Identification of the gene transcription repressor domain of Gli3

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Gli transcription factors are downstream targets of the Hedgehog signaling pathway. Two of the three Gli proteins harbor gene transcription repressor function in the N-terminal half. We have analyzed the sequences and identified a potential repressor domain in Gli2 and Gli3 and have tested this experimentally. Overexpression studies confirm that the N-terminal parts harbor gene repression activity and we mapped the minimal repressor to residues 106 till 236 in Gli3. Unlike other mechanisms that inhibit Gli induced gene transcription, the repressor domain identified here does not utilize Histone deacetylases (HDACs) to achieve repression, as confirmed by HDAC inhibition studies and pull-down assays. This distinguishes the identified domain from other regulatory parts with negative influence on transcription.

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1. Introduction

The morphogens of the Hedgehog (Hh) family are crucial for numerous developmental processes during embryogenesis as well as postnatally. In fact the Hh pathway is one of the four major signaling systems that are controlling the major developmental processes. Gli proteins are Zn-finger transcription factors and are targets as well as mediators of the Hh signaling pathway [1]. Mammals have three Gli genes encoding Gli1, Gli2 and Gli3 that are orthologs of the Drosophila transcription factor Cubitus interruptus (Ci). Ci is mainly a transcriptional activator in its full-length form that dominates in the presence of Hh. In the absence of Hh, a portion of Ci is proteolytically cleaved to produce an N-terminal gene repressor form. In a similar fashion Gli2 and Gli3 can undergo proteolysis to produce a gene repressor form. The full-length forms of Gli2 and Gli3 act as gene activators. A repressor form of Gli1 cannot be generated and Gli1 is considered to be a strong gene activator. The dominating role of Gli2 appears to be gene activation whereas Gli3 often has a gene repression role, mediated by the N-terminal part. In humans, several Gli3 morphopathies have been described, which can be broadly divided into two classes: Greig's syndrome caused by total loss of GLI3 function and Pallister–Hall syndrome (PHS)/other postaxial polydactylies that are presumed to be caused by abnormally high repressor generation. The first identified mutations causing PHS were found in the GLI3 gene [2]. Since then several GLI3 mutations have been identified in the same region (exons 12–14). Both original mutations are single nucleotide deletions that lead to frame shift and premature translational stop [2]. The produced peptide has 691 residues (compared to the 1596 residue full-length protein) but contain alternative residues in the last approximately 20 residues, encoded after the mutations [2]. It was shown that the corresponding peptide Gli3-PSH (residues 1–674) indeed has strong gene repressor activity, which may explain the phenotypes of these patients [3]. Due to its vast impact on cell differentiation and proliferation aberrant Hh signaling is involved in many cancers and several gene members of the pathway are either proto-oncogenes or tumor suppressors [4]. A thorough analysis of the Gli proteins is therefore important in order to understand the associated developmental biology and pathology as well as related carcinogenesis.

To further analyze the repressor function in the PHS part of Gli3 and to identify the specific repressor sequence, we made a series of GLI3 constructs and evaluated their activity in cellular gene regulation assays. This led to the identification of a specific repressor domain in GLI3 also conserved in Gli2 but not in Gli1. The repressor function of this domain is not dependent on histone deacetylases (HDAC) and therefore works through a different mechanism.

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Abbreviations: β-gal, β-galactosidase; Ci, Cubitus interruptus; DMEM, Dulbecco’s modified Eagle’s medium; FCS, fetal calf serum; HDAC, histone deacetylases; Hh, Hedgehog; PHS, Pallister–Hall syndrome; TSA, trichostatin-A.
2. Materials and methods

2.1. DNA constructs

Gli1, Gli1(1–407), Gli3, Gli3-PHS, Gli3ARD and Gli3-PHSARD all of human origin were cloned into pcDNA3.1His expression vector (some of these were described before [3,5]). The 12GliRE-luc and β-galactosidase (β-gal) constructs were described before [5]. The Gli3 repressor domain (residues 106–246) and shorter versions were subcloned into the pFA vector in frame with the DNA Binding Domain (DBD) of yeast Gal4 (Stratagene, La Jolla, CA, USA). As Gal4 reporter construct was used the pMN-Luc plasmid containing a thymidine kinase promoter with five tandem repeats of the yeast GAL4 binding sites that control expression of the firefly luciferase gene. For recruitment of HDAC in gene silencing we employed the C-terminal HDAC dependent repressor domain of the rat REST protein [6] cloned in frame with GAL4 DBD in pFA.

2.2. Cell culture

HEK293 cells were grown and transfected as previously described [7]. The cells were maintained in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% fetal calf serum (FCS) (PAA Laboratories, Pasching, Austria), streptomycin and penicillin (100 units/ml; Invitrogen, Carlsbad, CA, USA). Cells were grown at 37 °C and 5.0% CO2 in cell culture incubator. One day before transfection cells were plated into the required growth plates. Shh-Light2 cells were grown in the same medium as HEK293 supplemented with 4·18 (400 μg/ml; Sigma–Aldrich, St. Louis, MO, USA) and Zeocine (100 μg/ml; Invitrogen). At 24 h post transfection the medium was changed to low-serum medium (0.5% of FCS; PAA Laboratories).

2.3. Luciferase assays

Transfections for luciferase assays were performed in 24-well plates. Assessment of Gli1, Gli3 and Gli3-PHS in HEK293 cells was done as previously described [5,7]. Assessment in Shh-L2 cells was performed as described [8], using the incorporated luciferase gene as measurement of gene activation and the co-transfected β-gal as control. Transfections were done with the same amount of total DNA by using empty vector to compensate.

For measurement of the Gli3-RD deletion constructs we transfected HEK293 cells also using the β-gal construct as control. The amount of reporter plasmid (pMN-Luc) used was 300 ng per well and the effector plasmids (pFA Gal4 fusions with RD segments) were 30 ng per well. For normalization we used 100 ng of pCMV-β-gal. As a transfecting agent we used polyethylenimine (PEI; Sigma–Aldrich) 1 μg per well. DNA and PEI were mixed in 50 μl of DMEM. An additional 150 μl of DMEM was added to the DNA/PEI mixture and then applied to the cells. After 2 h the medium was exchanged for DMEM with 10% FCS. On the following day the medium was changed again and where required, trichostatin A (TSA) was added at 0.2, 0.5, and 1 μM. Cells were harvested after an additional 24 h. Firefly luciferase and β-gal assays were performed in Ascent FL fluoroskan with the Luciferase Assay Kit (BioThema, Darlå, Sweden) and Galacto-Light Plus System (Applied Biosystems, Foster City, CA, USA). In assays measuring HDAC induced gene silencing we used the REST expressing pFA vector as positive control of HDAC recruitment.

2.4. Immunoprecipitation and HDAC enzymatic assay

To test the association of Gli3-RD with HDAC we immunoprecipitated Gli3-RD and measured HDAC enzymatic activity of the precipitate. As a positive control we immunoprecipitated Sin3A that is known to be in a complex with HDAC1 and HDAC2.

We transfected HEK293 cells with Gli3-RD in 15 cm culture dishes. The DNA/PEI complex was prepared as follows: 30 μg of Gli3-RD DNA was mixed with 60 μg of PEI per plate in 500 μl of DMEM. After 10 min of incubation 9 ml of DMEM was added to the DNA/PEI mixture and then applied to the culture dish. After 2 h the medium was exchanged for DMEM with 10% FCS. On the following day, the medium was changed again and cells were lysed in PBS with 1% Triton X-100 (Sigma–Aldrich) after an additional 24 h.

For immunoprecipitation of Gli3-RD 5 μl of anti-Gal4 polyclonal antibodies (Santa Cruz Biotechnology, Santa Cruz, CA, USA) were incubated with 30 μl Protein G agarose (Amersham Biosciences, Bucks, UK). After 1 h 1 ml of cell lysate was added and immunoprecipitation was performed at 4 °C overnight. The immunoprecipitation of Gli3-RD was analyzed by Western blot using Gal4 monoclonal antibody (Santa Cruz Biotechnology). Sin3A was immunoprecipitated and detected as described above using an anti mSin3A antibody (Santa Cruz Biotechnology).

HDAC activity was measured using a fluorescent substrate Fluor de Lys (Biomol, Plymouth Meeting, PA, USA) that contains an acetylated lysine side chain. This substrate was incubated with immunoprecipitated Gli3-RD or mSin3A. If the immunoprecipitate contain HDAC’s the substrate is deacetylated and a fluorophore is produced. Assays were performed according to manufacturer’s instructions, and measured in Tecan GENios pro microplate spectrophotometer (Tecan Group, Männedorf, Switzerland) with the Magellan V5.03 system (Tecan Group).

3. Results and discussion

The Gli3-PHS part (residues 1–673) was shown to contain gene repressor activity [3]. Using a two-hybrid screening technology it was shown that almost the same part (residues 1–613) in Gli3 binds to Ski [9]. The Ski binding site on Gli3 was determined to the region from residue 152 to 397 using pull-down assays [9]. Since Ski is known to be a part of a gene repressor complex including HDAC, it was suggested that Gli3 exerts its repressor activity through binding of Ski and recruitment of HDAC [9]. However, most of the Ski binding region is conserved between all three Gli proteins, suggesting that Ski binding and HDAC recruitment is part of a general transcription termination signal common to all Gli proteins. Likewise, the SUFU binding site (BS) on Gli proteins (SYGH) is also found in all three Gli proteins [10]. Also SUFU is known to recruit HDAC through recruitment of SAP18 and Sin3A [11] and therefore, SUFU binding may also be regarded as a general mechanism to turn off Gli mediated transcription. Recently, two sites in Gli1 were identified as responsible for protein degradation [12]. One peptide (degron) was in the C-terminal part (DC) whereas the other was found in the N-terminal part (DN). In fact the DN part, contains DN and the SYGH peptide. Fig. 1A shows a schematic alignment of the N-terminal parts of mammalian Gli proteins, until the end of the Zn-fingers (corresponding to the PHS-domain), with indications of the respective domains describe above. From this work and a previous paper [3] it is suggested that the gene
repressor function is localized to the N-terminal (grey) part of Gli2 and Gli3. The physiological significance was shown by regulation of PTCH1 transcription [3]. We suggest that the other mechanisms with negative gene transcription activity (Ski BS, SUFU BS and DN peptide) that are common to all Gli proteins, are general means to terminate Gli induced transcription. At least two of these pathways (Ski and SUFU through interaction with SAP18 and Sin3A) probably recruit HDACs to terminate transcription and increase the degradation of the Gli protein.

As stated above it has previously been shown that the Gli3-PHS domain is a repressor of both basal and Gli3 induced transcription [3]. However, since Gli3 only induces a modest transcriptional activation we wanted to test the PHS domain together with the much stronger transcriptional activator Gli1. Therefore, in HEK293 cells Gli1, Gli3 and Gli3-PHS were transfected alone or in combinations together with a Gli-luciferase reporter construct [5]. As shown before [5,7] Gli1 is an effective activator of transcription whereas Gli3 only activates weakly (Fig. 1B). The transcriptional activation of Gli1 is strongly inhibited by co-expressing Gli3-PHS. Expression of full-length Gli3 also leads to repression of Gli1 induced transcription, although significant activity is seen. Gli3 on its own gives much lower activity but it appears that repression of Gli1 is a more pronounced effect (3–4 times Gli3 induction vs. 6 times repression). In other words, not only does the Gli3-PHS have repressor activity on its own [3] it also strongly repress Gli1 induced transcription. Expression of the Gli1 peptide corresponding to Gli3-PHS (residues 1–407) only weakly suppresses Gli1 induced transcription, which is likely to be due to competitive expression and suggest that the N-terminal of Gli1 does not exert any significant repressor function.

In order to analyze the constructs in a more in vivo-like setting, we turned to the Shh-Light2 (Shh-L2) cells that have a Gli-inducible luciferase reporter construct incorporated into the genome.[14]. Transfection of these cells is less efficient and the transcrip-
Transcriptional induction by Gli1 is much lower than in the HEK293 cells (Fig. 2). The induction posed by Gli3 is also lower but the difference is not as pronounced as in HEK293 cells. This may indicate that in the Shh-L2 cells the transcriptional regulation of the reporter is different from the vector-based one used in HEK293 cells. Alternatively, the differences could reflect differences in the ratios of the expressed Gli peptides. In the Shh-L2 cells Gli3 does not affect Gli1 induced transcription as much as in the HEK293 cells.

However, the pattern for both Gli3 and Gli3-PHS induced Gli1 repression is the same as in HEK293 cells, showing that the PHS domain is a strong repressor.

Based on the alignment we made deletion constructs of Gli3 and Gli3-PHS that lack residues 105–246 (Gli3\textsubscript{ARD} and Gli3-PHS\textsubscript{ARD}), corresponding to the grey area in Gli2 and Gli3 (Fig. 1A). Removal of this part enhances the gene transcription induction of Gli3 (Fig. 2). This is confirmed in the Gli1/Gli3\textsubscript{ARD} combination, though the effect is small. The effect is much more pronounced when comparing the repression of Gli3-PHS on Gli1 induced transcription to that of Gli3-PHS\textsubscript{ARD} (Fig. 2). In the latter case the repression is reduced almost 10 times. This clearly indicates that the particular sequence harbors significant gene transcription repression activity. In fact most (if not all) the repressor function is located in this part of the PHS domain.

We then proceeded to make constructs containing this repressor domain (RD) and parts of this in frame with Gal4 DBD and used the constructs to assess the minimal RD of Gli3. We used HEK293 cells that were also transfected with a Gal4 DBD responsive luciferase reporter. The DBD of the Gli3-RD fusion peptides bind to the reporter plasmid at the GAL4 binding sites. Compared to Gal4 alone (pFA vector) the N-terminus of Gli3 and the Gli3-RD significantly represses gene expression (Fig. 3). However, shorter versions of the Gli3-RD lose the ability to repress transcription and therefore we mapped the minimal RD of Gli3 between residues 106 and 235. When the RD is shortened beyond these residues the repression is reduced or lost.

The suggested Ski binding site on Gli3 has not been exactly mapped (it is in the region from residue 152 to 397\textsuperscript{[9]}) and may overlap with the identified repressor part. Since Ski and SUFU recruit HDACs to exert their inhibitory role, we wanted to test if the repressor function described here depends on the same mechanism or not. We transfected HEK293 cells with either Gli3-RD or the repressor domain of REST (that serve as positive control since it depend on HDAC to repress transcription) and tested the effect of the HDAC inhibitor TSA as shown in Fig. 4. Again the Gli3-RD suppresses transcription but there is not any effect of TSA up to 1 μM.

Fig. 2. Analyzes of the repressor function of the Gli3-PHS domain and the repressor domain (RD) in Shh-L2 cells. Shh-L2 cells (with an incorporated Gli responsive luciferase reporter gene) were transfected with Gli1, Gli3, Gli3-PHS, Gli3\textsubscript{ARD}, Gli3-PHS\textsubscript{ARD} or combinations of these to assess the effect on transcription alone or on the Gli1 induced transcription. The analyzes were performed at least three times and error bars indicate the standard deviations.

Fig. 3. Determination of the minimal repressor domain of Gli3. The repressor domain (residues 105–246) or parts of this were expressed together with the DBD of Gal4 and assessed for repression of Gal4 induced transcription in HEK293 cells (mock). Also a larger part of the Gli3 N-terminal part was measured since this is known to have significant repressor function (residues 1–480). The analyzes were performed three to five times and error bars show the standard deviations.
Sin3A is a transcriptional regulator known to recruit HDACs to the body. In this precipitate there was significant HDAC activity. precipitated Sin3A from the HEK293 cells using an mSin3A anti-HDAC binding (not shown). As a positive control we also immunoprecipitated both Gal4 DBD and the fusion protein showed any significant HDAC activity. Neither Gal4 DBD nor the fusion protein results from the precipitate. Neither Gal4 DBD nor the fusion protein showed any significant HDAC binding (not shown). As a positive control we also immunoprecipitated Sin3A from the HEK293 cells using an mSin3A antibody. In this precipitate there was significant HDAC activity. Sin3A is a transcriptional regulator known to recruit HDACs to induce gene silencing [15,16]. Both Ski and SUFU recruits HDACs through Sin3A. Thus, the repression induced by the Gli3-RD is through a different mechanism than that of Ski and SUFU.

When considering that HDACs are involved in more permanently shutting down a target gene, this also makes good sense. This mechanism is probably more useful when drastic measures are required, e.g. when Hh signaling ceases, or when other signals determine that Gli induced transcription has to end. Therefore, these signals (Ski/SUFU/HDAC) [9,11] are probably common to all Gli proteins. Also the degradation signal (DN) is of this general category and leads to removal of the targeted Gli protein and we suggest that the Ski and SUFU signals recruit HDACs to termi- nate Gli induced transcription at the Gli binding site on DNA (Fig. 1). In contrast our results clearly suggest that the gene transcription repression exerted by the N-termini of Gli2 and Gli3 is mediated by the repressor domain identified here and indicated with grey in Fig. 1A. We also suggest that the Ski binding site is strictly localized in the region common to all three Gli proteins, but a more exact mapping of the Ski binding site requires further experimentation. It remains to be investigated by which mechanism Gli3-RD regulates transcription. Perhaps the domain recruits other proteins than HDACs or interacts (physically or functionally) with the transcriptional machinery. It has been shown that Gli3 interacts with and regulate gene transcription via mediator [17] and perhaps the repressor domain it able to influence this interaction. However, the mediator binding site is localized at the C-terminal part of Gli3 [17] and the Gli3-PHS like repressor that is generated in vivo is not likely to bind mediator, but may exert its repressor function by an independent mechanism. It is clear that the repression is not dependent on HDACs and investigations of the mechanism of repression have been initiated.

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References

[1] Hooper, J.E. and Scott, M.P. (2005) Communicating with Hedgehogs. Nat. Rev. Mol. Cell Biol. 6, 306–317.
[2] Kang, S., Graham Jr., J.M., Olney, A.H. and Biesecker, L.G. (1997) Gli3 frameshift mutations cause autosomal dominant Pallister–Hall syndrome. Nat. Genet. 15, 206–208.
[3] Shin, S.H., Kogergerman, P., Lindström, E., Toftgård, R. and Biesecker, L.G. (1999) Gli2 mutations in human disorders mimic Droshophila Cubitus interruptus protein functions and localization. Proc. Natl. Acad. Sci. USA 96, 2880–2884.
[4] Rubin, L.L. and de Sauvage, F.J. (2006) Targeting the Hedgehog pathway in cancer. Nat. Rev. Drug Discov. 5, 1026–1033.
[5] Kogergerman, P., Grimm, T., Kogergerman, L., Krause, D., Undén, A.B., Sandstedt, B., Toftgård, R. and Zaphiropoulos, P.G. (1999) Mammalian suppressor-of-fused modules nuclear-cytoplasmic shuttling of Gli-1. Nat. Cell Biol. 1, 312–319.
[6] Huang, Y., Myers, S.J. and Dingle, R. (1999) Transcriptional repression by REST: recruitment of Sin3A and histone deacetylase to neuronal genes. Nat. Neurosci. 2, 867–872.
[7] Østerlund, T., Everman, D.B., Betz, R.C., Mosca, M., Nöthen, M.M., Schwarz, C.E., Zaphiropoulos, P.G. and Toftgård, R. (2004) The Fu gene and its possible protein isoforms. BMC Genomics 5, 49.
[8] Malowey, A., Finta, C., Østerlund, T. and Kogergerman, P. (2007) A possible role of mouse Fused (STK36) in Hedgehog signaling and Gli transcription factor regulation. J. Cell Commun. Signal. 1, 165–173.
[9] Dai, P., Shinagawa, T., Nomura, T., Harada, J., Kaul, S.C., Wadhwa, R., Khan, M.M., Akimaru, H., Sasaki, H., Colmenares, C. and Isshi, S. (2002) Ski is involved in transcriptional regulation. It remains to be investigated by which mechanism Gli3-RD regulates transcription. Perhaps the domain recruits other proteins