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A new role for Hedgehogs in juxtacrine signaling

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ABSTRACT

The Hedgehog pathway plays important roles in embryonic development, adult stem cell maintenance and tumorigenesis. In mammals these effects are mediated by Sonic, Desert and Indian Hedgehog (Shh, Dhh and Ihh). Shh undergoes autocatalytic cleavage and dual lipidation prior to secretion and forming a response gradient. Post-translational processing and secretion of Dhh and Ihh ligands has not previously been investigated. This study reports on the synthesis, processing, secretion and signaling activities of SHH, IHH and DHH preproteins expressed in cultured cells, providing unexpected evidence that DHH does not undergo substantial autoprocessing or secretion, and does not function in paracrine signaling. Rather, DHH functions as a juxtacrine signaling ligand to activate a cell contact-mediated HH signaling response, consistent with its localised signaling *in vivo*. Further, the LnCAP prostate cancer cell, when induced to express endogenous DHH and SHH, is active only in juxtacrine signaling. Domain swap studies reveal that the C-terminal domain of HH regulates its processing and secretion. These findings establish a new regulatory role for HHs in cell-mediated juxtacrine signaling in development and cancer.

1. Introduction

Hedgehog (Hh) genes encode signaling proteins controlling tissue patterning in the embryo and cell growth and differentiation in regenerating tissues (Ingham et al., 2011; McMahon et al., 2003; Varjosalo and Taipale, 2008). Hedgehog was originally identified in a genetic screen for patterning genes in *Drosophila melanogaster* (Nusslein-Volhard and Wieschaus, 1980; Varjosalo and Taipale, 2008; Wetmore, 2003). While *Drosophila* has only a single Hh gene, mammals have three: Desert (DHH), Indian (IHH) and Sonic (SHH). While these isoforms are redundant in some scenarios (Zhang et al., 2001), they largely show differential expression in tissues in the developing embryo and adult, and control induction of different sets of downstream target genes (Hooper and Scott, 2005; Pathi et al., 2001). It is important to note that both Shh and Ihh are expressed in endoderm development (van den Brink, 2007). In the mouse embryo, Shh is expressed and secreted from midline tissues, including the node, notochord, and floor plate, generating a morphogen signaling gradient for patterning of left–right and dorso–ventral axes of the developing embryo (Hooper and Scott, 2005; Varjosalo and Taipale, 2008). By contrast, Ihh is primarily expressed by pre-hypertrophic and early hypotrophic chondrocytes, signaling locally to both proliferating chondrocytes and the overlying perichondrial cells (St-Jacques et al., 1999; Vortkamp et al., 1996). Dhh expression is produced by Sertoli cells to control development of immediately adjacent Leydig cells required for male sexual differentiation (Bitgood et al., 1996; Clark et al., 2000; Kawai et al., 2011), and by Schwann

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cells to promote the development of the perineural sheath of during neuronal development (Bitgood and McMahon, 1995; Parmantier et al., 1999; Yoshimura and Takeda, 2012).

Hhs are expressed as unprocessed preproproteins that, based on studies of the *Drosophila* Hh protein, undergo processing and auto-catalytic cleavage (reviewed in Ryan and Chiang (2012) and Ingham et al. (2011)). In brief, human SHH is synthesized as a 462aa protein precursor of approximately 45 kDa designated as 'preproprotein'. This preproprotein is composed of a 23aa signal peptide ER targeting sequence, a 174 amino acid N-terminal signaling domain, and a 265aa C-terminal autoprocessing domain endowed with autoproteolysis and cholesterol transferase activity. Following cleavage of the signal peptide sequence at the extreme N-terminus, the 19 kDa N-terminal signaling fragment of Hh is autocatalytically cleaved from the C-terminus and a cholesterol moiety is added to the C-terminal end of the signaling fragment. A palmitate group is subsequently added at the N-terminus to produce a dual-lipidated molecule with high signaling capacity (Mann and Beachy, 2004). The processed N-terminal fragment would be retained in the cell by its cholesterol tail, except it is actively secreted by the synergistic actions of Disp and the secreted protein Scube2 (Burke et al., 1999; Tukachinsky et al., 2012). Secreted N-Hh forms a signaling gradient from the producing cells to responsive cells localized near or distant (Mimeault and Batra, 2010). Processed Hh accumulates in producing cells in Disp deficient mice and flies, and is able to activate the pathway in neighboring cells, but is not competent for long range signaling (Burke et al., 1999; Gallet et al., 2006). Studies of *Drosophila* Hh have identified specific residues in the C-terminal domain required for autoprocessing and lipid modifications (Hall et al., 1997; Lee et al., 1994; Porter et al., 1995), including a conserved cysteine, although prior to this study, cellular autoprocessing and secretion of the remaining mammalian Hh isoforms have not been investigated.

Mammalian Hh signaling, characterized largely in studies of recombinant N-terminal Shh, transduces target gene transcription and/or repression through control of activator and repressor forms of the Gli transcription factors. The Hh signaling response varies with the concentration and duration of the Hh signal. Hh signal transduction begins with the binding of Hh ligand with the transmembrane receptor, Patched (Ptch). Prior to Hh ligand binding, Ptch binds and inhibits the transmembrane protein, Smoothened (Smo), which is the positive transducer of the Hh pathway controlling the function of the Gli family of transcription factors. In the absence of Hh binding and repression of Smo, the Gli3 transcription factor is post-translationally processed to form a potent transcriptional repressor of Hh target genes. Hh ligand binding to Ptch relieves this inhibition of Smo, allowing Smo to activate a signaling cascade through the primary cilium, which results in activation of Gli2 and Gli3 to function as positive regulators of Hh target genes (Singla and Reiter, 2006). Among these primary target genes is Gli1, a most potent transcriptional activator that participates in this strong positive feedback loop for activation of target genes (Stecca and Ruiz, 2010). Other Gli-regulated Hh signaling components are involved in pathway repression, including Ptch1 and Hip (Hedgehog-interacting protein), establishing a negative feedback loop (Chuang and McMahon, 1999). The balance between pathway activation and repression in response to the concentration and duration of Hh signal determines the transcriptional program of the Shh-responding cell.

The N-terminal domains of the three mammalian isoforms are conserved and have similar binding affinities for the receptor Ptch (Pathi et al., 2001), suggesting their function in a common signal transduction pathway. Supporting their conserved functions, N-terminal recombinant Hh ligands activate expression of Gli1 and Ptch1 mRNA transcripts, as direct targets of Gli-mediated pathway activation. However, the N-terminal fragments of three mammalian Hh proteins have different potencies for Gli1 and Ptch1 activation, with N-SHH being most active and N-DHH being least active (Pathi et al., 2001). While recombinant N-terminal SHH, DHH, and IHH proteins are equipotent in their ability to induce Islet-1 expression in chick neural plate explants, the ligands are significantly different in their activities for induction of chondrocyte differentiation and nodal expression in the lateral plate mesoderm in the early chick embryo (Pathi et al., 2001). In support of these results, Echidna Hedghog (shhb) in zebrafish is around 3 times as effective at inducing slow muscle than Shh in zebrafish during development (Norris et al., 2000). These results suggest that the HH N-terminal signaling domains of the different isoforms have specific and individual functional capacities in cell and embryo functional assays.

In this study, we have investigated the processing, secretion and signaling functions of human SHH, DHH and IHH expressed as full length proteins. Current understanding of Hh function derives from genetic studies of *Drosophila* Hh and cell studies of recombinant N-terminal Hh proteins, primarily N-Shh, which lack the dual lipidation modifications and do not address roles for autoprocessing and secretion in the control of Hh functions. We have investigated the synthesis, processing, secretion and signaling activities of SHH, IHH and DHH preproteins expressed in cultured cells. We report unexpectedly that HH isoforms have remarkably different autoprocessing, secretion and signaling activities. Notably, expressed DHH, unlike SHH and IHH, does not undergo substantial autoprocessing or secretion and does not function in paracrine signaling. Rather, DHH functions in juxtacrine signaling to activate a cell contact-mediated Hh signaling response, consistent with its expression and localized signaling *in vivo*. We also show that the LnCAP prostate cancer cell, when induced to express endogenous DHH and SHH, is active only in juxtacrine signaling, and not paracrine signaling. Finally, domain swap studies reveal that the C-terminal domain of DHH regulates its processing and secretion, to promote its function as an autocrine signaling regulator. These findings challenge the assumption that HHs function only as secreted morphogens and establish a new regulatory role for HHs in cell-mediated juxtacrine signaling in development and cancer.