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Transforming Growth Factor-β-stimulated Clone-22 Is a Member of a Family of Leucine Zipper Proteins That Can Homo- and Heterodimerize and Has Transcriptional Repressor Activity*

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TGF-β-stimulated clone-22 (TSC-22) encodes a leucine zipper-containing protein that is highly conserved during evolution. Two homologues are known that share a similar leucine zipper domain and another conserved domain (designated the TSC box). Only limited data are available on the function of TSC-22 and its homologues. TSC-22 is transcriptionally up-regulated by many different stimuli, including anti-cancer drugs and growth inhibitors, and recent data suggest that TSC-22 may play a suppressive role in tumorigenesis. In this paper we show that TSC-22 forms homodimers via its conserved leucine zipper domain. Using a yeast two-hybrid screen, we identified a TSC-22 homologue (THG-1) as heterodimeric partner. Furthermore, we report the presence of two more mammalian family members with highly conserved leucine zippers and TSC boxes. Interestingly, both TSC-22 and THG-1 have transcriptional repressor activity when fused to a heterologous DNA-binding domain. The repressor activity of TSC-22 appears sensitive for promoter architecture, but not for the histone deacetylase inhibitor trichostatin A. Mutational analysis showed that this repressor activity resides in the non-conserved regions of the protein and is enhanced by the conserved dimerization domain. Our results suggest that TSC-22 belongs to a family of leucine zipper-containing transcription factors that can homodimerize and heterodimerize with other family members and that at least two TSC-22 family members may be repressors of transcription.

TGF-β-stimulated clone-22 (TSC-22),[1] a gene encoding a leucine zipper protein, was first isolated as a TGF-β-responsive gene from a mouse osteoblast cell line (1). TSC-22 is up-regulated by many stimuli that act via distinct signaling pathways: fibroblast growth factor 2, epidermal growth factor, dexamethasone, follicle-stimulating hormone, and different cytokines have all been shown to induce the expression of the gene (1–4). Kawamata *et al.* (5) reported that TSC-22 is up-regulated by an anti-cancer drug, vesnarinone, in a salivary gland carcinoma cell line. Using an antisense approach, they showed that TSC-22 has a growth inhibitory effect on this cell line and that it reduces tumor formation in nude mice. Moreover, TSC-22 is down-regulated in salivary gland tumors as compared with normal salivary gland tissue (6). Recently, we carried out a differential display screen to identify progesterone target genes in mammary carcinoma cells (7). Progestins are used to treat breast cancer and can induce growth inhibition in the mammary carcinoma cell line T47D by an unknown mechanism (8). We have found that TSC-22 is induced by progestins in T47D cells but not in two responsive cell lines that are not growth-inhibited by progestins (7).[2] Furthermore, we have found that TSC-22, when overexpressed in a distinct tumor cell line, also has a growth inhibitory action.[3] These results suggest that TSC-22 may be a negative regulator of proliferation and may have tumor suppressor activity.

TSC-22 has been highly conserved during evolution. The rat and the mouse genes are 100% identical at the amino acid level, while human TSC-22 is 98.5% identical (9, 10). In addition, the *Drosophila melanogaster* gene *shortsighted* (*shs*) or *bunched*, which plays an important role in oogenesis, and in eye, wing, and peripheral nervous system development, is very homologous (68% identity) in the leucine zipper and adjacent N-terminal region, which has been designated the TSC box (11, 12). Another related gene is delta sleep inducing peptide immunoreactive peptide (DIP), which was isolated serendipitously and contains the conserved leucine zipper and TSC box (13, 14). Recently, a synthetic peptide derived from the porcine DIP gene was shown to homodimerize via this leucine zipper (15).

Not much is known about the function of TSC-22 or any of its homologues. Since it contains a leucine zipper, TSC-22 has been hypothesized as being a transcription factor (1). Supporting this, nuclear localization was reported, although for the homologue *shs* cytoplasmic localization was observed (1, 11).

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The nucleotide sequence(s) reported in this paper has been submitted to the GenBank™/EBI Data Bank with accession number(s) AJ133115.

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[1] The abbreviations used are: TSC-22, TGF-β-stimulated clone-22; TGF-β, transforming growth factor-β; shs, shortsighted; DIP, delta sleep inducing peptide; GST, glutathione *S*-transferase; DBD, DNA-binding domain; HA tag, hemagglutinin tag; RD1 and RD2, repression domains 1 and 2; THG-1 and THG-2, TSC-22 homologous genes 1 and 2; EST, expressed sequence tag; 4i5g, 4×ICAM-5×GAL-TATA-luc reporter; 4i5g-250, 4×ICAM-5×GAL-250 bp-TATA-luc reporter; 5g4i, 5×GAL-4×ICAM-TATA-luc reporter; 5g-250–4i, 5×GAL-250 bp-4×ICAM-TATA-luc reporter; RARα, retinoic acid receptor α; TSA, trichostatin A; gal-2h, fusion construct of the THG-1 fragment cloned in the yeast two-hybrid screen with the GAL-DBD; bp, base pair(s); oligo, oligonucleotide; ICAM, intercellular adhesion molecule. GAL, DNA-binding site of yeast transcription factor GAL4; gal, DNA-binding domain of GAL4.

[2] H. A. Kester and B. van der Burg, unpublished observations.

[3] H. A. Kester, C. E. van den Brink, P. T. van der Saag, and B. van der Burg, manuscript in preparation.

However, TSC-22 does not belong to any of the known families of leucine zipper transcription factors, and it does not contain a classical DNA-binding domain such as those in the bZip or bHLH-Zip families. It has been hypothesized that TSC-22 might act as a repressor, by binding other leucine zipper-containing transcription factors, such as members of the AP-1 family, and inhibiting their DNA binding. Another report, however, showed that TSC-22 could bind to a specific DNA sequence *in vitro* (3).

Here, we report that TSC-22 forms homodimers via its leucine zipper domain. We identify the family member TSC-22 homologous gene-1 (THG-1) as another TSC-22 interacting partner. Both TSC-22 and THG-1 act as transcriptional repressors when fused to the DNA-binding domain of yeast transcription factor GAL-4. At least for TSC-22, this activity does not reside in the dimerization domain, but it is influenced by the presence of this domain. These data indicate that TSC-22 belongs to a homo- and heterodimeric family of leucine zipper-containing factors that repress transcription when sequestered to DNA.

EXPERIMENTAL PROCEDURES

Cell Culture and Transient Transfections—Monkey COS-1 cells were obtained from American Type Culture Collection (Rockville, MD). Cells were cultured in a 1:1 mixture of Dulbecco's modified Eagle's medium and Ham's F-12 medium (Life Technologies, Inc), buffered with bicarbonate and supplemented with 7.5% (v/v) fetal calf serum from Integro (Linz, Austria). For transient transfections, the cells were cultured in 24-well tissue culture plates. Cells were transfected using calcium phosphate coprecipitation with indicated amounts of luciferase reporter, SV2lacZ, and expression plasmids. pBluescript SK$^-$ plasmid was added to obtain a total amount of 1.6–2 μg of DNA/well. After 16 h, the medium was refreshed. Cells were harvested 24 h later and assayed for luciferase activity using the Luclite luciferase reporter gene assay kit and a TopCount liquid scintillation counter (Packard, Meriden, CT). Samples were corrected for transfection efficiency by measuring β-galactosidase activity as described previously (16). Trichostatin A (ICN, Costa Mesa CA) treatment was done for 24 h, with refreshment of medium and TSA after 12 h.

Plasmids—Fig. 1 shows the TSC-22 and THG-1 fusion constructs used. Details of the construction of the clones in this paper can be obtained from the authors upon request. TSC-22 sequences were obtained in part from American Type Culture Collection (Rockville, MD) (GenBank accession no. T07973, EST05864; Ref. 17), and in part as a polymerase chain reaction product from a differential display screen (7). The entire open reading frame and 5′-untranslated region of both clones was sequenced. Full-length THG-1 was obtained from American Type Culture Collection (Rockville, MD; GenBank accession no. AA212193; I.M.A.G.E. Consortium clone identification no. 725353; Ref. 18) and sequenced completely. A reporter plasmid containing four NFκB-binding sites from the ICAM-1 promoter was kindly provided by S. Wissink and the reporters TATA-luc and 5×GAL-TATA-luc by G. Folkers (19); the 250-bp fragment was cut by digestion with *Bam*HI and *Bgl*II from T7TS2 kindly provided by J. Joore. The pSG5-based expression plasmid with the c-Jun (obtained from D. Nathans) coding region inserted were provided by W. Kruijer. The GAL and ICAM sites containing plasmids were constructed using these plasmids by standard techniques; cloning details are available upon request. The CMV4 expression vector containing human RelA/p65 has been described previously (20); all 4×ICAM-containing promoters were activated by cotransfection of 20 ng of this expression vector, which induces these reporters 200-, 1000-, 1500-, and 70-fold (for 4i5g, 5g4i, 5g-250–4i, and 4i5g-250, respectively). The gal fusions were cloned in pSG424, GST fusions in pGEX2T, tag fusions in pSG5-hemagglutinin tag vector (21, 19, 22), and vpT$_{38-144}$ fusion in pSG5-hemagglutinin tag-VP16 vector kindly provided by G. Folkers. galVP16, galRARβ-EF, and galRARα were described before (21).

In Vitro Protein-binding Assay (GST Pull-down)—Extracts were made from COS-1 cells cultured in six-well dishes and transfected with 10 μg of expression plasmid. Cells were transfected by calcium phosphate coprecipitation as described above, and harvested as described before (19), in 30 μl 400 mM KCl, 20 mM Tris, pH 7.5, 20% (v/v) glycerol, 2 mM dithiothreitol, protease inhibitors phenylmethylsulfonyl fluoride (1 mM), leupeptin, aprotinin, pepstatin (all 1 μg/ml), and chymostatin

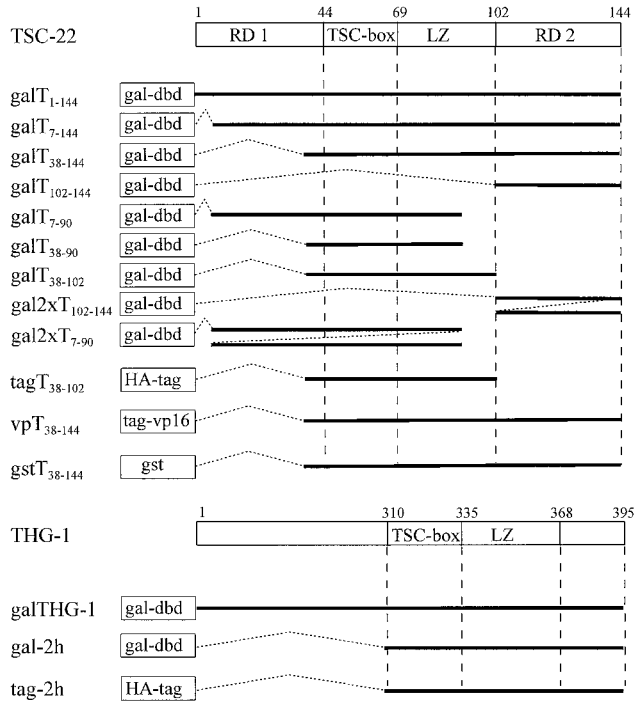

FIG. 1. **TSC-22 and THG-1 fusion constructs used.** *gal*, GAL4 DNA-binding domain; *gst*, glutathione *S*-transferase; *tag*, hemagglutinin-tagged pSG5 construct; *vp*, hemagglutinin-tagged VP16-activation domain-pSG5 construct; *LZ*, leucine zipper; *RD1* and *RD2*, repression domains 1 and 2.

(10 μg/ml). Alternatively, ^{35}S-labeled proteins were synthesized *in vitro* using the TnT coupled rabbit reticulocyte lysate system (Promega, Madison, WI) in the presence of [^{35}S]methionine according to the manufacturer's description.

Glutathione *S*-transferase fusion proteins were expressed in *Escherichia coli* BL21(plysS). Expression and purification with glutathione-coated beads (Amersham Pharmacia Biotech) was performed as described (16). The fusion proteins, loaded on Sepharose beads, were mixed subsequently with *in vitro* synthesized proteins or COS-1 extracts in binding buffer (250 mM NaCl, 50 mM Hepes, pH 7.5, 0.5 mM EDTA, 0.1% (v/v) Nonidet P-40, 0.2 mM phenylmethylsulfonyl fluoride, 1 mM dithiothreitol, 100 μg/ml bovine serum albumin), heated for 5 min at 42 °C, incubated for 2 h at 4 °C, washed extensively, resuspended in sample buffer, and analyzed by SDS-polyacrylamide gel electrophoresis or Western blotting with anti-tag antibody 12CA5 as described (19).

Electrophoretic Mobility Shift Assay (Gel Shift)—COS-1 cells were grown in six-well dishes and transfected with in total 10 μg of two expression plasmids and harvested as described above. A GAL-oligo was end-labeled as described before (21). Whole cell extracts (5 μl) were incubated with 10,000 cpm of probe (0.1–0.5 ng) and 1 μg of poly(dI-dC) for 30 min at room temperature in a total reaction mixture of 20 μl containing 20 mM Hepes, pH 7.5, 100 mM KCl, 0.2 mM EDTA, 20% (v/v) glycerol, 1 mM dithiothreitol, 1 μg/μl bovine serum albumin. Samples were loaded on a 4% polyacrylamide (29:1) gel, containing 0.5× TBE as running buffer.

Yeast Two-hybrid—TSC-22$_{38-144}$ was cloned in the yeast expression vector pGBT8 by inserting the *Bam*HI-*Sac*I fragment from galT$_{38-144}$ in *Bam*HI-*Sac*I-digested pGBT8, and used as bait in a yeast two-hybrid screen with the yeast strain YGH-1, which contains a *HIS3* and a *lacZ* reporter gene. The screen was performed with a reamplified mouse brain cDNA library as described (23, 24). Three to 10 days after transformation of yeast with 100 μg of cDNA library, DNA from His$^+$Gal$^+$ colonies was isolated and introduced in *E. coli* strain MH-4. All clones were sequenced and reintroduced into the yeast cells, together with the bait or with the empty vector pGBT8. Clones that gave rise to *HIS*$^+$*LacZ*$^+$ colonies only with bait were considered as true positives.

RESULTS

TSC-22 Forms Homodimers through Its TSC Box Leucine Zipper Region—Based on the sequence of the leucine zipper, TSC-22 may form homodimers (12). To test this, we performed

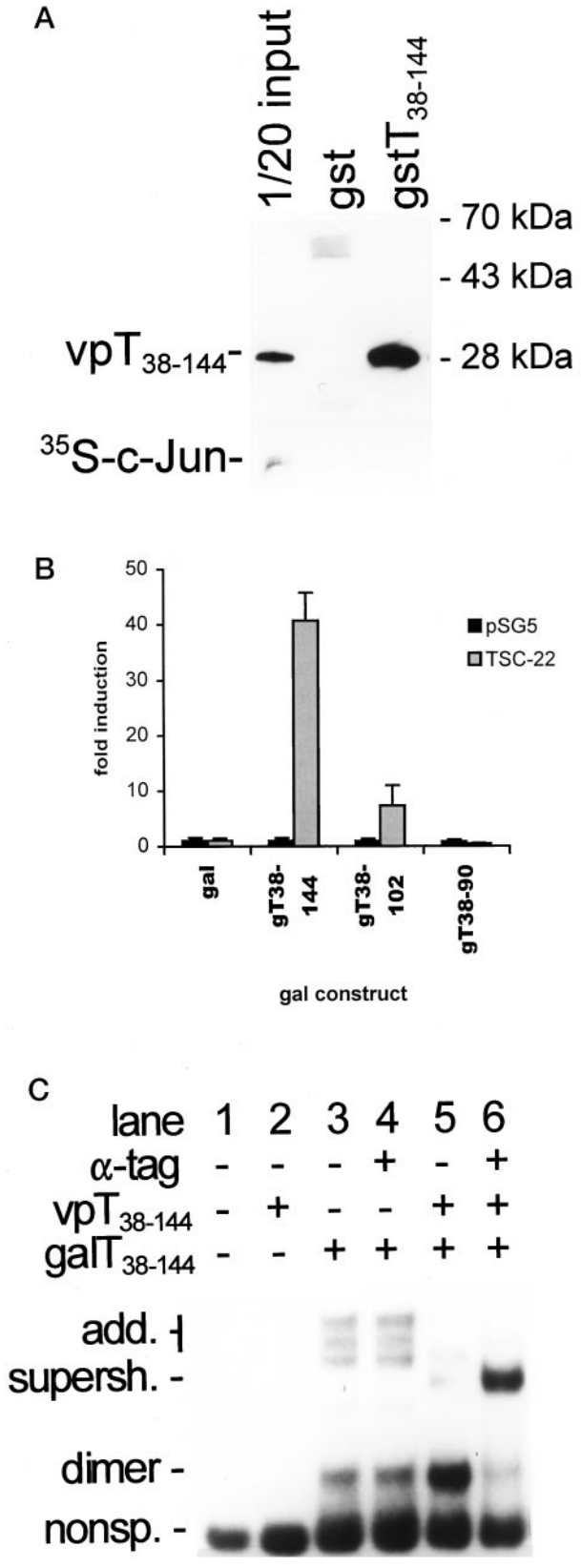

FIG. 2. **TSC-22 forms homodimers.** *A*, GST pull-down assay with GST and gstT_{38-144} to pull-down HA-tagged-T_{38-144} construct (vpT_{38-144}) with ^{35}S-labeled c-Jun as specificity control. Bound vpT_{38-144} was detected by Western blotting with α-tag antibody 12CA5. ^{35}S-Labeled c-Jun was detected by autoradiography. *B*, mammalian two-hybrid assay with gal-TSC-22 constructs (200 ng) and TSC-22 construct vpT_{38-144} or empty vector pSG5 (200 ng) in COS-cells. Values from three experiments (in duplicate) were normalized and averaged, and gal-DBD activity set at 1, indicated is also the standard error. *C*, gel shift assay with different COS-1 extracts, cotransfected with the indicated expression plasmids or empty vector, using a GAL-oligo. The positions of a nonspecific band (*nonsp.*), the dimer bound to the oligo, the dimer supershifted (*supersh.*) with the α-tag antibody 12CA5, and the additional complexes mentioned in the text (*add.*) are indicated.

GST pull-down experiments. In these experiments, we overexpressed a gstT_{38-144} fusion construct in *E. coli*, from which the protein can be easily purified with glutathione-coated beads. The gstT_{38-144}-bound beads were incubated with extract from COS-1-cells transfected with a hemagglutinin-tagged TSC-22 construct (vpT_{38-144}), and checked for vpT_{38-144} that remained bound to the beads on Western blot with an anti-tag antibody. Initially, no bound vpT_{38-144} was detected, but when the beads and extracts were incubated for 5 min at 42 °C prior to the 2-h binding step at 4 °C, a specific interaction of vpT_{38-144} with gstT_{38-144} (and not with GST alone), was observed. TSC-22 probably already forms dimers in the COS-1 cells and in *E. coli*, and these dimers first have to be disrupted (*e.g.* by elevated temperature) before any new dimers can be formed. The specificity control c-Jun (a leucine zipper-containing transcription factor of the AP-1 family) did not bind specifically to gstT_{38-144} (Fig. 2*A*), while the longer constructs gstT_{7-144} and vpT_{7-144} gave similar results as gstT_{38-144} and vpT_{38-144}, respectively (data not shown).

We also tested the ability of TSC-22 to form homodimers by means of a mammalian two-hybrid approach. Different GAL4 DNA-binding domain (GAL-DBD) constructs containing (parts of) TSC-22 (Fig. 1) were cotransfected with the expression plasmid vpT_{38-144} (which contains, apart from the HA tag, a VP-16 activation domain and amino acids 38–144 of TSC-22) or the empty expression vector pSG5. Strong activation of a 5×GAL-TATA-luc reporter construct (five GAL4 DNA-binding sites in front of an E1b TATA box and luciferase reporter gene) was observed with vpT_{38-144} but not with pSG5, indicating that TSC-22 can form homodimers *in vivo* (Fig. 2*B*). When a deletion mutant was used that only contained the TSC box and leucine zipper (galT_{38-102}) fused to the GAL-DBD, the reporter was still strongly activated. With a construct in which the leucine zipper is partially deleted (galT_{38-90}), the activity was completely abolished, indicating that this leucine zipper is indeed necessary for homodimer formation (Fig. 2*B*).

We studied the ability of TSC-22 to homodimerize in gel shift assays. In this assay, a radioactively labeled 17-bp oligonucleotide that contains the GAL4 DNA-binding site (GAL-oligo) is incubated with GAL-DBD fusion proteins. If this GAL-oligo is bound by a GAL-DBD fusion protein and separated on a nondenaturing polyacrylamide gel, it runs slower than an unbound GAL-oligo. After incubation of the GAL-oligo with extracts from COS-1 cells that were transfected with galT_{38-144} we clearly observed shifted bands (*lanes 3* and *4*) that were specific (competed by 100× excess unlabeled GAL-oligo and not by non-related oligo; data not shown), and not present in controls (incubations with extracts of COS-1-cells that were not transfected with gal constructs; *lanes 1* and *2*). With extracts of COS-1 cells cotransfected with galT_{38-144} and vpT_{38-144}, we also observed a clearly shifted band (Fig. 2*C*, *lane 5*, *band dimer*). This band was supershifted upon adding an antibody against the HA tag present in the vpT_{38-144} construct (Fig. 2*C, lane 6*, *band supersh.*), while bands from extracts containing galT_{38-144} only were not supershifted (*lane 4*). This indicates that the supershifted band must contain vpT_{38-144} bound to galT_{38-144} (the antibody binds the vpT_{38-144}·galT_{38-144}·GAL-oligo complex and causes it to migrate slower), again demonstrating that TSC-22 can homodimerize.

Interestingly, we noted additional slowly migrating complexes in the lanes with the galT_{38-144}-containing extract (Fig.

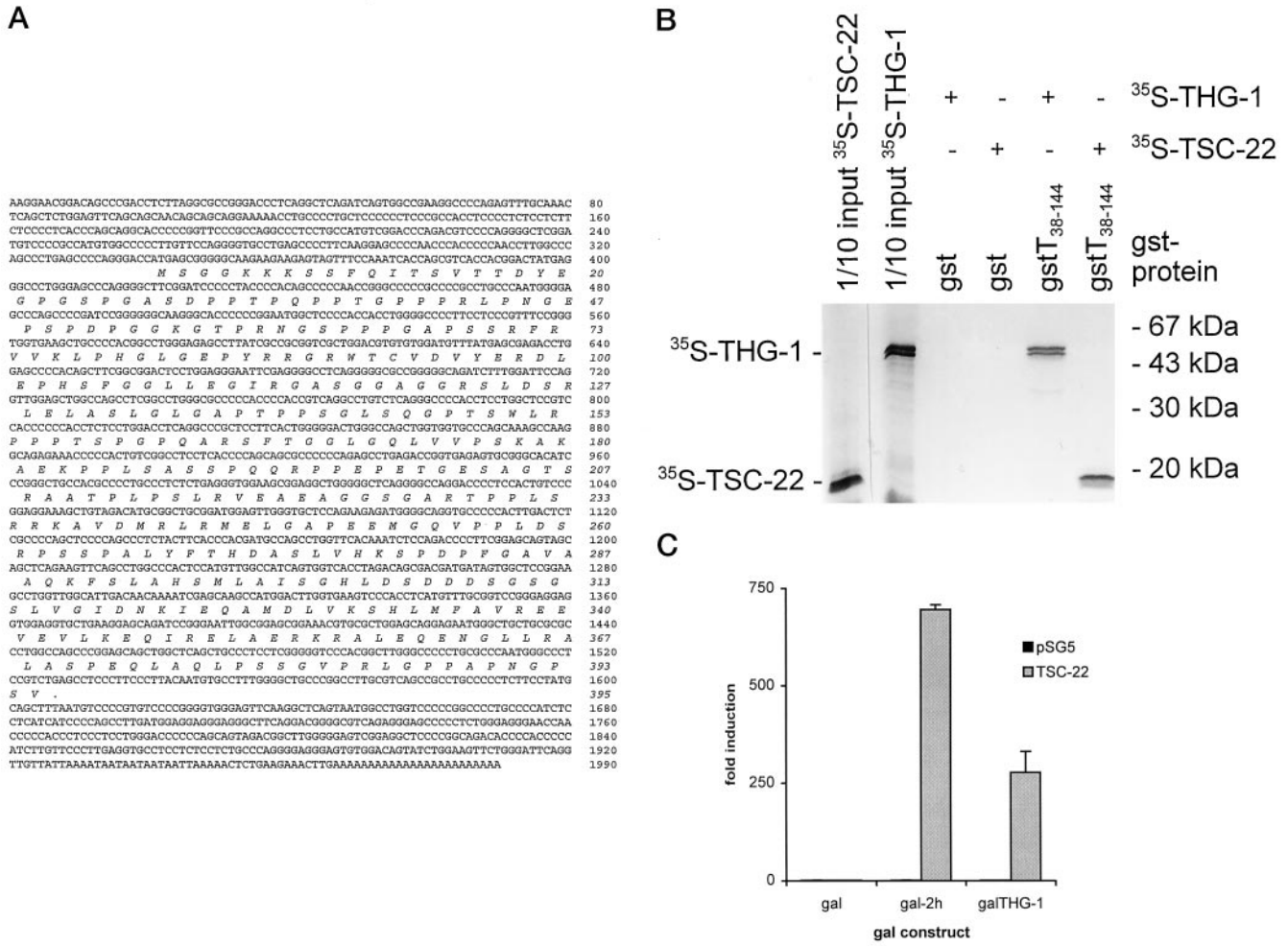

FIG. 3. **TSC-22 forms heterodimers with its homologue THG-1.** *A*, cDNA and protein sequence of THG-1. *B*, GST pull-down assay with GST and gstT_{38-144} on ^{35}S-labeled TSC-22 and THG-1. *C*, mammalian two-hybrid assay with gal-THG-1 constructs (200 ng) and TSC-22 construct vpT_{38-144} or empty vector pSG5 (200 ng) in COS cells. Values from three experiments (in duplicate) were averaged, and fold induction is expressed compared with combinations gal-pSG5, gal-2h-pSG5, or galTHG-1-pSG5. The standard error is also indicated.

2*C*, *lanes 3* and *4*). These appear to be competed by vpT_{38-144}, since in *lanes 5* and *6*, containing vpT_{38-144} in addition to galT_{38-144}, they are hardly or not visible. They do not arise from endogenous TSC-22 bound to galT_{38-144}, since this gives rise to faster migrating complexes (not shown). Possibly, there are other endogenous partners for TSC-22; some of them probably also bind via the leucine zipper.

Shibanuma *et al.* (1) suggested that TSC-22 might be able to bind to members of the AP-1 family. We tried to show interaction with AP-1 family member by means of direct interaction (galT_{38-144} with c-Jun-VP16) or competition (galT_{38-144} interaction with vpT_{38-144} competed by excess amounts of c-Jun, c-Fos, JunD, or JunB) in a mammalian two-hybrid approach or with GST pull-down experiments (c-Jun with gstT_{38-144}), but we never found an indication for interaction (results not shown and Fig. 2*A*). We conclude that TSC-22 can homodimerize, and that there are indications that it can also interact with other proteins, but these do not appear to be members of the AP-1 family.

TSC-22 Forms Heterodimers with Its Homologue THG-1—To try to find such TSC-22 interacting partners, we carried out a yeast two-hybrid screen. As a bait, we used amino acids 38–144 fused to a GAL-DBD in a suitable yeast vector, and screened a mouse brain cDNA library. Upon screening 40×10^6 transformants, we obtained 36 *HIS*$^+$ *LacZ*$^+$ colonies. Upon retransformation in yeast, most of these proved to be false positives. However, six of these initial candidates were fragments from a TSC-22 homologue, which we designated TSC-22 homologous gene-1 (THG-1). All of these clones started at the same amino acid (and therefore were probably derived from the same cDNA) and included the TSC box and the leucine zipper. No other leucine zipper proteins were found in the screen.

The observed interaction between TSC-22 and the THG-1 fragment was confirmed by GST pull-down and gel shift experiments (data not shown). Furthermore, in some cell lines, both THG-1 and TSC-22 are expressed, and, judged by immunofluorescence of COS-1 cells transfected with HA-tagged constructs of these genes, a similar subcellular localization was observed (mostly nuclear; results not shown). This suggests that the endogenous proteins are in a position to interact in mammalian cells; therefore, we cloned full-length THG-1. We identified and sequenced a human expressed sequence tag (EST; see "Experimental Procedures" for details) that contained an insert of 1990 bp (Fig. 3*A*). It contains an open reading frame of 395 amino acids, which includes the TSC box and leucine zipper region of THG-1, and which is preceded by a stop codon. It has a predicted size of 41 kDa, and upon cell-free translation with an expression construct containing this insert, two protein products of approximately 45 kDa are formed (Fig. 3*B*). We do not know the nature of the difference between these two bands; possibly there are kinases active in the cell-free extract, or posttranslational modifications or degradation are occurring. This protein was able to bind specifically in a GST pull-down to

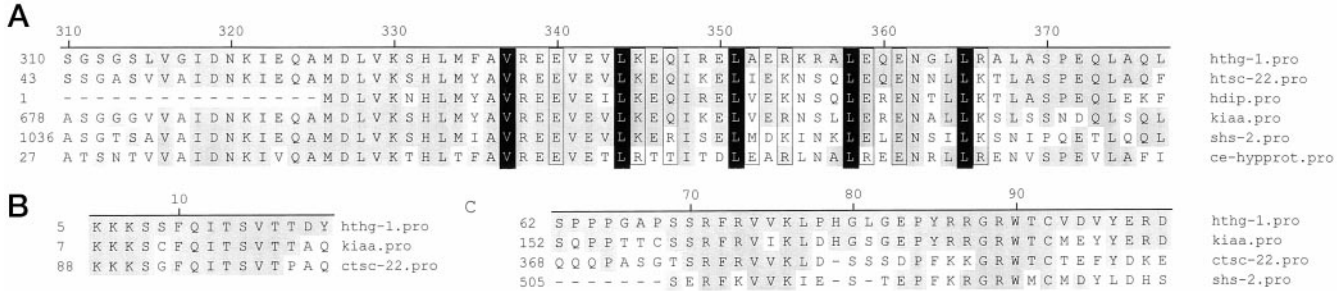

FIG. 4. **Conserved regions of TSC-22 family members.** *A*, alignment of TSC box-leucine zipper region of (*top* to *bottom*): human THG-1, human TSC-22, human DIP, human KIAA0669, *D. melanogaster* shs, and hypothetical protein from *C. elegans* (T18D3.7) (see Footnote 3). *Shaded* residues are residues conserved between THG-1 and family members, *black* residues are the central leucine zipper residues, and *boxed* residues are the amino acids important for the dimerization specificity (29).The *number* at *left* indicates the amino acid at which the alignment starts for the protein of that line. *B*, alignment of homologous region amino acids 5–19 of THG-1 of (*top* to *bottom*): human THG-1, human KIAA0669, and chicken TSC-22. *Shaded* residues are conserved residues between THG-1 and family members. *C*, alignment of homologous region amino acids 62–99 of THG-1 of (*top* to *bottom*): human THG-1, human KIAA0669, chicken TSC-22, and *D. melanogaster* shs. *Shaded* residues are conserved residues between THG-1 and family members.

TSC-22 (Fig. 3*B*). We also made fusion constructs of full-length THG-1 with the GAL-DBD and of GAL-DBD with the THG-1 fragment that interacted with TSC-22 in the yeast two-hybrid screen (gal THG-1 and gal-2h). With these clones we were able to show a strong activation of the 5×GAL-TATA-luc reporter after cotransfection with vpT_{38-144} (the VP16-activation domain-containing TSC-22 construct, Fig. 3*C*). This shows that THG-1 is able to interact with TSC-22 in mammalian cells.

The Dimerization Domain Is Highly Conserved in the TSC-22 Family of Leucine Zipper Proteins—We searched GenBank protein data bases using BLAST software with the TSC-22 and THG-1 protein sequences to find conserved regions and to identify additional family members. Apart from the known family members (TSC-22, THG-1, shs from *D. melanogaster*, human and pig DIP, and its probable mouse ortholog GILZ; Refs. 11, 13, 14, and 25), we also found the uncharacterized human KIAA0669 protein and a hypothetical protein from *Caenorhabditis elegans*, which had high homology in the TSC box-leucine zipper region. We also searched EST data bases with the TSC box-leucine zipper region (amino acids 44–122 of TSC-22), but we could not find evidence for a fifth TSC-22 homologue in mammals. Therefore, at least four mammalian paralogues exist that belong to the TSC-22 family of leucine zipper proteins, and homologues exist even in the distantly related species *D. melanogaster* and *C. elegans*.

An alignment of the TSC box-leucine zipper region of hTSC-22, THG-1, KIAA0669, hDIP, shs-2, and the hypothetical *C. elegans* protein is shown in Fig. 4*A*.[4] The central leucines (and one valine) of the zipper are all conserved (*black* residues). Furthermore, the charge of the amino acids that are important for the dimerization specificity of the leucine zipper (*boxed* residues) are conserved for the mammalian paralogues and shs. This configuration predicts that these proteins can homodimerize, or heterodimerize with any of the other family members (12, 26), which we could indeed show for TSC-22 and THG-1 (Figs. 2 and 3).

The conservation of the domains outside the TSC box-leucine zipper region is very limited between paralogues. For TSC-22, these domains are highly homologous in the chick, mouse, rat, and human orthologues, but they are not conserved in the known paralogues THG-1, KIAA0669, hDIP, or shs, apart from a few amino acids just C-terminal from the leucine zipper (Fig. 4*A*). The N-terminal region of THG-1 does contain two regions with homology to some of its paralogues (Fig. 4, *B* and *C*), although we could not find clear sequence motifs that would give a hint toward the function of these regions. Apparently, the TSC-22 family is a family of leucine zipper proteins with a highly conserved dimerization domain, which is coupled to different N- and C-terminal domains that are only conserved in a limited manner between paralogues.

TSC-22 and THG-1 Repress Transcription When Sequestered to DNA—Ohta *et al.* (3) showed that TSC-22 can bind specifically to DNA *in vitro*. In order to investigate whether some of the domains of TSC-22 may influence transcription, *i.e.* may be activation or repression domains, we tested constructs containing GAL-DBD with different parts of TSC-22 (see Fig. 1). Upon cotransfection with the reporter construct 5×GAL-TATA-luc in COS-1, T47D, or 293 cells, we never found a significant transcriptional activation (results not shown), showing that at least in these cells, no independent activation domain in TSC-22 is active.

Next we tested whether TSC-22 may have transcriptional repressor activity. We tested this on luciferase reporters containing four NFκB-binding sites from the ICAM-promoter (which can thus be activated by the NFκB transcription factor p65) and 5 GAL binding sites (which can thus bind GAL-DBD constructs) in different configurations, since this may influence repressor activity. On the reporter 4×ICAM-5×GAL-TATA-luc (4i5g), we observed a strong repressive effect when galTSC-22 was cotransfected in COS-1 cells (Fig. 5*A*; note that repressor activity is expressed as fold repression, *i.e.* high values mean strong repressor activity), as well as in T47D and 293 cells (data not shown). A TSC-22 expression plasmid lacking the GAL-DBD did not repress this reporter, nor was a 4×ICAM-TATA-luc reporter lacking GAL binding sites repressed by the galTSC-22 (data not shown), indicating that the repression is mediated via the GAL sites in the reporter. On a luciferase reporter in which the distance between the GAL sites and the TATA box was increased by 250 bp compared with 4i5g (4i5g-250), we noticed that galTSC-22 still repressed, but to a lesser extent than on 4i5g (Fig. 4*A*). When we reversed orientation between GAL and ICAM sites, 5×GAL-4×ICAM-TATA-luc (5g4i), repression decreased even more, but was still present (Fig. 4*A*). When we additionally increased the distance between the GAL sites and ICAM-TATA box, 5×GAL-250 bp-4×ICAM-TATA-luc (5g-250–4i), the repressive effect of galTSC-22 was gone (Fig. 4*A*). Apparently, TSC-22 does contain repressor activity when sequestered to DNA, but it is sensitive to promoter architecture, and both distance between repressor and activator/TATA box and orientation of activator and repressor seem to play a role.

[4] The accession numbers of proteins are as follows: human TSC-22, Q15714; human DIP, NP004080; human KIAA0669, BAA31644; *D. melanogaster* shs, AAC41608; hypothetical protein from *C. elegans* (T18D3.7), Q22544; and chicken TSC-22, BAA11565.

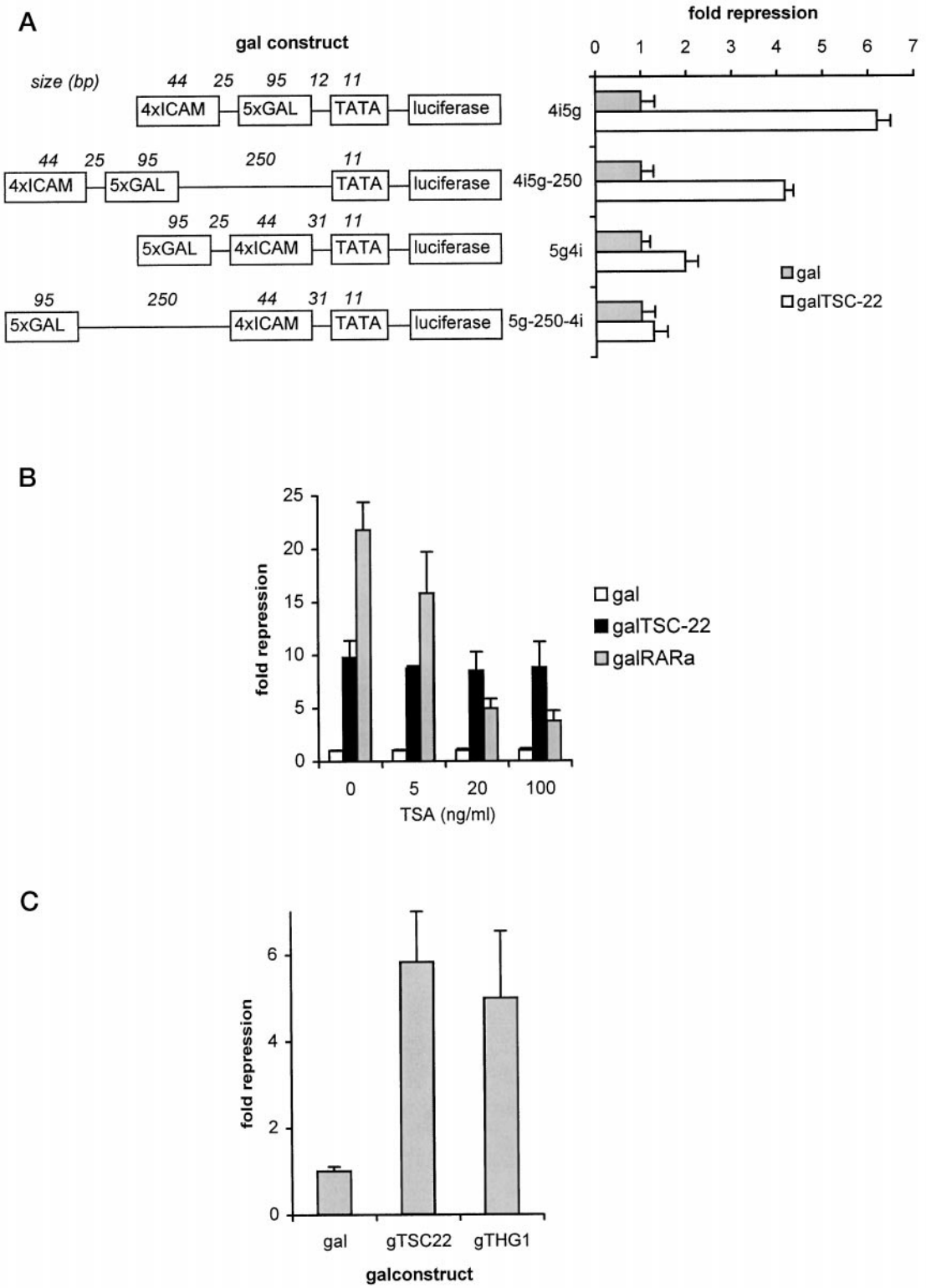

FIG. 5. **Repressor activity of TSC-22 and THG-1.** *A*, fold repression (activity of gal-DBD alone divided by galTSC-22) of galTSC-22 (200 ng) on different promoters in COS-1 cells, with gal-DBD alone set at 1. Values from three independent experiments (in duplicate) were normalized and averaged; the standard error is also indicated. At *left* is a schematic of the organization of the promoter elements in the different reporters used (with size in bp of the elements in *italics*). *B*, effect of TSA on repressor activity of galTSC-22 and full-length galRARα in COS-1 cells on the 4i5g reporter. Values from three experiments (in duplicate) were normalized and averaged, and gal-DBD alone was set at 1 for each concentration TSA. *C*, fold repression of galTHG-1 and galTSC-22 constructs (200 ng) on the 4×ICAM-5×GAL-TATA-luc reporter. Values from three experiments (in duplicate) were normalized and averaged, and gal-DBD alone was set at 1.

Many transcription factors, like unliganded retinoic acid receptor α (RARα), repress transcription through histone deacetylase-containing complexes, which can be inhibited by trichostatin A (TSA; Refs. 27–29). We wanted to test whether TSC-22-mediated repression also acts through such a complex, and could be inhibited by TSA. As a positive control, we tested a GAL-DBD construct containing full-length RARα (galRARα), which strongly represses the 4i5g reporter. This repression is

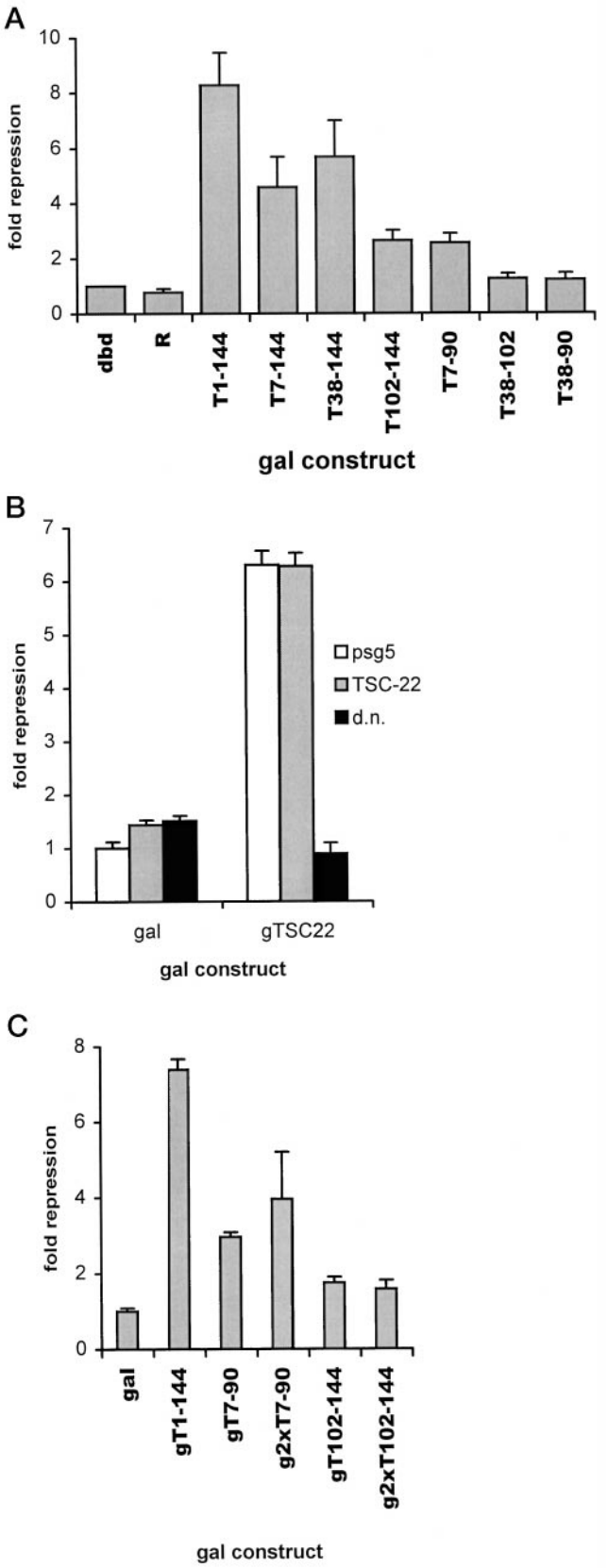

FIG. 6. **Role of different domains in repressor activity of TSC-22 in COS-cells.** *A*, fold repression of different galTSC-22 deletion constructs (20 ng) on the 4i5g reporter in COS-1 cells. gal-DBD alone was set at 1. Values are the mean of at least four independent experiments (in duplicate), the standard error is also indicated. *dbd*, gal-DBD alone; *R*, galRARβ-EF (transcriptionally inactive control; GAL-DBD fused to truncated RARβ); *numbers* on the *X* axis designate the different galTSC-22 deletion clones used. *B*, effect of overexpression of TSC-22 and tagT_{38-102} expression plasmids (200 ng) on repressor activity of galTSC-22 (200 ng) on the 4i5g reporter in COS-1 cells. Values from five experiments (in duplicate) were normalized and averaged, and activity of gal-DBD alone with pSG5 was set at 1. *C*, effect of artificial dimerization of RD1 and RD2 on repressor activity on the 4i5g reporter in COS-1 cells. 200 ng of gal-DBD expression vector used, values from four experiments (in duplicate) were normalized and averaged, and gal-DBD alone was set at 1.

suppressed by the histone deacetylase inhibitor TSA, in contrast to galTSC-22-mediated repression (Fig. 5*B*). These data therefore suggest that TSC-22 does not repress through histone deacetylase-containing complexes, but instead uses a distinct mechanism.

Next we tested whether THG-1 has a similar transcriptional activity as TSC-22. In cotransfections of galTHG-1 with the reporter construct 5×GAL-TATA-luc in COS-1, we did not find a significant transcriptional activation (results not shown); however, on the 4i5g reporter, we found a strong repressor activity of galTHG-1, comparable to that of galTSC-22 (Fig. 5*C*). Apparently, both TSC-22 and THG-1 have repressor activity and may contain independent repression domains.

TSC-22 Contains Independent Repression Domains in the Non-conserved Regions That Are Enhanced by the Dimerization Domain—We tested different galTSC-22 deletion constructs on the 4i5g reporter, to see whether TSC-22 contains separate repression domains (Fig. 6*A*). First we checked whether size of the fusion protein mattered, but this did not seem to be the case; the control galRARβ-EF (which misses binding sites for coactivators and corepressors and is therefore transcriptionally inactive) contains many more amino acids (204 amino acids fused to GAL-DBD) than $galT_{1-144}$ or its deletion constructs (at most 144 amino acids fused to GAL-DBD) but galRARβ-EF does not repress. Furthermore, we verified in gel shifts whether all constructs were expressed properly, and therefore differences in repressor activity could not be explained by differences in expression levels (data not shown). In COS-cells, only galTSC-22 deletion constructs $galT_{38-102}$ and $galT_{38-90}$, which lack the N- and C-terminal domains, showed no repression. It should be noted that the leucine zipper in $galT_{38-102}$ is still intact. Mutation of either the N- or the C-terminal domain also significantly reduces repressor activity ($galT_{38-144}$ *versus* $galT_{38-102}$ and $galT_{1-144}$ *versus* $galT_{38-144}$ or $galT_{7-144}$, in which the deletion of the first six amino acids already interferes with the function of the first repression domain). Since these two regions contain repressing activity, we designated them repression domain 1 and 2 (RD1 and RD2).

Although the TSC box leucine zipper region, the dimerization domain, is not sufficient for repression, it apparently plays a role, since adding this domain to the C-terminal repression domain increases the repression ($galT_{102-144}$ *versus* $galT_{38-144}$, Fig. 4*C*). When we interfere with the dimerization of $galT_{1-144}$ by overexpressing the deletion protein $tagT_{38-102}$ (which contains the TSC box and leucine zipper region but not RD1 and RD2 and therefore can homodimerize but not repress), the repressor activity of $galT_{1-144}$ is abolished (Fig. 6*B*), indicating an important function for this domain. With full-length TSC-22, the repressor activity is not influenced at all (Fig. 6*B*). This suggests that full-length TSC-22 homodimers have repressor activity, but if the repression domains of one of the partners are deleted, this repressor activity is strongly reduced.

However, constructs in which the repression domains are dimerized artificially (two copies of RD1 or RD2 in frame behind the GAL-DBD, designated $gal2{\times}T_{7-90}$ and $gal2{\times}T_{102-144}$, respectively) are not or hardly more efficient in repressing the 4i5g reporter than their single counterparts (Fig. 6*C*). This argues against the hypothesis that the enhancing activity of

the dimerization domain on the repressor activity is solely due to dimerization. Possibly, the TSC box-leucine zipper may have multiple roles in enhancing the repression domains, which are all necessary to enhance repressor activity. In conclusion, the repressor activity of TSC-22 resides in the N- and C-terminally located repression domains, and is enhanced by the centrally located dimerization domain, but this is probably not mediated solely through its dimerizing properties.

DISCUSSION

TSC-22 Is a Dimerizing Protein but Does Not Act on AP-1 Transcription Factors—It has been suggested that TSC-22 might be a repressor of AP-1 family members (1). Its mode of action would be similar to that of basic helix-loop-helix protein Id or leucine zipper protein CHOP; TSC-22 might interact with AP-1 family members and inhibit their DNA binding, and in this way repress the function of these transcription factors (30, 31). However, we did not find any leucine zipper protein other than TSC-22 or its homologue that binds to TSC-22, either in directed screening using GST pull-down or in mammalian two-hybrid screening, or in random screening using the yeast two-hybrid system. Therefore, we do not find any evidence for this hypothesis. Instead, we show that TSC-22 dimerizes with its family members, which strongly suggests that the endogenous protein will do likewise. These data suggest that TSC-22 acts in an autonomous fashion, and not by inhibiting DNA binding of AP-1 transcription factors through dimerization.

The Repressor Activity of TSC-22—The repressor activity of many repressors, like nuclear hormone receptors Mad or Rb, acts through histone deacetylase-containing complexes and can be inhibited by the histone deacetylase inhibitor TSA (27–29, 32–37). The activity of TSC-22 is not inhibited by this compound, indicating that TSC-22 does not repress transcription through this machinery. Furthermore, the TSC-22 repressor activity is remarkable in that it is very sensitive to promoter architecture, possibly due to distance and orientation effects.

The role of the dimerization domain is quite complex. It has an enhancing role on the repressor activity, but does not actively repress itself. Possibly, these repression domains act most strongly in a dimeric configuration. This also explains a remarkable observation made in the mammalian two-hybrid assays that the full-length TSC-22 constructs only weakly activate transcription, while deleting a repression domain in only one of the two partners strongly increases activation.[5] Note that an exogenous activation domain is added to one of the partners in this assay, hence the activation. However, only dimerization appears not to be sufficient to enhance the activity of the repression domains. Possibly, the dimerization domain has a second role, like inducing a conformational change, that is also needed to enhance repressor activity.

TSC-22 Is a Member of a Family of Interacting Leucine Zipper Proteins—In this paper, we show that TSC-22 can dimerize and repress transcription when sequestered to DNA. Ohta *et al.* (3) showed that TSC-22 can bind to a specific DNA-sequence, while Shibanuma *et al.* (1) reported a nuclear localization of TSC-22. Apparently, TSC-22 is a repressive transcription factor. Here we also show that the homologue THG-1 protein interacts with TSC-22, has repressor activity, and a truncated THG-1 is detected in the nucleus in COS-1 cells.[4] This therefore suggests that not only TSC-22 is a repressive transcription factor, but also some of its family members, including THG-1.

The central region, consisting of the TSC box and leucine zipper, is highly conserved between TSC-22 and its homologues THG-1, KIAA0669, DIP, and shs. This region appears to be involved in directing the protein to the appropriate intracellular compartment (mostly nuclear in COS-1 cells),[5] and is crucial in homodimerization of TSC-22. The isolation of THG-1 as a TSC-22-interacting protein is therefore not unexpected. Both TSC-22 and THG-1 can homo- and heterodimerize with each other. Recently, it was reported that a peptide derived from porcine DIP was also able to homodimerize (15). Therefore, at least three of the five family members identified so far are able to homodimerize, and at least two of them are able to bind to each other. The residues that are theoretically important for dimerization specificity are all conserved, and it would not be surprising if all of the family members interact with one another.

Functional Consequences of TSC-22 Family Member Interactions—The repression domains of TSC-22 identified in this paper are not conserved between family members. Frequently, no clear sequence similarity exists between repression domains of different repressors (38). This may be the case for the THG-1 and TSC-22 repression domains, although both contain regions that are rich in prolines (which is often found in repression domains, 38). For one or more of the other family members, it is, however, possible that they contain domains with totally different functions, *e.g.* transcriptional activation domains. This would add additional possibilities for regulating transcription, depending on the family members expressed in a specific cell. In line with this thinking, overexpression of solely the dimerization domain of TSC-22 severely reduces the repressor activity, indicating that dimerization partner may be important for TSC-22 repressor activity. Consequently, it is possible that, upon binding another partner, TSC-22 changes from a repressor into the silent partner of a positive acting complex. Therefore, for determining the function of TSC-22, it is crucial to investigate the function of the family members, since all of these may be able to interact with TSC-22 and may therefore influence its activity.

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Transforming Growth Factor-β-stimulated Clone-22 Is a Member of a Family of Leucine Zipper Proteins That Can Homo- and Heterodimerize and Has Transcriptional Repressor Activity

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