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Sonic Hedgehog activates the GTPases Rac1 and RhoA in a Gli-independent manner via coupling of Smoothed to G_i proteins

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Abstract

The vertebrate Hedgehog (Hh) pathway has essential functions during development and tissue homeostasis during normal physiology while its dysregulation is a common theme in cancer. The Hh ligands (Sonic, Indian, and Desert Hh), bind to the receptors Patched1 and 2, resulting in inhibition of their repressive effect on Smoothed (Smo). Smo is a 7-transmembrane protein, which was only recently proved to function as a GPCR with specificity toward G_i. In addition to G_i activation, Smo signals via its C-tail to inhibit Suppressor of Fused (Sufu), resulting in stabilization and activation of the Gli family of transcription factors, which execute a transcriptional response to the so-called “canonical Hh signaling”. In this presentation we illustrate two outcomes of Hh signaling that are independent of Gli transcriptional activity and, thus, are defined as “non-canonical”. One outcome is dependent on Smo coupling to G_i proteins and exerts changes in the actin cytoskeleton via stimulation of RhoA and Rac1. These cytoskeletal changes promote migration in fibroblasts and tubulogenesis in endothelial cells. The other non-canonical Hh pathway is independent of Smo, and consists of inhibition of Patched1-induced cell death.

Presentation Notes

Slide 1: Title page

This presentation shows that mammalian Hedgehog (Hh) signaling is much more complex than those events leading to Gli-mediated transcriptional changes. We present evidence that Hh ligands induce strong actin changes that modulate cell motility and morphology via stimulation of the small GTPases Rac1 and RhoA in a G_i-protein dependent manner.

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Slide 2: Assay for direct determination of coupling of Smoothened to heterotrimeric G proteins

The most direct assay for determination of activation of a heterotrimeric ($\alpha\beta\gamma$) G protein by a purported GPCR (commonly referred to as “coupling”) is the quantification of incorporation of GTP to the $G\alpha$ subunit. For this assay, we expressed Smoothened (Smo) together with different $G\alpha$ subunits representing the four families and $G\beta_1\gamma_2$ in Sf9 cells by means of baculoviral transduction. This allows for isolation of membranes highly enriched in Smo with specific combinations of G proteins. If Smo couples to a particular $G\alpha\beta\gamma$ combination, it will increase the rate of GDP release, which is quickly replaced by excess GTP[γ]S³⁵ present in the reaction mix. Proteins are rapidly solubilized under non-denaturing conditions and $G\alpha$ is immunoprecipitated with specific antisera. After extensive washing, GTP[γ]S³⁵ bound to $G\alpha$ is quantified by liquid scintillation, as described (1).

Slide 3: Smoothened selectively activates G_i protein members (2)

Using the GTP binding assay described before, we observed that Smo stimulates GTP incorporation in all members of the G_i family tested (G_{i1} , G_{i2} , G_{i3} , G_o , and G_z), as shown in the red bars. The black bars represent spontaneous GTP incorporation when the G proteins are expressed alone. Importantly, Smo was unable to couple to G proteins of the G_s , G_q and G_{12} families (aquamarine bars). It is worth mentioning that in mammals, most G_i members are sensitive to inhibition by a pertussis toxin (PTX), which ADP-rybosylates a Cys residue in $G\alpha$. The only exception is G_z , which is almost exclusively expressed in nervous tissue and platelets (3).

Slide 4: Modulation of Smo- G_i coupling by a small agonist and antagonists of Smo

The Smo/ G_{i2} pair was used to determine if the constitutive activity displayed by Smo can be modulated by small molecules that alter Gli transcriptional activity by binding to Smo. In the left panel, addition of purmorphamine, a Smo agonist (4), increased G_{i2} activation in a concentration-dependent manner, with an EC₅₀ of 1.4 μ M. The Smo inhibitors cyclopamine, KAAD-cyclopamine, and SANT-1 were able to dose-dependently reduce GTP[γ]S³⁵ incorporation to a level comparable to that of G_{i2} alone and with IC₅₀ similar to those reported for Gli transcription inhibition in cell-based assays (80 nM, 200 nM, and 15 nM, respectively) (5, 6). Tomatidine, an inactive cyclopamine analog, did not affect GTP incorporation at any concentration. These observations also suggest that the three Smo inhibitors behave pharmacologically as inverse agonists.

Slide 5: Non-canonical Hedgehog signaling

We define as “non-canonical” Hh signaling those events that occur in response to binding of a Hh ligand to its receptors Patched1 or Patched2 that are independent of Gli-dependent transcription. Mechanistically there are at least two types of non-canonical Hh signaling. A first class (Type I) that depends on Patched but is not exerted through modulation of Smo's activity and, therefore, is insensitive to cyclopamine and not mimicked by Smo agonists. The second class (Type II) is dependent on Smo and mediates cyclopamine-sensitive cellular responses other than Gli activation. Since Smo couples to G_i proteins, we hypothesized that

at least some Type II mechanisms will depend on Smo-G_i coupling and, therefore, be amenable to pertussis toxin (PTX) inhibition.

Slide 6: Type II non-canonical Hh signaling paradigm

We decided to study the role of Smo as a G_i-coupled receptor in fibroblast migration, which is stimulated by Sonic Hedgehog (Shh) in a Gli-independent manner (7).

Slide 7: Genetic models to study Hh-dependent fibroblast migration

We used NIH 3T3 fibroblasts and mouse embryonic fibroblasts (MEFs) of different genotype: Patched1 heterozygote (Ptc^{+/-}) cells, which are functionally identical to wild type cells and are responsive to Shh stimulation, Patched1-deficient (Ptc^{-/-}) cells, which present a constitutive activation of the canonical Hh pathway, and Smo^{-/-} cells, which cannot activate Gli in response to Shh.

Slide 8: Shh promotes fibroblast migration in a Smo-dependent fashion (8)

Addition of Shh to serum-starved wild type fibroblasts or Ptc^{+/-} MEFs increased the rate of migration in response to a scratch in the monolayer (micrographs and black bars). The direct Smo agonist purmorphamine (PUR) stimulated cell migration to a similar extent. The increase in motility by Shh and PUR was abolished by addition of KAAD-cyclopamine, a Smo inhibitor (white bars).

Slide 9: A Smo activity promotes fibroblasts migration

In the absence of Shh, the spontaneous rate of migration of Ptc^{-/-} MEFs is significantly higher than that of Ptc^{+/-} cells (A). This difference can be assigned entirely to the unrepressed activity of Smo, since addition of KAAD-cyclopamine (KAAD) reduces the spontaneous rate to that of Ptc expressing cells (A). In support for an essential role of Smo, the Smo-deficient MEFs are insensitive to a Smo agonist (PUR) or a Smo inverse agonist (KAAD) (B). These results confirm that activation of Smo is necessary and sufficient to promote fibroblasts migration (8). Since this paradigm is independent of Gli activity (7), it is likely to be a Type II non-canonical response.

Slide 10: Shh-induced fibroblast migration is mediated by G_i and phosphoinositide-3 kinase (PI3K)

Non-canonical signaling downstream of Smo may involve activation of G_i proteins and/or modulation of Sufu activity by the cytoplasmic C-tail of Smo. To address whether Smo promotes cell migration in a G_i-dependent manner, we pre-treated the cells with PTX. Inhibition of G_i protein activation completely abolished the effect of PUR (A), indicating that coupling of Smo to G_i is essential for Type II non-canonical Hh pathway.

We had previously reported that PI3K activity is essential for canonical Hh signaling (9). Since PI3K γ can be activated by free G $\beta\gamma$ subunits and it is involved in cell motility, we reasoned that activation of PI3K downstream of G_i might play a role in Shh-induced migration. As predicted, the PI3K inhibitor LY294002 (LY) also prevented the increased motility of PUR-treated cells (A). These data indicate that fibroblast migration induced by acute activation of Smo is dependent on G_i proteins and PI3K.

In support of the proposed mechanism, the increased basal migration observed in $Ptc^{-/-}$ cells by constitutive activation of Smo is also mediated by G_i proteins and PI3K, and the low motility of Smo-deficient MEFs is insensitive to PTX or LY294002 (B). According to these findings, $Ptc^{-/-}$ cells have higher levels of phosphorylated PDK1 and Akt1 under serum starvation conditions than $Smo^{-/-}$ MEFs, and this increase is due to increased Smo- G_i protein signaling since it is abolished by KAAD-cyclopamine or PTX (right panels).

Slide 11: Shh activates the small GTPases RhoA and Rac1

Cell motility takes place by the coordinated action of small GTPases of the Rho family, which modulate the actin cytoskeleton dynamics. Addition of Shh to fibroblasts induced a fast activation of both Rac1 and RhoA, as determined using pulldown assays, with different kinetics (A and B). Introduction of dominant negative mutants of the small G proteins (RacN17 and RhoN19) blocked Shh-induced migration and reduced basal motility as well (C and D).

Slide 12: Smo activation controls RhoA and Rac1

Remarkably, serum-starved $Ptc^{-/-}$ cells show a very high basal activity of RhoA and Rac1, comparable to that of $Ptc^{+/-}$ cells after stimulation with Shh (A and B). Addition of KAAD-cyclopamine reduced the basal activation of the small G proteins in $Ptc^{-/-}$ MEFs, demonstrating that is driven by unrepressed Smo.

Slide 13: G_i proteins mediate stimulation of RhoA and Rac1 by Shh

Since Smo coupling to G_i mediates cell migration in response to Shh, and Smo activates RhoA and Rac1, we sought to evaluate if G_i proteins were necessary intermediaries for activation of the small GTPases. Pre-treatment of NIH3T3 cells with PTX abolished Shh-induced stimulation of RhoA and Rac1 (A and B). Moreover, the PI3K inhibitor LY294002 also prevented activation of RhoA and Rac1, as was shown before for Shh-induced migration. These observations suggest that G_i proteins and PI3K link Smo to the actin cytoskeleton.

Slide 14: Activation of Rac1 does not require Gli transcriptional activity

While the timing of Rac1 activation suggested that it was not a transcriptional effect, we decided to completely rule out a possible contribution of the canonical Hh pathway to activation of the small GTPases and to fibroblast migration. To this end, we transduced $Ptc^{+/-}$ MEFs with an adenovirus encoding Gli3R-GFP (the 83 kDa Gli3 repressor) or GFP as control. Expression of Gli3R did not impair Rac1 activation (A) or fibroblast migration (B), confirming that these cellular responses to Shh are non-canonical.

Slide 15: The C-tail of Smo is required for Gli activation but not for coupling to G_i

We had previously demonstrated that Smo lacking the C-tail (Smo^{-C}), which is recognized by a N-terminus directed antibody (N-19) but not by a C-terminus targeted antibody (H-300) (A), retains full coupling activity towards G_{i1} and G_{i2} (B), which is cyclopamine-sensitive (C). However, deletion of the C-tail abrogated Smo's ability to stimulate a Gli-luciferase reporter in NIH3T3 cells, even on a constitutively active background (SmoM2^{-C}) (D).

Slide 16: Coupling of Smo to G_i is sufficient to stimulate small GTPases and migration of fibroblasts

An independent proof that Smo coupling to G_i is a separate function from activation of the Gli transcription factors came from rescue experiments in Smo-deficient MEFs. We re-introduced full-length SmoM2, a ciliary localization-deficient mutant (SmoM2-CLD) with WR mutated to AA, or a C-tail-deleted mutant (SmoM2- Δ C) in Smo^{-/-} cells. Only SmoM2 and SmoM2-CLD were able to stimulate the canonical Hh pathway after 24 h at high cell density (A). The latter was unexpected since the translocation to the primary cilium is a requirement for activation of the canonical pathway (10); however, the M2 mutation might conceivably overcome the need for the WR motif for ciliary accumulation. Regardless, SmoM2- Δ C did not restore canonical Hh signaling, but selectively rescued activation of Rac1 (B), RhoA (C), and cell migration (D) in Smo^{-/-} MEFs, demonstrating that coupling of Smo to G_i is not only necessary but sufficient to engage this non-canonical mode of Hh signaling.

Slide 17: Hh ligands also induce cytoskeletal changes in endothelial cells

Another example of Type II non-canonical signaling is the one reported by our group in endothelial cells (11). Endothelial cells cultured in 3D matrices have an intrinsic tendency to rearrange into tube-like structures, in a process known as tubulogenesis. Addition of Shh, Ihh, or Dhh to a 3D culture of endothelial cells in matrigel or collagen type I induced a stronger formation of tubes, both in number and branching (A and B). This effect was strictly dependent on Smo and G_i proteins, as was inhibited by KAAD-cyclopamine (KAAD) and PTX (B). Endothelial cells increased the level of active RhoA upon stimulation with any of the three Hh ligands (C) in a Smo- and G_i-dependent manner (D), akin to the one described in fibroblasts.

Slide 18: Type II non-canonical Hh signaling: linking Smo to small G proteins

We have provided here two examples of the existence of a Gli-independent function of the Hh family of proteins, which involves sequential activation of Smo, heterotrimeric G_i proteins and small GTPases of the Rho family to promote actin cytoskeleton remodeling. In fibroblasts this pathway is linked to migration, while in endothelial cells it promotes tubulogenesis (a pro-angiogenic function). Mechanistically, Smo operates independently of the cytoplasmic C-tail (otherwise essential for canonical signaling) to activate G_i and PI3K, which together or hierarchically conduce to RhoA and Rac1 activation.

Slide 19: Type I non-canonical Hh signaling: inhibiting Patched1-initiated apoptosis**Slide 20: Hh ligands reduce apoptosis in endothelial cells**

Endothelial cells are believed to be unresponsive to Hh because there are no evidences of Gli transcriptional activity either *in vitro* or *in vivo* (11-13). In our hands we confirmed that endothelial cells are incapable of activating the canonical Hh pathway (11). In addition, none of the three Hh isoforms stimulated proliferation in these cells (A). Nonetheless, Shh, Ihh, and Dhh were equally effective to reduce caspase-3 activation, a measure of apoptosis, after serum withdrawal (B).

Slide 21: The anti-apoptotic activity of Shh is independent of Smo

The reduction in caspase-3 activity by administration of Shh was insensitive to any of three Smo inhibitors: SANT-1, cyclopamine (CP), and KAAD cyclopamine (KAAD). Moreover, the direct Smo agonist purmorphamine (PUR) did not decrease caspase activation as Hh proteins do. Thus, modulation of Smo activity is not involved in Shh apoptotic activity, suggesting that is mediated by a Type I non-canonical pathway.

Slide 22: Shh prevents apoptosis in endothelial cells by inhibition of Patched1

In the last years it was reported that overexpression of Patched1 (PTCH-1 in human cells) induces apoptosis by recruitment of a pro-caspase complex to its last intracellular domain (14, 15). In order to determine if Shh reduces apoptosis in endothelial cells by inhibition of Patched1, we depleted PTCH-1 by 80-85% using two different siRNA duplexes (P1 and P2, shown in A). A siRNA dose-dependent experiment revealed a direct correlation between the expression level of PTCH-1 and caspase-3 activity in the absence of serum (B). Depletion of PTCH-1 was also accompanied by an increase in cell number, which was not the result of unrepressed Smo, as KAAD-cyclopamine could not block the effect of PTCH-1 depletion (C). In addition, Shh was unable to modulate caspase-3 activity in cells in which PTCH-1 had been depleted (D), indicating that the anti-apoptotic, pro-survival role of Shh in endothelial cells is exerted via inhibition of the pro-apoptotic function of PTCH-1, via a Type I signaling pathway.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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