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TGIF2 Interacts with Histone Deacetylase 1 and Represses Transcription*

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TG-interacting factor (TGIF) is a transcriptional repressor, which represses transcription by binding directly to DNA or interacts with transforming growth factor β (TGFβ)-activated Smads, thereby repressing TGFβ-responsive gene expression. Mutation of *TGIF* in humans causes holoprosencephaly, a severe genetic disorder affecting craniofacial development. Searching human expressed sequence tag data bases revealed the presence of clones encoding a TGIF-related protein (TGIF2), which contains two regions of high sequence identity with TGIF. Here we show that, like TGIF, TGIF2 recruits histone deacetylase, but in contrast to TGIF, is unable to interact with the corepressor CtBP. TGIF2 and TGIF have very similar DNA-binding homeodomains, and TGIF2 represses transcription when bound to DNA via a TGIF binding site. TGIF2 interacts with TGFβ-activated Smads and represses TGFβ-responsive transcription. TGIF2 appears to be a context-independent transcriptional repressor, which can perform similar functions to TGIF and may play a role in processes, which, when disrupted by mutations in TGIF, cause holoprosencephaly.

Homeodomain proteins play important roles in the regulation of many developmental processes in diverse species (1–3). TG-interacting factor (TGIF)[1] is a member of the TALE superfamily of homeodomain proteins, which have a three-amino acid loop extension (TALE) between helices 1 and 2 of the homeodomain (4, 5). In addition to TGIF, members of this family of proteins include Pbx1, Prep1, and the Meis proteins (6–10). TALE homeodomain proteins have been shown to act as transcriptional activators and repressors (10–12).

TGIF was identified by its ability to bind to a retinoid-responsive element from the cellular retinol-binding protein II gene, and the preferred DNA sequence to which TGIF binds was identified *in vitro* (4). Binding of TGIF to the cellular retinol-binding protein II retinoid response element precluded binding of retinoid receptors, resulting in transcriptional repression (4). Recent evidence has demonstrated that Meis2 and TGIF can bind to a similar sequence within the dopamine 1A receptor gene. D1A promoter activity is positively regulated by Meis2d and repressed by TGIF (13). We have demonstrated that TGIF contains multiple repression domains and can repress transcription by both HDAC-dependent and HDAC-independent mechanisms (14). TGIF interacts with HDACs via the carboxyl-terminal half of the protein and contains a PLDLS motif at its amino terminus, which is critical for the recruitment of the corepressor CtBP (carboxyl terminus-binding protein) (14, 15).

In addition to repressing transcription when bound to DNA directly via its homeodomain, TGIF can repress TGFβ-activated transcriptional responses (16). In response to TGFβ signals, Smad2 and Smad3 are directly phosphorylated by the TGFβ receptor and interact with Smad4, and the activated Smad complex then accumulates within the nucleus (17–19). Once in the nucleus, a TGFβ-activated Smad complex binds to DNA, either directly or via interactions with other DNA-binding proteins (20). TGFβ-activated Smad complexes activate target gene expression, in part, via interactions with general coactivators such as p300/CBP (21–23). In addition to interactions with coactivators, a nuclear Smad complex is able to interact with specific transcriptional repressors, which act to limit the extent of TGFβ-activated gene expression (16, 24–27). Smad corepressors such as TGIF and c-Ski or SnoN prevent full activation of TGFβ target genes partly by competing with coactivators for Smad interaction (16, 28). Interaction of TGIF with TGFβ-activated Smads displaces coactivators and represses transcription via the recruitment of a corepressor complex, which is likely to include chromatin modifiers, such as histone deacetylases and general corepressors such as the adenovirus E1A-interacting protein, CtBP (15, 16). Thus, the relative levels of Smad coactivators and corepressors within the cell may set the magnitude of the transcriptional response to TGFβ.

Recent evidence has demonstrated that loss of function mutations in human *TGIF* result in HPE, a severe genetic disease affecting craniofacial development (29, 30). The primary defect in HPE is incomplete cleavage of the ventral forebrain, suggesting that TGIF plays a critical role in early brain development (31, 32). In most cases, the *TGIF* mutations associated with HPE are deletion of a single copy of the gene with no loss of heterozygosity, suggesting a haploinsufficient phenotype (29, 30). The phenotypic penetrance of *TGIF* mutations is ~10%, suggesting that other proteins that can compensate for decreased TGIF function may exist. We were, therefore, interested to identify other proteins that may be able to perform similar functions to TGIF. By searching the human EST data base, we identified ESTs encoding a protein with a high degree of identity to the TGIF homeodomain. The gene encoding this protein, termed *TGIF2* for TG-interacting factor 2, has recently been characterized and its expression pattern is similar to that

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[1] The abbreviations used are: TGIF, thymine guanine-interacting factor; CtBP, carboxyl terminus-binding protein; TGFβ, transforming growth factor β; EGF, epidermal growth factor; HDAC, histone deacetylase; HPE, holoprosencephaly; TALE, 3-amino acid loop extension; EST, expressed sequence tag; HSV, herpes simplex virus; BMP, bone morphogenetic protein; MAP, mitogen-activated protein; GBD, Gal4p DNA binding domain; TK, thymidine kinase.

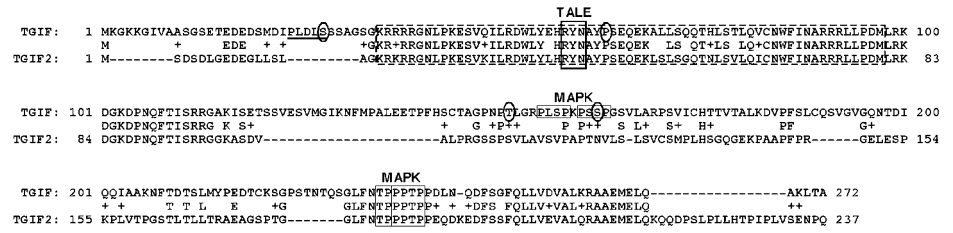

FIG. 1. **Sequence alignment of TGIF and TGIF2.** The predicted amino acid sequences of human TGIF and TGIF2 are *aligned*. Identities and similarities (+) are shown. The homeodomain is indicated by a *dashed outline*, and the 3-amino acid extension between helices 1 and 2 is shown (*TALE*). MAP kinase phosphorylation sites in TGIF and those conserved in TGIF2 are *boxed*, and the CtBP interaction motif in TGIF is *underlined*. Four residues in TGIF that are altered by missense mutations found in HPE patients are *circled* (S28C, P63R, T151A, and S162F).

of *TGIF* (33). Importantly, since TGIF mutations affect brain development, *TGIF2* mRNA is expressed in the human brain. However, no functional characterization of the TGIF2 protein has been described.

Interestingly, the *TGIF2* gene has been shown to be amplified in a proportion of cell lines derived from human ovarian carcinomas (33). This correlates with the finding that, in response to growth stimulatory signals, such as EGF, TGIF is phosphorylated by the Ras/MAP kinase pathway (34). This phosphorylation results in increased protein stability, and, in cells with oncogenic Ras mutations, the level of TGIF protein is significantly higher. Thus, increases in both TGIF and TGIF2 expression levels may be associated with tumorigenesis.

Here we demonstrate that TGIF2 can perform many of the same functions as TGIF. TGIF2 is a context-independent transcriptional repressor, which acts at least in part by recruiting HDACs but, unlike TGIF, cannot interact with CtBP. TGIF2 can repress transcription when bound directly to DNA and when brought to DNA via interactions with TGFβ-activated Smads.

EXPERIMENTAL PROCEDURES

Plasmids—Smad1 and Smad3 were expressed from within pCMV5, and all TGIF and TGIF2 constructs were present within modified pCMV5 vectors containing either a Flag epitope or two HA tags. Flag-HDAC1 and T7-CtBP were as described previously (35, 36). TGFβ receptor type I (37) and constitutively active type I BMP receptor (BMPRIA) were expressed from pCS2. The $(TG)_2$-thymidine kinase (TK)-luc and $(Gal)_5$-TK-luc reporters have been described elsewhere (14), and Gal4p DNA binding domain fusions were created in pM (CLONTECH). The *Renilla* luciferase transfection control (pCMV-RL) was obtained from Promega. A3-lux and PF1-luc reporters have been described elsewhere (38, 39). The TGIF2 clones were generated from an EST (GenBank™ accession no. AW411096). TGIF2 clones were analyzed by automated sequencing on an ABI 377 Prism sequencer.

Cell Culture and Transfections—Mink lung epithelial L17 cells were grown in minimal essential medium with nonessential amino acids supplemented with 10% fetal bovine serum. COS-1 cells were maintained in Dulbecco's modified Eagle's medium with 10% fetal bovine serum and transfected in 60-mm dishes using LipofectAMINE (Life Technologies, Inc.) according to the manufacturer's instructions. L17 cells were transfected in six-well plates using DEAE-dextran as described previously (16). TGFβ (R&D Systems) was added to a final concentration of 100 pM as indicated. EGF (10 nM) was added as indicated, and cells were pretreated with 50 μM PD98059 (New England Biolabs) for 30 min prior to EGF addition.

Reporter Assays—L17 cells were cotransfected with the appropriate firefly luciferase reporter and pCMV-RL, and luciferase activity was assayed after 40–48 h. For assays involving TGFβ addition, a TGFβ type I receptor expression vector (37) was cotransfected. Firefly luciferase was assayed using a luciferase assay kit (Promega) and *Renilla* luciferase activity was assayed with 0.09 μM coelenterazine (Biosynth) in 25 mM Tris, pH 7.5, 100 mM NaCl. Luciferase activities were assayed using a Berthold LB 953 luminometer.

Immunoprecipitation and Western Blotting—COS-1 cells were lysed by sonication in phosphate-buffered saline with 0.5% or 1% Nonidet P-40 supplemented with protease inhibitors (protease inhibitor mixture; Roche Molecular Biochemicals) and phosphatase inhibitors (50 mM NaF, 0.3 mM Na_3VO_4, 20 mM β-glycerophosphate). Following removal of cell debris by centrifugation, lysates were precleared with protein A-Sepharose and complexes precipitated on Flag-agarose (Sigma), or with protein A-Sepharose and a TGIF-specific rabbit antiserum. After washing, proteins were separated by SDS-polyacrylamide gel electrophoresis and transferred to Immobilon-P (Millipore). Blots were incubated with the appropriate antisera (anti-Flag M2 from Sigma; anti-HA (12CA5) from Roche Molecular Biochemicals; anti-T7 tag from Novagen; anti-Smad1 or anti-Smad2/3 from Upstate Biotechnology Inc.) and either horseradish peroxidase-conjugated goat anti-mouse or goat anti-rabbit antibody (Pierce). Proteins were visualized by ECL (Amersham Pharmacia Biotech). Cell extracts were treated with 20 units of alkaline phosphatase (Roche Molecular Biochemicals) for 30 min at 37 °C. Phosphatase inhibitors (20 mM NaF, 1 mM Na_3VO_4, 20 mM β-glycerophosphate) were included as indicated.

RESULTS

A TGIF-related Protein—Since mutation of TGIF in humans results in HPE with a relatively low penetrance, we wanted to know whether other TGIF-like proteins which might perform similar functions were present. Searching the EST data base revealed several human ESTs, which appeared to encode a protein with a high degree of similarity to the homeodomain of TGIF. We sequenced one of these ESTs (GenBank™ accession no. AW411096) and confirmed the similarity in the DNA binding domain. Recently, cDNAs encoding this protein (termed TGIF2, for TG-interacting factor 2) have been identified, and the *TGIF2* gene has been suggested to be amplified in some ovarian carcinoma-derived cell lines (33). TGIF and TGIF2 are highly conserved over their homeodomains and contain a 3-amino acid loop extension of identical amino acid sequence, suggesting that protein interactions via the homeodomain are likely to be similar. The third DNA binding helix is essentially identical between TGIF and TGIF2 (Fig. 1). It is, therefore, likely that both proteins will bind to the same DNA sequence. In addition, a region close to the carboxyl terminus of TGIF is highly conserved in TGIF2. This region of TGIF contains two MAP kinase sites, phosphorylation of which increases the half-life of the protein (34). Interestingly, the central region of TGIF, which plays a role in interactions with HDACs and with TGFβ-activated Smads, appears to be relatively dissimilar in TGIF2, and the amino-terminal PLDLS motif is absent from TGIF2 (Fig. 1).

TGIF2 Is Phosphorylated in Response to EGF Signaling—We created expression vectors encoding TGIF2 with an amino-terminal Flag epitope tag or T7 epitope tag. When transfected into either COS-1 or mink lung epithelial (L17) cells and analyzed by Western blotting, TGIF2 appeared to migrate as two forms of approximately 28 and 30 kDa and the upper band was sometimes further resolved into a closely spaced doublet (see Figs. 2 and 4*B*). Recent evidence has demonstrated that TGIF is phosphorylated at two pairs of MAP kinase sites, and phosphorylation of the carboxyl-terminal pair of sites in TGIF results in a shift to the slower migrating form of TGIF and increased protein stability (34). To determine whether the slower migrating bands represent phosphorylated forms of TGIF2, L17 cells were transfected with Flag-TGIF2 and extracts incubated at 37 °C with alkaline phosphatase. As shown

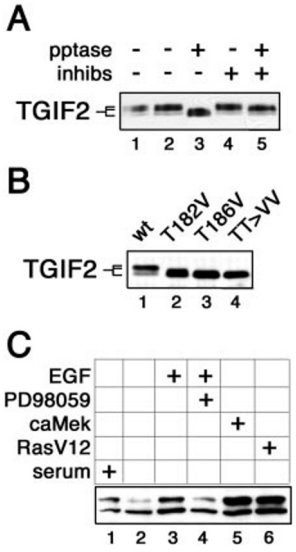

FIG. 2. **TGIF2 is phosphorylated by the Ras/MAP kinase pathway.** *A*, L17 cells were transfected with a Flag-TGIF2 expression vector and incubated in 10% serum. Cell extracts were treated with alkaline phosphatase (*pptase*) or a mixture of phosphatase inhibitors (*inhibs*) for 30 min at 37 °C as indicated (*lanes 2–5*), or without incubation at 37 °C (*lane 1*). *B*, Flag-TGIF2 and three mutant versions of TGIF2 expressed in L17 cells were analyzed by Western blotting. The TGIF2 mutants contain valines in place of threonine residues at positions 182 (T182V), 186 (T186V), or both 182 and 186 (TT → VV). *C*, L17 cells were transfected with Flag-TGIF2 alone, or together with vectors encoding a constitutively active Mek1 mutant (*caMek*) or oncogenic Ras (*RasV12*), with a valine at position 12. Cells were incubated in 0.1% serum, 10% serum, or 0.1% serum with the addition of 10 nM EGF for 30 min as indicated. The Mek inhibitor (PD98059) was added 30 min prior to addition of EGF. TGIF2 expression was visualized by Western blotting.

in Fig. 2*A*, phosphatase treatment resulted in a loss of the upper form of TGIF2, and this loss was prevented by the presence of phosphatase inhibitors. Thus, the upper TGIF2 bands appear to result from increased phosphorylation. Alteration to valine of either one of the two threonine residues present within the conserved carboxyl-terminal region of TGIF2 resulted in a loss of the slower migrating form of TGIF2 (Fig. 2*B*), although with the T182V mutation a band of slightly reduced mobility is visible.

To identify the kinase pathway responsible for this serum-inducible phosphorylation, cells were cotransfected with Flag-TGIF2, together with an expression vector encoding either constitutively active Mek or constitutively active Ras. As shown in Fig. 2*C* (*lanes 5* and *6*), both Ras and Mek expression caused an accumulation of hyperphosphorylated TGIF2 in serum-deprived cells. The addition of EGF also resulted in an increase in the upper form of TGIF2 when compared with serum-deprived cells (Fig. 2*C*, *lanes 2* and *3*), and this effect was blocked by the prior addition of the Mek inhibitor, PD98059 (*lane 4*). Together, these data suggest that, in response to the EGF-Ras-Mek pathway, TGIF2 is phosphorylated at two MAP kinase sites within the conserved carboxyl-terminal region.

TGIF2 Is a Transcriptional Repressor—TGIF is a context-independent transcriptional repressor, and one of its repression domains (RD-2b) appears to be conserved in TGIF2. To determine whether TGIF2 repressed transcription, we fused the entire coding sequence to the Gal4p DNA binding domain (GBD) and targeted this fusion protein to the HSV TK promoter via multiple Gal4p binding sites. Mink lung epithelial (L17) cells were cotransfected with the $(Gal)_5$-TK-luc reporter and increasing amounts of expression vectors encoding GBD/TGIF2, GBD/TGIF, or GBD alone. When compared with expression of the GBD alone, fusions to both TGIF and TGIF2 resulted in dramatic repression of luciferase activity from this reporter (Fig. 3*A*). In this assay, both TGIF proteins repressed transcription to a similar degree. To identify the region of TGIF2 responsible for this repression, we created a series of GBD/TGIF2 fusions, and each of these fusions was coexpressed in L17 cells with the $(Gal)_5$-TK-luc reporter (Fig. 3*B*). GBD/TGIF2-(103–237) repressed transcription to a level similar to that seen with the full-length fusion, suggesting that the TGIF2 repression domain is present in the carboxyl-terminal half of the protein. Deletion into this region to amino acid 177 resulted in a small decrease in repression, and further truncation to amino acid 105 almost completely abolished repression (Fig. 3*B*). Thus, the transcriptional repression activity of TGIF2 appears to be located between amino acids 103 and 237.

TGIF2 Represses Transcription via a TGIF Binding Site—The homeodomains of TGIF and TGIF2 are highly conserved, particularly in the third α helix, which is the major DNA-binding helix (Fig. 1). It is, therefore, likely that both proteins bind to the same DNA sequence. To test whether TGIF2 can repress transcription from a TGIF consensus binding site (CTGTCAA; Ref. 4), L17 cells were cotransfected with TGIF expression constructs and a luciferase reporter ($(TG)_2$-TK-luc) in which transcription is driven by the HSV TK promoter and two copies of a TGIF binding site. As shown in Fig. 4*A*, both TGIF and TGIF2 repressed transcription from this reporter, although TGIF2 appeared to repress less well than TGIF. The lower level of repression by TGIF2 may be due to lower expression levels of this protein in L17 cells (Fig. 4*B*) or to an intrinsic difference in the activity of the proteins. Carboxyl-terminal truncation of TGIF2 to either amino acid 177 or 105 resulted in a complete loss of repression (Fig. 4*A*), despite a high level of expression of the TGIF2-(1–177) construct (Fig. 4*B*). Thus, TGIF2 is able to repress transcription when bound to DNA via the homeodomain, and it appears that the conserved carboxyl-terminal region is a major determinant of this repression.

TGIF2 Interacts with HDAC1—TGIF interacts with both histone deacetylases and with the corepressor CtBP (14, 15). The PLDLS motif, which is important for the recruitment of CtBP to TGIF, is not conserved in TGIF2. To determine whether TGIF2 interacts with CtBP, COS-1 cells were transfected with expression constructs encoding HA-TGIF2 and T7 epitope-tagged CtBP. Immunoprecipitation via the amino-terminal HA epitope appeared to disrupt TGIF-CtBP interaction (data not shown), so immunocomplexes were precipitated using a TGIF-specific antiserum (which recognizes the conserved homeodomain; data not shown). CtBP was clearly detectable in precipitates from cells transfected with HA-TGIF (Fig. 5*A*). In contrast, little CtBP was detected in complexes precipitated via HA-TGIF2 or a mutant form of TGIF in which the PLDLS motif is disrupted (HA-TGIF(S28C)).

In addition to CtBP, TGIF interacts with histone deacetylases. To determine whether TGIF2 interacts with HDAC1, COS-1 cells were cotransfected with vectors expressing Flag-HDAC1 and either HA-TGIF2 or HA-TGIF. As shown in Fig. 5*B*, TGIF as well as TGIF2 were present in immunocomplexes precipitated via the Flag epitope present on HDAC1. This interaction was specific, as no TGIF2 precipitated in the absence of cotransfected HDAC1. Thus, it appears that TGIF2 interacts with HDAC1 but, consistent with the lack of a PLDLS-like motif, is unable to interact with CtBP. This suggests that the major way in which TGIF2 represses transcription is by recruiting histone deacetylases.

TGIF2 Interacts with TGFβ-activated Smads—TGIF interacts with TGFβ-activated Smads and acts as a Smad transcriptional corepressor (16). We were, therefore, interested to determine whether TGIF2 interacted with the mediator of TGFβ

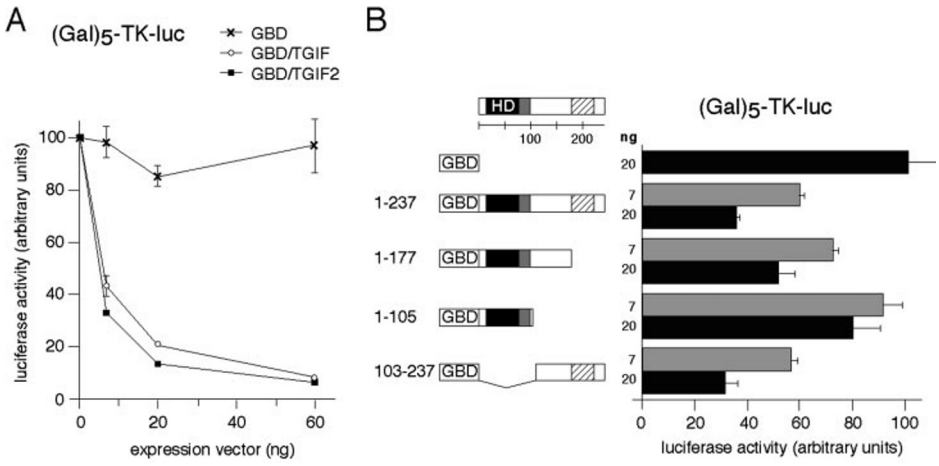

FIG. 3. **TGIF2 is a transcriptional repressor.** *A*, The entire TGIF2 coding sequence was fused to the Gal4p DNA binding domain to create GBD/TGIF2. L17 cells were transfected with a luciferase reporter in which transcription is activated by the HSV TK promoter and five copies of a Gal4p DNA binding site ($(Gal)_5$-TK-luc), together with expression vectors encoding GBD/TGIF2, GBD/TGIF, or the GBD alone. 36 h later luciferase activity was measured from cells transfected with increasing amounts of each expression vector and is presented as the mean ± S.D. of triplicate transfections. *B*, L17 cells were cotransfected with $(Gal)_5$-TK-luc and expression vectors (7 or 20 ng) encoding GBD/TGIF2, or fusions of the GBD to the indicated regions of TGIF2. The mean luciferase activity ± S.D. from triplicate transfections is shown (in arbitrary units).

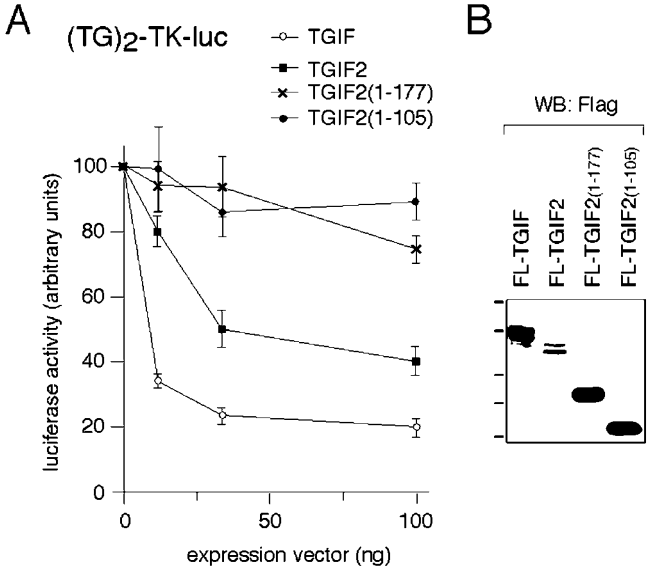

FIG. 4. **TGIF2 represses transcription via a TGIF binding site.** *A*, L17 cells were cotransfected with a reporter ($(TG)_2$-TK-luc) in which the luciferase gene is regulated by the HSV TK promoter and two copies of a TGIF binding site (CTGTCAA) and increasing amounts of expression vectors encoding Flag-tagged TGIF, TGIF2, or deletion mutants of TGIF2 (encoding amino acids 1–177 or 1–105). *B*, relative expression levels of each of the Flag tagged expression constructs used in *A* were determined by Western blotting (*WB*) with a Flag antiserum. The relative positions of molecular size markers are shown (49, 36.4, 24.7, 19.2, and 13.1 kDa). Cells were transfected and assayed for luciferase activity as in Fig. 4. Activities are presented in arbitrary units (mean ± S.D. triplicate transfections).

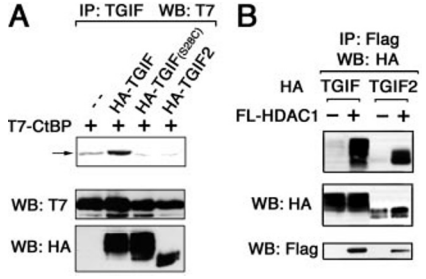

FIG. 5. **TGIF2 interacts with HDAC1.** *A*, COS-1 cells were cotransfected with expression vectors encoding HA-tagged TGIF and TGIF2 expression constructs and T7-tagged CtBP. Cells were lysed by sonication, and proteins were precipitated with a TGIF-specific serum and separated by SDS-polyacrylamide gel electrophoresis. Immunocomplexes were analyzed by Western blot (*WB*) using a T7-specific antiserum. A portion of each lysate was analyzed for expression of transfected proteins by direct Western blot (below). *IP*, immunoprecipitation. *B*, COS-1 cells were transfected and analyzed as in *A*. Proteins were precipitated via the Flag epitope present on HDAC1 and precipitates analyzed with an HA-specific antiserum. Expression of transfected proteins in the lysate was monitored by direct Western (*bottom*). TGIF and TGIF2 were tagged at their amino termini with two HA epitopes.

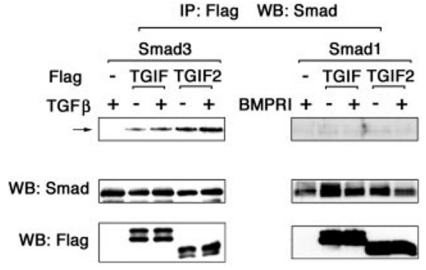

FIG. 6. **TGIF2 interacts with Smad3.** COS-1 cells were cotransfected with expression vectors encoding Flag-TGIF or Flag-TGIF2, together with either Smad3 or Smad1. TGFβ was added as indicated for 1 h prior to cell lysis, or a constitutively active BMP type I receptor (*BMPRI*) was coexpressed with Smad1 as shown. Proteins were precipitated on Flag-agarose, and the presence of Smad proteins detected with a Smad1-specific or Smad2/3-specific antiserum. Protein expression levels were monitored by direct Western blot (*WB*) of a portion of the cell lysate (*bottom*).

signals, Smad3 (40). COS-1 cells were cotransfected with expression vectors encoding Smad3 and Flag-TGIF, Flag-TGIF2, or a control vector. Cells were either treated with TGFβ for 1 h prior to lysis or left untreated. As shown in Fig. 6, Smad3 was clearly detectable in Flag immunoprecipitates from cells expressing either Flag-TGIF or Flag-TGIF2. No Smad3 was visible in control precipitates, and the amount of Smad3 coprecipitating with TGIF or TGIF2 was slightly enhanced by TGFβ treatment. When similar experiments were performed using the BMP-responsive Smad1 (41), together with a constitutively active BMP type I receptor, no interaction of Smad1 was observed with either TGIF or TGIF2 (Fig. 6). Thus, it appears that both TGIF and TGIF2 interact specifically with Smad3, but not with the BMP pathway-specific Smad1.

TGIF2 Is a Smad Transcriptional Corepressor—The above results suggest that, in response to TGFβ signals, TGIF2 interacts with Smad3. To determine the effect of TGIF2 on TGFβ-activated transcriptional responses, L17 cells were cotransfected with increasing amounts of a TGIF2 expression construct, together with the A3-lux reporter and a FAST2 expression vector. A3-lux contains three copies of the activin response element from the *Xenopus mix.2* gene (42). The activin response element is bound by FAST winged helix transcription factors, and when FAST2 is expressed in mammalian cells, luciferase activity from the A3-lux reporter is strongly induced by TGFβ (43–45). As shown in Fig. 7*A*, increasing expression of TGIF2 resulted in repression of TGFβ-induced

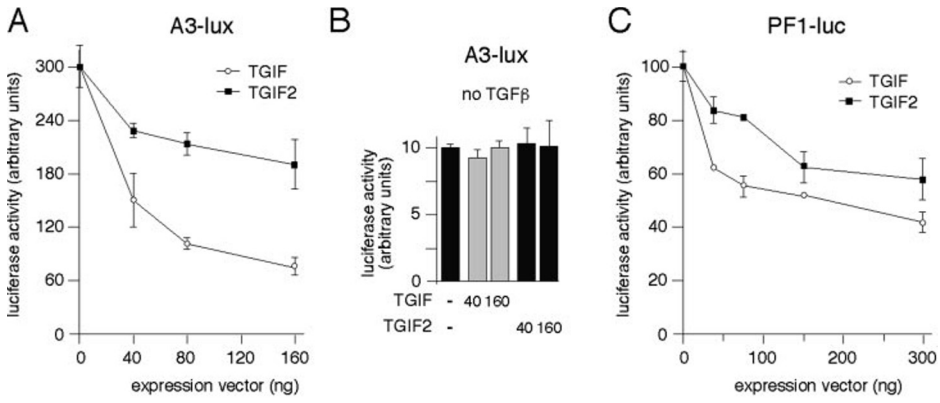

FIG. 7. **TGIF2 represses TGFβ-activated transcription.** *A*, L17 cells were cotransfected with the A3-lux reporter and expression vectors encoding FAST2, TGFβ receptor type I, and increasing amounts of either TGIF or TGIF2. TGFβ was added 18 h prior to cell lysis, and luciferase activity was determined. *B*, L17 cells were transfected as in *A*, except that the TGFβ receptor type I was omitted and TGFβ was not added. *C*, L17 cells were transfected and treated with TGFβ as in *A*, except that FAST2 was absent and the PF1-luc reporter was used in place of A3-lux. In all cases, luciferase activity is presented in arbitrary units as the mean ± S.D. of triplicate transfections.

activity from the A3-lux reporter. Expression of TGIF2 had no effect on this reporter in the absence of added TGFβ (Fig. 7*B*). Repression of A3-lux by TGIF2 was less potent than that observed with TGIF. However, this lower level repression may be due to differences in expression levels of the two TGIF proteins when transfected into L17 cells (see Fig. 4*B*). To further analyze the effect of TGIF2 on TGFβ-activated transcription, we tested the PF1-luc reporter, in which luciferase gene expression is activated by the TGFβ-inducible element from the *PAI-1* promoter (39). As shown in Fig. 7*C*, in the presence of TGFβ signaling both TGIF and TGIF2 repressed luciferase activity from this reporter. In this case, the difference in repression by TGIF and TGIF2 appeared to be less significant. Together, these results suggest that TGIF2 interacts with TGFβ-activated Smads and is able to repress the activation of TGFβ-responsive transcription.

DISCUSSION

TGIF is a transcriptional repressor, which can interact with TGFβ-activated Smads and repress expression of TGFβ target genes (16). TGIF also binds directly to DNA, thereby repressing a different set of gene responses, independent of TGFβ signals and the Smad proteins (4, 13, 14). Transcriptional repression by TGIF is dependent on its ability to recruit other transcriptional corepressors, including HDACs and CtBP (14, 15). Here we demonstrate that the related protein, TGIF2, is a transcriptional repressor which can work in the same pathways as TGIF and, like TGIF, represses transcription by recruiting HDACs.

Mutations in human *TGIF* result in holoprosencephaly (HPE; Ref. 29). The primary defect in HPE is in the formation of the ventral forebrain, and this is often associated with disruption of midline facial structures (31, 46, 47). In general, *TGIF* mutations in HPE are deletions of one allele, although several missense mutations which result in partial loss of function have been identified (29, 30). Interestingly, no loss of heterozygosity has been observed, suggesting that relatively small changes in TGIF levels can have dramatic phenotypic outcomes. *TGIF* mutations are not fully penetrant with respect to the HPE phenotype, and ~10% of TGIF deletions cause HPE. However, the range and severity of the HPE phenotypes associated with *TGIF* mutants is similar to that seen with mutations at other HPE loci, such as *sonic hedgehog* (31, 32, 48). This variability of phenotype and penetrance suggests the presence of genetic modifiers of TGIF function or of other factors that may be able to compensate for decreased TGIF activity. We were, therefore, interested to identify candidate proteins which could perform similar functions to TGIF. Based on the sequence similarity between TGIF and TGIF2, it is to be expected that TGIF2 would bind to a TGIF site. However, outside the homeodomain and a region at their carboxyl termini, the two proteins share little sequence similarity.

We show that TGIF2 carries out similar functions to TGIF; TGIF2 appears to be a context-independent transcriptional repressor, which works by recruiting HDAC1. However, TGIF2 lacks the CtBP interaction motif that is present in TGIF, and is unable to interact with CtBP. Thus, it appears that TGIF2 cannot repress transcription via the HDAC-independent mechanism of CtBP recruitment, which may act to target TGIF to certain polycomb group proteins. It is possible that the two modes of repression identified for TGIF (via HDACs or via CtBP) may be important at different locations, or act sequentially at the same genes. Alternatively, both activities may be part of the same general corepressor complex. However, if these two modes of repression have specific functions, perhaps repressing different sets of target genes, TGIF2 would only carry out one of these roles. Thus, TGIF2 may be a specialized form of TGIF, which is dedicated to HDAC-dependent transcriptional repression.

In addition to repressing transcription from a cognate TGIF site, TGIF2 interacts with TGFβ-activated Smads and represses transcription of TGFβ-responsive genes. Thus, TGIF2 can repress both TGFβ-dependent and -independent transcription. HPE mutations in *TGIF* appear to affect its repressor functions. However, we do not know whether such mutations result in HPE due to effects on the TGFβ-dependent roles of TGIF or on other TGFβ-independent functions. Our results suggest that TGIF2 may be able to compensate for the effects of decreased TGIF levels on both TGFβ-dependent and -independent transcriptional regulation. Thus, if there is some variation in expression levels of TGIF2, it is possible that decreased TGIF function may have more severe phenotypic outcomes in some individuals than in others. In this context it should be noted that loss of one *TGIF* allele might result in less than a 50% reduction in overall TGIF function, since one functional *TGIF* allele plus two copies of *TGIF2* will remain.

At least 12 separate HPE loci have been mapped, and the genes responsible for the HPE phenotype have been identified at 4 of them (31, 32). The human *TGIF2* gene is located on chromosome 20q11, a region to which no candidate HPE loci have been mapped (33). Interestingly, only one of the four HPE missense mutations identified in TGIF alters a residue that is conserved in TGIF2 (P63R, see Fig. 1). It will now be of interest to determine whether decreased TGIF function results in HPE due to effects on TGFβ-activated transcription or on other TGFβ-independent responses.

Northern analysis of human *TGIF* and *TGIF2* expression demonstrated that both genes are expressed at low levels in adult brain (4, 33, 49). *In situ* hybridization on developing mouse embryos revealed more extensive expression of TGIF transcripts in the brain during embryogenesis (49). However, it remains to be determined whether TGIF2 is expressed in the developing forebrain. Importantly, our characterization of TGIF2 suggests that loss of a single copy of TGIF may, at least

in some tissues, result in an even smaller reduction in overall TGIF function than previously assumed. The fact that HPE mutations are found in TGIF but not in TGIF2 may suggest that the HPE phenotype results from loss of a TGIF-specific function, such as CtBP interaction. In this context, it is of interest that one of the HPE mis-sense mutations found in TGIF specifically prevents interaction with CtBP (15). However, we cannot rule out the possibility that TGIF may play a more important role in brain development simply due to differences in expression levels or patterns.

Phosphorylation of TGIF by the Ras/MAP kinase pathway results in increased protein levels within the cell, due to an increase in the half-life of the protein (34). TGIF2 is also phosphorylated by this pathway, although we have no clear evidence for alterations in TGIF2 stability resulting from phosphorylation. However, it is possible that signal regulated alterations in the level of expression of one or both TGIF proteins may contribute to the variable penetrance of the HPE phenotype. In addition, there may be other effects of MAP kinase-mediated phosphorylation on TGIF or TGIF2 function.

Recent evidence has suggested that the TGIF2 gene is amplified in certain ovarian tumor-derived cell lines (33). It is tempting to speculate that increased TGIF function may play a role in tumor progression by decreasing the ability of TGFβ signals to prevent cell cycle progression. Indeed, increased expression of TGIF in a human keratinocyte cell line has been shown to interfere with TGFβ-activated expression of the $p15^{Ink4b}$ CDK inhibitor gene, and to decrease TGFβ-induced growth inhibition (34). The observed amplification of the *TGIF2* gene and concomitant increase in mRNA expression in ovarian tumor cell lines is relatively modest. However, MAP kinase-mediated phosphorylation of TGIF in response to oncogenic Ras results in increased protein stability, thereby increasing steady state levels of TGIF. Thus, amplification of the TGIF2 gene together with effects of Ras activation on TGIF may result in a synergistic increase in overall TGIF levels and activity. It will, therefore, be of interest to determine whether TGIF and TGIF2 protein levels are increased in tumor-derived cell lines.

In summary, we have demonstrated that TGIF2 is a transcriptional repressor that can repress transcription when bound directly to DNA, or when recruited by TGFβ-activated Smads. These results raise important questions about the role of both TGIF proteins in brain development and in tumorigenesis.

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REFERENCES

1. McGinnis, W., Levine, M. S., Hafen, E., Kuroiwa, A., and Gehring, W. J. (1984) *Nature* **308,** 428–433
2. McGinnis, W., Garber, R. L., Wirz, J., Kuroiwa, A., and Gehring, W. J. (1984) *Cell* **37,** 403–408
3. Gehring, W. J., Affolter, M., and Burglin, T. (1994) *Annu. Rev. Biochem.* **63,** 487–526
4. Bertolino, E., Reimund, B., Wildt-Perinic, D., and Clerc, R. (1995) *J. Biol. Chem.* **270,** 31178–31188
5. Burglin, T. R. (1997) *Nucleic Acids Res.* **25,** 4173–4180
6. Kamps, M. P., Murre, C., Sun, X. H., and Baltimore, D. (1990) *Cell* **60,** 547–555
7. Moskow, J. J., Bullrich, F., Huebner, K., Daar, I. O., and Buchberg, A. M. (1995) *Mol. Cell. Biol.* **15,** 5434–5443
8. Nourse, J., Mellentin, J. D., Galili, N., Wilkinson, J., Stanbridge, E., Smith, S. D., and Cleary, M. L. (1990) *Cell* **60,** 535–545
9. Berthelsen, J., Zappavigna, V., Mavilio, F., and Blasi, F. (1998) *EMBO J.* **17,** 1423–1433
10. Berthelsen, J., Zappavigna, V., Ferretti, E., Mavilio, F., and Blasi, F. (1998) *EMBO J.* **17,** 1434–1445
11. Asahara, H., Dutta, S., Kao, H. Y., Evans, R. M., and Montminy, M. (1999) *Mol. Cell. Biol.* **19,** 8219–8225
12. Jacobs, Y., Schnabel, C. A., and Cleary, M. L. (1999) *Mol. Cell. Biol.* **19,** 5134–5142
13. Yang, Y., Hwang, C. K., D'Souza, U. M., Lee, S. H., Junn, E., and Mouradian, M. M. (2000) *J. Biol. Chem.* **275,** 20734–20741
14. Wotton, D., Lo, R. S., Swaby, L. A., and Massague, J. (1999) *J. Biol. Chem.* **274,** 37105–37110
15. Melhuish, T. A., and Wotton, D. (2000) *J. Biol. Chem* **275,** 39762–39766
16. Wotton, D., Lo, R. S., Lee, S., and Massague, J. (1999) *Cell* **97,** 29–39
17. Heldin, C.-H., Miyazono, K., and ten Dijke, P. (1997) *Nature* **390,** 465–471
18. Zhang, Y., and Derynck, R. (1999) *Trends Cell Biol.* **9,** 274–279
19. Massagué, J., Hata, A., and Liu, F. (1997) *Trends Cell Biol.* **7,** 187–192
20. Massagué, J., and Wotton, D. (2000) *EMBO J.* **19,** 1745–1754
21. Feng, X.-H., Zhang, Y., Wu, R.-Y., and Derynck, R. (1998) *Genes Dev.* **12,** 2153–2163
22. Janknecht, R., Wells, N. J., and Hunter, T. (1998) *Genes Dev.* **12,** 2114–2119
23. Pouponnot, C., Jayaraman, L., and Massagué, J. (1998) *J. Biol. Chem.* **273,** 22865–22868
24. Sun, Y., Liu, X., Eaton, E. N., Lane, W. S., Lodish, H. F., and Weinberg, R. A. (1999) *Mol. Cell* **4,** 499–509
25. Sun, Y., Liu, X., Ng-Eaton, E., Lodish, H. F., and Weinberg, R. A. (1999) *Proc. Natl. Acad. Sci. U. S. A.* **96,** 12442–12447
26. Stroschein, S. L., Wang, W., Zhou, S., Zhou, Q., and Luo, K. (1999) *Science* **286,** 771–774
27. Luo, K., Stroschein, S. L., Wang, W., Chen, D., Martens, E., Zhou, S., and Zhou, Q. (1999) *Genes Dev.* **13,** 2196–2206
28. Wotton, D., and Massague, J. (2001) *Curr. Top. Microbiol. Immunol.* **254,** 145–164
29. Gripp, K. W., Wotton, D., Edwards, M. C., Roessler, E., Ades, L., Meinecke, P., Richieri-Costa, A., Zackai, E. H., Massague, J., Muenke, M., and Elledge, S. J. (2000) *Nat. Genet.* **25,** 205–208
30. Overhauser, J., Mitchell, H. F., Zackai, E. H., Tick, D. B., Rojas, K., and Muenke, M. (1995) *Am. J. Hum. Genet.* **57,** 1080–1085
31. Muenke, M., and Beachy, P. A. (2000) *Curr. Opin. Genet. Dev.* **10,** 262–269
32. Wallis, D. E., and Muenke, M. (1999) *Mol. Genet. Metab.* **68,** 126–138
33. Imoto, I., Pimkhaokham, A., Watanabe, T., Saito-Ohara, F., Soeda, E., and Inazawa, J. (2000) *Biochem. Biophys. Res. Commun.* **276,** 264–270
34. Lo, R. S., Wotton, D., and Massague, J. (2001) *EMBO J.* **20,** 128–136
35. Schaeper, U., Boyd, J. M., Verma, S., Uhlmann, E., Subramanian, T., and Chinnadurai, G. (1995) *Proc. Natl. Acad. Sci. U. S. A.* **92,** 10467–10471
36. Yang, W.-M., Yao, Y.-L., Sun, J.-M., Davie, J. R., and Seto, E. (1997) *J. Biol. Chem.* **272,** 28001–28007
37. Cárcamo, J., Weis, F. M. B., Ventura, F., Wieser, R., Wrana, J. L., Attisano, L., and Massagué, J. (1994) *Mol. Cell. Biol.* **14,** 3810–3821
38. Chen, X., Weisberg, E., Fridmacher, V., Watanabe, M., Naco, G., and Whitman, M. (1997) *Nature* **389,** 85–89
39. Hua, X., Liu, X., Ansari, D. O., and Lodish, H. F. (1998) *Genes Dev.* **12,** 3084–3095
40. Zhang, Y., Feng, X.-H., Wu, R.-Y., and Derynck, R. (1996) *Nature* **383,** 168–172
41. Graff, J. M., Thies, R. S., Song, J. J., Celeste, A. J., and Melton, D. A. (1994) *Cell* **79,** 169–179
42. Chen, X., Rubock, M. J., and Whitman, M. (1996) *Nature* **383,** 691–696
43. Labbé, E., Silvestri, C., Hoodless, P. A., Wrana, J. L., and Attisano, L. (1998) *Mol. Cell* **2,** 109–120
44. Liu, B., Dou, C., Prabhu, L., and Lai, E. (1999) *Mol. Cell. Biol.* **19,** 424–430
45. Liu, F., Pouponnot, C., and Massagué, J. (1997) *Genes Dev.* **11,** 3157–3167
46. Golden, J. A. (1998) *J. Neuropathol. Exp. Neurol.* **57,** 991–999
47. Roessler, E., and Muenke, M. (1998) *J. Inherit. Metab. Dis.* **21,** 481–497
48. Nanni, L., Ming, J. E., Bocian, M., Steinhaus, K., Bianchi, D. W., Die-Smulders, C., Giannotti, A., Imaizumi, K., Jones, K. L., Campo, M. D., Martin, R. A., Meinecke, P., Pierpont, M. E., Robin, N. H., Young, I. D., Roessler, E., and Muenke, M. (1999) *Hum. Mol. Genet.* **8,** 2479–2488
49. Bertolino, E., Wildt, S., Richards, G., and Clerc, R. G. (1996) *Dev. Dyn.* **205,** 410–420