Dual degradation signals control Gli protein stability and tumor formation

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Abstract

Regulated protein destruction controls many key cellular processes with aberrant regulation increasingly found during carcinogenesis. Gli proteins mediate the transcriptional effects of the Sonic hedgehog pathway, which is implicated in up to 25% of human tumors. Here we show that Gli is rapidly destroyed by the proteasome and that mouse basal cell carcinoma induction correlates with Gli protein accumulation. We identify two independent destruction signals in Gli1, D\textsubscript{N} and D\textsubscript{C}, and show that removal of these signals stabilizes Gli1 protein and rapidly accelerates tumor formation in transgenic animals. These data argue that control of Gli protein accumulation underlies tumorigenesis and suggest a new avenue for antitumor therapy.

Results and Discussion

While expression of either Gli1 or Gli2 in the epidermis of transgenic mice induces BCCs [Fig. 1a], we have observed a considerable delay in the appearance of Gli-dependent tumors. Analysis of transgenic mice expressing Gli2 revealed an average latency of 7 mo before tumor appearance [Fig. 1b]. We ruled out changes in transcription of the transgene with age as a cause of the tumors, as similar levels of RNA are seen in both age groups as measured by quantitative PCR [Fig. 1c]. This suggested the existence of keratinocytes of additional processes, whose loss or dysregulation is required to permit Gli activity and direct tumor formation. Our previous studies indicated that differential accumulation of Gli protein plays an important role in restricting Shh target gene induction in interfollicular epithelium [Oro and Higgins 2003]. Indeed, we detected no transgenic Gli protein in normal skin, whereas we found high levels in the BCC tumors [Fig. 1d]. Cultured explants of primary keratinocytes from normal skin also contained little detectable Gli protein [Fig. 1e]. However, treatment of these cells with the proteasome inhibitor MG132 caused full-length Gli2 protein to accumulate many fold within 3 h, confirming the presence of an active Gli2 protein destruction mechanism. These data support the conclusion that proteasome-dependent Gli protein destruction underlies the latency in Shh target gene response.

To study the molecular mechanisms that govern Gli protein degradation, we chose to focus our initial studies on Gli1, which, unlike Gli2 or Gli3, is primarily a transcriptional activator and is not processed to a repressor form [Dai et al. 1999; von Mering and Basler 1999]. In this way, Gli protein function and degradation could be examined independently of proteolytic processing and transcriptional repressor regulation. We tested Gli1 stability in a variety of in vitro settings and found that Gli1 is degraded by the proteasome. In Xenopus egg extracts, a system where the ubiquitin–proteasome system (UPS) is known to be active to control β-catenin and IkB stability [Winston et al. 1999; Margottin-Goguet et al. 2003], 35S-labeled Gli1 protein is destroyed in a proteasome-dependent manner, with a half-life of 40 min [Fig. 1f]. Similar kinetics are seen in a variety of cultured normal and cancer cells, including the Shh-responsive NIH 3T3 cells [Fig. 1g; Taipale et al. 2000]. We ruled out degradation of Gli1 by other mechanisms such as lysosomal degradation [Dai et al. 2003], as cathepsin and lysosome inhibitors [E64 and chloroquine, respectively] had no effect on Gli1 levels [Fig. 1h]. The efficacy of these inhibitors was confirmed in primary human keratinocytes where they inhibit the EGF-dependent lysosomal destruction of EGFR [Fig. 1h]. These data provide strong support for destruction of vertebrate Gli1 by the UPS.

To identify signals that allow Gli1 to interact with the UPS, we were guided by the previous finding in Drosophila that the βTrCP locus is required for Gli processing [Jiang and Struhl 1998]. The degron DSGXXS, recognized by βTrCP, is present in vertebrate regulatory pro-
Two destruction signals in Gli proteins

While βTrCP-dependent degradation clearly plays a role in Gli1 destruction, the Gli1ΔC mutation only partially altered the destruction kinetics of Gli1 protein in cultured cells. At 3 h after cycloheximide addition, destruction of Gli1ΔC was decreased by only ∼25% relative to wild-type Gli1 (47.1% ± 6% vs. 21% ± 5%, Avg. ± SEM) [Fig. 3b]. This result suggested that Gli1ΔC stabilized Gli1. As with the DC degron, degron DN mutations (Gli1ΔN1–116, referred to as Gli1ΔN) alone had modest effects on Gli destruction kinetics in vitro (3 h: 40.1% ± 6% vs. 21% ± 5%, Avg. ± SEM) [Fig. 3b]. However, Gli1 lacking both degrons [double mutant; Gli1ΔNΔC] became stable, possessing destruction kinetics similar to that with addition of proteasome inhibitor [Figs. 3b, 1g], indicating that the destruction signal may be found in many Gli proteins.

We next determined whether degron DN functioned independently of degron DC. We tested whether βTrCP binding depends on DC. Consistent with the notion of distinct signals, coimmunoprecipitation studies showed that βTrCP bound equally well to both wild-type Gli1 and the Gli1ΔDC mutant [Fig. 3c]. Moreover, we tested whether degron DN could confer instability to a heterologous protein. Green Fluorescent Protein (GFP) is a stable protein with a long half-life. Addition of amino acids 1–208, a region that encompasses degron DN sequences, destabilized GFP in a proteasome-dependent fashion, giving it a half-life of 180 min [Fig. 3d]. Together, these data suggest the two destruction signals function independently.

Degron DN is immediately adjacent to the binding site for Sufu [Fig. 3a], a powerful negative regulator of the Shh pathway, suggesting that the degron might work in conjunction with Sufu. Consequently, we tested whether DN mutations affected the known Sufu functions of transcriptional corepression and Gli sequestration in the cytosol [Ding et al. 1999; Kogerman et al. 1999; Cheng and Bishop 2002]. Gli1ΔDC bound to Sufu as well as wild-type Gli1 in GST pull-down [Fig. 3e] assays. Also, Gli1

Figure 1. Onset of BCC formation correlates with Gli protein accumulation. (a) Clinical appearance of focal BCCs induced in transgenic animals expressing Gli1 in the skin epithelium with the keratin 5 promoter. (b) Bar graph showing representative onset of tumors in K5-Gli2 mice. (c) Quantitative PCR of Gli2 RNA levels from skin of wild-type or Gli2 transgenic animals of indicated age. Gli2 RNA levels in each sample were normalized to those of GAPDH. Error bars are standard error of the mean (SEM). (d) Immunofluorescence with anti-HA [red] antibody showing protein accumulation only in BCC tumor (arrowhead), not in interfollicular epidermis [arrow]. (Green) Anti-laminin 5, [blue] Hoechst. (e) Western blot of lysates from explanted K5-Gli2 keratinocytes demonstrating the rapid accumulation of Gli2 protein with the addition of the proteasome inhibitor MG132, but not with DMSO. (f) Autoradiogram of 35S-labeled Gli1 mixed with Xenopus oocyte extract. Gli1 is degraded in a proteasome-dependent manner with a half-life of ∼40 min. (g) Western blot of HA-Gli1 in NIH 3T3 cells showing rapid, proteasome-dependent destruction. The nonspecific band demonstrates equal protein loading. (h) HA-Gli1 protein is rapidly degraded (C) via a process inhibited by proteasome inhibitors [M], but not cathepsin or lysosomal inhibitors E64 [E] or chloroquine [Q], respectively. The efficacy of the E64 and chloroquine used in this experiment was confirmed by their ability to inhibit ligand-dependent lysosomal destruction of EGFR in primary human keratinocytes.
Huntzicker et al.

Figure 2. Degron DC (DSGVEM) mediates Gli1 destruction via the βTrCP destruction complex. 
(a, left) Alignment of chordate Gli sequences showing conserved DC sequence. The box details key residues that bind βTrCP. (Right) βTrCP-binding sequences from other vertebrate proteins. 
(b) Reciprocal communoprecipitation of HA-Gli1 or HA-Gli1ΔDC with myc-βTrCP. Note the lack of βTrCP binding in the mutant. The characteristic mobility shift of immunoprecipitated Gli1 is not an artifact of βTrCP overexpression as this shift is observed even in its absence. 
(c) Degradation of transfected Gli1 in NIH 3T3 cells. Note the small but significant delay in destruction kinetics of the mutant versus wild-type protein. The densitometry of both assays is shown to the right and is representative of three independent experiments. Equal sample loading and transfer was confirmed by post-staining of the experimental membranes with Coomassie blue. The 150–250-kDa region of the membranes is shown. 
(d) Western blot of Gli1 or Gli1DC mutants, increasing amounts of transfected βTrCP. Note the decreased steady-state levels of wild-type, but not mutant Gli1. The difference is quantified below and is representative of three independent experiments. Error bars are standard error of the mean (SEM). 
(e) Communoprecipitation assay of 6X-His-tagged ubiquitin and HA-Gli1Δ398 containing degron DC or HA-Gli1Δ398ΔDC mutant. Ubiquitinylated Gli1 (top panel) is detected in the wild-type Gli1 C terminus, but not the ΔDC mutant, in the presence of βTrCP (bottom panel).

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Two destruction signals in Gli proteins

Figure 3. Degron DN mediates Gli destruction independent of DC or Sufu function. (a) Alignment showing the conserved N-terminal region containing degron DN. A solid line indicates the most highly conserved region that is deleted in the DC mutant, while the boxed area shows the Sufu-binding site, which is retained in the DC mutant. (b) Destruction assays of HA-Gli1 in NIH 3T3 cells showing the effects of single DC, DN, and double mutants in comparison to wild-type (WT) Gli1 in the presence and absence of MG132. The densitometry of blots is shown to the right and is based on three independent experiments. Note that results are plotted on the base 2 logarithmic scale. Error bars are standard error of the mean (SEM). (c) Coimmunoprecipitation of wild-type and mutant Gli with βTrCP. Note that the DN mutation does not affect the binding of βTrCP to degron DN. (d) Changes in levels of green fluorescent protein (EGFP) fused to Gli1 N-terminal residues (top), or EGFP (bottom), in the presence of cycloheximide (left) or MG132 (right). The amount of fusion protein is identical at t = 0, but the exposure time for the left and right panels differs to avoid signal saturation. The densitometry is shown to the right with results plotted on a linear scale. The results are representative of three independent experiments. Error bars are SEM. (e) Coprecipitation assays with GST-Sufu and lysates from cells containing wild-type or mutant Gli proteins. Note that the Gli1DN mutation leaves Sufu binding intact. (f) Immunofluorescence of Gli1 shows similar subcellular localization of wild-type and mutant Gli1 proteins in the absence (left) or presence (right) of the Crm1-inhibitor leptomycin B. (g, left) Luciferase transcription assays of wild-type, single, and double-mutant Gli1 proteins. Error bars are SEM. Western blot (right) of levels of Gli1 protein in luciferase assay and quantitation (middle) of protein levels normalized for loading and transfer efficiency determined by immunoblot for nuclear pore complex (NPC).

Material and methods

Destruction assays

Xenopus egg extracts. Xenopus egg cytoplasmic extracts were prepared fresh as previously described [Reimann et al. 2001]. Substrate proteins were in vitro translated in the presence of [35S]methionine using the TnT IVT system (Promega). IVT protein was added to egg extract to 10% of final volume. Destruction assays were conducted in a final volume of 2–10 µL, and stopped by addition of 2x Sample buffer and snap-freezing in liquid nitrogen. In some experiments, MG-132 (Calbiochem) was added to a final concentration of 1 mM.

NIH 3T3. NIH 3T3 cells were transfected as described above. Two days after transfection cycloheximide was added to final concentration of 20 µg/mL and samples were harvested in 2x Sample buffer at various time points. Alternatively, cycloheximide was added at various time points.

umor formation [Supplementary Fig. 1]. As with other key regulatory proteins such as myc, p53, IκB, and β-catenin, there appears to be a finely balanced control of Gli1 protein levels to allow for proper target gene induction while preventing epithelial tumor formation. Our data suggest that the BCC tumors observed in the K5Gli2 transgenic mice likely arise as a result of secondary changes that lead to Gli2 stabilization rather than as a result of gradual saturation of the destruction machinery. Arguing against saturation is the lack of increased protein in adjacent normal tissue or in the explanted cells from older animals. Furthermore, with the addition of proteasome inhibitors, we see rapid accumulation of Gli2 protein.

This suggests that halting the destruction of Gli proteins is an early step in the tumor process and that cellular changes that allow Gli1 protein accumulation may contribute to human carcinogenesis [Kinzl et al. 1988]. Similarly, targeted therapies that delay the onset of Gli accumulation may have potent antitumor properties.

Our study illustrates how two destruction signals cooperate to prevent Gli protein accumulation, target gene induction, and subsequent tumor formation. While a role for βTrCP has been implicated in Ci processing, the present study is the first to demonstrate that it acts by directly binding Gli to facilitate ubiquitinylination and destruction. Interestingly, while Ci and Gli1 are both directed by PKA and βTrCP to interact with the proteasome, the end result differs in that Gli1 is degraded but not cleaved. This could be due to either the particular amino acid sequence of the degron or to surrounding amino acids that influence βTrCP/UPS function. The identified Gli degron differs significantly from that of β-catenin, Em1, and IκB in that it lacks a second serine shown to be important for sequential phosphorylation and contains a phosphomimetic glutamic acid residue [Amit et al. 2002; Moshe et al. 2004]. Future studies will focus on whether these sequence differences are sufficient to account for the different final disposition of the protein. This study further identifies a novel degron, DN, that shares little identity with other known degradation signals. The conserved sequences in this degron are found in both Gli2 and Gli3, and removal of the region containing them has been associated with activation of Gli2 [Sasaki et al. 1999; Mill et al. 2003]. Our data suggest that a portion of this activation may be due to Gli2 protein stabilization via degron DN rather than simply loss of transcriptional repressor activity.
Supplemental Material and then recombined into the bovine keratin 5 promoter by Gateway cloning. Transgene copy number was determined by quantitative real-time PCR [Brilliant Sybr Green; Stratagene] using DNA isolated from transgenic mouse tails. We used primers specific to the 3’-region of human Gli1 [F: GC CGTGCCTAAAGCTCCAGTGACA; R: AG AGTGCAGCTGTCGCTGCTCC]. These primers did not amplify mouse Gli1. A 10-fold dilution series of transgenic plasmid diluted into a constant amount of nontransgenic mouse DNA was used as a standard to determine transgene copy number in a given amount of tail DNA. Mouse GAPDH [GAPDH F: TCTTCTT GTGCACTGGCCACCTCGTCCC; R: GACT GTGCCGTTGTAATTGGCCCTGAGTC] and mouse Gli2 primers [F: CCTCCCTGG GAAGAGACTTGGCTCTCA; R: TCAAT GCTCTCAACCTGGCCCTCAAC] were used as controls for DNA loading and quality. Copy number results are expressed as copies per diploid genome. Expression analysis of transgene expression was performed by quantitative real-time RT–PCR [Brilliant Sybr Green; Stratagene] according to the manufacturer’s instructions. RNA was isolated from right hind-limb tissue using Trizol reagent [Invitrogen]. Mouse Keratin 5 primers [F: CTCAGGAAACCATCTAGT CTGGGCCAGTC; R: CACCCCGAAGCCA AAGCCACTACCA] were used to control for RNA loading and quality. Template quantity was determined using the delta-delta CT method according to the manufacturer’s instructions.

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Two destruction signals in Gli proteins

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