Sticky Proteins for Stem Cells

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Lipids are usually known as a form of storage of metabolic energy, as components of biological membranes and as signalling substances. Another, much less recognised function of lipids is their covalent attachment to proteins. Many cytoplasmic proteins are modified by the addition of lipid moieties that modulate their interactions with other proteins and facilitate their association with cellular membranes.

An extracellular glycoprotein of the Wnt family was recently purified and shown to maintain haematopoietic stem cells (HSCs) in their self-renewing state. The protein bears a palmitoyl residue on an N-terminal cysteine side chain, and this modification turned out to be critical for its signalling properties.[1]

Background: Wnt Signalling

The products of *wnt* genes, the Wnt proteins, are secreted factors with molecular weights of about 40 kDa that stimulate cells in vitro in an autocrine and in a paracrine manner. These proteins control a variety of processes during animal development.[2a] The first *wnt* gene to be identified (*int-1*, now called *wnt-1*) was discovered in 1982 as a protooncogene of the mouse.[3] Its homologue in *Drosophila melanogaster*, the *wingless* gene, controls segment polarity and wing development in the fly as a nonclassical morphogen.[4] Since these discoveries were made, *wnt* genes of various species, from the nematode *Caenorhabditis elegans* to vertebrates, have been characterised; nineteen human *wnt* genes have been reported to date.[2]

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Wnt signals control proliferation and differentiation during embryonic development and morphogenesis.[2a] The so-called classical or canonical Wnt signalling pathway (Figure 1) activates target genes through stabilisation of a cytoplasmic protein, β-catenin, the mammalian homologue of the *Drosophila* segment polarity gene product Armadillo.[5] This pathway represents a ubiquitous cell communication system and essentially involves the four downstream components Frizzled, Dishevelled, glycogen synthase kinase-3β (GSK-3β), and β-catenin. In brief, the Wnt cascade is activated by binding of secreted Wnt glycoproteins to membrane receptors of the Frizzled and low-density lipoprotein receptor-related protein families. In the presence of co-receptors like proteoglycans, the cytoplasmic phosphoprotein Dishevelled[6] is activated, which leads to the inhibition of the serine/threonine protein kinase GSK-3β. As a result, the phosphorylation of one of the target proteins of this enzyme, β-catenin, is suppressed. β-Catenin, which is degraded in its phosphorylated form by the ubiquitin system, accumulates and is translocated to the nucleus, where it interacts with transcription factors of the LEF/TCF family and activates the transcription of Wnt target genes. The destruction complex that leads to β-catenin degradation in the absence of the Wnt signal contains Axin as a scaffold protein, the tumour suppressor gene product APC and GSK-3β.

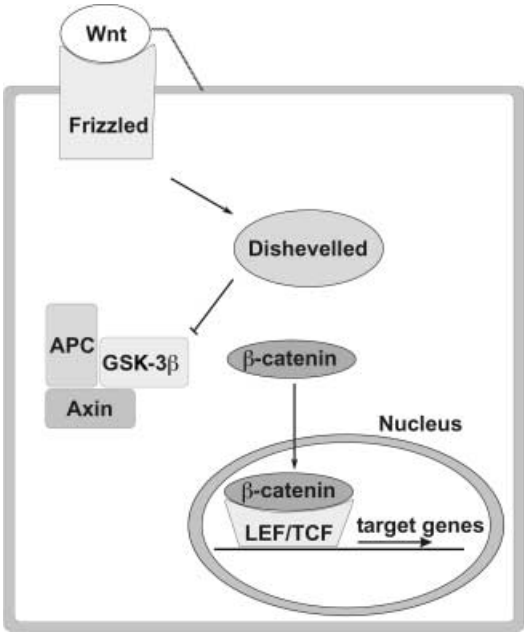

Figure 1. *Overview of canonical Wnt signalling (modified from ref. [12]). In the absence of extracellular Wnt protein, cytoplasmic β-catenin binds to the destruction complex composed of adenomatosis polyposis coli (APC), Axin, and glycogen synthase kinase-3β (GSK-3β). Binding to this complex leads to β-catenin phosphorylation, ubiquitinylation and degradation by the proteasome. Wnt binding to receptors like Frizzled causes dissociation of the destruction complex, which results in stabilisation of β-catenin and its translocation to the nucleus, where it triggers the transcription of target genes by interacting with transcription factors, such as those of the LEF/TCF family (LEF, lymphoid enhancer factor; TCF, T-cell factor).*

One key player in the canonical Wnt cascade is β-catenin, a protein that was initially discovered as a binding protein for the cytoplasmic domain of the cell adhesion protein E-Cadherin, which mediates Ca^{2+}-dependent cell–cell interactions. Such contacts connect E-Cadherin to the actin filaments of the cytoskeleton. Defects in the canonical Wnt pathway are not only associated with morphological alterations and defects in organ development. Mutations in the genes encoding APC or β-catenin can lead to the constitutive stabilisation of transcriptionally active β-catenin and to tumour development, particularly in the gastrointestinal tract.[7, 8]

Recent investigations have led to the discovery of additional components and to a more detailed view of this highly complex pathway. In addition to the

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canonical pathway, several subpathways can be distinguished: 1) the planar cell polarization pathway, which leads to an asymmetric orientation of the cytoskeleton and to the polarised phenotype of cells in epithelial tissues, 2) the Wnt/Ca^{2+} pathway, which leads to phospholipase C activation and Ca^{2+} release, and 3) a pathway that controls asymmetric cell division and spindle orientation.[9]

Palmitoylation of Wnt

A milestone in the analysis of Wnt proteins was the isolation of a purified member of the Wnt family in its active form. This isolation was achieved by using the culture medium of cells expressing recombinant murine Wnt3a protein as the protein source and purifying Wnt3a in the presence of the zwitterionic detergent 3-[(cholamidopropyl)dimethylammonio]-propanesulfonate in several chromatographic steps. The purification started with binding of the protein to Blue Sepharose. Elution with a solution of increasing ionic strength led to a 2500-fold enrichment. Size exclusion chromatography and cation exchange afforded a protein sample that was more than 95% pure according to the results of Coomassie staining analysis. Various functional assays, such as stabilisation of cytosolic β-catenin as a measure of Wnt-dependent signal transduction, showed that the protein remained functionally active during purification. The morphological changes caused by Wnt3a in a cell line derived from mouse mammary gland cells were similar to those induced by *wnt* gene transfection. The fact that detergent was required during purification, as well as other observations, pointed to a hydrophobic character of the protein, although no hydrophobic stretches could be deduced from the amino acid sequence. Metabolic labelling with radiolabelled palmitate indicated the presence of a fatty acid modification of the protein. Treatment of the protein with an acylprotein thioesterase removed its hydrophobic property, as indicated by phase separation experiments, and blocked the ability of the protein to stabilise β-catenin. Mass spectrometric analysis of Wnt-3a, and also of *Drosophila* Wnt8 expressed and purified in the same way, led to the discovery that the *N*-terminal cysteine residue is the modified amino acid. Mutation of this cysteine residue to alanine led to a loss of activity in a β-catenin stabilisation assay, but this loss was not entirely complete in a transfection assay. Earlier experiments with chimeric proteins also indicated the functional importance of this part of the molecule.[10] Inside the cell, the majority of Wnt proteins is associated with secretory vesicles. Since secreted Wnt proteins associate mainly with the cell surface, only small amounts of the soluble proteins exist in the medium of cultured cells. Therefore, the expression and purification protocol constitutes a major advance in this area of research, and the demonstration of lipid attachment to a Wnt protein explains the tight association of these proteins with membranes.

Background: Haematopoietic Stem Cells

Every second the human body has to produce several million blood cells to survive. Erythrocytes, granulocytes and lymphocytes have only a limited life span and have to be continuously generated from the pluripotent haematopoietic stem cells (HSCs) of the bone marrow. Division of one of these cells leads to a daughter stem cell with the capability of self-renewal, and to a progenitor cell that differentiates into one of the different types of blood cells. The identity of the factors required for stem cell self-renewal is not clear[11] and researchers have had limited success in preventing differentiation of HSCs in long-term cultures.[12]

Wnt3a and HSC Renewal

The purified Wnt3a protein was able to induce proliferation of HSCs. The opposite outcome, differentiation instead of proliferation, was observed when medium was taken from cultures of Wnt3a-expressing cells and added to cultured HSCs, a result clearly due to the presence of factors with differentiation-inducing properties. After transplantation of Wnt3a-treated HSCs into mice followed by lethal irradiation, it was found that all the mice contained donor-derived cells, which indicates that self-renewal of HSCs induced by Wnt-3a also occurs in vivo.

The role of the Wnt pathway in the renewal of HSCs has been described in an additional communication[13]. Expression of constitutively active β-catenin maintained long-term cultures of He cells in an immature state and allowed cell growth for up to four weeks, while control cells were not able to survive 48 h. The cells maintained the features of HSCs, were functional according to the results transplantation analysis and responded to Wnt signalling. Wnt signalling resulted in the up-regulation of HoxB4 and Notch 1, which have been implicated in stem cell renewal before. This work provides further evidence for the requirement of Wnt signalling for the renewal of haematopoietic[12, 14] and other stem cells.[11]

Palmitoylation of Extracellular Proteins

Many intracellular proteins are posttranslationally modified by covalent lipid attachment, which includes the ubiquitous palmitoylation of cysteine residues.[15, 16] Palmitoylation of extracytoplasmic proteins, however, is highly unusual, presumably as a result of the redox potential outside the cytosol favouring cystine bridges instead of free cysteine residues. The morphogens of the Hedgehog (hh) family are remarkable exceptions to this rule; they are palmitoylated[17] and trigger a communication pathway that has similarities with the Wnt cascade. hh proteins are secreted tissue patterning factors that control a variety of processes during development, such as left-right asymmetry. Three mammalian hh genes are known as *sonic*, *indian* and *desert hedgehog*. Like Wnt proteins, hh proteins are implicated in stem cell renewal, and aberrant Hedgehog signalling is associated with cancer.[18] Both in the Wnt and in the hh pathway, the extracellular signal prevents the phosphorylation-dependent proteolysis of an intracellular key effector, with the consequence that the silencing of target genes is abolished by the signal. Binding of hh proteins to the transmembrane protein Patched stops it from blocking another transmembrane protein (Smoothened) with homology to Frizzled, and leads to the transduction of the signal

inside the cell. In the absence of the hh signal, the *Drosophila* intracellular zinc finger protein Cubitus interruptus, a homologue of the Gli protein found in mammals, is phosphorylated by protein kinase A and proteolytically processed to form a transcriptional suppressor. In the absence of phosphorylation, the uncleaved protein acts as an activator of hh target gene transcription.[19]

In addition to this similarity between the Wnt and hh pathways, both types of protein are lipid modified. The Sonic Hedgehog protein is modified by two lipid residues that are important for signalling in neurons and the development of the brain.[20, 21] Mature Hedgehog proteins have a molecular weight of about 20 kDa, bear a cholesterol residue connected by an ester linkage to their C-terminal carboxy group and are palmitoylated on an N-terminal cysteine residue. There is evidence that the cholesterol modification restricts the spatial distribution of the Hedgehog signal by interacting with membranes. While truncated forms of this protein without cholesterol are still active in vitro and in vivo, palmitoylation or the presence of another hydrophobic element in the N-terminal part of the protein appears to be essential for activity, at least in some tissues.

Enzymology

Something is already known about proteins that have the correct topology to introduce and to remove palmitic residues from extracytoplasmic proteins. The acyltransferase Skinny Hedgehog, which transfers palmitic acid to Sonic hh in the lumen of exocytotic vesicles, has been described.[22] A similar factor for Wnt modification has not been unequivocally characterised, but early observations pointed to one of the *Drosophila* segment polarity gene products, Porcupine. This protein is required for the generation of active Wnt signals and is homologous to membrane-bound acyltransferases like Skinny Hedgehog.[23]

Are proteins required for removal of extracellular thioester-bound fatty acids? In contrast to the myristoylation of *N*-terminal glycine residues or the prenylation of cysteine side chains near the C terminus of a protein, the covalent attachment of palmitic acid through a thioester linkage is a labile and reversible modification. Nevertheless, the phenotype of patients with inherited defects in the lysosomal cleavage of protein-bound thioesters indicates that this cleavage reaction requires enzyme catalysis in vivo. Deficiency of lysosomal palmitoyl-protein thioesterase I leads to a severe human disease with infantile onset: Type I of the Neuronal Ceroid Lipofuscinoses.[24] It is not clear whether Sonic Hedgehog is really cleaved by the enzyme that is deficient in this disease and there are hints that the thioester linkage can rearrange to an amide-linked palmitoyl residue.[17] However, the topology and substrate specificity of this enzyme point to such a role and palmitoylated proteins located in the cytoplasm are cleaved by cytoplasmic acylprotein thioesterase I.[15]

Outlook

The recent discovery of this posttranslational modification will lead to further investigation of its function. By travelling long distances, morphogens form concentration gradients and, in this way, allow tissue patterning.[4] Remarkably, Wnt and Hedgehog proteins are known to associate tightly with membranes and can be transported through cells as passengers of transport vesicles, also called Argosomes.[25] It is to be expected that the reversible addition of a hydrophobic building block to morphogens of the Wnt and Hedgehog family is closely related to their function.

The details of the role of Wnt proteins in the maintenance of stem cells of various origin remain an exciting topic. A potential application of purified and active Wnt proteins is the expansion of donor HSCs for transplantation purposes, although our understanding of the molecular details of the action of these proteins in tissues such as the skin[26] is still in its infancy.

Keywords: lipids · palmitoylation · posttranslational modification · proteins · stem cells

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