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A Single Aspartate Coordinates Two Catalytic Steps in Hedgehog Autoprocessing

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Supporting Information

ABSTRACT: Hedgehog (Hh) signaling is driven by the cholesterol-modified Hh ligand, generated by autoprocessing of Hh precursor protein. Two steps in Hh autoprocessing, N−S acyl shift and transesterification, must be coupled for efficient Hh cholesteroylation and downstream signal transduction. In the present study, we show that a conserved aspartate residue, D46 of the Hh autoprocessing domain, coordinates these two catalytic steps. Mutagenesis demonstrated that D46 suppresses non-native Hh precursor autoprocessing and is indispensable for transesterification with cholesterol. NMR measurements indicated that D46 has a pK_a of 5.6, ∼2 units above the expected pK_a of aspartate, due to a hydrogen-bond between protonated D46 and a catalytic cysteine residue. However, the deprotonated form of D46 side chain is also essential, because a D46N mutation cannot mediate cholesteroylation. On the basis of these data, we propose that the proton shuttling of D46 side chain mechanistically couples the two steps of Hh cholesteroylation.

Post-translational modification by cholesterol activates Hedgehog (Hh) family proteins,[1] which are secreted signaling ligands with crucial roles in development and cancer.[2,3] Cholesteroylation of Hh slows the rate of extracellular diffusion, giving rise to finely regulated signaling gradients during embryogenesis.[4] Mutagenesis studies show that abolishing cholesteroylation prevents Hh ligand secretion, resulting in the degradation of premature Hh ligand, and blockade of downstream signaling events.[5−8] In humans, defects in Hh cholesteroylation are associated with holoproencephaly (HPE), a congenital syndrome that impairs brain development.[9−11] Cholesteroylation of Hh is carried out by the autoprocessing activity of Hh precursor[12,13] (Figure 1A).

The 45 kDa Hh precursor is composed of two domains, an N-terminal signaling domain (HhN) and a C-terminal autoprocessing domain (HhC) (Figure 1A and 1B). HhC has two functional segments, a Hint (Hedgehog/intein) module and a sterol-recognition region (SRR) (Figure 1B). During Hh autoprocessing, HhN is cleaved from the precursor and covalently linked to cholesterol at the C-terminus. This transformation is catalyzed by HhC in two steps (Figure 1A):

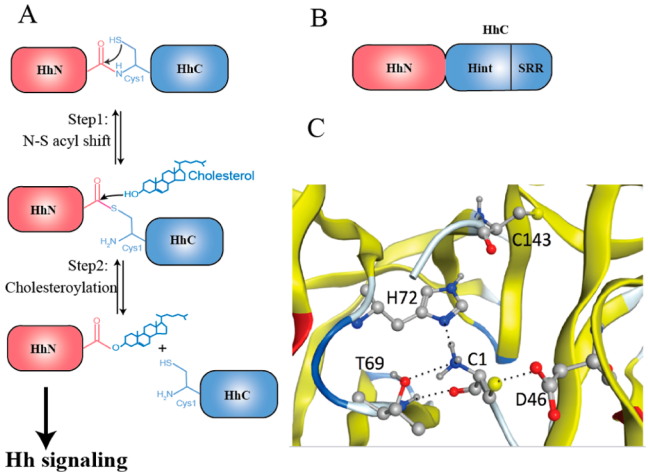

Figure 1. Catalytic steps, domain structure, and active site in Hedgehog autoprocessing. (A) Two steps in Hh autoprocessing mechanism. Hh = Hedgehog; HhN = N-terminal domain of Hh; HhC = C-terminal domain of Hh. (B) Domain organization of the Hh precursor protein. SRR = sterol recognition region which binds cholesterol. (C) Active site of the Hint domain, composed of C1, D46, T69, H72, and C143. Dashed lines denote hydrogen bonds.

1. N−S acyl shift—the conserved Cys 1 of HhC carries out a nucleophilic attack on the carbonyl of the last glycine residue of HhN (G-1), resulting in a thioester intermediate. An identical step occurs in intein-mediated protein splicing, also catalyzed by the Hint module.[13]
2. Transesterification—the hydroxyl group of a cholesterol molecule bound to SRR attacks the thioester, displacing and cholesteroylating HhN.

These two steps of Hh autoprocessing are well coordinated. When the Hh autoprocessing reaction products were analyzed by MS, only cholesteroylated HhN was observed.[3,13] If the two steps are not closely coupled, the thioester intermediate can react with nucleophiles other than cholesterol, precluding production of the lipidated HhN ligand required for proper Hh signaling.

Hh autoprocessing is dependent on the autocatalytic activity of the Hint domain (Figure 1B). The crystal structure of

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Drosophila melanogaster (Dme) Hint domain[13] shows an active site composed of conserved polar residues: C1, D46, T69, H72, and C143 (Figure 1C). C1 is the nucleophile attacking the scissile bond carbonyl (step 1), supported by loss of activity in C1A mutants.[14] T69 and H72 form the signature "TXXH" motif important for N−S acyl shift in both Hh and inteins. D46 and C143 do not appear to have a mechanistic counterpart in inteins but are known to be crucial for autoprocessing.[13,15] C143 can form an internal disulfide with C1, proposed to be important for Hh folding.[13] However, the mechanistic role of D46 is poorly understood.

D46 Inhibits Unproductive Autoprocessing of Hh Precursor. We used a recently developed FRET-based assay for Hh autoprocessing[16] to probe the role of D46. The key construct, C-H-Y, has cyan (C) and yellow (Y) fluorescent proteins[17] fused to the N- and C-termini of the *Dme* HhC domain. In the Hh precursor state, the CFP and YFP are close to each other and the fusion protein exhibits FRET; this FRET is lost when the fluorescent proteins are separated from each other through normal autoprocessing in the presence of cholesterol (Figure 2A). C-H-Y also reacts with non-native

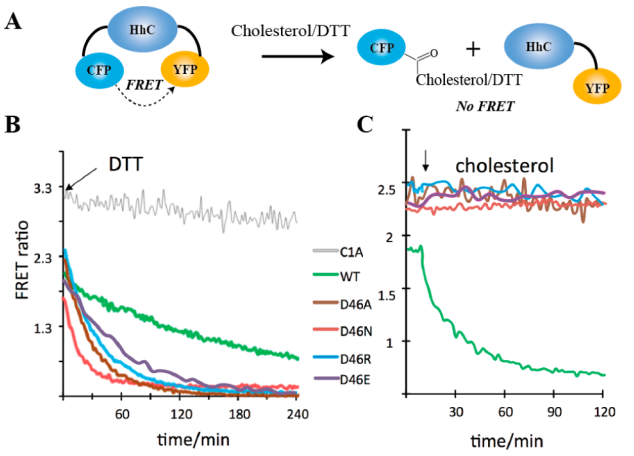

Figure 2. FRET-based DTT-cleavage and autoprocessing assays demonstrate D46 inhibits premature Hh precursor cleavage and is indispensable for cholesteroylation. (A) Schematics of the FRET assays. (B) D46 WT has much slower DTT-mediated N-terminal cleavage than D46A, D46N, D46E, and D46R mutants. (C) Cholesteroylation is abolished in all four D46 mutants.

nucleophiles, such as dithiothreitol (DTT), which can substitute for cholesterol in attacking the thioester intermediate. We compared the wild-type to four variants: the alanine point mutant, D46A; an isosteric mutant, D46N; a charge reversed mutant, D46R; and D46E, which changes the side chain by only a methylene group. All four expressed as soluble proteins in *E. coli* and exhibited strong FRET signals consistent with proper folding.

We first assayed the reactivity of C-H-Y and the D46 point mutants toward DTT as a means of assessing the first step of autoprocessing, N−S acyl shift (Figure 2B). In the presence of DTT (0.2 M), the D46 WT reacted at a rate of $(8.4 \pm 0.2) \times 10^{-5}$ s^{-1}, indicating the presence of an internal thioester. As a negative control for the assay, we used a C1A mutant, where a hydrogen atom replaces the native thiol group. This mutant was insensitive to added DTT, as apparent from its stable FRET signal. Interestingly, all four D46 mutations *accelerated* the non-native reaction toward DTT compared with WT (Figure 2B), with rates of $(54 \pm 4) \times 10^{-5}$ s^{-1} for D46A, $(83 \pm 5) \times 10^{-5}$ s^{-1} for D46N, $(55 \pm 3) \times 10^{-5}$ s^{-1} for D46N, and $(26 \pm 1) \times 10^{-5}$ s^{-1} for D46E. Gel-based cleavage assay showed similar results (Figure S4). Thus, the native D46 has a role in restricting non-native autoprocessing, most likely by manipulating the N−S acyl shift equilibrium or by blocking access to the thioester intermediate. This autoinhibitory effect on promiscuous cleavage is fitting for a coordination residue, which should control the first step before the second step is ready to proceed.

D46 Is Crucial for the Transesterification Step. Using the FRET assay, we next probed the effect of D46 mutations on second step of Hh autoprocessing. The WT protein exhibited a rapid decline in the FRET ratio following addition of cholesterol (0.25 mM), in accord with our earlier study.[16] By contrast, there was *negligible* change in FRET for D46N, D46E, D46R and D46A, indicating that these mutations abolish cholesteroylation activity (Figure 2C). The lack of activity with cholesterol is not a consequence of defective N−S acyl shift (first step), because the robust cleavage mediated by DTT indicates that thioester formation is intact (Figure 2B). Therefore, D46 not only restricts off pathway activity, but is essential for transesterification to cholesterol, suggesting that D46 likely mediates crucial interactions with the substrate. Indeed, extending the native side chain in D46E by a methylene group blocks activity toward cholesterol. The D46N mutant has a side chain very similar to the WT except for the negative charge of aspartate. The fact that D46N can not mediate cholesteroylation suggests that the deprotonated aspartate side chain is important for transesterification. We suspected that the acid−base equilibrium of D46 is crucial for Hh autoprocessing, leading us to examine the pK_a of D46 with solution NMR.

pK_a of D46 Is Elevated. A strikingly high pK_a value of 5.6 was obtained by NMR titration in the precursor (Figure 3A and Figure S1), composed of 5 HhN residues and Hint domain (Table S1). A similar pK_a of 5.8 was observed in the Hint domain (Figure S3), indicating that the presence of HhN residues do not affect D46 pK_a. Both values are about two units above) the normal pK_a of aspartate side chain. High pK_a of active-site aspartate usually points to the mechanistic importance of the protonated aspartic side chain.

pK_a Values of D46 and C1 Are Coupled. Next we examined the structural basis of the elevated pK_a of D46. Because D46 and C1 are close in the 3D structure (Figure 1C), we examined how C1A mutation affects the pK_a of D46. Interestingly, the C1A mutation decreases the D46 pK_a to 4.2 (Figure 3B), close to the regular pK_a value of aspartate, demonstrating that D46 pK_a is enhanced by the absolutely conserved C1 side chain. We then tested whether D46 in turn influences the side chain of C1. We monitored cysteine ^{13}CB chemical shift in ^{1}H−^{13}C HSQC using a specifically cysteine ^{13}CB labeled sample (Figure S2).[18] In the WT precursor, C1 has a pK_a of 5.1 (Figure 3C), more than 3 units lower than the normal pK_a of cysteine, ~8.3. The D46A mutation increased C1 pK_a to 6.3 (Figure 3D). Therefore, the elevated pK_a of D46 is coupled to the decreased pK_a of C1.

The pK_a coupling suggests a direct interaction between D46 and C1, and can be explained by a hydrogen bond between C1 thiolate and D46 carboxyl (Figure 3E). As a general rule, a positive charge lowers the pK_a of nearby ionizable groups, whereas a nearby negative charge raises the pK_a. In Figure 3E, the negative charge of the C1 thiolate increases D46 pK_a, while the positive partial charge of the carboxyl hydrogen decreases the C1 pK_a. Because D46 inhibits spurious Hh precursor

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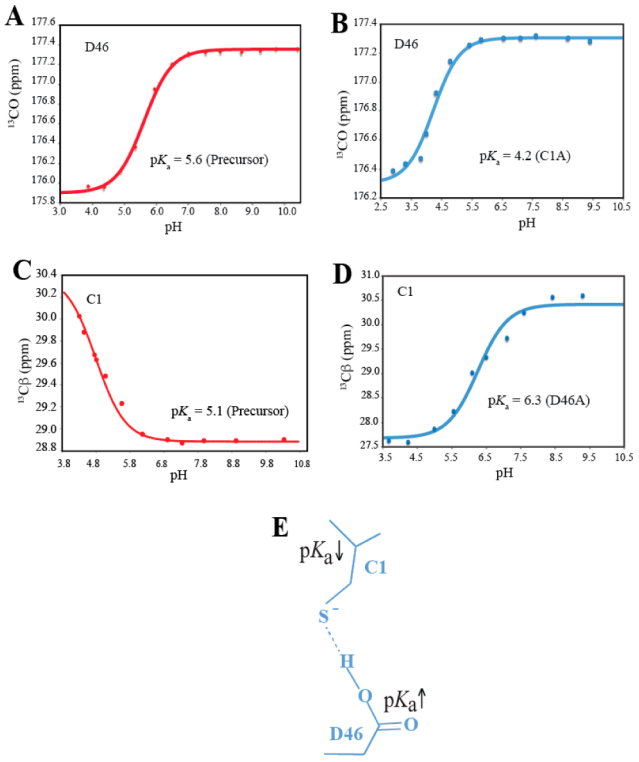

Figure 3. pK_a coupling between D46 and C1. (A) D46 pK_a is elevated to 5.6, determined by ^{13}CO chemical shift titration with HB(CB)CO (see Figure S1). (B) C1A mutation lowers D46 pK_a to normal. (C) C1 has a depressed pK_a of 5.1 and a pK_a of 6.3 with a D46A mutation (D), based on ^{13}CB chemical shift changes with pH. (E) The structural basis for pK_a shift and coupling between C1 and D46.

reactivity (Figure 2B), D46 likely holds C1 thiolate in a nonproductive conformation or alternatively stabilizes the thioester, before cholesteroylation can proceed, serving the role of a coordination residue. Similar pK_a coupling has been observed between a conserved aspartate (but not homologous to D46) and C1 in an intein.[19] pK_a matching has also been observed for catalytic interactions in other enzymes.[20]

D46 Coordinates the Two Steps through Side-Chain Proton Shuttling. On the basis of the above data, we propose a novel mechanism for D46 coordination:

1. D46 carboxyl stabilizes the C1 thiolate but holds it in an inactive conformation, pointing away from scissile bond carbonyl (Figure 4). D46 should therefore inhibit N−S acyl shift mediated Hh cleavage by DTT. Indeed, as demonstrated in Figure 2B, D46 has a much slower rate of DTT cleavage than four D46 mutants. D46 should also have an elevated pK_a, due to the nearby negative charge of C1 thiolate, confirmed by NMR titration (Figure 3A,B).
2. We hypothesize that when cholesterol binds to Hh, the hydroxyl of cholesterol interacts with D46, lowering its pK_a due to the partially positive charge of the hydroxyl proton. D46 deprotonates, liberating C1 thiolate to carry out the N−S acyl shift. D46 may donate its proton to a nearby water molecule, H72, or the incipient amine group of C1.
3. In a coupled step, the newly deprotonated D46 side chain is poised to serve as a general base to activate the hydroxyl group of bound cholesterol for attack at the thioester.

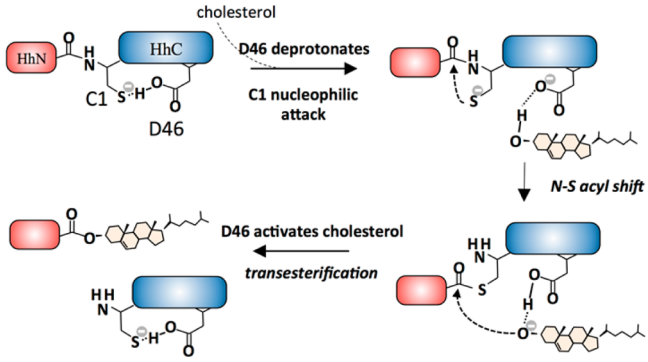

Figure 4. Coordination mechanism of D46 in the two catalytic steps of Hh autoprocessing, in which the protonation and deprotonation of D46 plays a pivotal role.

This scheme links N−S acyl shift and transesterification through proton shuttling by D46, and suppresses premature N−S acyl shift and thioester cleavage, ensuring the fidelity of Hh autoprocessing. Further, the mechanism explains the unique conservation of this aspartate side chain in Hh Hint domains/modules.

Many enzymes catalyze multistep reactions. Without proper coordination in these enzymes, side reactions will occur and prevent the formation of the intended product. The coordination mechanism of D46 in Hh autoprocessing provides an example of how conformational change of a side chain coupled with proton shuttling can drive the precise progression of complicated steps at an active site. Even in nonenzymatic systems, such as the chlorine transporters (CLC), similar behavior is observed. In CLC, an active site glutamate cycles through ionization states at three sites.[21] At two sites, the negatively charged glutamate side chain replaces bound chlorines; at a third site, it gains a proton, which neatly accounts for the exchange stoichiometry of 2 Cl^- for 1 H^+ in these transporters.

Hh signaling, driven by the Hh ligand, plays vital roles in both embryogenesis and cancer. Hh autoprocessing, which generates the Hh ligand, thus occupies a unique position at the very origin of the Hh signaling cascade. Abnormal Hh autoprocessing in development leads to congenital diseases. Recently, we provided evidence that abnormal Hh autoprocessing may be linked to zinc deficiency[22] and metabolism of antiprostate cancer drugs.[23] Our results in this paper improve our understanding of Hh's unique autoprocessing mechanism while providing a foothold[18] to correct aberrant Hh levels in developmental disorders and cancer.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/jacs.6b06928.

Additional methods and data, including Figures S1−S4 and Table S1 (PDF)

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Notes

The authors declare no competing financial interest.

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