Wnts have a structure resembling a hand with “thumb” and “index” fingers that grasp the cysteine rich domains of Frizzled receptors at two distinct binding sites. In the present work we show that the WIF domain of Wnt Inhibitory Factor 1 is also bound by Wnts at two sites. Using C-terminal domains of Wnt5a and Wnt7a and arginine-scanning mutagenesis of the WIF domain we demonstrate that, whereas the N-terminal, lipid-modified “thumb” of Wnts interacts with the alkyl-binding site of the WIF domain, the C-terminal domain of Wnts (Wnt-CTD) binds to a surface on the opposite side of the WIF domain.

1. Introduction

Wnts are large, lipid-modified extracellular growth factors that play critical roles in embryonic development and stem cell proliferation of Metazoa [1,2]. Wnts exert their effects through the activation of different intracellular signal transduction pathways [3].

Three distinct but interconnected signaling pathways (the canonical Wnt/β-catenin pathway, the non-canonical Wnt/planar cell polarity pathway and the Wnt/calcium pathway) have been shown to mediate signaling through Wnt-Frizzled interactions [4,5]. Canonical Wnt signals are transmitted through Frizzled family receptors which form a co-receptor complex with the LDL receptor Related Proteins LRP5/6. In the case of the Wnt/calcium pathway binding of Wnts to the co-receptor complex leads to an increase in intracellular calcium and activation of calmodulin-regulated signaling cascade, whereas in the Wnt/planar cell polarity pathway c-jun N-terminal kinase is the effector molecule [6].

The extracellular Wnt-binding domains of Frizzled receptors, usually called Fz domains, have been shown to bind multiple Wnts with high affinity, the specificity of Wnt signaling depends on the affinities between various Wnt-Frizzled pairs [7,8].

Domains related to the Fz domain of Frizzled receptors were also identified in Ror type receptor tyrosine kinases [9–11], raising the possibility that these receptor tyrosine kinases may also be directly involved in Wnt signaling. This prediction was confirmed by several studies: Ror receptor tyrosine kinases do mediate signaling cascades in response to Wnt stimulation [12–14].

The Fz domain is not the only domain type that has high affinity for Wnts: the WIF domain of Wnt Inhibitory Factor 1 (WIF-1) exerts its action by sequestering Wnts [15]. The extracellular part of receptor tyrosine kinases of the Ryk family was also shown to contain a WIF domain raising the possibility that these receptor tyrosine kinases may also be involved in Wnt-binding [16]. This prediction was confirmed by subsequent studies: there is increasing evidence that Ryks are also involved in Wnt-binding and Wnt...
In the present work we have tested this hypothesis using C-terminal domains of Wnt5a and Wnt7a and arginine-scanning mutagenesis of the WIF domain. Our studies suggest that whereas the N-terminal, lipid-modified domains of Wnts interact with the alkyl-binding site of the WIF domain, the C-terminal domains bind to a surface on the opposite side of the WIF domain.

2. Materials and methods

See Supplementary file.

3. Results

3.1. Characterization of the C-terminal domains of Wnt5A and Wnt7a

The C-terminal domains of Wnt5a and Wnt7a, secreted by Pichia pastoris were found to be N-glycosylated (Supplementary Fig. 1), in harmony with the fact that both proteins are predicted by the NetNGlyc 1.0 Server (http://www.cbs.dtu.dk/services/NetNGlyc/) to contain N-glycosylated Asn-Xaa-Ser/Thr sequons at equivalent positions: at GLRGC/C24 in Wnt5a-CTD and at GRACG/K/C24 in Wnt7a-CTD. Treatment of the recombinant proteins with Endoglycosidase H resulted in a significant decrease in their apparent Mr values of Wnt5a-CTD (∼33 kDa → 18 kDa) and Wnt7a-CTD (∼30 kDa → 16 kDa).

Although the three dimensional structures of Wnt5A and Wnt7a are unknown, their homology with XWnt8 of Xenopus permitted the construction of homology models based on the crystal structure of the latter protein (see Supplementary file). In the crystal structure of XWnt8 and the homology models of Wnt5A and Wnt7a (Fig. 1A–C), a characteristic feature of the C-terminal cytokine-like ‘finger’ domains is the presence of two long, twisted antiparallel β-strand connected by a β-hairpin structure that is stabilized by several disulfide bridges [28–30].

In the XWnt8-Frizzled-8 complex, the two domain Wnt structure resembles a hand, where the N-terminal and C-terminal domains provide the “thumb” and “index” fingers that grasp the Frizzled receptor at two distinct binding sites [28]. The lipid group at the tip of the N-terminal thumb is inserted into a deep groove in the Wnt-binding domain of the receptor, whereas the tip of the C-terminal ‘index finger’ interacts with residues on the opposite side of the receptor [28]. Although three dimensional structures of ternary Wnt-receptor complexes have not yet been solved, mutational analysis of mouse Wnt3a revealed that the linker connecting the N- and C-terminal domains participates in LRP6 binding [31].

The activity of Wnts is modulated by a variety of extracellular proteins that interfere with the formation of the Wnt-receptor complexes [32–34]. Dickkopf proteins bind to LRP5/6 preventing the association of these co-receptors with Wnts, whereas Wnt Inhibitory Factor 1, Cerberus and secreted Frizzled-related proteins bind directly to Wnts blocking their binding to Wnt receptors.

Wnt Inhibitory Factor 1 is an extracellular protein containing a WIF domain and five EGF repeats, the WIF domain being primarily responsible for the Wnt inhibitory activity of the protein [15]. In our earlier work we have solved the three dimensional structure of the WIF domain of human WIF-1 by NMR spectroscopy and identified an alkyl-binding site that we suggested to serve as a binding site for the essential lipid groups of Wnts [35]. In an attempt to localize the Wnt-binding site of the WIF domain of Wnt Inhibitory Factor 1 we have subjected the WIF domain of Wnt Inhibitory Factor 1 to structure-guided arginine-scanning mutagenesis [36]. These studies have confirmed that the alkyl-binding site of the WIF domain is critical for its interaction with Wnts: substitution of residues known to interact with the aliphatic moiety of the alkyl-ligand resulted in significantly decreased affinity for Wnts [36]. Nevertheless, none of these mutations resulted in complete elimination of the binding of the WIF domain to Wnts, suggesting that – in analogy with the Wnt-Frizzled interaction – Wnts may bind to the WIF domain at additional sites.
Studies with deglycosylated forms of Wnt5a-CTD and Wnt7a-CTD revealed that they have higher affinity for the WIF domain than the glycosylated forms (Table 1) suggesting that the carbohydrate moieties attached to the N-glycosylation sites bury some sites that may interact with the WIF domain. Nevertheless, the fact that the glycosylated forms of Wnt5a-CTD and Wnt7a-CTD have significant affinity for the WIF domain indicates that critical parts of the interaction site remain exposed. Since in the homology models of Wnt5a-CTD and Wnt7a-CTD the N-glycosylation sites are at the ‘bottom’ of the protruding cytokine-like folds (Fig. 1E and F), it seems likely that residues of these finger-like structures may be most critical for the interaction with the WIF domain.

3.3. Identification of residues involved in the interaction of the WIF domain with the C-terminal domains of Wnt5a and Wnt7a

In our earlier work we have shown that substitution of residues of the alkyl-binding site of the WIF domain decreases the affinity of the WIF domain for Wnts, suggesting that the alkyl-binding site contributes significantly to the WIF–Wnt interaction, possibly...
through the interaction with the lipid moiety present in the N-terminal part of Wnts [36].

As a further test of the assumption that the surface around the alkyl-binding site of the WIF domain interacts with the N-terminal part of Wnts, in the present work we have studied the interaction of Wnt5a-CTD and Wnt7a-CTD with some alkyl-binding site mutants of the WIF domain. These studies have shown that the Ile49Arg, Phe51Arg and Phe66Arg mutations that significantly lowered the affinity of the WIF domain for full-length Wnts [36] had practically no effect on the interaction of WIF with Wnt5a-CTD and Wnt7a-CTD: in competition assays the mutants were as efficient as the wild type protein in preventing binding of Wnt-CTDs to immobilized wild type WIF (Table 2). This observation suggests that the WIF–Wnt interaction mediated by Wnt-CTDs does not involve the alkyl-binding site of the WIF domain.

In order to localize the region of the WIF domain that interacts with Wnt-CTDs we have selected additional residues for arginine-scanning mutagenesis (the list of residues subjected to mutagenesis is shown in Supplementary Table 1). Although we have selected a total of thirteen positions of the WIF domain for arginine-scanning mutagenesis, the effect of the Asp116Arg and Leu106Arg mutations on the WIF-Wnt interaction could not be studied because P. pastoris failed to secrete these mutant proteins. Inspection of the crystal structure (2YCN.pdb) of the WIF domain [37] revealed that a hydrogen bond connects the carboxyl oxygen of the Asp116 side chain to the backbone nitrogen of Ser108. A possible explanation why the mutant protein failed to be expressed is that substitution of Asp116 with an arginine disrupts this hydrogen bond and this may cause misfolding and degradation of the WIF domain.

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Our solution competition assays with the remaining eleven mutants have revealed that substitution of residues Leu123, Ile155 and Ile172 by arginine resulted in a detectable decrease in the affinity for Wnt5a, the most significant effect being observed in the case of the Ile155Arg mutant (Table 3). Inspection of the structure of the WIF domain reveals that residue Ile155 is distant from the alkyl-binding site of the WIF domain (Fig. 3), consistent with the notion that the Ile155Arg mutation of WIF affects interactions that do not involve the N-terminal domain of Wnt5a.

To test the assumption that the Ile155 residue of WIF is located in the region that serves as a Wnt-CTD-binding site we have monitored the influence of this mutation on the interaction of WIF with Wnt5a-CTD and Wnt7a-CTD. In harmony with this assumption our competition assays revealed that the Ile155Arg mutation impaired the ability of the mutant protein to compete with immobilized wild type WIF domain for both Wnt5a-CTD and Wnt7a-CTD (Fig. 4).

As a further test of the influence of the Ile155Arg substitution on the WIF–Wnt interaction we have assayed the binding of full-length Wnt5a, Wnt7a, Wnt5a-CTD and Wnt7a-CTD to Ile155Arg mutant WIF on sensor chips (Table 4). In the case of full-length Wnt5a the Ile155Arg mutation of WIF caused an increase in $K_d$ ($K_d = 8 \times 10^{-10} \text{M}$ in Wnt5a to $K_d = 1.55 \times 10^{-9} \text{M}$), but a more drastic reduction in affinity for Wnt5a-CTD was observed. Although Wnt5a-CTD has significant affinity for wild type WIF domain (Fig. 2, Table 1), no interaction could be detected by SPR using immobilized Ile155Arg mutant WIF.

The Ile155Arg mutation of WIF also caused some increase in the $K_d$ for the interaction with full-length Wnt7a.
(K_d = 3.46 x 10^{-10} M → K_d = 8.73 x 10^{-10} M) but the decrease in affinity for Wnt7a-CTD was more dramatic (K_d = 8.4 x 10^{-9} M → K_d = 1.64 x 10^{-7} M).

Our observation that the Ile155Arg mutation of WIF results in elimination of detectable binding to Wnt5a-CTD, whereas Wnt7a-CTD retains some affinity for the mutant (Table 4) suggests...
that in the case of Wnt7a-CTD additional side chains contribute significantly to the interaction.

In harmony with the notion that both the alkyl-binding site and the Wnt-CTD binding site of the WIF domain are essential for the high affinity of Wnts for WIF, WIF variants with double mutations (e.g. Ile155Arg-Ile49Arg, in which residues at both the alkyl-binding site and Wnt-CTD-binding site are substituted) have more drastically reduced affinity for Wnt5a (Fig. 5, Table 3).

### Table 4

| Interacting proteins                  | $K_0$ (M) | $k_a$ (M/s) | $k_d$ (1/s) |
|---------------------------------------|-----------|-------------|-------------|
| WIF Ile155Arg – Wnt5a                | 1.55 ± 0.15 × 10⁻¹ | 3.85 ± 0.44 × 10³ | 5.98 ± 0.41 × 10⁻⁴ |
| WIF Ile155Arg – Wnt7a                | 8.73 ± 0.34 × 10⁻¹ | 5.53 ± 8.82 × 10³ | 4.84 ± 0.14 × 10⁻⁴ |
| WIF Ile155Arg – Wnt5a-CTD            | nd        | nd          | nd          |
| WIF Ile155Arg – Wnt7a-CTD            | 1.64 ± 0.12 × 10⁻⁷ | 4.41 ± 0.19 × 10³ | 7.23 ± 0.68 × 10⁻⁴ |

* nd – no detectable binding.

### 3.4. Modeling the interaction of the WIF domain with Wnts

Protein–protein docking with the ClusPro 2.0 server provided some insight into the possible structures of the Wnt5a-WIF and Wnt7a-WIF complexes that are consistent with the results of our mutagenesis and protein–protein interactions studies. In these docking experiments Wnt5a and Wnt7a were used as receptors and the crystal structure of the WIF domain (2YGN.pdb) was used.

![Fig. 5](image-url)

*Fig. 5. Effect of wild type and mutant WIF domains on the binding of Wnt5a to immobilized wild type WIF domain, monitored by surface plasmon resonance. Sensorgrams of the interactions of immobilized WIF domain with Wnt5a (20 nM) (A) preincubated with 0 μM, 0.25 μM, 0.5 μM, 2 μM and 4 μM of wild type WIF domain; (B) preincubated with 0 μM, 4 μM, 8 μM, 24 μM of WIF-I172R-F51R; (C) preincubated with 0 μM, 4 μM, 8 μM and 24 μM of WIF-I155R-I49R. Wnt5a was preincubated with various concentrations of wild type or mutant WIF domains for 30 min at room temperature and were injected over CM5 sensorchips containing immobilized wild type WIF domain (at 0 s on the abscissa). The binding phase lasted 240 s, followed by a washing phase. Association and dissociation rate constants were calculated by analysis of the sensorgrams of the binding and washing phases, respectively. In each case SPR response decreased parallel with the increase of WIF concentration. Panel (D) shows the concentration dependence of the inhibitory effect of WIF domains as monitored by changes in observed association rate, $k_{obs}$. Symbols: wild type WIF domain – - - - ; WIF-I155R-I49R - ▽ - ▽ - ; WIF-I172R-F51R - ○ - ○ -.
as the ligand. The docking algorithms of ClusPro evaluate billions of putative complexes, retaining those with favorable surface complementarities [38,39]. Fig. 6 shows the structure of one of the predicted Wnt5a-WIF complexes that might illustrate how the C-terminal ‘finger’ of Wnt5a interacts with the Wnt-CTD binding site of the WIF domain, while the N-terminal ‘thumb’ of Wnt5a binds to the alkyl-binding site of the WIF domain.

In view of the homology of the extracellular ligand-binding domain of Ryk receptor tyrosine kinases with the WIF domain of Wnt Inhibitory Factor 1 [15] and the fact that Ryk tyrosine kinases serve as receptors for Wnts [17–22] our results may also have relevance for structure–function aspects of these receptor tyrosine kinases. Although the 3D structure of the WIF domain of Ryk receptor tyrosine kinase is not yet known, its homology with the WIF domain of Wnt Inhibitory Factor 1 permitted us to build a homology model for the domain (Fig. 7). Comparison of the predicted structure of Ryk’s WIF domain (Fig. 7) with the structure of the WIF domain of Wnt Inhibitory Factor 1 (Fig. 3) identified Ryk residues Ile74, Leu76, Tyr91, Thr173 (the structural equivalents of Ile49, Phe51, Phe66 and Ile155 of WIF-1) as the most likely Wnt-binding binding sites in Ryk receptor tyrosine kinases.

4. Discussion

In the present work we have shown that – in addition to the alkyl-binding site – a second site at the opposite surface of the WIF domain is critical for its interaction with Wnts. The fact that the Ile155Arg substitution at this second site drastically reduces binding to Wnt5a-CTD and Wnt7a-CTD clearly establishes that the C-terminal cytokine-like ‘finger’ domain of Wnts binds to this second site of the WIF domain. Conversely, the fact that mutations affecting the alkyl-binding site of the WIF domain weaken the WIF–Wnt interaction [36], but have no influence on the interaction of WIF with Wnt5a-CTD or Wnt7a-CTD is consistent with the notion that the alkyl-binding site may be involved in the binding of the lipid-modified, N-terminal ‘thumb’ of Wnts.

Understanding the structural basis of the Wnt–WIF interaction is of major importance as it might permit the design of WIF variants with improved therapeutic properties.

The therapeutic importance of Wnt Inhibitory Factor 1 may be illustrated by the fact that epigenetic silencing of WIF-1 is associated with aberrant activation of the Wnt pathways in a variety of...
cancers, whereas restoration of its expression inhibits tumor progression [32–34]. The significance of WIF-1 as a tumor suppressor is also supported by the recent observation that a Cys294Phe mutation of the WIF1 gene predisposes members of a large family to multiple early onset cancers including prostate, breast, colon, and several other uncommon cancers [40]. Since the Cys294Phe mutation disrupts a conserved disulfide bond of the fourth EGF domain of the WIF-1 protein it seems plausible to assume that the misfolded protein is unable to fulfill its function as a negative regulator of various Wnts, leading to aberrant activation of the various Wnt-Fzd-LRP5/LRP6 signaling pathways.

In view of the growing interest in developing therapies that exploit inhibitors of the Wnt pathway [41,42] it is important to point out that inhibition of Wnt signaling by transfection with vectors coding for WIF-1 or treatment with recombinant WIF domain reduced tumor growth in several models [32–33,43].

We believe that understanding the structure of Wnt–WIF complexes promotes the rational design of WIF variants, permitting more specific targeting of Wnt family members and more specific targeting of distinct forms of cancer.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.febslet.2015.08.031.

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