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Mammalian Groucho Homologs: Redundancy or Specificity?

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Abstract The proteins termed TLE in humans, Grg in mice and Groucho in *Drosophila* constitute a family of transcriptional corepressors. In mammalians there are five different genes encoding an even larger number of proteins. Interactions between these TLE/Grg proteins and an array of transcription factors has been described. But is there any specificity? This review tries to make a case for a non-redundant function of individual TLE/Grg proteins. The specificity may be brought about by a tightly controlled temporo-spatial expression pattern, post-translational modifications, and subtle structural differences leading to distinct preferences for interacting transcription factors. A confirmation of this concept will ultimately need to come from genetic experiments. J. Cell. Biochem. 95: 670–687, 2005.

Key words: Groucho; TLE; Grg; corepressor; transcriptional regulation

The *Drosophila* corepressor protein Groucho is the founding member of the family of Gro/TLE proteins. Groucho is involved in multiple developmental processes in *Drosophila*, including neurogenesis, sex determination, and segmentation [Fisher and Caudy, 1998; Chen and Courey, 2000]. In mammals, the Gro/TLE family consists of four proteins of similar molecular weight and structure, termed transducin-like enhancer of split 1-4 (TLE1-4) in humans and groucho gene-related protein (Grg1-4) in mice. In addition, there is one shorter protein, named amino-terminal enhancer of split (AES) in humans for its homology to the amino-terminus of TLE1-4, and Grg5 in mice.

Grg and TLE proteins are broadly, but specifically expressed in multiple developing organs in mammals [Stifani et al., 1992a; Leon and Lobe, 1997].

The Grg/TLE proteins cannot bind to DNA themselves, but modulate the process of transcription by physical interaction with transcription factors. They can either down regulate the expression of target genes of transcriptional activators, enhance the transcriptional repression effect of transcriptional repressors, or convert transcriptional activators into repressors [Grbavec et al., 1998; Imai et al., 1998; Levanon et al., 1998; Ren et al., 1999; Eberhard et al., 2000; Javed et al., 2000; Tetsuka et al., 2000; Wang et al., 2000, 2004; Brantjes et al., 2001; Dasen et al., 2001; Gao et al., 2001; Muhr et al., 2001]. Grg/TLE corepressors were shown to interact with multiple transcription factors, such as Tcf/HMG box transcription factors, Runt domain proteins, HES proteins, Hesx1, NF-κB, PRDI-BF1, PU.1, HNF3β, Hex, and the androgen receptor (AR) [Grbavec and Stifani, 1996; Grbavec et al., 1998; Imai et al., 1998; Levanon et al., 1998; Thirunavukkarasu et al., 1998; Ren et al., 1999; Javed et al., 2000; Tetsuka et al., 2000; Wang et al., 2000, 2004; Brantjes et al., 2001; Dasen et al., 2001; Gao et al., 2001; Yu et al., 2001; Linderson et al., 2004; Swingler et al., 2004]. Through these interactions Grg/TLE cofactors can modulate

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multiple developmentally important processes, such as neural system development and patterning, bone formation, hematopoiesis, myogenesis, and intestinal development. In this review we attempt to highlight some mechanisms of function of Grg/TLE proteins and briefly outline what is known about the role and function of these proteins in specific developmental pathways in mammals.

STRUCTURE AND FUNCTION: HOW Grg/TLE PROTEINS MEDIATE REPRESSION

Grg/TLE 1 through 4 consist of five domains (Fig. 1). The amino-terminal glutamine-rich region (Q domain), a region rich in glycine/proline (GP domain), a CcN domain containing a nuclear localization sequence, as well as putative cdc2 and casein kinase II (protein kinase CK2) phosphorylation sites, a region rich in serine/proline residues (SP domain), and the carboxyl-terminal region with multiple tryptophane and aspartic acid tandem repeats (WD40 domain) [Stifani et al., 1992a; Miyasaka et al., 1993; Fisher and Caudy, 1998; Parkhurst, 1998]. Three of these domains, the Q, CcN, and WD40 domains are most highly conserved. While the Q domain is important for homo- and heterodimerization of Grg/TLE proteins and tetramerization in Gro, the GP domain is essential for interaction of Gro with histone deacetylases (HDACs) [Pinto and Lobe, 1996; Chen et al., 1998; Grbavec et al., 1998; Brantjes et al., 2001]. WD40, SP, GP, and Q domains contain regions important for interaction with transcription factors (see Table I).

The short members of the Grg/TLE family, Grg5 in mouse and AES in humans are composed only of Q and GP domains [Mallo et al., 1993; Miyasaka et al., 1993]. The main part of the Q domain is highly conserved, but the GP domain and carboxy-terminus of Q the domain differ slightly from those of the long Grg/TLE proteins.

Grg/TLE proteins cannot bind to DNA by themselves, but by binding to different transcription factors they can be docked to certain DNA regions. Grg/TLE proteins were shown to dimerize via their conserved aminoterminal Q domain and form large multiprotein complexes associated with the nuclear chromatin [Pinto and Lobe, 1996; Palaparti et al., 1997; Grbavec et al., 1998]. TLE can bind the amino-terminal domain of histone H3, which contains potential

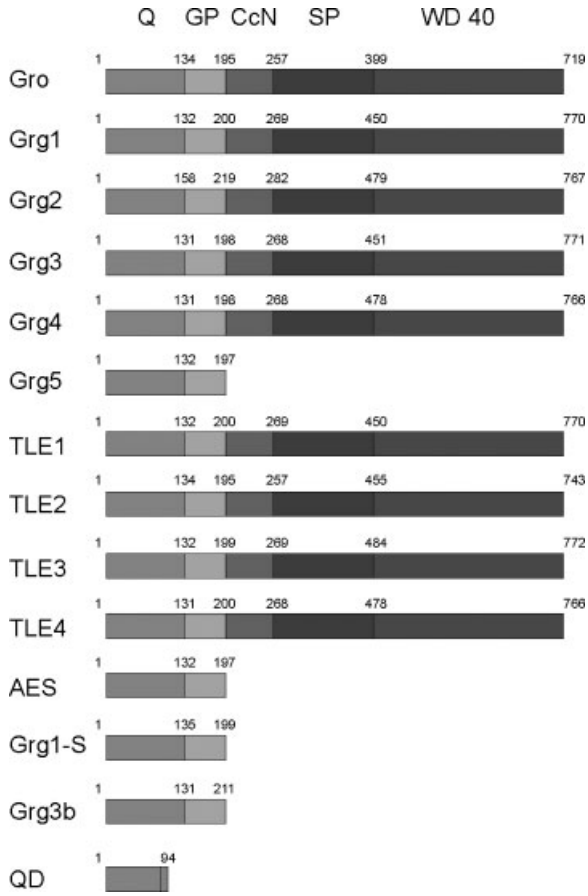

Fig. 1. Schematic representation of the structure of members of the TLE/Grg protein family. Gro, *Drosophila* Groucho protein; Grg 1 through Grg5, mouse Groucho homologs; TLE1 through 4 and AES, human Groucho homologs; Grg1-S, Grg3b and QD, shorter splice variants of Grg/TLE proteins. Q, glutamine rich domain; GP, glycine/proline rich domain; CcN, domain containing putative phosphorylation sites and putative nuclear localization signal; SP, serine/proline rich domain; WD40, domain containing series of tandem repeats of tryptophane and aspartic acid residues. Numbers indicate amino acids.

phosphorylation and acetylation sites [Palaparti et al., 1997]. Therefore, it seems likely that Grg/TLE proteins can form multimeric complexes which influence histone architecture and hence alter chromatin structure. For example Grg/TLE could recruit histone deacetylases (HDAC), which remove acetyl residues from histones, thus rendering chromatin more compact and transcriptionally inactive. This model is probable because *Drosophila* Groucho and a yeast TLE-homolog Tup1, were shown to interact with HDACs [Chen et al., 1999; Watson et al., 2000]. The GP domain is essential for Groucho–HDAC interaction [Chen et al., 1999]. In agreement with this observation it could be demonstrated that the truncated form of

TABLE I. Interaction Partners of Grg/TLE Proteins

Grg/TLE	Transcription factor	Domain of the factor responsible for effective interaction	Domain of TLE responsible for interaction	Tissue/process	Assay	References
Grg5	Runx2	Last 141 aa; VWRPY motif dispensable	Q and GP domain	Developing bone	Y2H; RA; CoIP	Wang et al. [2004]
Grg3, Grg3b	Runx2				RA	Wang et al. [2004]
TLE2	Runx2	Last 12 aa incl. VWRPY motif		Osteoblasts	RA	Thirunavukkarasu et al. [1998]
TLE1	Runx1	C-terminus			PD; RA; Y2H; IVA	Levanon et al. [1998]
TLE1, TLE2	Runx1	C-terminus			Y2H; RA	Javed et al. [2000]
TLE1	Runx3	C-terminus			PD; Y2H; RA; IVA	Levanon et al. [1998]
TLE1	LEF-1				IVA	Levanon et al. [1998]
TLE1	Runx1	C-terminus (aa 454–480)	aa 344–399 in SP domain	Hematopoiesis	PD; IVA	Imai et al. [1998]
TLE1, TLE3	Runx2	C-terminus (aa 241–523 or aa 468–528)—binds both Q and WD40 on TLE; VWRPY dispensable	Q (aa 1–135); WD40 Q was not tested WD40 (aa 490–774)		PD; Y2H PD	McLarren et al. [2000]
Grg5	Tcf-1		Q (aa 4–106)		Y2H	Brantjes et al. [2001]
Grg1, TLE2, Grg3, Grg4	Tcf-1, LEF-1, XTcf-3, Tcf-4				RA	Brantjes et al. [2001]
AES, TLE1, TLE2	PRDI-BF1/Blimp-1	aa 331–398	Q		PD	Ren et al. [1999]
AES	Androgen receptor (AR)	N-terminus (aa 1–559)	Full length		PD; Y2H; RA	Yu et al. [2001]
AES	TFIIE			Basal transcription machinery	PD	Yu et al. [2001]
AES	p65 subunit of NF-kappaB	Vicinity of p65 transactivation domain			PD; Y2H; CoIP	Tetsuka et al. [2000]
TLE1	p65 subunit of NF-kappaB				CoIP	Tetsuka et al. [2000]
Grg1	Hesx1	N-terminal highly conserved helical motif FXLXXIL in eh-1 domain	GP WD40	Pituitary organogenesis	PD; RA; ISH; TG	Dasen et al. [2001]
TLE1	HES6	C-terminal WRPW		Myogenic differentiation	PD; Y2H; mammalian Y2H	Gao et al. [2001]
TLE1	HNF3β	C-terminal highly conserved region II (aa 361–388) FNHPF sequence			PD; Y2H	Wang et al. [2000]
Grg5, Grg4	Six3	eh1-like motif in Six domain conserved Phenyloalanine 88 is important	Q	Eye development	PD; Y2H; CoIP	Zhu et al. [2002]
Grg4	Six6				PD; CoIP	Zhu et al. [2002]
TLE1, AES	SIX3, SIX6	Six domain, WDR motif (in Six3); six domain (in Six6)	Q	Eye development	Y2H	Lopez-Rios et al. [2003]
Grg4	Pax5	Octapeptide motif aa 179–186 C-terminal transactivation domain aa304–358	SP Q	B-cell development	Y2H; RA	Eberhard et al. [2000]

Grg4	Pax1, Pax3, Pax6				PD; CoIP	Eberhard et al. [2000]
Grg4	PU.1					Linderson et al. [2004]
TLE1	PRH/Hex	aa 1–98 containing Eh-1 motif: T_{30}PFYIEDILG$_{39}$ Mutation F32E dramatically decreases binding affinity	Hematopoiesis		Y2H; PD; interaction in hematopoietic cells	Swingler et al. [2004]
AES	Bit1		Apoptosis		PD; Y2H	Jan et al. [2004]
TLE1	Hes-1	Carboxyl-terminal region, containing WRPW motif	Q aa 1–135 SP aa 370–455		Y2H; PD	Grbavec et al. [1998, 1999]
TLE2	Hes-1	C-terminus, containing WRPW motif	Q SP aa 316–403		Y2H; PD	Grbavec et al. [1998]
TLE2	Hes-5	aa 1–166			PD	Grbavec et al. [1998]
Grg4	Nkx2.2$_{a,b}$, Nkx6.1$_{a,b}$, Nkx2.9$_b$, Nkx6.2$_b$	TN domain (similar to eh1 domain in engrailed)	Neural tube patterning		a, PD; b, RA	Muhr et al. [2001]

Y2H, yeast-two-hybrid assay; RA, transcriptional reporter assay; PD, pull-down assay; CoIP, coimmunprecipitation; IVA, in vitro association; TG, transgenic mouse model; ISH, in situ hybridization.

Xenopus XGrg4, comprising only Q and GP domains can bind to HDAC-1 [Brantjes et al., 2001]. Opposing this concept, however is the observation that short members of Grg/TLE family, Grg5 and AES failed to bind HDAC-1, and HDAC-1 and -3, respectively [Brantjes et al., 2001; Yu et al., 2001]. This phenomenon could be explained by slight differences in GP domain sequence between long and short Grg/TLE proteins, which could result in different binding affinities for HDACs [Brantjes et al., 2001]. In addition this could at least partially explain why Grg5 is able to act as a dominant negative form of long Grg/TLE proteins. In this model Grg5 could di/tetramerize with other Grg proteins via its Q domain and thus interact with transcription factors, but then fail to interact with HDACs and hence fail to repress transcription. However Grg5 and AES can also act as corepressors and this shows that either the short proteins can interact via GP domain with HDACs other than HDAC-1 and HDAC-3, or that influencing chromatin structure by recruitment of HDACs is not a general and only mechanism for Grg/TLE mediated repression. They could repress transcription, e.g., by influencing the basic transcription machinery as it has been shown that AES can interact with basic transcription factor TFIIE [Yu et al., 2001].

An additional mechanism of Grg/TLE mediated transcriptional repression is the specific inhibition of modification in the activation domain of a transcription factor. An example of this mechanism is the influence on phosphorylation of Pax2. It was demonstrated that Grg4 can specifically inhibit the phosphorylation of the activation domain of this transcription factor [Cai et al., 2003].

THE DOUBLE FACE OF SHORT GROUCHO PROTEINS

Human AES (amino-terminal enhancer of split) and mouse Grg5 are short members of Grg/TLE family. They are comprised of only a Q domain responsible for homo- and heterodimerization of Grg/TLEs and a glycine-proline-rich GP domain [Mallo et al., 1993; Miyasaka et al., 1993]. Other short forms result of an alternative splicing of long Grg/TLE mRNA. Grg1-S is a short form of Grg1 and is composed of the Q domain and a large part of the GP domain with an additional short sequence not translated in

the long form of Grg1 [Lepourcelet and Shivdasani, 2002]. Grg3b is short form of Grg3 and consists of the Q and GP domains [Leon and Lobe, 1997]. A shortened version of TLE4 has been reported consisting only of Q domain and hence was named QD [Milili et al., 2002]. While long Grg/TLE proteins function exclusively as corepressors, the role of short isoforms remains controversial.

It is believed that Grg5 and AES act as dominant negative form of long Grg/TLE corepressors. A couple of reports favor this concept. It was shown that Grg5 increases transactivation activity of HNF3β and Runx2, de-represses Tcf-mediated transcriptional activation, and alleviates repression mediated by PRH [Wang et al., 2000, 2002; Brantjes et al., 2001; Swingler et al., 2004]. Grg5 also reduces Grg4-mediated enhancement of Nkx-dependent repression in vitro [Muhr et al., 2001]. The QD protein inhibits TLE4-Pax5 binding and the lack of Grg5 in Grg5-deficient Runx2 heterozygous mutant mice enhances the phenotypical effect caused by Runx2 insufficiency, showing that Grg5 is a functional Runx2 activator in vivo [Milili et al., 2002; Wang et al., 2004].

On the other hand, Grg5 or AES do not inhibit the repression mediated by interactions between Grg4 and Pax5, PRDI and BF1, or HES1 and TLE [Ren et al., 1999; Eberhard et al., 2000; McLarren et al., 2001]. AES represses transcription mediated by NF-κB or the androgen receptor (AR) [Tetsuka et al., 2000; Yu et al., 2001]. Grg1-S represses β-catenin/Tcf-mediated gene activation [Lepourcelet and Shivdasani, 2002]. In addition AES was shown to act as a repressor when fused to the Gal4 DNA-binding domain [Ren et al., 1999]. In contrast, however, the Q domains of AES and TLE1 can act as dominant negative forms of TLE [Ren et al., 1999]. This suggests that the Q domain by itself could be responsible for an anti-repressive function of Grg5/AES, most probably mediated by binding to the Q domain of long Grg/TLE proteins and thus inhibiting their ability to form functional di/tetramers, or inhibiting their ability to bind to transcription factors.

In this model the Q domain binds other Grg/TLE proteins and the lack of a GP domain in case of QD protein which was shown in Groucho to be essential for interaction with HDACs could cause an inability of the multimer to interact with proteins responsible for chromatin architecture and could therefore cause inability to repress transcription [Chen et al., 1999; Milili et al., 2002]. In Grg5 the GP domain differs from GP domains of Grg1 through 4 and this differences could result in a lack of repressive interaction with chromatin structures potentially because of an inability to recruit HDAC-1 [Brantjes et al., 2001]. The truncated form of Grg1, Grg1-S, acts as a corepressor, because it contains a GP domain almost identical that of Grg1, hence able to mediate the interaction with chromatin components in a fashion similar to the Grg1 GP domain. AES was also shown to act as a corepressor, but its GP domain differs from that of long Grg/TLE proteins much in the same way as the one of Grg5. AES does not interact with HDAC-1 or HDAC-3 [Ren et al., 1999; Tetsuka et al., 2000; Yu et al., 2001]. It is conceivable, however, that modifications within the GP domain of AES cause it to interact with HDACs other than HDAC-1 and -3 or with other factors influencing chromatin structure, for example with a members of basal transcription machinery [Yu et al., 2001].

The exact mechanism of how the short Grg/TLE proteins influence Grg/TLE-mediated repression still remains to be resolved.

ROLE OF PHOSPHORYLATION STATE IN Grg/TLE-MEDIATED REPRESSION

Protein phosphorylation is involved in regulating Grg/TLE function. Within their CcN domain Grg/TLE proteins contain evolutionarily conserved consensus phosphorylation sites for a number of kinases [Stifani et al., 1992b]. It was demonstrated that the phosphorylation state of Grg/TLE proteins increases after induction of differentiation in neural and chondrocytic cells [Husain et al., 1996; Yao et al., 1998; Nuthall et al., 2002a]. The process leading to an increase in phosphorylation was shown to consist of series of events. At first protein kinase CK2 phosphorylates TLE1 at S239 [Nuthall et al., 2004]. As CK2 is an ubiquitously and constitutively active kinase and TLE1 becomes phosphorylated at S239 immediately after translation, it seems likely that phosphorylation of TLE1 at S239 is not correlated with any specific function of TLE1, but represents a general regulatory event [Sarno et al., 2001; Nuthall et al., 2004]. This phosphorylation is necessary for cofactor-activated phosphorylation (CAP) which TLE1 undergoes as an effect of interaction with HES-1. CAP in turn

increases transcription repression activity of TLE1 [Nuthall et al., 2002a]. The hyperphosphorylation of TLE is correlated with its strong association to the nuclear compartment through interaction with chromatin. The other transcription factors, such as RUNX1, Pax5, and BF-1 were also shown to induce Grg/TLE hyperphosphorylation [Eberhard et al., 2000; Nuthall et al., 2002a].

Another Grg/TLE phosphorylation process that results in the effects opposite to the one described above has also been reported. It was shown that protein kinase cdc2 hyperphosphorylates TLE proteins at mitosis most probably weakening the association of TLE proteins with nuclear components. This in turn may inactivate the repressive function of TLE during cell division [Nuthall et al., 2002b].

The two above processes and the fact that each TLE1 protein possesses at least four potential phosphorylation sites theoretically allows for 16 variations of phosphorylation patterns on a single TLE1 protein. This demonstrates how complex the regulation of Grg/TLE activity by phosphorylation might be and how important it is to resolve this mechanism for a complete understanding of the global mechanism of action of Grg/TLE corepressors.

Grg/TLE PROTEINS INTERACT WITH Runt DOMAIN FACTORS: OSTEOGENESIS

Runt homology transcription factors, Runx1 through 3, are essential gene regulatory proteins controlling lineage commitment and development [Speck et al., 1999]. Runx1 was shown to play a crucial role in hematopoiesis, Runx2 is a key factor in the process of skeletal development and Runx3 plays a role during neurogenesis and possibly as a gastrointestinal tumor suppressor [Castilla et al., 1996; Ito, 1996; Okuda et al., 1996; Wang et al., 1996; Komori et al., 1997; Mundlos et al., 1997; Otto et al., 1997; Guo et al., 2002; Inoue et al., 2002; Levanon et al., 2002; Li et al., 2002]. Grg/TLE family members are coexpressed with Runx factors in a variety of cell types and were shown to interact with them physically and downregulate the expression of a number of Runx-dependent genes [Dehni et al., 1995; Simeone et al., 1995; Okuda et al., 1996; Aronson et al., 1997; Imai et al., 1998; Levanon et al., 1998; Thirunavukkarasu et al., 1998; Javed et al., 2000; McLarren et al., 2000; Yao et al., 2000]. Therefore, Grg/TLE proteins via interaction with individual Runx transcription factors could have important influence on Runx-regulated processes.

The Runx-dependent process, in which the role of Grg/TLE has been characterized in most detail, is osteogenesis. A key factor in this process is Runx2. It is required for differentiation and function of osteoblasts, for chondrocyte differentiation towards hypertrophy, and for bone matrix production by mature osteoblasts [Ducy et al., 1997; Komori et al., 1997; Otto et al., 1997; Inada et al., 1999; Kim et al., 1999]. Grg/TLE proteins are coexpressed with Runx2 in skeletal cells [Thirunavukkarasu et al., 1998]. The induction of the osteocalcin (*OC*) gene correlates with downregulation of the level of Grg/TLE in mice skeletal tissues between E14 and birth and Grg/TLEs were shown to inhibit Runx2 dependent activation of *OC* gene transcription [Javed et al., 2000]. In particular TLE2 was demonstrated to downregulate transactivation abilities of Runx2 in reporter assays [Thirunavukkarasu et al., 1998]. The factor, which can antagonize Runx2–Grg/TLE interaction, is HES-1. HES-1 can bind both Runx2 and Grg/TLE [Grbavec and Stifani, 1996; Grbavec et al., 1998; McLarren et al., 2000]. HES-1 is coexpressed with Runx2 in different skeletal cells and together they take part in the regulation of osteoblast-specific genes, for example osteopontin, providing antagonistic inputs to the expression control of this gene [Ducy et al., 1997; Matsue et al., 1997]. While HES-1 represses Opn, Runx2 activates expression [Ducy et al., 1997; Matsue et al., 1997; Harada et al., 1999]. The region in Runx2 responsible for Grg/TLE binding overlaps with the one responsible for the interaction with HES-1 [McLarren et al., 2000]. Thus, HES-1 could perturb TLE–Runx interaction both by competing with Grg/TLE for the binding site on Runx2 and by titrating Grg/TLE away from Runx2. Finally binding of HES-1 to Runx2 enhances Runx2 transcriptional activity [McLarren et al., 2000].

Another protein which can enhance Runx2 transcriptional activity in vitro is Grg5, a dominant negative form of long Grg/TLE proteins [Wang et al., 2004]. To investigate a potential Grg5 effect on Runx2 in vivo, Runx2 heterozygous mice were crossbred with Grg5 null mice [Wang et al., 2004]. Depletion of Grg5 alone, with normal activity of Runx2, causes postnatal growth retardation in about 50% of

the mice. After 4–5 weeks of age most mice recover from growth retardation. The Grg5 null phenotype is characterized by a long bone growth plate defect, which includes shorter zones of proliferating and hypertrophic cartilage and decreased trabecular bone formation. The growth plate defect is associated with reduced Indian hedgehog (Ihh) expression and signaling [Wang et al., 2002]. In Grg5 null Runx2+/− mice, the lack of Grg5 function combined with the heterozygous loss of Runx2 activity resulted in a growth deficiency which was more pronounced than would have been expected, if Grg5 and Runx2 contributed to growth independently. This finding suggests that Grg5 and Runx2 interact with each other in vivo and that their combined activity is necessary for the activation of another factor important for bone and cartilage development. It is highly probable that the factor regulated by Grg5–Runx2 interaction is Ihh [Wang et al., 2004].

The other factors in skeletal tissue that might be regulated by Grg/TLE proteins in their transcriptional activity are Lef1 and Tcf4. Both are expressed in skeletal tissues and were shown to interact with Grg/TLE proteins [Hartmann and Tabin, 2000; Brantjes et al., 2001].

The role of Grg/TLE protein in the context of interaction with another Runt-domain protein, Runx3, remains to be elucidated. Grg/TLE could play role in intestinal function or neurogenesis, where Runx3 function was shown to be important, since Grg/TLE can downregulate Runx3-mediated expression in reporter assays [Castilla et al., 1996; Javed et al., 2000; Guo et al., 2002; Inoue et al., 2002; Levanon et al., 2002; Li et al., 2002].

The interaction of Grg/TLE with the third Runx family transcription factor, Runx1, and its input in hematopoiesis is discussed below.

HEMATOPOIESIS—Pax5, Runx1

The involvement of Grg4/TLE4 in B-cell development has been extensively described.

In the hematopoietic lineage TLE4 mRNA is expressed only in B-cells and the level of Grg4 or TLE4 expression decreases following B-cell activation [Milili et al., 2002; Linderson et al., 2004]. This suggests that Grg4/TLE4 could be an important regulator of B-cell development. Pax5 is a critical B-cell lineage commitment factor which restricts cellular development to the B-lymphoid pathway by suppressing alternative cell fates [Nutt et al., 1999; Rolink et al., 1999]. Pax5 was shown to act both as an activator and a repressor [Nutt et al., 1998]. The repressive function of Pax5 is most likely mediated by Grg4, as Grg4 can physically interact with Pax5 and inhibit its transcriptional activity in cell cultures [Eberhard et al., 2000]. Grg4 and Pax5 interact via the Q domain of Grg4 that binds to the C-terminal transactivation domain of Pax5 and the SP domain of Grg4 that binds to the Pax5 octapeptide motif. Additionally it was observed that Grg4 WD40 repeats are also required for repression of Pax5 activity which suggests that this protein-protein interaction motif recruits an additional factor into the Grg4–Pax5 complex [Eberhard et al., 2000].

Corecruitment of Grg4 by PU.1, another factor important for B-cell commitment, and Pax5 to the HS 1,2 enhancer and J-chain promoter can be essential for downregulation of IgH (immunoglobulin heavy-chain) and J-chain [Linderson et al., 2004]. As Grg4 expression levels decrease upon B-cell activation, the repression of those genes can be relieved and aid terminal differentiation.

In addition to the down/up-regulation of its expression levels, the Grg4/TLE4 function in pro- and pre-B cells may be regulated by interaction with its shorter, dominant-negative forms. Although it was shown that Grg5 does not influence the effect of Grg4 on Pax5-mediated repression, a short form (QD) of TLE4 was identified in the human B-cell lineage and is able to inhibit TLE4-Pax5 binding in vitro [Eberhard et al., 2000; Milili et al., 2002]. It would be interesting to find whether such alternative splice variants of Grg4 exist in the mouse B-cell lineage and how they modulate the process of gene regulation mediated by Grg4, Pax5, and PU.1.

The interaction with TLE corepressors was shown for the PRDI-BFI/Blimp-1 protein, another factor involved in B-cell development. This transcriptional repressor is required for normal B-cell differentiation and it was shown that PRDI-BFI/Blimp-1 repression of the IFN-β promoter is enhanced by interactions with TLE1, TLE2, and AES [Turner et al., 1994; Ren et al., 1999].

TLE1 can interact physically with a protein called proline-rich homeodomain (PRH) , also known as hematopoietically expressed (Hex)

[Swingler et al., 2004]. PRH functions as a transcriptional repressor in hematopoietic, liver, thyroid, and embryonic stem cells [Tanaka et al., 1999; Brickman et al., 2000; Pellizzari et al., 2000; Guiral et al., 2001]. TLE1 interacts with PRH in hematopoietic cells and increases PRH-mediated repression. This repression can in turn be attenuated by Grg5.

Grg/TLE proteins can have an influence on hematopoiesis via interaction with Runx1. Runx1, termed also AML1, Cbfa2 or PEBP2αB is essential for fetal liver hematopoiesis in mice [Ito, 1996; Okuda et al., 1996; Wang et al., 1996]. In humans the *Runx1* gene is frequently targeted by chromosomal translocations which lead to acute myeloid leukemia [Miyoshi et al., 1991; Ito, 1996]. TLE1 specifically interacts with Runx1 in vitro and in vivo and inhibits Runx1-induced transactivation of a number of hematopoietic lineage-specific genes, such as T cell receptor (TCR) α and β enhancers, M-CSF receptor or neutrophil elastase [Imai et al., 1998; Levanon et al., 1998]. Additionally downregulation of Runx1 and upregulation of Grg2, Grg1, and Grg4 by the E2A-HLF oncoprotein resulting from the chromosomal translocation t(17;19)(q22;p13) in leukaemic pro-B cells points to the involvement of Grg/TLE family proteins in proper development of B-lymphocytes [Dang et al., 2001].

MYOGENESIS

Myogenesis is regulated by some members of basic helix-loop-helix (bHLH) family of transcription factors [Molkentin and Olson, 1996; Yun and Wold, 1996]. One of myogenic bHLH factors is MyoD [Hasty et al., 1993; Nabeshima et al., 1993; Rudnicki et al., 1993; Rawls et al., 1998]. It has an antagonist, MyoR, a myogenic repressor which is abundantly expressed in undifferentiated myoblasts in culture, but is downregulated during differentiation [Lu et al., 1999]. In addition, certain HES family proteins (mammalian homologs of *Drosophila* Hairy and Enhancer of Split) were shown to be involved in myogenic processes. HES-1 inhibits the activity of MyoD causing an inhibition of myogenesis [Sasai et al., 1992]. The other factor, HES6, seems to promote myoblast differentiation, although its mode of action is not clear yet. HES6 downregulates MyoR in myoblast cell cultures [Gao et al., 2001]. The repression ability of HES6 may depend on its interaction with TLE1 or other TLE family members, as it was shown that TLE1 and HES6 interact in vitro via the C-terminal WRPY motif of HES6 and this WRPY motif is necessary for HES6 mediated repression [Gao et al., 2001].

As it is known that TLE family members can interact with other HES proteins, for instance HES-1, it would be interesting to investigate the effect on myogenesis of transcriptional repression mediated by different TLE-HES combinations [Grbavec and Stifani, 1996; Grbavec et al., 1998]. It would also be interesting to analyze whether—in analogy to the developing nervous system—HES-mediated phosphorylation of TLE proteins occurs during myogenesis and how it regulates TLE corepressor activity [Nuthall et al., 2002a].

INVOLVEMENT OF Grg/TLE IN EYE DEVELOPMENT—Six3 AND Six6

Six3 and Six6 are the only members of the Six gene family expressed during the early stages of visual system development [Oliver et al., 1995; Jean et al., 1999; Lopez-Rios et al., 1999; Toy and Sundin, 1999]. On the basis of phylogenetic analysis they were included in the same Six gene subclass as the *Drosophila* optix gene [Jean et al., 1999; Seo et al., 1999; Seimiya and Gehring, 2000]. There is ample data showing the importance of Six3 and Six6 activity for eye formation. The mis-expression of Six3 in transgenic mouse embryos caused the induction of ectopic optic vesicle-like structures or lenses, whereas the over-expression of Six6 increased the eye size in *Xenopus* [Zuber et al., 1999; Bernier et al., 2000; Lagutin et al., 2001]. In humans, loss-of-function mutations in the *SIX3* gene cause holoprosencephaly type II, whereas SIX6 has been associated with anophthalmia and pituitary defects [Gallardo et al., 1999; Wallis et al., 1999; Pasquier et al., 2000].

Groucho family members were shown to be co-expressed, interact with and modulate the activity of Six3/Six6 transcription factors in the developing eye. The expression patterns of Grg4 and Grg5 in mouse embryos are similar to the one of Six3, which is expressed in the ventral forebrain and developing optic vesicle [Oliver et al., 1995; Koop et al., 1996; Zhu et al., 2002]. Grg3 is expressed in the neural layer of the retina and the lens at embryonic day E12.5 and in neuroblastic layer of the retina at E16.5 similar to the expression pattern of Six3 [Leon

and Lobe, 1997; Zhu et al., 2002]. Grg4 and Grg5 were shown to interact physically with Six3 and Six6 and TLE1 and AES with SIX3 and SIX6 [Zhu et al., 2002; Lopez-Rios et al., 2003]. The Q domain of Grg/TLE proteins interacts with the eh1-like motif within the highly conserved Six domain of the Six3/Six6 proteins [Zhu et al., 2002; Lopez-Rios et al., 2003]. Furthermore SIX3 interacts with TLE proteins via the WDR domain [Lopez-Rios et al., 2003]. The interaction with Grg/TLEs is important for Six3-mediated repression in vitro and in vivo [Zhu et al., 2002]. Both Grg4 and Grg5 enhance Six3-mediated auto-repression in cell assays and interaction of Six3 with Grg proteins was shown to be relevant for photoreceptor differentiation in the developing rat retina [Zhu et al., 2002]. For other mammalian proteins involved in eye development besides Six3 and Six6, no interaction with Grg/TLE family proteins has been reported. An interaction of Grg4 and Grg5 with Six2 and Six4 could be detected neither by GST pull-down experiments nor yeast two-hybrid analyses [Zhu et al., 2002]. The repression of γE/F crystallin by Pax6 was shown not to be mediated by Grg4 [Kralova et al., 2002].

All of these point to a crucial role of Grg/TLE corepressors in a developing visual system.

The reports about the involvement of Grg/TLE-mediated Six3/Six6 regulation in eye development in other vertebrates, for example lens morphogenesis and crystallin regulation in chicks, retina development in medaka fish and eye and forebrain formation in zebrafish suggest the existence of similar mechanisms in mammals [Kobayashi et al., 2001; Zhu et al., 2002; Lopez-Rios et al., 2003].

NERVOUS SYSTEM DEVELOPMENT

The process in which the role of Grg/TLE proteins has been described in most detail is neurogenesis.

During the development of the mammalian central nervous system neural progenitor cells located in ventricular zone of the neural tube start to proliferate and ultimately differentiate into neurons as a response to both intrinsic and extrinsic cues. The process of commitment of progenitor cells into neural fate and their final differentiation into mature neurons is controlled, in part, by either positive or negative regulatory proteins belonging to two separate families of transcription factors containing the bHLH motif. Proteins promoting neural differentiation include transcriptional activators that are referred to as proneural proteins (reviewed in [Kageyama and Nakanishi, 1997; Anderson, 1999]). These proneural proteins are divided into the Neurogenin, Ash, Ath, and NeuroD sub-families [Guillemot et al., 1993; Lee et al., 1995; Ma et al., 1996; Ben-Arie et al., 1997; Fode et al., 1998; Ma et al., 1998]. The other family of bHLH factors consists of transcriptional repressors which negatively regulate neuronal differentiation. It comprises a number of factors homologous to *Drosophila* Hairy/Enhancer of split (HES). In *Drosophila* the combined activity of HES proteins and Groucho negatively regulate the expression of neurogenic genes in response to signaling through the Notch pathway [Delidakis et al., 1991; Knust et al., 1992; Jennings et al., 1994; Paroush et al., 1994; Fisher et al., 1996; Heitzler et al., 1996].

The best characterized mammalian homologue of *Drosophila* HES is HES-1, a strong antineural repressor. Persistent expression of Hes-1 inhibits neuronal differentiation in the developing telencephalon and, conversely, targeted disruption of Hes-1 causes premature neuronal differentiation and up-regulation of proneural genes [Ishibashi et al., 1994, 1995; Ohtsuka et al., 1999; Tomita et al., 1999].

Recent studies on brain developmental processes show that Hes-1 interacts with TLE1 to negatively regulate postmitotic neuronal differentiation in the central nervous system. First the temporal and spatial expression patterns of Hes-1 and TLEs are very similar. Both proteins are highly expressed during the progenitor-to-neuron transition while later on their expression decreases during the developmental maturation of postmitotic neurons [Sasai et al., 1992; Yao et al., 1998]. Second—the in vivo experiments with constitutive ectopic expression or depletion of these proteins produced similar effects. Constitutive expression of TLE1 in murine postmitotic neurons inhibits neuronal development in the embryonic forebrain leading to a loss of cortical and striatal neurons of telencephalon [Yao et al., 2000]. Similarly persistent Hes-1 expression inhibits neuronal differentiation in the developing telencephalon, while its targeted disruption causes premature neuronal differentiation and up-regulation of proneural genes [Ishibashi et al., 1994, 1995;

Ohtsuka et al., 1999; Tomita et al., 1999]. Interestingly neither constitutive TLE1 expression in postmitotic neurons nor Hes-1 depletion significantly perturbed spinal cord or peripheral nervous system development [Ishibashi et al., 1995; Yao et al., 2000].

Third TLE1 and Hes-1 interact physically in vitro and this interaction is required for both nuclear matrix association of Hes-1 and its transcriptional repression activity [Grbavec and Stifani, 1996; McLarren et al., 2001; Ju et al., 2004]. TLE1 was shown to be a part of a repressor complex mediating HES-1 dependent MASH1 repression in neural stem cells and in 239 cells. This complex consists of about 13 polypeptides associated with TLE1. Nine of them were identified. These are non-muscle myosin II heavy chain, TopoIIβ, Rad50, PARP-1, nucleolin, HSP70, p54nrb, β-actin, and nucleophosmin. Ju et al. [2004] put forward a model how the HES-1-TLE1-PARP-1 trio regulates neuronal differentiation by control of *MASH1* gene transcription. In a first step, as an answer to the Notch signaling pathway HES-1 recruits TLE1 and thus the TLE1-dependent repressor complex to the promoter of MASH1 causing a repression of this gene in proliferating neural stem cells. In a second step induction of Ca^{2+}/CaMKII dependent program activates a member of the repression complex, PARP-1 (poly(ADP-ribose) polymerase 1), which poly (ADP-ribosyl)ates TLE1 and some other repression complex members causing them to dissociate from HES-1 and thus derepressing the *MASH1* gene. CaMKII also induces phosphorylation of HES-1 on S126 permitting it to recruit coactivators, including CBP, and finally to activate MASH1 which is important for neuronal differentiation [Ju et al., 2004].

Fourth Hes-1 mediates hyperphosphorylation of TLE by protein kinase CK2, which is correlated with an increase in the affinity of TLE proteins to the nuclear compartment and TLE-mediated transcriptional repression [Nuthall et al., 2002a]. Hes1–TLE interaction is not influenced by Grg5, but can be negatively regulated by interaction with Runx2 or by Hes6 [McLarren et al., 2001; Gratton et al., 2003]. Hes6 forms heterodimers with Hes-1. Hes-1-Hes6 heterodimers interact poorly with TLE, and probably reduce the interaction of Hes-1 homodimers with TLE depleting Hes-1 of its critical corepressor and negatively regulates its function [Gratton et al., 2003].

Taken together this information points to a crucial negative role of the TLE1-Hes-1 duo in neural fate determination drama.

The other members of the mammalian Gro homolog family also seem to exert an influence on brain development, although their role is not yet as well characterized as that of TLE1. All of these proteins are expressed in CNS and their temporal and spatial expression patterns are both complementary and combinatorial pointing to a non-abundant role of TLE proteins in brain development [Yao et al., 1998]. TLE2 is expressed in areas of the developing embryonic brain and spinal cord containing postmitotic neurons [Grbavec et al., 1998]. It is also more abundantly expressed in the neonatal and adult nervous system than other *TLE* genes and the level of TLE2 decreases during early stages of in vitro differentiation, while levels of TLE3 and TLE4 remain constant and TLE1 level is rapidly increasing [Stifani et al., 1992b; Husain et al., 1996; Yao et al., 1998]. These data suggest that TLE2 oppositely to TLE1 plays a role in maturation and survival processes of postmitotic neurons rather than in determination and differentiation of proneural cells [Grbavec et al., 1998]. As TLE2 is co-expressed with HES-1 and HES-5 in the developing mammalian nervous system and can physically interact with the latter, it seems very likely that the TLE2 corepressor achieves its function via interaction with those transcription factors [Akazawa et al., 1992; Sasai et al., 1992; Grbavec et al., 1998]. The other member of Grg/TLE family—TLE3, similar to TLE1 is expressed in cortical neurons of more external layers, while TLE4 is present in differentiating and differentiated neurons in more internal layers of cortical plate and in neural progenitor cells in early neurogenesis [Dehni et al., 1995; Koop et al., 1996; Yao et al., 1998]. The mouse homologue of human TLE4, Grg4, seems to be involved in repression of Fgf8 by Lmx1b, being important factors in formation and maintenance of isthmus organizer activity [Matsunaga et al., 2002].

DEVELOPMENT OF PITUITARY GLAND—INTERACTION OF Grg1 WITH Hesx1

During the development of the pituitary gland, two highly related paired-like homeodomain factors, a repressor Hesx1/Rpx and an activator, Prop-1 are expressed in sequential, overlapping temporal patterns [Dattani

et al., 1998; Martinez-Barbera et al., 2000; Thomas et al., 2001]. Repressive actions of Hesx1 could be required for the initial pituitary organ commitment, and progression beyond the appearance of the first pituitary (POMC) lineage requires loss of Hesx1 expression and the action of Prop-1 [Gage et al., 1996; Hermesz et al., 1996; Sornson et al., 1996]. Grg1 was shown to be broadly expressed throughout Rathke's pouch where its expression pattern coincides with Hesx1 between E9.5 and E12. Additionally Grg1 and Hesx1 were demonstrated to physically interact with each other [Dasen et al., 2001]. Grg1 is thought to act as a corepressor with HESX1 to antagonize the action of PROP1 [Dasen et al., 2001]. In addition a homozygous mutation within eh1 of human HESX1, associated with evolving hypopituitarism, impairs the function of HESX1 as a transcriptional repressor and this reduction in transcriptional repression is mediated by impaired interaction with Grg/TLE corepressor [Carvalho et al., 2003]. The other Grg/TLE family genes *Grg5*, *Grg3*, and *Grg4* are also expressed in the pituitary gland, but their expression pattern is not as broad as the one of Grg1, but dynamic and regionally restricted. Grg3 and Grg5 are localized to the dorsal aspects of the pituitary gland and Grg4 is localized in the infundibulum. Prop-1 is required to restrict expression of Grg3, but not of Grg5 [Brinkmeier et al., 2003].

The expression pattern of Grg3 overlaps with that of NKX3.1, a transcription factor comprising an eh1 domain [Treier et al., 1998; Brinkmeier et al., 2003]. This domain was shown to be important for interaction with Grg/TLE proteins. The other eh1-containing transcription factors are also expressed in the pituitary gland. These are the TCF/Lef family proteins, Hes-1, Six3, Six6 which have been shown to interact with Grg/TLE proteins in other tissues (Table I) [Treier et al., 1998; Brantjes et al., 2001; Dasen et al., 2001; Kobayashi et al., 2001; Scully and Rosenfeld, 2002; Zhu et al., 2002]. It therefore seems most likely that Grg/TLE family members expressed in pituitary gland are involved in the regulation of gene expression mediated by those factors opening up a huge field for investigations.

NEURAL TUBE

Grg/TLE corepressors play an important role in neural tube patterning. This process is controlled by a graded Sonic hedgehog (Shh) signaling regulating the expression of progenitor HD proteins which are divided into two classes. Class I consists of Dbx1, Dbx2, Pax6, Pax7, Irx3 and these proteins are expressed in the absence of Shh signaling. Class II contains Nkx6.1, Nkx6.2, Nkx2.2, and Nkx2.9 the expression of these factors depending on Shh signaling. Cross-regulatory interactions between complementary pairs of class I and class II HD proteins seem to be responsible for the establishment of progenitor cell identity and sharp boundaries between adjacent domains ensuring that cells within individual domains express distinct combinations of HD proteins [Ericson et al., 1997; Briscoe et al., 2000; Sander et al., 2000]. It appears that once an individual domain with an individual constellation of HD proteins is established, the consequent activation of expression of downstream transcription factors drives the cells to the certain neural-type fate [Sharma et al., 1998; Tanabe et al., 1998; Pierani et al., 1999; Briscoe et al., 2000]. Still the mechanism of those events is not well known.

Muhr et al. [2001] proposed a model in which the pattern of neuronal generation in the ventral neural tube is achieved through the spatially controlled repression of transcriptional repressors—a derepression strategy for neural cell specification. In other words, in certain domains the HD proteins of one HD class repress HD proteins of the other class, thus permitting the expression of certain subclass determinants. The repression mediated by HD proteins most probably involves corepressors from Grg/TLE family. First, it was shown that Grg3 and Grg4 are expressed in the neural tube. In E10.5 mouse neural tube Grg3 is expressed ventrally and Grg4 is expressed in ventral and intermediate levels and more dorsally [Miyasaka et al., 1993; Koop et al., 1996; Leon and Lobe, 1997; Muhr et al., 2001]. At E15.5 high levels of Grg4 and Pax2 are coexpressed in the dorsal half of neural tube at a time when Pax2 positive interneurons have migrated from the ventricular zone [Cai et al., 2003]. Grg1 expression was not detected in the neural tube and Grg5 is expressed primarily in post-mitotic neurons [Muhr et al., 2001].

Second, Gro/Grg proteins can interact physically with HD proteins. Grg4 was shown to interact in vitro with all members of class II HD proteins [Muhr et al., 2001]. This interaction

enhances class II HD factors repression ability and is mediated by TH domain which shows sequence similarity to eh1 motif present in *Drosophila* Engrailed (En) [Smith and Jaynes, 1996; Muhr et al., 2001]. Gro binds Dbx1, Dbx2 and Pax7, class I HD proteins, but fails to bind Pax6 and Irx3 which do not possess a TN motif [Ericson et al., 1997; Briscoe et al., 2000; Muhr et al., 2001]. It was also shown that a reduction in Gro/TLE activity blocks the ability of class II proteins to repress class I protein expression [Muhr et al., 2001].

All the above points to an important role of interaction between Grg/TLE corepressors and HD transcription factors in the process of neural tube patterning and neural cell fate determination.

PLACENTA

Some of the Grg/TLE family members are expressed in the placenta. In humans strong expression of TLE3 mRNA was detected. The mRNA of TLE1 and TLE2 was also detected in placenta, but their expression was weaker compared to that of TLE3. The expression of TLE4 mRNA was not observed [Stifani et al., 1992a]. In the mouse Grg1 is expressed only in maternal decidual cells. Grg2 is expressed exclusively in trophoblast giant cells at E8.5, but at E10.5 it is expressed in giant cells as well as in a few cells scattered in the spongiotrophoblast layer. Grg3 is expressed in all layers of the placenta. Grg3 mRNA is strongly expressed in giant cells and weakly in the ectoplacental cone at E8.5. At E10.5 it is expressed in giant cells, spongiotrophoblast, labyrinth and in scattered cells in the decidua [Nakayama et al., 1997]. It is supposed that little or no Grg4 or Grg5 is expressed in mouse placenta [Mallo et al., 1993].

The above data suggest that the most important corepressor from Grg/TLE family in the development of human or mouse placenta is Grg3/TLE3.

Grg/TLE could influence the development of placenta via interaction with HES transcription factors.

Mammalian HES transcription factors, related to *Drosophila* hairy and Enhancer of split (E(spl)), are effectors of the Notch signaling pathway [Akazawa et al., 1992; Sasai et al., 1992; Takebayashi et al., 1994, 1995]. As an effect of Notch signaling HES proteins accumulate and downregulate expression of certain genes. One of putative target genes for HES factors in placenta is Mash-2, as it is a homologue of *Drosophila* Achaete-scute which in fruitfly is a target for hairy and E(spl). Grg/TLE proteins have already been shown to interact with HES-1, HES-5, and HES-6 and act as a corepressor of Hes-1 [Grbavec et al., 1998; Gao et al., 2001].

In mouse placenta Notch-2 is the only Notch receptor expressed. Of the HES family at E10.5 only HES-2 and HES-3 are expressed in all cell layers: labyrinth, spongiotrophoblast, giant cells, and decidua. Of the Grg/TLE family Grg2 and Grg3 are most abundantly expressed in the placenta. The expression pattern of HES-2 and HES-3 overlaps with the one of Grg2 and Grg3 and coincides with the decrease of Mash-2 expression. Therefore, it is proposed that in the response to Notch-2 activation, HES-2/3 interact with Grg2/3 to downregulate the expression of Mash-2.

INTESTINE

Grg1-S, a short form of Grg1, consisting only of the Q and GP domains, is strongly expressed in developing mouse gut and in adult small intestine. It was demonstrated that Grg1-S represses β-catenin/Tcf-mediated gene activation in vitro and in vivo [Lepourcelet and Shivdasani, 2002]. The proper action of the β-catenin/Tcf4 complex is crucial for correct development of the intestine demonstrated by the fact, that Tcf-4-deficient mice develop abnormal small intestines and that β-catenin/Tcf4 is constitutively active in colon carcinoma [Korinek et al., 1997, 1998]. These informations suggest that Grg1-S could play an important role in gut development and function.

ROLE OF Grg/TLE IN APOPTOSIS

Recently it was reported that AES is an important factor mediating apoptosis caused by loss of cell attachment to the ECM, a process known as anoikis. Bit1, a mitochondrial protein, when released from mitochondria forms a complex with AES and together they induce apoptosis. The Bit1/AES pro-apoptotic pathway is selectively suppressed by integrin-mediated cell attachment. Particularly it is regulated by $\alpha5\beta1$ integrin, which binds fibronectin, but not by the collagen binding integrins $\alpha1\beta1$ and $\alpha2\beta1$. This pathway is also atypical in that caspase activation is not involved [Jan et al.,

2004]. Overexpression of TLE1 is inhibiting Bit1/AES complex formation and Bit1/AES mediated apoptosis. TLE2 did not counteract Bit1/AES apoptotic effect to such an extent as TLE1 did [Jan et al., 2004]. It was also shown that elevated TLE1 expression is unfavorable prognostic sign in lymphoma [Shipp et al., 2002]. TLE1 may protect lymphoma cells against apoptosis caused by loss of cell attachment. It is not known, however, whether an upregulation of *TLE* genes is a general mechanism to prevent apoptosis during carcinogenesis.

SUMMARY

TLE/Grg corepressor proteins take part in many crucial processes. As more and more knowledge accumulates on the function of the different proteins it becomes clear that their role in development is non-redundant. One of the most novel and maybe most interesting facets of the different functional aspects of TLE/Grg proteins is their involvement in apoptosis. This observation provides a link between these proteins and not only physiological development of the organism, but also malignant growth. Experiments that further elucidate role and function of this protein family are urgently awaited. Resolving the three-dimensional structure as well as generating mouse strains deficient in the expression of these proteins will provide important insights into role and function of TLE/Grg proteins.

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