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Characterization of a Novel Mammalian Groucho Isoform and Its Role in Transcriptional Regulation*

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The Wnt/β-catenin/Tcf pathway serves important functions in embryonic development and is constitutively activated in human colorectal cancer. The nuclear output of Wnt signaling is mediated by a complex between DNA-binding proteins of the TCF family and the transcriptional coactivator β-catenin. Groucho proteins act to repress transcriptional activation by β-catenin-Tcf complexes, probably by interacting directly with Tcf transcription factors. We have identified several splice forms of the mouse Groucho *Grg1* gene expressed in the developing intestine. Prominent among these is a novel and abundant isoform, Grg1-S, which we characterize in this report. Grg1-S has highest homology with the TLE family of large Groucho proteins but features only the amino-terminal Q and glycine- and proline-rich domains typical of the Groucho/AES subfamily. Grg1-S is expressed in development and in several adult mouse tissues. Expression in the adult small intestine is highest at the base of the crypts of Lieberkuhn. Grg1-S acts to antagonize β-catenin activity in *Xenopus* axis duplication and luciferase reporter assays in mammalian cells. Taken together, these findings suggest that Grg1-S may operate in conjunction with β-catenin and Tcf factors to regulate vertebrate gut epithelial cell differentiation.

Wnt signaling regulates many developmental processes, including embryonic polarity and cell fate specification, by virtue of distinct signal transduction mechanisms (1, 2). In the absence of Wnt signals, the multifunctional protein β-catenin is continually phosphorylated and thereby targeted for degradation. Activation of the canonical Wnt pathway retards β-catenin turnover and makes it available to interact with Tcf/LEF transcription factors and transactivate target genes. Two principal modes of regulation are recognized for Tcf-dependent genes as follows: the first controls β-catenin stability and, consequently, its intracellular concentration; the second modulates their expression at the level of DNA-protein complexes (3).

Tcf/LEF proteins are bipartite factors that bind DNA and may act as either transcriptional repressors or activators depending on alternative protein associations (4). Transcriptional activation is driven in part by β-catenin, whereas repression is mediated by corepressors that may interact either with β-catenin (5–7) or directly with Tcf proteins (8–11). An important class of Tcf corepressors belongs to the Groucho protein family (12–14), which includes several highly conserved members. Groucho proteins were originally implicated in *Drosophila* neuronal and sex determination (15, 16) and are classified into two subgroups. The first group, designated Groucho/transducin-like enhancer-of-split (TLE),[1] contains the five domains illustrated in Fig. 1*A*. Two of these, the amino-terminal, glutamine-rich Q domain and a carboxyl-terminal string of tandem WD40 repeats, are highly conserved and are separated by a variable region including glycine- and proline-rich (GP), casein kinase II and cdc2 phosphorylation sites, nuclear localization sequence (CcN), and serine- and proline-rich (SP) domains. Several distinct mammalian genes are recognized and are named transducin-like enhancer (TLE) in man or Groucho-related gene (Grg) in the mouse. The second group, designated amino-terminal enhancer-of-split (AES), contains the products of distinct gene loci and harbors only Q and GP domains. Besides a difference in protein size, the two Groucho subgroups share limited conservation within the GP domain and carboxyl-terminal portions of the Q domain. The mechanism by which Groucho proteins repress Tcf-dependent genes is poorly understood and may involve association with histone deacetylases (16, 17). Besides the Tcf/LEF family, Groucho proteins also serve as corepressors for many other transcription factors (15).

The role of Tcf/β-catenin gene regulation is well established in human colorectal cancer (18–20) and is increasingly appreciated in development and homeostasis of the vertebrate intestine. Sox 17, an early regulator of vertebrate endoderm differentiation, interacts physically with β-catenin to repress Wnt signaling (21), and Tcf4 knockout mice have severely reduced cell proliferation in prospective crypts in the small intestine mucosa (22). Although such observations contribute toward an emerging picture of Wnt signaling in normal and malignant gut epithelial differentiation, the place of Groucho proteins in this scheme has been explored in much less detail. Characterizing the spectrum of Groucho proteins expressed in the developing intestine is one prerequisite to this end. Additionally, accumulating evidence highlights the considerable extent to which Tcf/β-catenin gene regulation makes physiologic use of mRNA splicing variants. All Tcf genes encode a large coterie of complex variants (3) that may regulate pathway functions either subtly (23) or profoundly. In intestinal crypts Tcf4 stimu-

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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) AY155195–155200.

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[1] The abbreviations used are: TLE, transducin-like enhancer-of-split; GP, glycine- and proline-rich; SP, serine- and proline-rich; Grg, Groucho-related gene; AES, amino-terminal enhancer-of-split; RT, reverse transcriptase; HA, hemagglutinin; UTR, untranslated region; ORF, open reading frame; PBS, phosphate-buffered saline; dpc, days post-coitum.

lates endogenous expression of a dominant negative form of Tcf1 that provides negative feedback for β-catenin gene regulation (24), whereas in colon tumors it preferentially activates a full-length LEF1 for apparent positive feedback (25).

To identify Groucho family members expressed in the fetal gut, we used degenerate PCR cloning, followed by screening a developing mouse gut cDNA library, and we isolated multiple independent isoforms encoded by the mouse *Grg1* gene. The variety of spliced forms suggests the possibility of complex transcriptional regulation by *Grg1*. One novel Grg1 variant, which is expressed abundantly and designated Grg1-S, defines a third subgroup within the Groucho protein family. Grg1-S acts to repress β-catenin/Tcf-mediated gene activation *in vitro* and *in vivo*. It is expressed in the developing gut and in the adult small intestine, where it is most highly detected near the base of the crypts of Lieberkuhn. These findings define a new class of active Groucho proteins and suggest that Grg1-S may be a physiologic regulator of Tcf-dependent and other genetic pathways.

EXPERIMENTAL PROCEDURES

Cloning of Grg1 mRNA Species—A 442-bp fragment was first obtained by RT-PCR on reverse-transcribed mRNA from 14.5 dpc mouse small intestine, using degenerate primers corresponding to the conserved WD domain of Groucho proteins, 5′-CARATGCARCCNGTNCCNTTYCCN-3′ and 5′-TCCCANACNGCRATRTTNCCRTC-3′. A search against the Celera mouse genome data base pointed to errors in the mGrg1 sequence deposited in GenBank™ (GenBank™ accession number gi: 6755802) and indicated 100% match with the *mGrg1* gene locus; the deduced peptide sequence is identical to that of human TLE1 (GenBank™ accession number gi: 13640384). A 193-bp subfragment (probe 193), amplified using the PCR primers 5′-TAAGGTGTGGGACATCAGCC-3′ and 5′-TGTCAGCTCTGCCTTTATGC-3′, was used to screen a 13.5-dpc mouse gut cDNA library (26) and to recover one clone containing part of the open reading frame and the 3′-UTR (Fig. 1*A*). We extended this clone in the 5′-direction by RT-PCR and then used a 250-bp fragment derived from its 5′ terminus to screen the same fetal gut cDNA library and isolate Grg1-LΔGPWD and the full-length Grg1-S clones (Fig. 1*A*). Grg1-S and previously identified Grg1 transcripts are identical in the short portion of the 5′-UTR that is described for Grg1-L (GenBank™ accession number gi: 6755802). A primer specific to this UTR (5′-GGAGGATAGAGCTATCCCG) was then used in conjunction with one complementary to the 3′-UTR of Grg1-L (5′-GACAGGCAGCAGGTAGCTCC-3′) for RT-PCR on reverse-transcribed mRNA from the 14.5-dpc mouse gut. Several amplified fragments hybridized with probe 193 on Southern analysis (Fig. 3*C*), and each of these RT-PCR products was subcloned into the PCR2.1 vector (Invitrogen) and sequenced. This led us to identify the presumptive full-length Grg1-L transcript and three splice variants of differing relative abundance, which we designate Grg1-LΔWD, Grg1-LΔGP, and Grg1-LΔLZ2 according to the missing subdomains.

Plasmid Expression Constructs—The open reading frame (ORF) of Grg1-L was subcloned by PCR into the *Bam*HI and *Sac*II sites of pCDNA3 (Invitrogen) in-frame with a carboxyl-terminal Myc epitope using primers 5′-TCGGGATCCATGTTCCCGCAGAGCCGC-3′ and 5′-GAACCGCGGGTAGATGACCTCATAAACGGTAG-3′. The Grg1-S ORF was cloned into pCDNA3 using the reverse primer 5′- GAACCGCGGATCTGGCTTGCCCGGCCTC-3′ and the same forward primer, and into the *Hin*dIII and *Not*I sites of the expression plasmid pMH (Roche Molecular Biochemicals) in-frame with a carboxyl-terminal hemagglutinin (HA) epitope using a similar PCR strategy.

mRNA Expression Analysis—Total RNA was extracted from whole mouse embryos or from the colon, jejunum, ileum, duodenum, and stomach of adult and 14.5-dpc fetal mice using Trizol reagent (Invitrogen). 30 μg of RNA from each source was resolved on denaturing formaldehyde gels, transferred onto Hybond N nylon membranes (Amersham Biosciences), and hybridized in Church buffer (27) at 65 °C with the following probes (Fig. 1*A*): 3′Grg1-L (3′-UTR of Grg1-L generated by restriction digestion from a cDNA library-derived clone), 3′Grg1-S (3′-UTR specific to Grg1-S, generated by PCR with the primers GATAACAGGAGCTGTCTGTC and CATTGCACATTCCTAATACAGTG), and 5′Grg1, which is common to both Grg1-S and Grg1-L in the amino-terminal coding region. Adult mouse tissues were evaluated on a commercial blot (Clontech). Membranes were washed to a final stringency of 0.1× SSC and 0.1% SDS for 30 min at 65 °C and exposed to autoradiography film. For *in situ* hybridization, antisense RNA probes were labeled with digoxigenin or, in independent experiments, with ^{35}S-UTP, and hybridizations were performed as described previously (26, 28).

Transfections and Luciferase Reporter Assays—293 cells were grown in Dulbecco's modified Eagle's medium (Invitrogen) supplemented with 10% fetal bovine serum (HyClone) and seeded in 12-well tissue culture plates at a density of 10^5 cells/well. The next day cells were transfected over 5 h using LipofectAMINE reagent (Invitrogen) and 2.12 μg of plasmid DNA as follows: 100 ng of the reporter OT (containing 3 Tcf consensus binding sites upstream of firefly luciferase cDNA) or OF (mutated in the Tcf-binding site), 800 ng of XTcf3 or hTcf4, 200 ng of β-catenin, 20 ng of pRl-TK *Renilla* as an internal transfection control, and 0.2–1 μg of Grg1-S and/or Grg1-L, always completed to 1 μg with the empty vector if necessary. Cells were harvested 36 h later in passive lysis buffer (Promega), and the luciferase activity was monitored using a dual luciferase assay system (Promega).

Immunofluorescence—COS-7 cells were seeded at 10^5/ml on chamber slides (Nalge Nunc International) and transfected with Myc epitope-tagged forms of either Grg1-S or Grg1-L using LipofectAMINE (Invitrogen) for 5 h. 24 h later cells were rinsed in phosphate-buffered saline (PBS), fixed in 3% paraformaldehyde, permeabilized with 0.1% Triton X-100, and treated with 50 mM NH_4Cl. After a blocking step in PBS supplemented with 10% each of goat and fetal bovine sera, slides were incubated sequentially with 2 μg/ml purified anti-Myc antibody (Clone 9E10, Roche Molecular Biochemicals) diluted in PBS, 5% fetal calf serum, and with 0.5 μg/ml fluorescein isothiocyanate-conjugated goat anti-mouse IgG (Transduction Laboratories) for 1 h each. Nuclei were stained with 4,6-diamidino-2-phenylindole.

Coimmunoprecipitation—COS-7 cells were seeded at 10^5/ml and transfected with either HA epitope-tagged Grg1-S and/or Myc epitope-tagged Grg1-L over 5 h using LipofectAMINE. 300 μl of cell lysates, prepared as described previously (14), were cleared by centrifugation and incubated overnight at 4 °C with 50 μl of either anti-HA (clone 12AC5) or anti-Myc (clone 9E10) hybridoma supernatants, followed by 50 μl of protein G-agarose beads (Sigma) for 3 h. Beads were washed 3 times in 1 ml of lysis buffer, and bound proteins were analyzed by SDS-PAGE and immunoblotting according to standard procedures (29).

Xenopus Axis Duplication Assay—Capped mRNAs were synthesized *in vitro* using linearized plasmid templates and the mMESSAGE mMACHINE kit (Ambion, Austin, TX). *Xenopus* embryos were collected, fertilized, cultured, and staged as described previously (30). Synthetic mRNAs (4.6 nl) were injected in the equatorial region of one blastomere on the prospective ventral side at the 4-cell stage. Embryos were incubated at 19 °C until untreated sibling tadpoles reached Nieuwkoop-Faber stage 39, fixed overnight in 0.1 M MOPS, 2 mM EGTA, 1 mM $MgSD_4$ $7H_2O$, 3.7% formaldehyde (30), scored for axis duplication, and stored in ethanol. Uniform criteria were applied to distinguish between partial and complete axis duplications as described in the legend to Fig. 6.

Accession Numbers—All cDNA sequences are deposited in GenBank™, with accession numbers AY155195 to AY155200.

RESULTS

The Mouse Grg1 Gene Encodes Multiple Splice Variants—Groucho proteins function as corepressors of several transcription factors, including those of the Tcf family (12, 13) and may hence regulate gut epithelial differentiation. To identify Groucho family genes expressed in the developing gut, we used a combination of cDNA library screening and RT-PCR on mRNA harvested from the 14.5 dpc mouse small intestine. Several distinct transcripts (Fig. 1*A*, for details see "Experimental Procedures") share nearly identical sequences, implying that they are mRNA splicing variants of the same gene. Based on data base searches, we conclude that each of these transcripts is encoded by the mouse *Grg1* gene, and we have named them accordingly (Fig. 1*A*). Grg1-L (for "long") encodes the longest protein containing all the typical Groucho/TLE domains, whereas four other transcripts correspond to alternative splice forms that are predicted to encode variant Groucho/TLE proteins. Because the latter species were isolated by RT-PCR and likely comigrate with the full-length Grg1-L mRNA on Northern analysis, their precise abundance and biological significance are presently unclear.

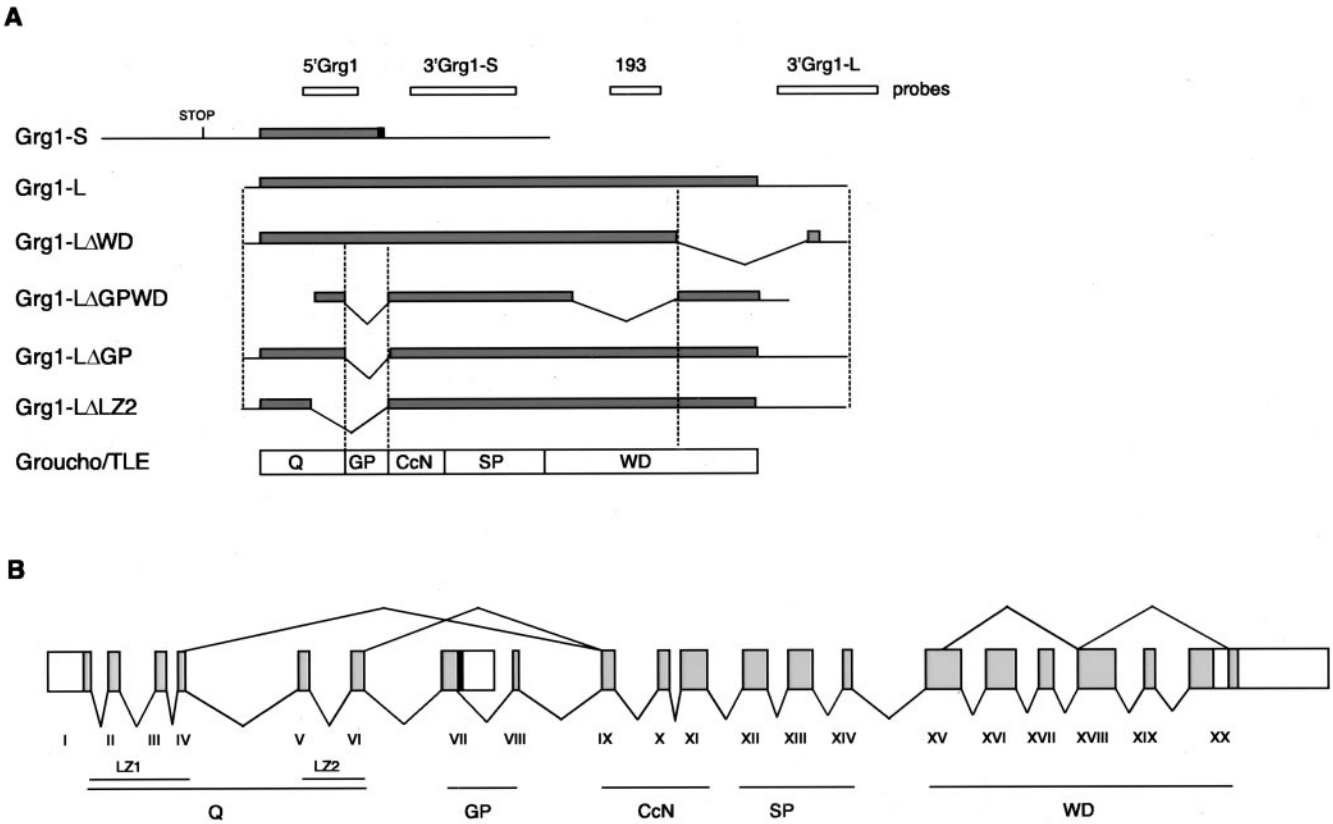
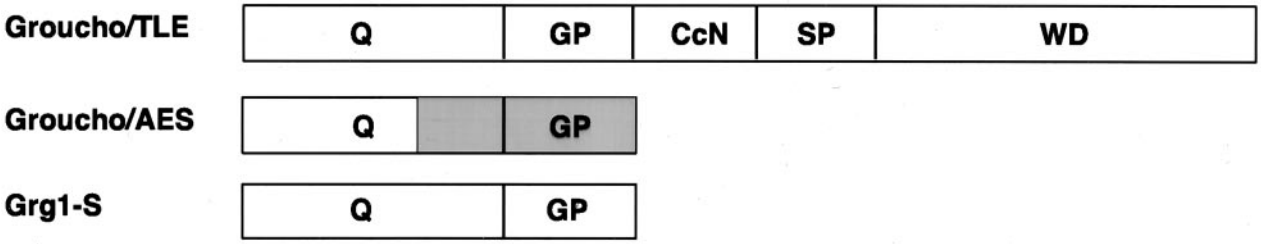


FIG. 1. **The mouse *Grg1* gene encodes multiple splice variants.** *A*, schema of *mGrg1* transcripts isolated from a fetal gut cDNA library (Grg1-LΔGPWD and Grg1S) and by RT-PCR cloning (Grg1-L, Grg1-LΔWD, Grg1-LΔGP, and Grg1-LΔLZ2). Open reading frames are depicted by *gray boxes* and are positioned relative to the characterized Groucho/TLE subdomains. Grg1-L encodes a 770-amino acid protein that contains all five typical domains; Grg1-LΔLZ2 encodes a 650-residue protein missing the second leucine zipper and the GP domain; Grg1-LΔGP (696 amino acids) lacks only the GP domain; and the predicted 653-residue Grg1-LΔWD protein carries a deletion of the last 122 amino acids in the WD domain. Clone Grg1-LΔGPWD, which is missing a 5′ end, features the following two internal splices: one removing the GP domain and the second removing amino acids 479–660 from the WD domain. All positions are numbered relative to the Grg1-L peptide sequence. Grg1-S is a 2063-bp transcript that features a unique 3′-UTR and a longer 5′-UTR and encodes a 199-residue protein with the same amino terminus as the other variants. The probes 3′Grg1-L, 3′Grg1-S, 5′Grg1, and 193 used for Northern or Southern analysis are also represented (*white boxes*). *B*, inferred organization of the *mGrg1* gene locus. *Gray boxes* correspond to coding regions, *white boxes* to untranslated regions, and the *lines* to distinct splice positions. Exons 1–4 encode the first 78 Q domain residues, including the first leucine zipper motif; exons 5 and 6 encode the second leucine zipper; exons 7 and 8 encode the GP domain, and the intervening intron is included in the *Grg1-S* 3′-UTR. The *black box* represents the last 8 amino acids of Grg1-S, which are not present in Grg1-L. The CcN, SP, and WD domains are encoded by exons 9–11, 12–14, and 15–20, respectively.

In contrast, one transcript, isolated as a 2063-bp full-length clone from a mouse fetal gut cDNA library, encodes a short protein containing Q and GP domains that are identical to those of Grg1-L. This species, which we designate Grg1-S (for "short"), contains a long 5′-UTR including an in-frame stop codon and is characterized by a novel 3′-UTR that restricts the ORF to only the Q and GP domains. A search of the mouse genome data base (www.celera.com) with Grg1-S and Grg1-L sequences allowed us to infer the structure of the mouse *Grg1* locus on chromosome 4 and the origins of each of the different splice forms (Fig. 1*B*). The gene spans ~100 kb and is composed of at least 20 exons, the first 7 of which produce Grg1-S. Reported Grg1-L clones are up to 2 kb shorter than the 4.3-kb length predicted by Northern analysis, indicating that a large portion of the 5′-UTR remains uncharacterized. Moreover, oligonucleotides from the 3′ end of Grg1-L and position 443 in the 5′-UTR of Grg1-S fail to amplify a product by RT-PCR, whereas in combination with other oligonucleotides, the reverse primer for this experiment readily amplified the complete Grg1-L open reading frame depicted in Fig. 1*A*. We hence suspect that Grg1-L results from use of an alternative, noncoding first exon.

mGrg1-L shares highest homology with human TLE1, showing 87.5% nucleotide and 95.6% amino acid identity, which suggests that these genes may be orthologous. In comparing mGrg1-S with the human genome sequence, we identified two related loci, one of which corresponds to the *TLE1* gene on chromosome 9 and reveals exon-intron organization similar to that described for *mGrg1* (Fig. 1*B*). The second locus, which is on the X chromosome (PAC 323B6: emb Z83841), shows 87.4% nucleotide identity, lacks introns, and with an ORF disrupted by a stop codon, most likely represents a pseudogene. By using oligonucleotides complementary to the 3′-UTR of a presumptive human Grg1-S mRNA species, we obtained an RT-PCR product from the 293 human cell line, and DNA sequencing confirms that it corresponds to a human Grg1-S transcript. In Northern analysis of human cell lines, this product recognizes a single band (data not shown). Thus, the human *TLE1* locus also enables transcription of Grg1-S. High conservation between mouse and man within the ORF and the entire 3′-UTR suggests an important role for this newly identified mRNA.

Grg1-S Defines a New Subtype of Groucho Proteins—Groucho proteins are divided broadly into two subgroups, which are the products of distinct genes. Groucho/TLE proteins such as Grg1-L feature five recognized domains, whereas Groucho/AES proteins contain only two domains related to Q and GP (Fig. 2). The AES group shares less homology with the TLE group in the second half of the Q domain and in the GP domain, and the implication that these subfamilies thus serve distinct cellular functions has received experimental support (13, 31). In contrast, Grg1-S is nearly identical to the entire NH_2 terminus of

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Grg1-S -------MFPQSRHPTPHQAAGQPFKFTIPESLDRIKEEFQFLQAQYHSLKLECEKLASEKTEMQRHYVMYYEMSYGLNIEMHKQTEIAK 83
Grg1-L -------MFPQSRHPTPHQAAGQPFKFTIPESLDRIKEEFQFLQAQYHSLKLECGKLASEKTEMQRHYVMYYEMSYGLNIEMHKQTEIAK 83
TLE1   -------MFPQSRHPTPHQAAGQPFKFTIPESLDRIKEEFQFLQAQYHSLKLECEKLASEKTEMQRHYVMYYEMSYGLNIEMHKQTEIAK 83
XGrg4  MIRDLSKMYPQTRHPAAPHQPAQPFKFTISESCDRIKEEFQFLQAQYHSLKLECEKLASEKTEMQRHYVMYYEMSYGLDIEMHKQAEIVK 90
mAES1  M-GGAGRRSPSCPRRASSHLPQQ-LKFTTSDSCDRIKDEFQLLQAQYHSLKLECDKLASEKSEMQRHYVMYYEMSYGLNIEMHKQAEIVK 132
XGrg5  MMFPQS----SSRHSGSSHMPQQ-LKFTTSDSCDRIKDEFQLLQAQYHSLKLECDKLAGEKSEMQRHYVMYYEMSYGLNIEMHKQAEIVK 85

Grg1-S RLNTICAQVIPFLSQEHQQQVAQAVERAKQVTMAELNAIIGQQ-LQAQHLSHGHGPPVPLTPHPSGLQPPGIPPLGGSASLLALSSALSG 172
Grg1-L RLNTICAQVIPFLSQEHQQQVAQAVERAKQVTMAELNAIIGQQQLQAQHLSHGHGPPVPLTPHPSGLQPPGIPPLGGSASLLALSSALSG 173
TLE1   RLNTICAQVIPFLSQEHQQQVAQAVERAKQVTMAELNAIIGQQQLQAQHLSHGHGPPVPLTPHPSGLQPPGIPPLGGSAGLLALSSALSG 173
XGrg4  RLNAICAQVIPFLSQEHQQQVVQAVERAKQVTMAELNAIIGQQQLQAQHLSHAHGLPVPLTPHPSGLQPPAIPSIGSSAGLLALSSALGG 180
mAES1  RLNGICAQVLPYLSQEHQQQVLGAIERAKQVTAPELNSII-RQQLQAHQLSQLQALALPLTPLPVGLQPPSIPAVSAGTGLISLS-ALGS 220
XGrg5  RLHGICAQVLPYLSQEHQQQVLGAIERAKQVTAPELNSII-RQ-LQVHQLSQLQALALPLTSLPMGLQAPSIP-ISASSGLISLS-ALGS 171

Grg1-S QSHLAIKDDKKHHDAERHRGERPGKPD. 200
Grg1-L QSHLAIKDDKKHHDAERHRDREPGTSNSLLVPDSLRS---- 210
TLE1   QSHLAIKDDKKHHDAEHHRDREPGTSNSLLVPDSLRG---- 210
XGrg4  QSHIPIKDEKKHHDSDHQRDRDSIKSSSVSPSASFRA---- 217
mAES1  QIHIS-KEDKNGHDGDTIQEDDGEKSD. 247
XGrg5  QGHLP-KEDKNGHEGDKRPDDDGDKSD. 198
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FIG. 2. **Grg1-S defines an additional subgroup in the Groucho protein family.** Schematic representation of the three Groucho protein subgroups, with *clear areas* showing highly conserved regions and *gray shading* indicating areas less conserved between the AES and TLE subfamilies. The Q and GP domains of Grg1-S and Grg1-L are identical, as shown in the sequence alignment between assorted proteins of the Groucho/TLE family, XGrg4 (GenBank™ accession number gi: U18775) and hTLE1 (GenBank™ accession number gi: M99435), or of the Groucho/AES family, mAES-1 (GenBank™ accession number gi: X73359) and XGrg5 (GenBank™ accession number gi: U18776). Residues conserved in Grg1-S are *shaded in black*. The last 8 amino acids of Grg1-S differ from Grg1-L because of alternative splicing.

Grg1-L, with the exception of the last 8 amino acids generated by alternative splicing within exon 7 (Figs. 1*B* and 2). This agrees with its apparent origin as a splicing variant; however, Grg1-S is more closely related to the AES subfamily in size. These features distinguish Grg1-S from Groucho/TLE and AES forms described previously (16) and define it as a novel, naturally occurring Groucho protein subtype.

Expression of Grg1-S and Grg1-L mRNAs—To ascertain the level of Grg1-S mRNA expression, we performed Northern analysis. In adult mouse tissues the 5′Grg1 probe, common to most isoforms, identifies discrete 4.3- and 2.3-kb mRNA species of roughly equal abundance, with predominant expression in liver and lung and detectable expression in heart, brain, kidney, and testis (Fig. 3*A*, *middle panel*). In contrast, the specific 3′Grg1-S probe identifies only the 2.3-kb transcript, in much the same distribution (Fig. 3*A*, *left panel*). Interestingly, this tissue expression pattern strongly resembles that previously identified for mTcf4 (26). A probe specific for the 3′-UTR of Grg1-L identifies only the 4.3-kb transcript (Fig. 3*A*, *right panel*). The common 5′ probe is the only one to detect a weak 2.3-kb signal in skeletal muscle, which may reflect cross-hybridization with some other transcript.

Northern analysis at different stages of mouse fetal development identifies the same two major mRNA species of 2.3 and 4.3 kb with nearly constant levels between 11.5 and 15.5 dpc (Fig. 3*B*). However, in contrast to adult tissues, where the two transcripts are present in nearly equal proportion, the level of Grg1-L mRNA slightly exceeds that of Grg1-S (Fig. 3*B*, *middle panel*). The 3′Grg1-S probe recognizes an additional 9-kb transcript of unclear significance (Fig. 3*B*, *left panel*). Taken together, these observations indicate that Grg1-S is a major mRNA species encoded by the mouse *Grg1* locus and expressed in several adult tissues. Grg1-S and Grg1-L mRNA levels do not appear to be developmentally regulated in the midgestation mouse embryo.

mRNAs represented through hybridization with the 3′Grg1-L probe should be interpreted cautiously because all the Grg1-L mRNAs have an identical 3′-UTR and could be quite similar in size. To estimate the relative abundance of the splice variants detected by RT-PCR on the 14.5-dpc mouse gut, we analyzed the products of this RT-PCR by Southern analysis (Fig. 3*C*) prior to their cloning. Grg1-LΔGP and full-length Grg1-L are the two predominant isoforms, whereas variants missing either the second leucine zipper and GP domain or a portion of the WD domain are relatively minor species.

Grg1-S exhibits nearly similar expression along the rostrocaudal axis of the developing (14.5 dpc) and adult mouse gut (Fig. 4, *A* and *B*), with possibly lower levels in the colon. However, the 9-kb transcript detected weakly in the whole embryo is more readily observed in the developing gut, raising the possibility that Grg1 encodes two major fetal intestinal mRNA species with common 3′-UTR sequences. To localize Grg1-S mRNA in the gut with greater precision, we performed *in situ* hybridization on sections of the adult small intestine using a specific 3′-UTR probe and observed strongest expression at the base of the crypts of Lieberkuhn (Fig. 4*C*). Cells that reside here constitute the proliferative compartment of the intestinal mucosa, maintain a brisk cellular turnover throughout life, respond to epithelial injury, and are the target of defects in mice lacking *Tcf4* gene function (22, 32, 33).

Grg1-S Is Located in Both Cytosol and Nucleus and Physically Interacts with Itself and with Grg1-L—Groucho/TLE proteins contain a nuclear localization signal within the CcN domain and are present in the cell nucleus (34), whereas Groucho/

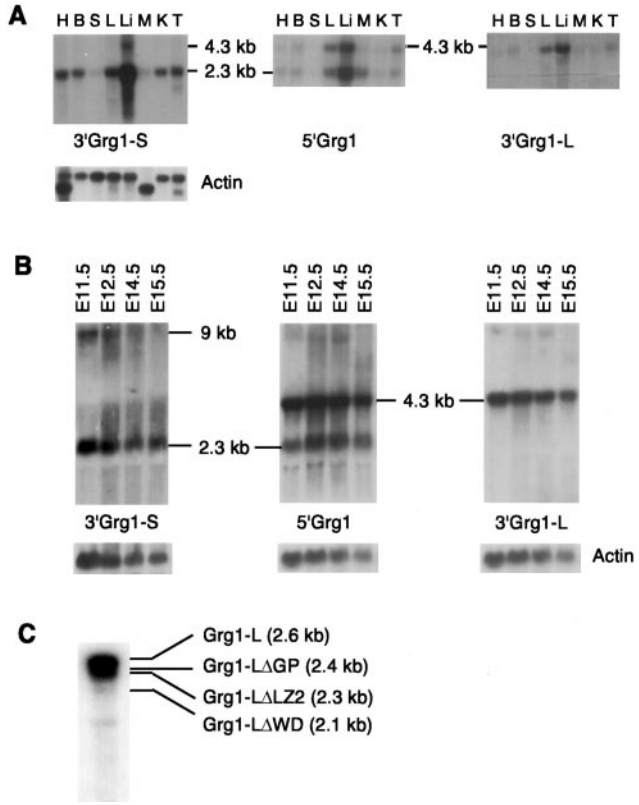

FIG. 3. **Expression pattern of mouse *Grg1-S* and *Grg1-L*.** Northern analysis was performed sequentially with three different DNA probes: 3′Grg1-L specific to the *Grg1-L* 3′-UTR, 3′Grg1-S specific to the *Grg1-S* 3′-UTR, and 5′Grg1 common to all Grg1 transcripts (see Fig. 1*A*). *A*, expression in adult mouse tissues (*H*, heart; *B*, brain; *S*, spleen; *L*, lung; *Li*, liver; *M*, skeletal muscle; *K*, kidney; and *T*, testis). *B*, expression in mouse whole embryos from 11.5 to 15.5 dpc. An actin probe confirmed equal RNA loading, and transcript sizes are indicated in kilobases (*kb*). *C*, relative expression levels of different Grg1-L splice variants. RT-PCR was performed on 14.5-dpc mouse gut RNA and blotted with probe 193 (see "Experimental Procedures" and Fig. 1*A*). Sizes of the PCR products and identity of the corresponding splice variants, determined by cloning, are indicated.

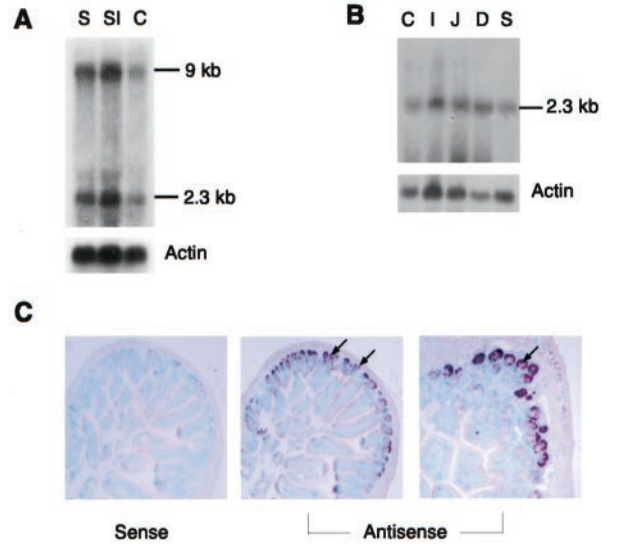

FIG. 4. **Expression of *Grg1-S* in the gastrointestinal tract.** Northern analysis with the *Grg1-S* 3′-UTR probe on total RNA extracted from mouse gut tissues. *A*, fetal gut at 14.5 dpc (*S*, stomach; *SI*, small intestine; *C*, colon). *B*, adult gut (*C*, colon; *I*, ileum; *J*, jejunum; *D*, duodenum; and *S*, stomach). *C*, mRNA *in situ* hybridization on sections of adult mouse small intestine (jejunum) stained with a digoxigenin-labeled antisense riboprobe corresponding to the *Grg1-S* 3′-UTR (*right two panels*) or the corresponding sense probe (*left panel*), and counterstained with methyl green. *Arrows* point to the regions of highest Grg1-S mRNA expression near the base of the crypts of Lieberkuhn. Original magnification, *left two panels*, ×100; *right panel*, ×200. Experiments with ^{35}S-UTP-labeled riboprobes yielded the same conclusions.

AES proteins may be found either in the cytosol or in the nucleus (13, 35). To determine the subcellular locations of Grg1-L and Grg1-S, we transfected COS cells with Myc epitope-tagged expression constructs and assessed protein expression by indirect immunofluorescence (Fig. 5*A*). Whereas Grg1-L is exclusively and predictably nuclear, Grg1-S is found in both the cytosol and nucleus despite the absence of a CcN domain. This bicompartmental distribution is observed at even the lowest levels of Grg1-S transfection. Grg1-S thus defines not only a novel subtype of Groucho proteins but also shows unexpected subcellular localization. Cotransfection of Grg1-L and Grg1-S did not visibly modify the location of either protein, nor did cotransfection of Grg1-S with different Tcf-family members result in movement of the cytosolic Grg1-S fraction to the nucleus (data not shown).

Groucho proteins can form homo- and hetero-oligomers through interactions that require the Q domain and are necessary for transcriptional regulation (36, 37). To test the ability of Grg1-S to interact with itself or with Grg1-L, we performed coimmunoprecipitation experiments after overexpressing hemagglutinin (HA) epitope-tagged Grg1-S with Myc epitope-tagged forms of either Grg1-S or Grg1-L in COS cells (Fig. 5, *B* and *C*). Both Grg1-S and Grg1-L are present in Grg1-S immune complexes (Fig. 5, *B*, *lane 3* and *C*, *lane 1*), and Grg1-S is identified in both Grg1-S and Grg1-L complexes (Fig. 5, *B*, *lane 6*, and *C*, *lane 4*). Thus, Grg1-S possesses the capacity for both nuclear localization and protein interactions to form Grg1-S homopolymers and Grg1-S-Grg1-L heteropolymeric complexes.

Grg1-S Functions as a Repressor of β-Catenin-mediated Gene Activation—Groucho proteins interact with Tcf factors as negative regulator of the β-catenin/Tcf transcriptional pathway (13). Based on the findings described above, we considered two possible functions: (i) that Grg1-S could repress β-catenin/Tcf-mediated gene activation, or (ii) that it may behave as a dominant antagonist of repression mediated by other Groucho proteins such as Grg1-L. We first performed axis duplication assays in *Xenopus* embryos, where β-catenin accumulates early on the prospective dorsal side and formation of the dorsoventral axis requires activation of β-catenin/Tcf target genes (13, 38). Forced expression of β-catenin mRNA on the future ventral side leads to formation of a secondary longitudinal axis that is readily recognized at later developmental stages (Fig. 6*A*) and allows interrogation of the biological function of Grg1-S.

In accordance with published results, Grg1-L behaves as a weak but consistent antagonist of β−catenin/Tcf activation (Fig. 6*B*, reduction of complete axis duplications from 15 to 4.3%); this activity is observed only with the relatively low dose of 50 pg of β-catenin mRNA. The repressive effect is not released upon coinjection of Grg1-S mRNA; instead, introducing Grg1-S (0.5 or 1 ng) and Grg1-L (1 ng) mRNAs together inhibits β-catenin-induced axis duplication almost completely (from 15% fully duplicated embryos to 0.8 and 0%, respectively, and from 44% partial duplications to 35.6 and 4.6%, respectively). Grg1-S by itself also represses β−catenin-mediated axis duplication in *Xenopus* embryos (reduction of partial duplications from 44 to 24.6% and of complete duplications from 15 to 2.1%). Thus, Grg1-S does not act as a negative regulator (derepressor) of Grg1-L function but rather mimics its activity. These effects are dose-dependent and could result from additive or cooperative interactions.

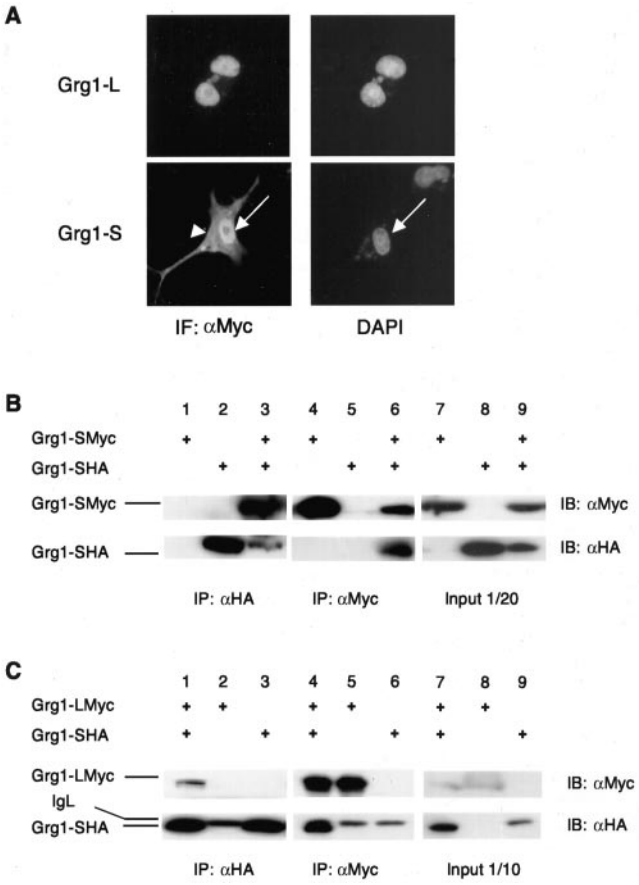

FIG. 5. **Subcellular localization and physical associations of Grg1-S and Grg1-L.** *A*, COS cells were transfected with Myc epitope-tagged Grg1-L or Grg1-S constructs and processed for immunofluorescence (*left panels*); the nucleus is revealed in corresponding 4,6-diamidino-2-phenylindole (*DAPI*)-stained images (*right panels*). Grg1-L is detected exclusively in the nucleus (*arrow*), whereas Grg1-S is detected in both cytosol (*arrowhead*) and nucleus, even upon transfection of minimal amounts of Grg1-S plasmid. *B* and *C*, lysates of COS cells transfected with the indicated epitope-tagged constructs were immunoprecipitated (*IP*) with either anti-HA (*lanes 1–3*) or anti-Myc antibodies (*lanes 4–6*), and the immunoprecipitated proteins were analyzed by immunoblotting (*IB*) with the indicated antibodies. Myc-tagged Grg1-S is immunoprecipitated by anti-HA antibody when the two epitope-tagged Grg1-S forms are cotransfected (*lane 3*) but not when either is transfected alone (*lane 1*). Likewise, HA-tagged Grg1-S is detected in Myc immunoprecipitants in the converse experiment, revealing the presence of Grg1-S homopolymers in cells. Similarly, experiments with epitope-tagged Grg1-L constructs reveal heteropolymerization of Grg1-S and Grg1-L (*C*). Protein expression levels (*lanes 7–9*) were determined by immunoblotting 1/20 (*B*) or 1/10 (*C*) of the total cell extract used for the immunoprecipitation.

To determine the specificity of the observed effects, we coinjected each mRNA with *Siamois*, an endogenous target and downstream effector of β-catenin/Tcf signaling in *Xenopus* embryos (39). Neither Grg1-S nor Grg1-L blocks the axis duplication induced by *Siamois*. These results show that Grg1-repressive function selectively targets the upstream activity of β-catenin, presumably in conjunction with endogenous Tcfs. XTcf3 is essential to *Xenopus* axis formation and probably interacts with endogenous Groucho proteins to prevent association with β-catenin and hence repress ectopic axis formation (40). We therefore propose that Grg1-S exerts its apparent transcriptional repressive effects by interfering with β-catenin/XTcf3-induced gene activation.

To test this hypothesis, we performed luciferase reporter assays in 293 cells using the reporter construct OT, which contains three consensus Tcf-binding sites (41). Cotransfection of β-catenin and XTcf3 activates the reporter to levels that are not observed with a control reporter (OF) containing three defective Tcf-binding sites or with transfection of either construct alone. Cotransfection of Grg1-L (data not shown) or Grg1-S (Fig. 7) represses the transactivation provided by β-catenin in a dose-dependent manner, consistent with the repressive function revealed in the *Xenopus* experiments. Based on these findings and the prominent expression of Grg1-S in intestinal crypts, we also tested if Grg1-S could antagonize transcriptional activation mediated by Tcf4. Indeed, forced expression of Grg1-S in 293 cells inhibits Tcf4/β-catenin-induced reporter gene activation (Fig. 7). Repression in this instance is of the same magnitude as with XTcf3 but is not enhanced with increasing amounts of Grg1-S.

In normal *Xenopus* embryos, base-line transcriptional repression by XTcf3 is thought to be countered by accumulation of β-catenin on the prospective dorsal side (38, 40). Furthermore, dorsal overexpression of XGrg-4, a Groucho/TLE protein, represses the *Siamois* promoter and partially ventralizes embryos (13). Although we thus considered the possibility that Grg1-S might influence *Xenopus* axis formation directly, injection of Grg1-S on the dorsal side does not affect the endogenous axis (data not shown), revealing its lack of intrinsic ventralizing function. Native *Xenopus* Groucho proteins (42, 43) possibly already saturate potential protein interaction sites or perhaps Grg1-S fails to undergo required modifications on the prospective dorsal side.

DISCUSSION

Groucho proteins act as corepressors of a wide variety of transcription factors (44–47) and thus regulate many developmental and tumorigenic pathways. For example, they act as negative regulators of Wnt signaling, apparently by countering β-catenin/Tcf-mediated activation of target genes (16). Inappropriate stabilization of β−catenin leads to cellular transformation and may be the central deregulation in colorectal and other human cancers (18, 48–50). Tcf4 activity is negatively regulated in part by its own induction of a dominant inhibitory Tcf1 isoform in gut and mammary epithelia (24) and reinforced by selective activation of a promoter that drives expression of full-length LEF1 in colonic tumors (25). Other mammalian Tcf/LEF genes also encode multiple splice variants (3). Although the significance of much of this alternative mRNA splicing remains unclear, it is important to characterize the full spectrum of structurally distinct and biologically active Tcf and Groucho proteins. This is especially true because Groucho proteins are already recognized in two distinct forms and may interact in complex transcriptional regulatory networks within several distinct pathways. Here we report the presence of several splicing variants of the *mGrg1* gene and characterize Grg1-S, a new type of Groucho protein with unique features and intrinsic transcriptional repression activity.

Mouse *Grg1* is the first Groucho gene reported to encode multiple splice forms. In adult tissues, the two major mRNA species are Grg1-L and Grg1-S. Grg1-L is most closely related to human TLE-1, whereas Grg1-S is a newly identified transcript that encodes a third subgroup of naturally occurring Groucho isoforms. Grg1-S is related in size and structure to the AES subfamily but is identical to the amino terminus of Grg1-L and encompasses the entire Q and GP domains. Its functions are best considered in the context of the established roles of the five characterized Groucho/TLE subdomains. For example, the Q domain has been implicated previously (36, 51) in mediating Groucho polymerization, and its retention in Grg1-S is consistent with our detection of both Grg1-S-Grg1-L and homomeric Grg1-S complexes. Such complexes may be important in Grg1-S biological functions, as oligomerization is required for Groucho/TLE-mediated repression (36, 51–53). In contrast, ab-

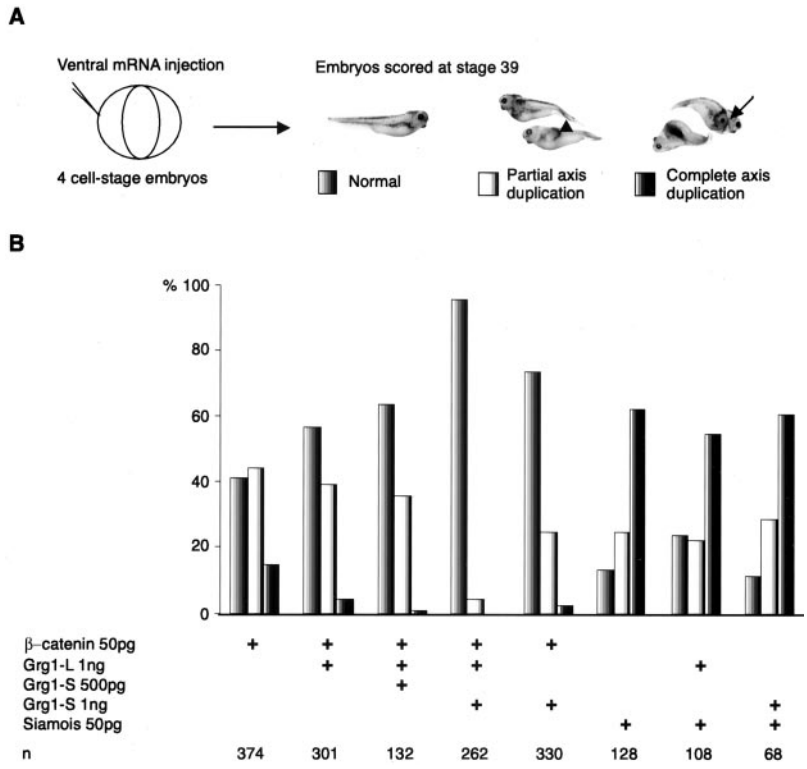

FIG. 6. **Grg1-S inhibit β-catenin-induced axis duplication in *Xenopus* embryos.** *A*, capped mRNAs were injected in the equatorial region of one ventral blastomere of 4 cell-stage *Xenopus* embryos, and duplicated axes were scored at stage 39 according to three groups as follows: complete axis duplication, where a second head with differentiated eyes is identified unambiguously (*arrows*); partial axis duplication, where the secondary axis is incomplete and additional eyes are not detected (*arrowheads*); and normal embryos, without overt evidence of duplicated axes. *B*, histogram showing the percentage of embryos with normal or duplicated axes after injection of the indicated mRNAs. The results are pooled from several independent experiments, which gave consistent results, and the total number of scored embryos (*n*) is indicated.

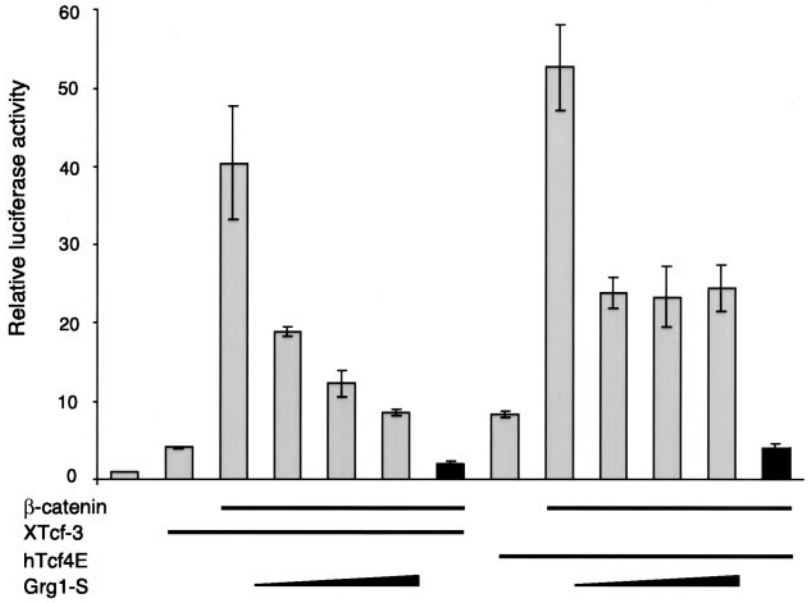

FIG. 7. **Grg1-S inhibits reporter gene activation induced by β-catenin and XTcf3 or hTcf4.** Transfection of the indicated plasmids was performed in duplicate in 293 cells, and cell lysates were assayed for firefly luciferase activity. Results shown are from one study and are representative of at least three independent experiments. Addition of Grg1-S plasmid abrogates the luciferase activity induced by β-catenin and either XTcf3 or hTcf4E. The reporter construct OF (*black box*), which carries mutations in the Tcf-binding sites, is not activated. Luciferase values are corrected for transfection efficiency using an internal control transfection of pRl-TK, a plasmid expressing *Renilla* luciferase under control of the thymidine kinase promoter. Ratios of firefly:*Renilla* luciferase were determined and normalized to values from those samples in which the OT reporter was transfected alone and arbitrarily set at 1. Results are expressed as the mean ± S.E.

sence of the carboxyl-terminal WD domain might preclude association with many other cellular factors (15, 16).

Subcellular localization has been reported previously (13) for the AES-like protein Grg5 and for an artificially truncated form of XGrg4 that contains only the Q and GP domains and hence resembles Grg1-S; both are excluded from cell nuclei. In contrast, Grg1-S lacks a CcN domain, yet localizes measurably to the nucleus in mammalian cells, similar to mouse Grg (35). Together with its lack of a consensus nuclear localization signal, this finding raises the possibility that Grg1-S nuclear transport is driven by association with other cellular proteins or after other modifications. TLE1, the human ortholog of Grg1-L, displays different affinities for the nuclear compartment depending on its phosphorylation state (54), and the same may hold for Grg1-S. We have noted that Myc epitope-tagged Grg1-L and Grg1-S migrate with higher than predicted molecular mass on SDS-PAGE (data not shown), which suggests the presence of post-translational changes. It remains to be determined whether such modifications are relevant to Grg1-S function and whether subcellular localization may be subject to external influences.

Groucho/TLE proteins interact with some DNA-binding factors such as Engrailed, Hairy, Runt-related factor and AML1 at least partly through the carboxyl-terminal WD domain (55–58), whereas mouse Grg5 associates with Tcf1 through the most amino-terminal ~100 amino acids (31). However, biological effects of Groucho proteins, and indeed of other transcriptional coregulators, can vary with cellular, protein, and promoter context. For instance, AES subfamily proteins behave as corepressors (37, 59, 60) or coactivators (13). Grg5 potentiates the

repressive effect of TLE1 on HNF3β transcriptional activity (61) and is thus the only short, AES-type Groucho protein reported to interfere with large Groucho function. We therefore tested the possibilities that Grg1-S may function either to repress transcription directly or to interfere with Grg1-L activity. First, we used coimmunoprecipitation to demonstrate that Grg1-S can associate physically with Grg1-L and hence potentially modulate its activity. Second, in both axis duplication assays in *Xenopus* embryos and luciferase reporter assays in cultured mammalian cells, Grg1-S by itself causes transcriptional repression of Tcf/β-catenin-dependent gene activation. Brantjes *et al.* (31) recently reported that an artificially truncated form of XGrg4 that contains only the Q and GP domains represses Tcf/β-catenin-dependent reporter activation; this result is consistent with our findings with Grg1-S, a naturally occurring Groucho isoform. Combining Grg1-L and Grg1-S leads to slightly increased repression, which is likely explained by an additive effect, and there is no evidence for synergy or cross-modulation between these two isoforms. The Groucho GP domain may mediate interaction with histone deacetylases and is required for efficient repression (17). Thus, like some of its AES relatives, Grg1-S carries sequences that endow it with intrinsic transcriptional repression function. However, we cannot exclude the formal possibility that overexpressed Grg1-S actually potentiates the function of some endogenous repressor, including Grg1-L, perhaps by being recruited to transcription factor-bound complexes at target gene promoters. Our studies also do not address the properties of Grg1-S in relation to other Groucho-regulated signaling pathways.

Although Grg1-S inhibits β-catenin/Tcf-mediated reporter gene activation in 293 cells, we could not elicit the same effect in SW480 colon cancer cells, in which Tcf signaling is activated constitutively (48). One possibility is that different cell types facilitate alternative post-translational modifications that impart specificity in protein-protein interactions. Consistent with this idea are the following: (i) mGrg-5 interacts functionally with Tcf1 but not with Lef-1 in COS cells (13); (ii) interaction of Grg4 with Pax5 is associated with altered Grg4 phosphorylation states (53); and (iii) interaction between Hes1 and RUNX1 leads to hyperphosphorylation of *Drosophila* Groucho and promotes transcriptional repression through increased chromatin association (62).

Besides Grg1-S, the alternative internal splicing described for *mGrg1* in Fig. 1*A* generates additional transcripts whose roles are not known. Additionally, the gene probably employs alternative promoters and at least one untranslated first exon. Two independent factors suggest this to be the case. (i) The 4.3-kb size estimated for Grg1-L RNA by Northern analysis exceeds that predicted from the position of the polyadenylated tail and points to heterogeneity in the 5′-UTR. (ii) Putative promoter regions are identified 0.8 (transcription start site program TSSW prediction score, 12.25) and 5.5 kb (TSSW prediction score, 18.53) (genomic.sanger.ac.uk/gf/gf.shtml) upstream of the Grg1-S initiation codon. Our expression and functional data further indicate that Grg1-L and Grg1-S mRNAs are present at comparable abundance, in the same distribution, and the corresponding proteins show equivalent activity in relation to Tcf/β-catenin gene regulation.

Most colorectal cancers harbor *APC* or *β-catenin* mutations (63), which initiate tumors. As Tcf-mediated gene transcription may depend critically on a balance between the opposing activities of β-catenin and Groucho, it will be interesting to determine the status of Grg1-S, Grg1-L, and other Groucho factors in human colon cancers. The distribution of *Grg1-S* mRNA in adult mouse tissues closely resembles that of mTcf4 (26, 64); its prominence at the base of intestinal crypts is striking, and it can repress Tcf4-induced transcriptional activation. These observations point to a potential role for Grg1-S in gut epithelial physiology, especially in light of the requirement for Tcf4 in maintaining crypt stem cells (22). Independently, TLE1, a presumptive human Grg1-L ortholog, may also play a role in vertebrate gut development through interactions with the transcription factor HNF3β, as HNF3β-null mice show defective gut endoderm invagination (65, 66). To investigate this potential role, we attempted to generate transgenic mice that overexpress Grg1-S in differentiated villus enterocytes, under control of the rat *Fabpi* and *Fabpl* promoters (67). None of six independent transgenic founder mice or their F1 progeny expressed detectable levels of epitope-tagged Grg1-S protein in the intestine. Thus, either high Grg1-S expression in differentiated gut epithelial cells is lethal, or this experiment may require the use of other promoters that drive higher levels of transgene expression.

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REFERENCES

1. Cadigan, K. M., and Nusse, R. (1997) *Genes Dev.* **11,** 3286–3305
2. Miller, J. R., Hocking, A. M., Brown, J. D., and Moon, R. T. (1999) *Oncogene* **18,** 7860–7872
3. Hurlstone, A., and Clevers, H. (2002) *EMBO J.* **21,** 2303–2311
4. Barker, N., Morin, P. J., and Clevers, H. (2000) *Adv. Cancer Res.* **77,** 1–24
5. Bauer, A., Chauvet, S., Huber, O., Usseglio, F., Rothbacher, U., Aragnol, D., Kemler, R., and Pradel, J. (2000) *EMBO J.* **19,** 6121–6130
6. Tago, K., Nakamura, T., Nishita, M., Hyodo, J., Nagai, S., Murata, Y., Adachi, S., Ohwada, S., Morishita, Y., Shibuya, H., and Akiyama, T. (2000) *Genes Dev.* **14,** 1741–1749
7. Sakamoto, I., Kishida, S., Fukui, A., Kishida, M., Yamamoto, H., Hino, S., Michiue, T., Takada, S., Asashima, M., and Kikuchi, A. (2000) *J. Biol. Chem.* **275,** 32871–32878
8. Waltzer, L., and Bienz, M. (1998) *Nature* **395,** 521–525
9. Brannon, M., Brown, J. D., Bates, R., Kimelman, D., and Moon, R. T. (1999) *Development* **126,** 3159–3170
10. Sampson, E. M., Haque, Z. K., Ku, M. C., Tevosian, S. G., Albanese, C., Pestell, R. G., Paulson, K. E., and Yee, A. S. (2001) *EMBO J.* **20,** 4500–4511
11. Snider, L., Thirlwell, H., Miller, J. R., Moon, R. T., Groudine, M., and Tapscott, S. J. (2001) *Mol. Cell. Biol.* **21,** 1866–1873
12. Cavallo, R. A., Cox, R. T., Moline, M. M., Roose, J., Polevoy, G. A., Clevers, H., Peifer, M., and Bejsovec, A. (1998) *Nature* **395,** 604–608
13. Roose, J., Molenaar, M., Peterson, J., Hurenkamp, J., Brantjes, H., Moerer, P., van de Wetering, M., Destree, O., and Clevers, H. (1998) *Nature* **395,** 608–612
14. Levanon, D., Goldstein, R. E., Bernstein, Y., Tang, H., Goldenberg, D., Stifani, S., Paroush, Z., and Groner, Y. (1998) *Proc. Natl. Acad. Sci. U. S. A.* **95,** 11590–11595
15. Fisher, A. L., and Caudy, M. (1998) *Genes Dev.* **12,** 1931–1940
16. Chen, G., and Courey, A. J. (2000) *Gene* (*Amst.*) **249,** 1–16
17. Chen, G., Fernandez, J., Mische, S., and Courey, A. J. (1999) *Genes Dev.* **13,** 2218–2230
18. Morin, P. J. (1999) *Bioessays* **21,** 1021–1030
19. Polakis, P., Hart, M., and Rubinfeld, B. (1999) *Adv. Exp. Med. Biol.* **470,** 23–32
20. Wong, N. A., and Pignatelli, M. (2002) *Am. J. Pathol.* **160,** 389–401
21. Zorn, A. M., Barish, G. D., Williams, B. O., Lavender, P., Klymkowsky, M. W., and Varmus, H. E. (1999) *Mol. Cell* **4,** 487–498
22. Korinek, V., Barker, N., Moerer, P., van Donselaar, E., Huls, G., Peters, P. J., and Clevers, H. (1998) *Nat. Genet.* **19,** 379–383
23. Pukrop, T., Gradl, D., Henningfeld, K. A., Knochel, W., Wedlich, D., and Kuhl, M. (2001) *J. Biol. Chem.* **276,** 8968–8978
24. Roose, J., Huls, G., van Beest, M., Moerer, P., van der Horn, K., Goldschmeding, R., Logtenberg, T., and Clevers, H. (1999) *Science* **285,** 1923–1926
25. Hovanes, K., Li, T. W., Munguia, J. E., Truong, T., Milovanovic, T., Lawrence Marsh, J., Holcombe, R. F., and Waterman, M. L. (2001) *Nat. Genet.* **28,** 53–57
26. Lee, Y. J., Swencki, B., Shoichet, S., and Shivdasani, R. A. (1999) *J. Biol. Chem.* **274,** 1566–1572
27. Sambrook, J., and Russel, D. W. (2001) *Molecular Cloning: A Laboratory Manual*, 3rd Ed., Vol. 3, Appendix A1–12, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY
28. Magi-Galluzzi, C., Mishra, R., Fiorentino, M., Montironi, R., Yao, H., Capodieci, P., Wishnow, K., Kaplan, I., Stork, P. J., and Loda, M. (1997) *Lab. Invest.* **76,** 37–51
29. Bonifacino, J. S., Dasso, M., Harford, J. B., Lippincott-Schwartz, J., and Yamada, K. M., eds (1998) *Current Protocols in Cell Biology*, Sec 6.1.1 and 6.2.1, John Wiley & Sons, Inc., New York
30. Shoichet, S. A., Malik, T. H., Rothman, J. H., and Shivdasani, R. A. (2000)

Proc. Natl. Acad. Sci. U. S. A. **97,** 4076–4081
31. Brantjes, H., Roose, J., van De Wetering, M., and Clevers, H. (2001) *Nucleic Acids Res.* **29,** 1410–1419
32. Potten, C. S., Booth, C., and Pritchard, D. M. (1997) *Int. J. Exp. Pathol.* **78,** 219–243
33. Stappenbeck, T. S., Wong, M. H., Saam, J. R., Mysorekar, I. U., and Gordon, J. I. (1998) *Curr. Opin. Cell Biol.* **10,** 702–709
34. Stifani, S., Blaumueller, C. M., Redhead, N. J., Hill, R. E., and Artavanis-Tsakonas, S. (1992) *Nat. Genet.* **2,** 119–127
35. Mallo, M., Gendron-Maguire, M., Harbison, M. L., and Gridley, T. (1995) *Dev. Dyn.* **204,** 338–347
36. Chen, G., Nguyen, P. H., and Courey, A. J. (1998) *Mol. Cell. Biol.* **18,** 7259–7268
37. Ren, B., Chee, K. J., Kim, T. H., and Maniatis, T. (1999) *Genes Dev.* **13,** 125–137
38. Larabell, C. A., Torres, M., Rowning, B. A., Yost, C., Miller, J. R., Wu, M., Kimelman, D., and Moon, R. T. (1997) *J. Cell Biol.* **136,** 1123–1136
39. Brannon, M., Gomperts, M., Sumoy, L., Moon, R. T., and Kimelman, D. (1997) *Genes Dev.* **11,** 2359–2370
40. Molenaar, M., van de Wetering, M., Oosterwegel, M., Peterson-Maduro, J., Godsave, S., Korinek, V., Roose, J., Destree, O., and Clevers, H. (1996) *Cell* **86,** 391–399
41. da Costa, L. T., He, T. C., Yu, J., Sparks, A. B., Morin, P. J., Polyak, K., Laken, S., Vogelstein, B., and Kinzler, K. W. (1999) *Oncogene* **18,** 5010–5014
42. Choudhury, B. K., Kim, J., Kung, H. F., and Li, S. S. (1997) *Gene* (*Amst.*) **195,** 41–48
43. Molenaar, M., Brian, E., Roose, J., Clevers, H., and Destree, O. (2000) *Mech. Dev.* **91,** 311–315
44. Paroush, Z., Finley, R. L., Jr., Kidd, T., Wainwright, S. M., Ingham, P. W., Brent, R., and Ish-Horowicz, D. (1994) *Cell* **79,** 805–815
45. Dehni, G., Liu, Y., Husain, J., and Stifani, S. (1995) *Mech. Dev.* **53,** 369–381
46. Grbavec, D., Lo, R., Liu, Y., and Stifani, S. (1998) *Eur. J. Biochem.* **258,** 339–349
47. Yao, J., Liu, Y., Lo, R., Tretjakoff, I., Peterson, A., and Stifani, S. (2000) *Mech. Dev.* **93,** 105–115
48. Korinek, V., Barker, N., Morin, P. J., van Wichen, D., de Weger, R., Kinzler, K. W., Vogelstein, B., and Clevers, H. (1997) *Science* **275,** 1784–1787
49. Morin, P. J., Sparks, A. B., Korinek, V., Barker, N., Clevers, H., Vogelstein, B., and Kinzler, K. W. (1997) *Science* **275,** 1787–1790
50. Rubinfeld, B., Robbins, P., El-Gamil, M., Albert, I., Porfiri, E., and Polakis, P. (1997) *Science* **275,** 1790–1792
51. Pinto, M., and Lobe, C. G. (1996) *J. Biol. Chem.* **271,** 33026–33031
52. Choi, C. Y., Kim, Y. H., Kwon, H. J., and Kim, Y. (1999) *J. Biol. Chem.* **274,** 33194–33197
53. Eberhard, D., Jimenez, G., Heavey, B., and Busslinger, M. (2000) *EMBO J.* **19,** 2292–2303
54. Husain, J., Lo, R., Grbavec, D., and Stifani, S. (1996) *Biochem. J.* **317,** 523–531
55. Jimenez, G., Paroush, Z., and Ish-Horowicz, D. (1997) *Genes Dev.* **11,** 3072–3082
56. Imai, Y., Kurokawa, M., Tanaka, K., Friedman, A. D., Ogawa, S., Mitani, K., Yazaki, Y., and Hirai, H. (1998) *Biochem. Biophys. Res. Commun.* **252,** 582–589
57. Javed, A., Guo, B., Hiebert, S., Choi, J. Y., Green, J., Zhao, S. C., Osborne, M. A., Stifani, S., Stein, J. L., Lian, J. B., van Wijnen, A. J., and Stein, G. S. (2000) *J. Cell Sci.* **113,** 2221–2231
58. McLarren, K. W., Theriault, F. M., and Stifani, S. (2001) *J. Biol. Chem.* **276,** 1578–1584
59. Tetsuka, T., Uranishi, H., Imai, H., Ono, T., Sonta, S., Takahashi, N., Asamitsu, K., and Okamoto, T. (2000) *J. Biol. Chem.* **275,** 4383–4390
60. Yu, X., Li, P., Roeder, R. G., and Wang, Z. (2001) *Mol. Cell. Biol.* **21,** 4614–4625
61. Wang, J. C., Waltner-Law, M., Yamada, K., Osawa, H., Stifani, S., and Granner, D. K. (2000) *J. Biol. Chem.* **275,** 18418–18423
62. Nuthall, H. N., Husain, J., McLarren, K. W., and Stifani, S. (2002) *Mol. Cell. Biol.* **22,** 389–399
63. Sparks, A. B., Morin, P. J., Vogelstein, B., and Kinzler, K. W. (1998) *Cancer Res.* **58,** 1130–1134
64. Barker, N., Huls, G., Korinek, V., and Clevers, H. (1999) *Am. J. Pathol.* **154,** 29–35
65. Ang, S. L., and Rossant, J. (1994) *Cell* **78,** 561–574
66. Weinstein, D. C., Ruiz í Altaba, A., Chen, W. S., Hoodless, P., Prezioso, V. R., Jessell, T. M., and Darnell, J. E., Jr. (1994) *Cell* **78,** 575–588
67. Sweetser, D. A., Birkenmeier, E. H., Hoppe, P. C., McKeel, D. W., and Gordon, J. I. (1988) *Genes Dev.* **2,** 1318–1332