712–722 (2007)

Functional Domains of the TGF-β-Inducible Transcription Factor Tieg3 and Detection of Two Putative Nuclear Localization Signals Within the Zinc Finger DNA-Binding Domain

Björn Spittau,* Ziyuan Wang, Dagmara Boinska, and Kerstin Krieglstein

Center of Anatomy, Department of Neuroanatomy, University of Göttingen, Germany

Abstract The recently identified TGF-β-inducible early gene 3 (*Tieg3*) belongs to the gene family of Sp1/Klf-like transcription factors and is upregulated immediately after TGF-β treatment. To explore the molecular mechanisms of Tieg3-mediated transcriptional control, GAL4-based luciferase assays were performed in order to determine regulatory domains within the Tieg3 protein. Using EGFP-fusion proteins, we monitored the intracellular localization and mapped putative nuclear localization signals (NLS). We provide evidence that the amino-terminus of Tieg3 is essential to repress the transcription and that the loss of the mSin3A interacting domain (SID) disrupts the repressive effects of Tieg3 in the oligodendroglial cell line OLI-neu. Herein we also demonstrate that the zinc finger containing DNA-binding domain (DBD) alone is able to activate the transcription of a reporter gene. Sequence analysis of the zinc finger region revealed no similarities to known activation domains. Analysis of the subcellular localization disclosed Tieg3 as a nuclear protein. Further, we identified the DBD as being essential for the nuclear localization of Tieg3. We detected two closely located putative bipartite NLS within the second and third zinc finger, which are conserved among the members of the Tieg family of proteins. Together these results may help to increase the understanding of Tieg3-mediated transcriptional control and to characterize this TGF-β-induced Sp1/Klf-like transcription factor. J. Cell. Biochem. 101: 712–722, 2007.
© 2007 Wiley-Liss, Inc.

Key words: Tieg3, TGF-β; GAL4-based transcriptional assay; EGFP fusion proteins; nuclear localization; Sp1/Klf-like transcription factor

The recently identified TGF-β-inducible early gene 3 (*Tieg3*) belongs to the gene family of Sp1/Klf-like transcription factors that play crucial roles in a variety of aspects of mammalian development [Suske, 1999]. Individual members of this family are separated in three subgroups based upon structural and functional features [Kaczynski et al., 2003]. Up to now, more than twenty-five members in mammalians have been identified and the number is still growing. The Sp1/Klf family is defined by a highly conserved DNA-binding domain (DBD) located at or very close to the carboxyl terminus. This DBD consists of three Cys_2His_2-type zinc finger motifs that are similar to the motifs found in the transcription factor Sp1 and in the Drosophila protein Krüppel. The zinc fingers mediate binding to GC-rich promoter sequences, often referred to as Sp1-sites. An independent subgroup of Sp1/Klf proteins is the family of Tieg proteins, originally identified as TGF-β early response genes. TIEG1/KLF10 [Subramaniam et al., 1995] and TIEG2/KLF11 [Cook et al., 1998] represent the human isoforms, whereas Tieg1/Klf10 [Yajima et al., 1997; Fautsch et al., 1998] and the recently cloned transcription factor Tieg3 [Wang et al., 2004] are the murine isoforms. Initially, Tieg proteins were introduced as transcriptional repressors inhibiting proliferation and inducing apoptosis [Tachibana et al., 1997; Chalaux et al., 1999; Bender et al., 2004], but recent results demonstrate that Tieg

Grant sponsor: Deutsche Forschungsgemeinschaft.

*Correspondence to: Björn Spittau, Center of Anatomy, Department of Neuroanatomy University of Göttingen, Kreuzbergring 36, 37075 Göttingen.
E-mail: bspitta@gwdg.de

Received 30 August 2006; Accepted 8 November 2006
DOI 10.1002/jcb.21228

© 2007 Wiley-Liss, Inc.

proteins can also function as transcriptional activators [Noti et al., 2004; Neve et al., 2005]. To act as a site-specific transcription factor it is important for a protein to posses three essential features: a functional nuclear localization signal (NLS) for nuclear import, a DBD to bind to specific promoter sites and repression and/or activation domains to regulate the transcription of target genes. Recent studies helped to develop a better understanding of the mechanisms of Tieg-mediated transcriptional control. GAL4-based transcriptional assays showed that human TIEG1 and TIEG2 contain three amino terminal repression domains [Cook et al., 1999]. An alpha-helical motif is located within the first repression domain and is critical for the interaction with the co-repressor mSin3A [Zhang et al., 2001]. The E3 ubiquitin ligase SIAH1 binds to TIEG1/KLF10 and targets this Sp1/Klf transcription factor for proteosomal degradation [Johnsen et al., 2002b]. This could explain the rapid turnover of Tieg proteins and may serve to regulate the duration and extent of Tieg response.

In the present study, we have examined the transcriptional regulatory potential of Tieg3 using GAL4-based transcriptional assays. We show that the amino terminus of Tieg3 mediates transcriptional repression, whereas the DBD containing three zinc fingers is able to activate transcription. Moreover, we demonstrate that the carboxy terminal part downstream of the zinc fingers has the strongest activating potential, although this region does not contain a known activation domain. In addition, we demonstrate that, like other members of the Tieg family, Tieg3 is a nuclear protein. Using EGFP-Tieg3 fusions, we provide evidence that the DBD of Tieg3 is essential for its nuclear localization. Further deletions suggest that this subcellular localization is mediated by at least one putative bipartite NLS within the zinc finger motifs of this Sp1/Klf transcription factor. The results of this work may help to extend the characterization of the Sp1/Klf transcription factor Tieg3.

MATERIALS AND METHODS

Antibodies and Reagents

For the detection of FLAG-tagged Tieg3 proteins, a polyclonal rabbit anti-FLAG-antibody (Sigma) was used as primary antibody. Immunofluorescence was determined after incubating the cells with a FITC-conjugated mouse anti-rabbit secondary antibody.

Cell Lines and Transfection

Cells of the oligodendroglial precursor cell line OLI-neu were cultured in Dulbecco's Modified Eagle Medium (Invitrogen) supplemented with 10% fetal bovine serum, N1 supplement (Sigma) and Insulin (Sigma). HeLa cells were cultured in Dulbecco's Modified Eagle Medium (Invitrogen) with 10% fetal bovine serum. For GAL4-based transcriptional assays 50,000 cells/well of a 48-well plate (Nunc) were transfected with the Effectene Transfection Reagent Kit (Qiagen) according to the manufacturer's protocol. For the detection of the intracellular localization of the EGFP-Tieg3 (fragment) fusion proteins 30,000 cells/well of a 24-well plate (Nunc) were transfected by the LipofectAMINE procedure (Invitrogen) according to the manufacturer's instructions.

Plasmid Constructions

GAL4-Tieg3 chimeric constructs were made by fusing Tieg3 fragments with the carboxyl terminus of the GAL4 DBD (amino acids 1–147). For this purpose, fragments of Tieg3 were generated by PCR from a Tieg3 cDNA template, as described previously [Wang et al., 2004]. The PCR products were digested with EcoRI and BamHI or BamHI alone and the digested products were separated on a 1% agarose gel and purified using the Gene Clean II Kit (BioGene). After gel extraction, fragments were ligated into the corresponding sites of the cytomegalovirus-driven mammalian expression vector pM1 (BD Clontech), which contains the GAL4 DBD. The plasmids are pM-Tieg3 (AA 1–502), pM-Tieg3ΔDBD (AA 1–378), pM-Tieg3ΔSID (AA 123–502), pM-Tieg3ΔRD (AA 379–502), pM-Tieg3ZF2/3 (AA 408–502), pM-Tieg3ZF3 (AA 444–502), and pM-Tieg3cterm (AA 467–502). The pFLAG-Tieg3 expression plasmid was generated by inserting the HindIII/BamHI digested full length Tieg3 cDNA into the pFLAG-CMV2 expression vector (Sigma).

Plasmids expressing EGFP-Tieg3 fusion proteins were constructed by inserting the EcoRI/BamHI digested DNA fragments encoding Tieg3 and deletions into the pEGFP-C2 expression vector (Clontech). The DNA fragments were the same as described for the construction of the GAL4 constructs. The plasmids are

pEGFP-Tieg3 (AA 1–502), pEGFP-Tieg3ΔDBD (AA 1–378), pEGFP-Tieg3ΔRD (AA 379–502), pEGFP-Tieg3ZF2/3 (AA 402–502), and pEGFP-Tieg3ZF3 (AA 444–502). All plasmid constructs were verified by DNA sequencing.

GAL4-Based Transcriptional Assays

For GAL4 transcriptional assays, the reporter plasmid was pFR-Luc (Promega), which contains five GAL4-binding sites upstream of the luciferase gene. The expression plasmid pCMV-βGal coding for β-galactosidase was used to normalize the transfection efficiency. Five hundred nanograms of the pM1-derived expression plasmids were cotransfected with 100 ng of the reporter plasmid pFR-Luc and 100 ng of pCMV-βGal. After 4 h, the DNA complexes were removed and the cells were incubated for additional 20 h. Finally, the cells were harvested with Passive Lysis Buffer (Promega) and the luciferase activity was determined with an EG&Berthold Lumat 9507 Luminometer.

Direct and Indirect Fluorescence Microscopy

After transfection with the pFLAG-Tieg3 expression plasmid, OLI-neu cells were washed three times with phosphate buffered saline (PBS) and fixed with methanol for 10 min at room temperature. The FLAG-tagged Tieg3 was detected using a polyclonal anti-FLAG-antibody (Sigma) according to the manufacturer's instructions. The nuclei were counterstained with DAPI (1:1,000) for 5 min at room temperature and coverslips were mounted with VectaSHIELD® Mounting Medium.

For the determination of the intracellular localization of the EGFP-Tieg3 fusion proteins OLI-neu cells and HeLa cells were seeded on collagen coated glass cover slips. After transfection with pEGFP-C2-derived expression plasmids (as described above) and an incubation time of 24 h, the cells were washed with PBS. Glass cover slips were mounted with VectaSHIELD® Mounting Medium containing DAPI (1:1,000) to counterstain the nuclei. Autofluorescence was visualized immediately by immunofluorescence microscopy (Zeiss).

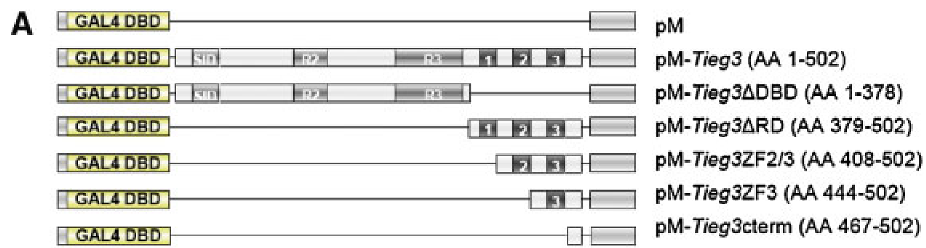
RESULTS

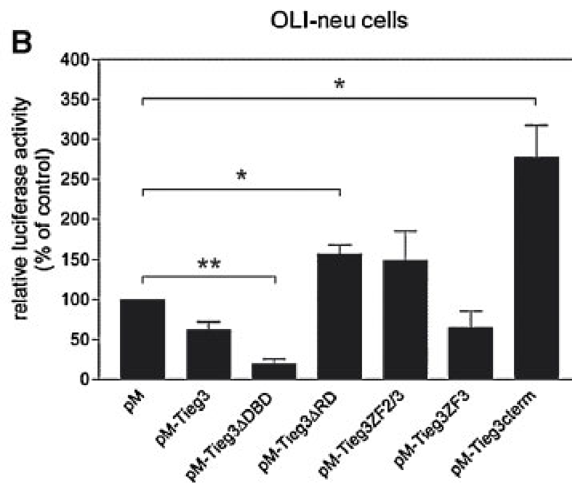
The Transcriptional Regulatory Domains of Tieg3

To determine the transcriptional repression and/or activation domains of Tieg3, the yeast transcription factor GAL4 fusion system was used. GAL4 fusion proteins have little background interference in mammalian cells because of their yeast origin. In addition, the GAL4 DBD directs fusion proteins to the nucleus, alleviating the concern that deletion mutants might disrupt the natural NLS. Sequence analysis of Tieg3 revealed a DBD with three C_2H_2 zinc fingers at the carboxy-terminal part of the protein and three amino-terminal proline-rich repression domains, which are considered as hallmarks of the Tieg family [Cook et al., 1999]. A series of Tieg3 deletions fused to the GAL4 DBD (AA 1–147) were created as shown in Figure 1A. These fusion plasmids were cotransfected into OLI-neu cells with a reporter construct containing five GAL4-binding sites upstream of the firefly luciferase gene.

The GAL4-Tieg3 (pM-Tieg3) fusion had no significant effect on the reporter gene, but showed a repression of transcription (Fig. 1B). Transfection with pM-Tieg3ΔDBD, a construct coding for Tieg3 lacking the DBD, revealed a significant repression of the firefly luciferase gene compared to the GAL4 DBD alone (Fig. 1B), as described previously [Wang et al., 2004]. This result indicates that Tieg3 AA 1-378 contain a repression domain that is able to repress transcription. To answer the question if this repression is mediated by the putative mSin3A interacting domain (SID) of the Tieg protein family, a deletion mutant without the SID was generated (Fig. 2A). As shown in Figure 2B, the repressive effects observed with pM-Tieg3 wild-type and pM-Tieg3ΔDBD abolished when cells were transfected with pM-Tieg3ΔSID. This result indicates that the SID is essential for Tieg3-mediated repression in OLI-neu cells.

Although the amino terminal part of the Tieg proteins is well characterized, the transcriptional regulatory potential of the DBD, containing the three zinc fingers, remains to be elucidated. There are results leading to the conclusion that the DBD of the Sp1/Klf proteins is important for the transcriptional regulation of target genes [Song et al., 2002b]. Transfection of OLI-neu cells with pM-Tieg3ΔRD, coding for AA 379-502, resulted in a significant activation of transcription (Fig. 1B), suggesting that the DBD contains a putative activation domain. Note that the presence of the SID seems to inhibit the putative activation domain within the carboxy-terminal part since the construct





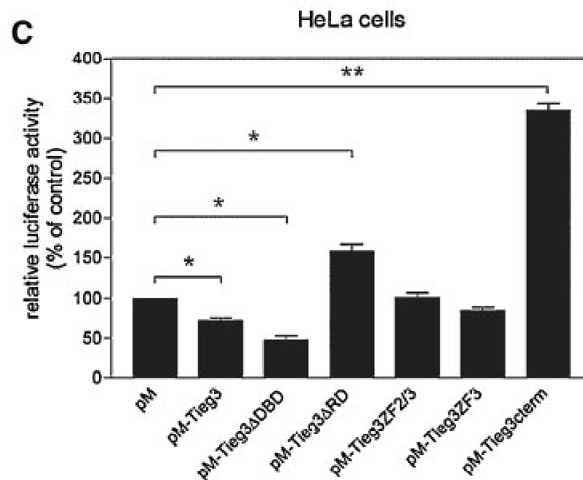


Fig. 1. The transcriptional regulatory potential of specific portions of the Tieg3 protein. **A**: Schematic representation of the full-length GAL4DBD-Tieg3 and a series of Tieg3 deletion constructs. The locations of the three putative repression domains and of the three zinc fingers (DBD) are indicated. **B**: Results of GAL4-based transcriptional assays in OLI-neu cells. Cells were transiently transfected with 500 ng of the GAL4 fusion constructs, 100 ng pFR-Luc and 100 ng pCMV-βGal. Twenty four hours post-transfection, cells were harvested and subjected to luciferase activity measurement. **C**: To alleviate the concern that the regulatory effects of Tieg3 are cell line-specific, HeLa cells were transfected under the same conditions. Note that the results are comparable with the effects observed in OLI-neu cells. To correct differences in transfection efficiency, all values were normalized to β-galactosidase values. Data represent the means of three independent experiments ± SEM. *P* values derived from Student's *t*-test are $*P < 0.05$ and $**P < 0.01$ compared to the control (pM).

pM-Tieg3 displayed a repressive effect (Fig. 1B). To further characterize the activation domain, a series of carboxy-terminal deletions of Tieg3 fused to GAL4 DBD was created (Fig. 1A). Among them, pM-Tieg3-cterm (AA 467–502) has the strongest activating effect. The construct pM-Tieg3ZF2/3 (AA 408–502) showed a transcriptional activation, which is not

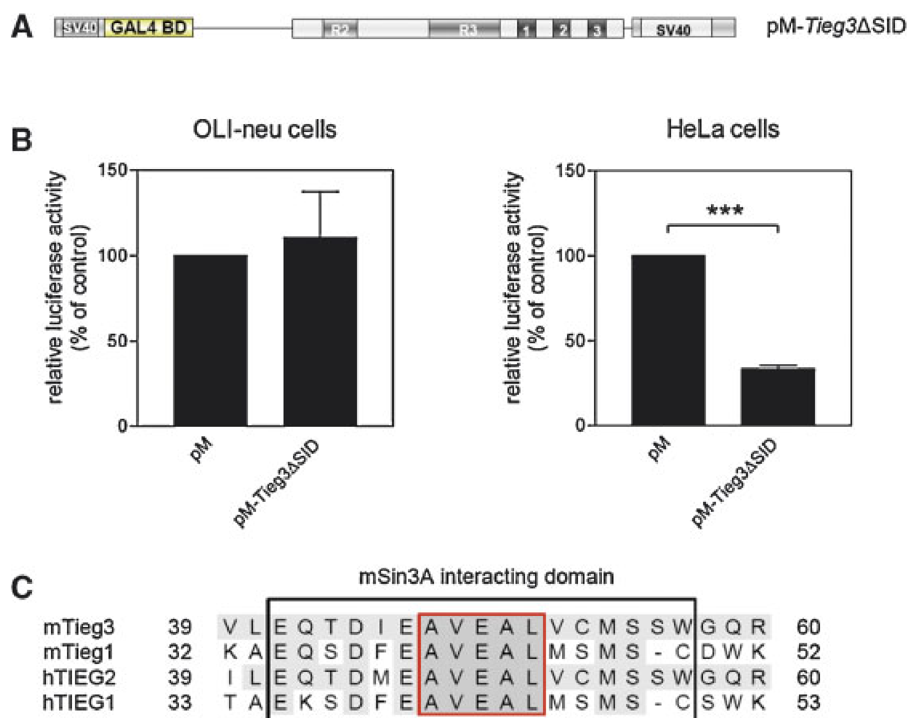


Fig. 2. The role of the mSin3A interacting domain (SID) in Tieg3-mediated repression. **A**: Schematic representation of the construct pM-Tieg3ΔSID, lacking the amino terminal SID. **B**: Transfection of OLI-neu cells with the plasmid pM-Tieg3ΔSID resulted in a loss of repression. This indicates that the Tieg3-mediated repression in OLI-neu cells is mSin3A-dependent. Lack of the SID did not abrogate the repressive effects in HeLa cells. Data are given as means of three independent experiments. *P* values derived from Student's *t*-test are $^{***}P < 0.001$ compared to the control (pM). **C**: The amino acid residues mediating the interaction with the co-repressor mSin3A are conserved within the murine (mTieg1 and mTieg3) and human (hTIEG1 and hTIEG2) Tieg proteins.

significant, compared with the control. Transfection with the construct pM-Tieg3ZF3 did not result in a significant repression or activation of transcription (Fig. 1B).

To ensure that these results are not cell line specific we performed the same experiments in HeLa cells. The influence of Tieg3 wild-type was similar to the effects demonstrated in OLI-neu cells. Again, Tieg3 served as a transcriptional repressor. A significant transcriptional repression was observed by transfecting the cells with pM-Tieg3ΔDBD (Fig. 1C). The Tieg3 DBD alone (pM-Tieg3ΔRD) was able to activate the transcription of the firefly luciferase gene (Fig. 1C). The effects of the Tieg3 DBD deletion mutants were comparable with those observed in OLI-neu cells. The strongest activation of transcription was obtained after transfection with the construct pM-Tieg3-cterm resulting in a three-fold increase of transcription. Interestingly, transfection of HeLa cells with the SID lacking pM-Tieg3ΔSID construct still resulted in a significant repression, indicating that the mechanism of transcriptional repression in HeLa cells is not mSin3A-dependent (Fig. 2B).

The TGF-β Inducible Transcription Factor Tieg3 Is Localized to the Nucleus

Previous studies have shown that members of the Tieg family of transcription factors are nuclear proteins [Tachibana et al., 1997; Cook et al., 1998]. To identify the subcellular localization of Tieg3, OLI-neu cells were transiently transfected with the Tieg3 expression plasmid pFLAG-Tieg3, coding for the full-length Tieg3. Twenty-four hours post-transfection FLAG-tagged Tieg3 was visualized by immunocytochemistry staining followed by immunofluorescence microscopy. The nuclei were counterstained with DAPI to discriminate between nucleus and cytoplasm. Cells transfected with the empty control vector showed

almost no signal (data not shown). In contrast, cells transfected with the pFLAG-Tieg3 expression plasmid displayed a nuclear accumulation of Tieg3 (Fig. 3A). For proteins larger than 45 kDa it is not possible to pass the nuclear membrane by passive diffusion [Miller et al., 1991]. Since Tieg3 (54 kDa) is too large to diffuse into the nucleus it must be expected that Tieg3 contains at least one functional NLS or is transported via interaction with a protein containing a NLS.

The Zinc Finger DNA-Binding Domain of Tieg3 Is Essential for Nuclear Localization

To map regions of Tieg3 required for its nuclear localization, full-length Tieg3 and several deletions were fused in-frame to EGFP. The fusion genes were expressed in OLI-neu and HeLa cells and cellular localization was monitored by autofluorescence. DAPI staining was utilized to define the nucleus, and a two-color merge was used to assess subcellular localization. Figure 3B gives an overview of the EGFP fusion constructs. Fluorescence of EGFP alone is shown in Figure 3C (panel A) and is present throughout the cell, as described previously [Stauber et al., 1995]. In contrast, the EGFP-full-length Tieg3 fusion protein accumulated in the nucleus (Fig. 3C, panel B). Interestingly, sequence analysis revealed that Tieg3 does not contain a typical core or monopartite NLS. In the founding paper of Tieg3, two putative NLS were supposed to be important for nuclear accumulation [Wang et al., 2004]. One NLS directly upstream of the amino-terminal SID and another NLS within the second zinc finger at the carboxyl-terminus. To prove the role of these putative NLSs in Tieg3 nuclear localization, cells were transiently transfected either with the construct pEGFP-Tieg3ΔDBD (AA 1–378) or with pEGFP-Tieg3ΔRD (AA 379–502). An exclusively cytoplasmic localization was observed after transfection with pEGFP-Tieg3ΔDBD (Fig. 3C, panel C). In contrast, the DBD of Tieg3 was sufficient to translocate EGFP to the nucleus (Fig. 3C, panel D). This demonstrates that the putative amino-terminal NLS of Tieg3 does not play a role in nuclear

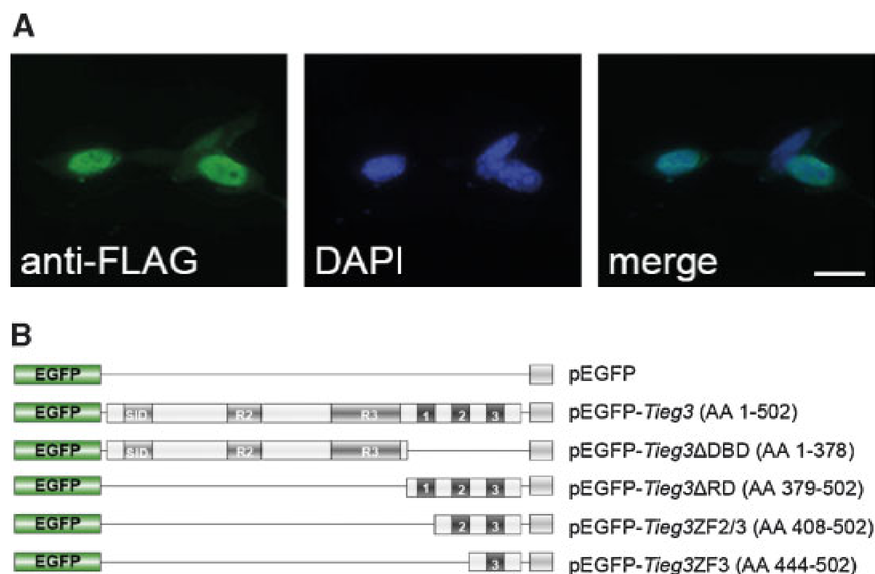


Fig. 3. The Sp1/Klf transcription factor Tieg3 is a nuclear protein. **A**: OLI-neu cells were transfected with the expression plasmid pFLAG-Tieg3 coding for the full-length Tieg3. The FLAG-tagged Tieg3 fusion proteins were stained with an anti-FLAG antibody as primary antibody and a FITC-conjugated secondary antibody. To discriminate between nucleus and cytoplasm the nuclei were counterstained with DAPI. Overlay of anti-FLAG staining and DAPI displays a nuclear localization of Tieg3. The scale bar indicates 20 μm. **B**: Schematic representation of the EGFP fusion constructs. Tieg3 wild-type and a series of deletions were fused to the carboxyl-terminus of EGFP. **C**: Localization of EGFP fusion proteins in OLI-neu cells. Whereas wild-type EGFP is located in the cytoplasm and in the nucleus (**panel A**), the EGFP-Tieg3 fusion protein translocated EGFP to the nucleus (**panel B**). With the exception of the DNA-binding domain lacking EGFP-Tieg3ΔDBD fusion protein, all Tieg3 deletions showed a nuclear localization. **D**: Localization of the EGFP fusion proteins in HeLa cells. The intracellular distributions of Tieg3 and the deletions are the same as seen in the oligodendroglial cell line OLI-neu, indicating that the mechanism of nuclear import of Tieg3 is not cell line specific. The scale bars indicate 20 μm.

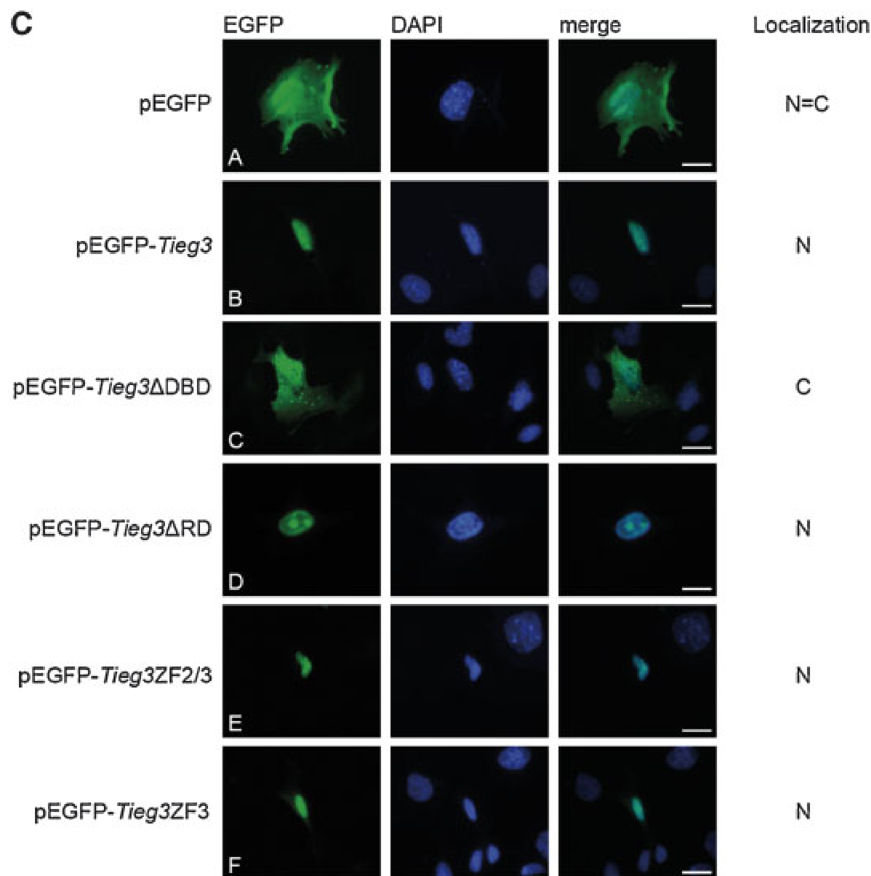


Fig. 3. (*Continued*)

localization and that the DBD is essential for Tieg3 nuclear accumulation. To further delineate the NLS within the DBD, deletion mutants towards the carboxyl-terminus were generated. Deletion of the first zinc finger (pEGFP-Tieg3ZF2/3) still revealed a nuclear localization of the EGFP fusion protein (Fig. 3C, panel E). Interestingly, transfection with the construct pEGFP-Tieg3ZF3, coding for the third zinc finger and the carboxyl-terminal end of Tieg3, was also able to translocate EGFP to the nucleus. The results after transfection of HeLa cells with the EGFP-Tieg3 fusion constructs are depicted in Figure 3D. The intracellular localization of the fusion proteins was the same as in OLI-neu cells, suggesting a general mechanism for nuclear transport of Tieg3 in both cell lines. Taken together these results indicate that the DBD is essential for the nuclear localization of Tieg3 and that the intact structure of the DBD is not important for nuclear accumulation of this Sp1/Klf protein. The second and the third zinc finger contain amino acid residues that weakly resemble bipartite NLSs. These sequences contain two clusters of the basic amino acids lysine and arginin, where two basic amino acids are followed by an eight amino acid spacer and another three basic amino acids out of five (Fig. 4B). Comparison of these residues with the classical bipartite NLS of Nucleoplasmin (Fig. 4A) shows that the putative Tieg3 NLSs are domains for nuclear import with different requirements in the number and distribution of the basic amino acid residues.

DISCUSSION

The aim of the present work was (1) to characterize the residues of Tieg3 responsible for its role in transcriptional regulation and (2) to display the intracellular localization and the part of the Tieg3 protein being essential for this localization. TIEG2/KLF11, a human Sp1/Klf protein with high similarity to Tieg3, is the best

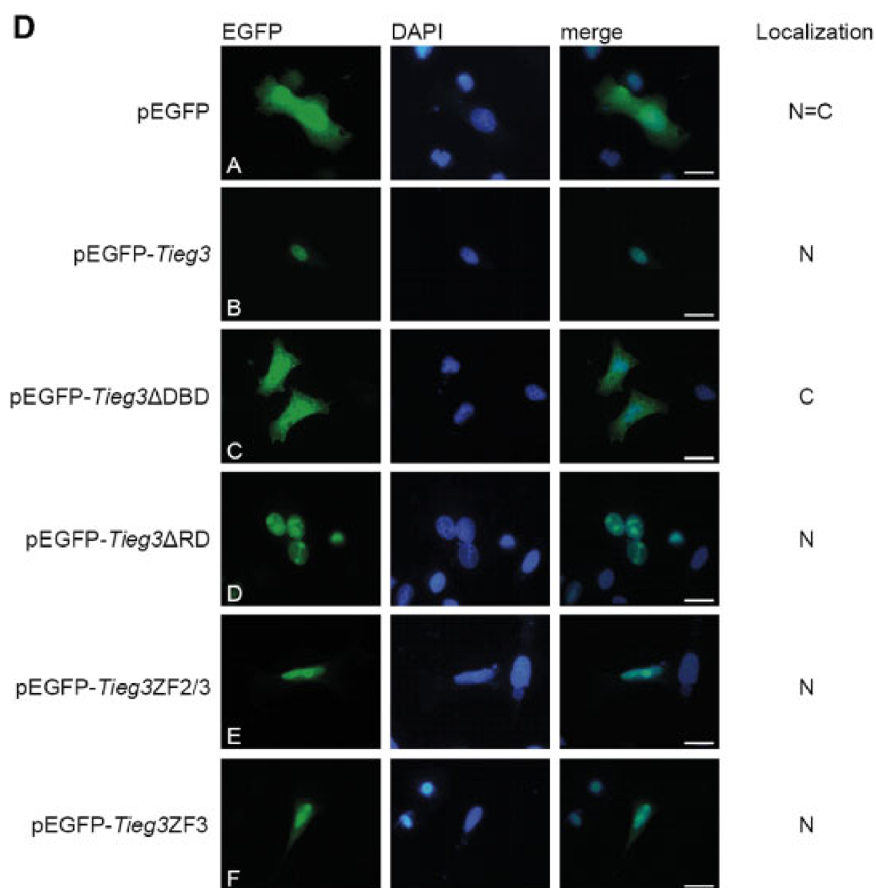


Fig. 3. (*Continued*)

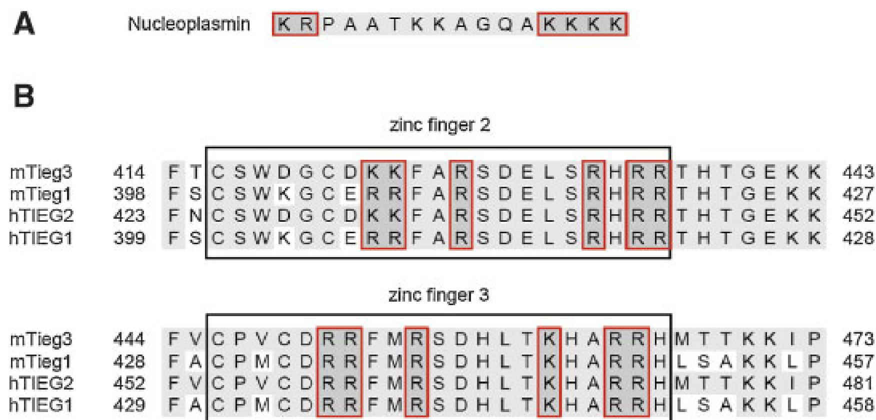


Fig. 4. The basic amino acid residues of the two putative bipartite NLSs are conserved within the Tieg family. **A**: The classical bipartite NLS of Nucleoplasmin. The dark shaded boxes mark the basic residues. **B**: Amino acid residues of the two putative bipartite NLSs of Tieg3 using the single-letter code. The corresponding amino acid residues of the murine Tieg1 and the human isoforms TIEG1 and TIEG2 are shown. The big boxes highlight the second and third zinc finger of Tieg3 and the murine and human members of the Tieg family. The dark shaded boxes mark the basic amino acids within the second and third zinc finger, which are conserved within all Tieg proteins. Note that the two putative NLSs weakly resemble classical bipartite NLSs.

characterized member of the Tieg family and contains three amino-terminal repression domains which are the defining feature of this protein family [Cook et al., 1999]. It has been shown that TIEG2/KLF11 represses the transcription through the recruitment of the mSin3A-histone deacetylase complex. The interaction of TIEG2/KLF11 with mSin3A is mediated by an alpha-helical motif located within the first repression domain of TIEG2/KLF11 [Zhang et al., 2001]. Sequence analysis revealed that this SID is highly conserved within the Tieg family (Fig. 2C), suggesting that the recruitment of histone deacetylase complexes via binding to mSin3A is a general mechanism of transcriptional repression by Tieg proteins. In this study we demonstrated that the amino-terminal end of Tieg3 containing the three repression domains is essential for the inhibition of transcription in OLI-neu cells, as well as in HeLa cells. Lack of the SID results in the loss of transcriptional repression in OLI-neu cells, suggesting that binding to mSin3A is the dominant mechanism for Tieg3-mediated repression in this cell line. Interestingly, lack of the SID did not abrogate the repressive effects in HeLa cells. This result allows the conclusion that the Tieg3-mediated transcriptional repression in HeLa cells is not mSin3A-dependent and that the repression domains R2 and R3 may be responsible for the observed repressive effects. Cook et al. [1999] have shown for TIEG2/KLF11 that each repression domain alone is able to silence gene transcription. No other transcription regulating binding partners for TIEG2/KLF11 have been found so far and both domains share no homology with known regulatory domains. Hence, the mechanism of R2- and R3-mediated repression remains elusive.

Although Tieg proteins were originally identified as transcriptional repressors, recent studies have shown that they are also able to activate transcription. For example, TIEG1 has been shown to enhance TGF-β signaling by transcriptional activation of SMAD2 [Johnsen et al., 2002a]. In myeloid cells, TIEG1 binds to the CD11d promoter and acts as an inducer of gene transcription [Noti et al., 2004]. TIEG2/KLF11 plays a critical role in the function of endocrine pancreas by binding to the insulin promoter and activating the transcription of the insulin gene [Neve et al., 2005]. However, a transcriptional activation domain within the Tieg proteins has not been identified so far. Here we report that the carboxyl-terminal end of Tieg3, containing the DBD with three zinc fingers is able to activate the transcription in OLI-neu and HeLa cells. We further demonstrate that the carboxyl terminus downstream of the zinc finger motifs shows the strongest transcriptional activation. In the context of the full-length protein, the N-terminal repression domains and the C-terminal activator domain have a counteracting activity. This effect could be mediated by protein folding resulting in intramolecular interactions of these two domains. Truncation of the full-length protein and expression of the isolated domains (pM-Tieg3ΔDBD and pM-Tieg3ΔRD) might rescue both domains unmasking their regulatory potentials. Phosphorylation or other post-translational modifications of Tieg3 could also contribute to the function of the repression and activation domains. Thus, Tieg3 might act as a context-dependent transcriptional repressor or activator.

Several activation domains have been identified on the basis of their amino acid compositions, including glutamine-, proline-, serine/threonine-, and acidic residue-rich domains [Mitchell and Tjian, 1989; Triezenberg, 1995]. Other domains, however, do not fall into these categories, suggesting that a diverse array of domains can activate transcription. Other studies have shown that many "atypical" activation domains interact with specific co-activators to regulate the basal transcription machinery or chromatin structure to increase transcription initiation. Interestingly, the Sp1/Klf transcription factor BTEB3/FKLF2 binds to mSin3A to act as a repressor, but is also able to activate transcription. It has been shown that BTEB3/FKLF2 binds to the co-activators PCAF (p300/CREB binding protein associated factor) and p300/CBP, proteins with intrinsic acetylase activity. These interactions are mediated via the zinc finger region of BTEB3/FKLF2 and are essential for BTEB3/FKLF2-mediated transcriptional activation. Moreover, it has been shown that both, PCAF and p300/CBP acetylate BTEB3/FKLF2 within the zinc finger domain [Song et al., 2002b]. The modification of transcription factors by acetylation has been shown to regulate the activation function at multiple levels, including DNA binding, interaction with other proteins and stability [Kouzarides, 2000]. It is important to identify the interacting proteins of Tieg3 to further increase the

understanding of Tieg-mediated transcriptional activation in mammalian cells.

In this study we further demonstrate that wild-type Tieg3 is exclusively localized to the nucleus. To detect the essential region for this localization within the Tieg3 protein, we initially fused either the wild-type gene or Tieg3 gene deletions to the heterologous EGFP gene and determined the subcellular localization of the respective fusion proteins in OLI-neu and HeLa cells. As expected, the full-length EGFP-Tieg3 fusion protein was localized efficiently to the nucleus. Transfection with the deletion constructs revealed that the Tieg3 DBD containing the three zinc fingers is important for nuclear accumulation. We further demonstrated that the third zinc finger alone is able to direct EGFP to the nucleus. Analysis of the amino acid sequence of the DBD revealed the presence of two putative bipartite NLS that map to amino acids 423–436 and 451–465. However, the spacing of the basic residues in the putative Tieg3 bipartite NLSs differs from that seen in the classical bipartite NLS of Nucleoplasmin where the clusters of basic amino acids are separated by 10 amino acids. It has been hypothesized that the two clusters of basic amino acids in a bipartite NLS are brought to a juxtaposed position due to protein folding and are subsequently recognized by the nuclear import machinery [Boulikas, 1993]. Although the DBD with its three zinc fingers is highly conserved within the Sp1/Klf family the occurrence of NLSs varies. For nuclear accumulation of EKLF/KLF1 the intact structure of the three zinc fingers is essential [Pandya and Towes, 2002; Quadrini and Bieker, 2002], KLF13/RFLAT-1 contains two functional and independent basic NLS; one immediately upstream of the zinc finger DBD and the other located within the zinc fingers [Song et al., 2002a]. The common feature of many Sp1/Klf family members is that the NLSs are located in close proximity to or within the DBD of these transcription factors. For other members of the Tieg subfamily a nuclear localization has been reported [Cook et al., 1998], but no functional NLS has been described so far. In this study we provide evidence that Tieg3 contains two putative bipartite NLSs that are located within the DBD. Sequence analysis revealed that the basic residues of these putative NLSs are conserved within the zinc fingers of all other members of the Tieg family (Fig. 4B), suggesting that these basic amino residues are involved in nuclear transport of all Tieg proteins. However, all these residues do not resemble classical NLSs. Since many nuclear proteins lack a classical NLS [Boulikas, 1994], it has to be discussed whether Tieg3 binds to Importin-β or other proteins to be directly transported to the nucleus. The nuclear localization of Tieg3 and all the other members of the Tieg family are consistent with the role of Tieg proteins in transcriptional control and regulation of cell death and proliferation. Therefore, a precise characterization of the two putative NLSs and the detection of binding partners are in progress to elucidate the mechanisms of the nuclear localization of the TGF-β-inducible transcription factor Tieg3.

ACKNOWLEDGMENTS

The authors thank Detlef Doenecke for critically reading the manuscript.

REFERENCES

Bender H, Wang Z, Schuster N, Krieglstein K. 2004. TIEG1 facilitates transforming growth factor-β-mediated apoptosis in the oligodendroglial cell line OLI-neu. J Neurosci Res 75:344–352.

Boulikas T. 1993. Nuclear localization signals (NLS). Crit Rev Eukaryot Gene Expr 3:193–227.

Boulikas T. 1994. Putative nuclear localization signals (NLS) in protein transcription factors. J Cell Biochem 55:32–58.

Chalaux E, Lopez-Rovira T, Rosa JL, Pons G, Boxer LM, Bartrons R, Ventura F. 1999. A zinc-finger transcription factor induced by TGF-β promotes apoptotic cell death in epithelial Mv1Lu cells. FEBS Lett 457:478–482.

Cook T, Gebelein B, Mesa K, Mladek A, Urrutia R. 1998. 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. J Biol Chem 273:25929–25936.

Cook T, Gebelein B, Belal M, Mesa K, Urrutia R. 1999. Three conserved transcriptional repressor domains are a defining feature of the TIEG subfamily of Sp1-like zinc finger proteins. J Biol Chem 274:29500–29504.

Fautsch MP, Vrabel A, Rickard D, Subramaniam M, Spelsberg TC, Wieben ED. 1998. Characterization of the mouse TGFβ-inducible early gene (TIEG): Conservation of exon and transcriptional regulatory sequences with evidence of additional transcripts. Mammalian Genome 9:838–842.

Johnsen SA, Subramaniam M, Katagiri T, Janknecht R, Spelsberg TC. 2002a. Transcriptional regulation of Smad2 is required for enhancement of TGFβ/Smad signaling by TGFβ inducible early gene. J Cell Biochem 87:233–241.

Johnsen SA, Subramaniam M, Monroe DG, Janknecht R, Spelsberg TC. 2002b. Modulation of transforming growth factor β (TGFβ)/Smad transcriptional responses through targeted degradation of TGFβ-inducible early gene-1 by human seven in absentia homologue. J Biol Chem 277:30754–30759.

Kaczynski J, Cook T, Urrutia R. 2003. Sp1- and Krüppel-like transcription factors. Genome Biol 4:206.

Kouzarides T. 2000. Acetylation: A regulatory modification to rival phosphorylation? EMBO J 19:1176–1179.

Miller M, Park MK, Hanover JA. 1991. Nuclear pore complex: Structure, function and regulation. Physiol Rev 71:904–949.

Mitchell PJ, Tjian R. 1989. Transcriptional regulation in mammalian cells by sequence-specific DNA binding proteins. Science 245:371–378.

Neve B, Fernandez-Zapico ME, Ashkenazi-Katalan V, Dina C, Hamid YH, Joly E, Vaillant E, Benmezroua Y, Durand E, Bakaher N, Delannoy V, Vaxillaire M, Cook T, Dallinga-Thie GM, Jansen H, Charles MA, Clement K, Galan P, Hercberg S, Helbecque N, Charpentier G, Prentki M, Hansen T, Pedersen O, Urrutia R, Melloul D, Froguel P. 2005. Role of transcription factor KLF11 and its diabetes-associated gene variants in pancreatic beta cell function. PNAS 102:4807–4812.

Noti JD, Johnson AK, Dillon JD. 2004. The zinc finger transcription factor transforming growth factor beta-inducible early gene-1 confers myeloid-specific activation of the leukocyte integrin CD11d promoter. J Biol Chem 279:26948–26958.

Pandya K and Towes TM. 2002. Basic residues within the Kruppel zinc finger DNA binding domains are the critical nuclear localization determinants of EKLF/KLF-1. J Biol Chem 277:16304–16312.

Quadrini KJ, Bieker JJ. 2002. Krüppel-like zinc fingers bind to nuclear import proteins and are required for efficient nuclear localization of erythroid Krüppel-like factor. J Biol Chem 277:32243–32252.

Song A, Patel A, Thamatrakoln K, Liu C, Feng D, Clayberger C, Krensky AM. 2002a. Functional domains and DNA-binding sequences of RFLAT-1/KLF13, a Krüppel-like transcription factor of activated T lymphocytes. J Biol Chem 277:30055–30065.

Song C, Keller K, Murata K, Asano H, Stamatoyannopoulos G. 2002b. Functional interaction between coactivators CBP/p300, PCAF, and transcription factor FKLF2. J Biol Chem 277:7029–7036.

Stauber R, Gaitanaris GA, Pavlakis GN. 1995. Analysis of trafficking of Rev and transdominant Rev proteins in living cells using green fluorescent protein fusions: Transdominant Rev blocks the export of Rev from the nucleus to the cytoplasm. Virology 213:439–449.

Subramaniam M, Harris SA, Oursler MJ, Rasmussen K, Riggs BL, Spelsberg T. 1995. Identification of a novel TGF-β-regulated gene encoding a putative zinc finger protein in human osteoblasts. Nucl Acids Res 23:4907–4912.

Suske G. 1999. The Sp-family of transcription factors. Gene 238:291–300.

Tachibana I, Imoto M, Adjei PN, Gores GJ, Subramaniam M, Spelsberg TC, Urrutia R. 1997. Overexpression of the TGFβ-regulated zinc finger encoding gene, TIEG, induces apoptosis in pancreatic epithelial cells. J Clin Invest 99:2365–2374.

Triezenberg SJ. 1995. Structure and function of transcriptional activation domains. Curr Opin Gen Dev 5:190–196.

Wang Z, Peters B, Klussmann S, Bender H, Herb A, Krieglstein K. 2004. Gene structure and evolution of Tieg3, a new member of the Tieg family of proteins. Gene 325:25–34.

Yajima S, Lammers C, Lee S, Hara Y, Mizuno K, Mouradian MM. 1997. Cloning and characterization of murine glial cell-derived neurotrophic factor inducible transcription factor (MGIF). J Neurosci 17:8657–8666.

Zhang J, Moncrieffe MC, Kaczynski J, Ellenrieder V, Pendergast FG, Urrutia R. 2001. A conserved α-hellical motif mediates the interaction of Sp1-Like transcriptional repressors with the corepressor mSin3A. Mol Cell Biol 21:5041–5049.