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Structure Report

Crystal structure of a hemojuvelin-binding fragment of neogenin at 1.8 Å

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ABSTRACT

Neogenin is a type I transmembrane glycoprotein with a large ectodomain containing tandem immunoglobulin-like and fibronectin type III (FNIII) domains. Closely related to the tumor suppressor gene DCC, neogenin functions in critical biological processes through binding to various ligands, including netrin, repulsive guidance molecules, and the iron regulatory protein hemojuvelin. We previously reported that neogenin binds to hemojuvelin through its membrane-proximal fifth and sixth FNIII domains (FN5–6), with domain 6 (FN6) contributing the majority of critical binding interactions. Here we present the crystal structure of FN5–6, the hemojuvelin-binding fragment of human neogenin, at 1.8 Å. The two FNIII domains are orientated nearly linearly, a domain arrangement most similar to that of a tandem FNIII-containing fragment within the cytoplasmic tail of the β4 integrin. By mapping surface-exposed residues that differ between neogenin FN5–6 and the comparable domains from DCC, which does not bind hemojuvelin, we identified a potential hemojuvelin-binding site on neogenin FN6. Neogenin FN5, which does not bind hemojuvelin in isolation, exhibits a highly electropositive surface, which may be involved in interactions with negatively-charged polysaccharides or phospholipids in the membrane bilayer. The neogenin FN5–6 structure can be used to facilitate a molecular understanding of neogenin's interaction with hemojuvelin to regulate iron homeostasis and with hemojuvelin-related repulsive guidance molecules to mediate axon guidance.

1. Introduction

Neogenin is a type I transmembrane glycoprotein expressed in multiple tissues including brain, kidney, liver, and skeletal muscle (Meyerhardt et al., 1997; Vielmetter et al., 1997). Closely-related to the tumor suppressor molecule DCC (deleted in colorectal cancer) (Vielmetter et al., 1994), neogenin is composed of four immunoglobulin (Ig)-like domains followed by six fibronectin type III (FNIII) domains, a transmembrane region, and a cytoplasmic domain (Vielmetter et al., 1994; Vielmetter et al., 1997). Neogenin functions in a variety of developmental and metabolic processes (Wilson and Key, 2007), and several ligands have been identified, including netrin, repulsive guidance molecules (RGMs) (Matsunaga and Chedotal, 2004; Matsunaga et al., 2004; Rajagopalan et al., 2004), and the iron regulatory protein hemojuvelin (Zhang et al., 2005).

While netrin-1 and neogenin mediate chemoattractive axon guidance, the neogenin/RGMa interaction functions specifically in axon repulsion (Wilson and Key, 2006). Neogenin has also been implicated as a dependence receptor (Bredesen et al., 2005), such that it triggers apoptosis in the absence of a ligand RGM molecule, whereas the ligand-bound state inhibits this effect (Matsunaga and Chedotal, 2004; Matsunaga et al., 2004). Downstream signaling elicited by the binding of neogenin to RGMa involves the Rho family of small GTP-binding proteins, which regulate cytoskeletal dynamics by controlling actin filaments and causing growth cone collapse (Conrad et al., 2007). Pre-incubation of netrin-1 inhibits this signaling, indicating either that netrin-1 occludes the RGMa-binding site on neogenin, or that a different signaling cascade is initiated to counteract the Rho-mediated signaling (Conrad et al., 2007).

In hepatocytes and perhaps also skeletal muscle, neogenin is involved in iron homeostasis through interactions with hemojuvelin, also known as HFE2 or RGMc (Zhang et al., 2005). Hemojuvelin is a glycosylphosphatidylinositol (GPI)-anchored protein that shares sequence similarity with RGMa and RGMb, which, unlike hemojuvelin (RGMc), are expressed predominantly in the nervous system (Schmidtmer and Engelkamp, 2004). Hemojuvelin is an upstream

Abbreviations: DCC, deleted in colorectal cancer; Ig, immunoglobulin; FNIII, fibronectin type III; RGM, repulsive guidance molecule; BMP, bone morphogenetic protein; Ihog, interference hedgehog.

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modulator of hepcidin, a peptide hormone that regulates iron flux in mammals (Lin et al., 2005). Interaction with neogenin has been suggested to initiate retrograde trafficking of membrane-bound hemojuvelin to the Golgi and trans-Golgi network for further processing before soluble hemojuvelin is released from the cell (Maxson et al., 2009; Zhang et al., 2007; Zhang et al., 2008). The ratio of membrane-bound and soluble forms of hemojuvelin is believed to be important for determining the amount of signal sent to the nucleus through the bone morphogenetic protein (BMP)/hemojuvelin pathway, which regulates hepcidin expression levels (Babitt et al., 2006).

We previously described biochemical studies using neogenin ectodomain deletion mutants to localize the hemojuvelin-binding site to the two membrane-proximal FNIII domains (FN5–6) (Yang et al., 2008). The FN5–6 fragment was as effective as the intact neogenin ectodomain in competing with cell membrane neogenin both *in vitro* (Zhang et al., 2008) and *in vivo* (Zhang et al., 2009), suggesting that the FN5–6 region contains the hemojuvelin-binding region on neogenin. While FN5 did not bind detectably to hemojuvelin, FN6 alone bound hemojuvelin, although more weakly than FN5–6, suggesting a potential contribution from the domain linking region in the binding interaction (Yang et al., 2008).

Here we report the crystal structure of the hemojuvelin-binding fragment of human neogenin, FN5–6, at 1.8 Å resolution. Each domain adopts the canonical FNIII fold, with the two domains arranged nearly linearly, surprisingly similar to the arrangement of a pair of tandem FNIII domains from the cytoplasmic tail of the β4 integrin. The neogenin FN5 domain displays a highly positively-charged surface, a feature shared with DCC FN5 and other proteins known to bind heparan sulfate (Bennett et al., 1997; McLellan et al., 2006). In addition to the possibility of interacting with negatively-charged carbohydrate or protein ligands, we suggest that the positive surface on the neogenin FN5 domain may promote interactions with negatively-charged phospholipids to facilitate exposure of the hemojuvelin-binding FN6 domain to hemojuvelin proteins on the surface of another cell. To gain insight into which portion of neogenin FN5–6 interacts with hemojuvelin, we mapped non-conserved residues from the comparable domains of DCC, which does not bind hemojuvelin, onto the neogenin FN5–6 structure. One side of the FN6 domain, comprising strands C, C′, F, and G, contains a high concentration of non-conserved surface residues, suggesting that this face of the molecule contains the potential hemojuvelin-binding site.

2. Materials and methods

2.1. Crystallization and data collections

Neogenin FN5–6, corresponding to the fifth and sixth FNIII domains of human neogenin (residues 853–1054) plus a C-terminal 6x-His tag, was expressed in baculovirus-infected insect cells and purified from supernatants as previously described (Yang et al., 2008). This version of neogenin FN5–6 was previously referred to as sFNIII 5–6 to distinguish it from a longer version of these domains (FNIII 5–6; residues 851–1103). The longer version bound to hemojuvelin with ~18-fold higher affinity than FN5–6 (Yang et al., 2008), but did not crystallize, presumably due to disorder of the C-terminal extension. The best crystals were obtained from FN5–6 purified from culture media supplemented with 0.5 mg/L tunicamycin (Sigma) to inhibit addition of N-linked glycans. Crystallization screening was done using a Mosquito nanoliter handling system (TTP LabTech) with drops containing 200 nL protein plus an equal volume of reservoir solution. Initial crystals grew in mother liquor containing 0.1 M Tris, pH 8.5, 0.2 M ammonium sulfate, 25% PEG-3350 at 20 °C. Larger crystals were obtained in a Qiagen 24-well screw-top hanging drop plate using the same mother liquor. A single crystal was cryo-preserved in mother liquor supplemented with 5% glycerol and a native data set was collected on an R-AXIS-VI rotating anode X-ray generator (Rigaku) at 100 K.

2.2. Structure determination and model refinement

Data were processed by Denzo and scaled using Scalepack (Otwinowski and Minor, 1997) in the orthorhombic space group $C222_1$ (a = 52.6, b = 112.9, c = 80.9 Å). The calculated Matthews coefficient (V_M = 2.5 Å^3/Da) (Matthews, 1968) suggested a solvent content of 51% and one molecule per asymmetric unit. The structure was solved by molecular replacement using the program Phaser (McCoy et al., 2007) and search models derived from NMR structures of individual domains of neogenin (PDB codes 1X5J and 1X5K) in which residues not present in our construct were deleted. Solvent-flattened electron density maps for model building were generated using the program DM (CCP4, 1994). After rigid body refinement, the model was iteratively improved using cycles of refinement using CNS (Brunger et al., 1998) and manual rebuilding using COOT (Emsley and Cowtan, 2004) into $2F_o$–F_c annealed omit maps. The final model (R_{cryst} = 20.0% and R_{free} = 23.4%) consists of neogenin residues 853–899 and 903–1052 (residues 900–902 were disordered), and 293 water molecules (Table 1). For analyses of contacts and buried surface areas, FN5 was defined as residues 853–949, and FN6 was defined as residues 952–1052. The CCP4 program Areaimol (CCP4, 1994; Lee and Richards, 1971; Saff and Kuijlaars, 1997) was used to calculate buried surface area using a 1.4 Å probe and to identify interacting residues using the following criteria: a distance of <3.5 Å and a hydrogen bond angle of >90° for hydrogen bonds and a maximum distance of 4.0 Å for van der Waals interactions. Figures were prepared by Pymol (DeLano, 2002).

Table 1
Data collection and refinement statistics.

Unit cell	Space group	$C222_1$
Cell dimensions	a, b, c (Å)	52.6, 112.9, 80.9
Data collection		
	Resolution (Å)	32.8–1.8 (1.86–1.80)
	[a]R_{merge} (%)	5.9 (37.8)
	Completeness	99.3 (98.2)
	$I/\sigma I$	28.2 (4.2)
	Mean redundancy	3.8 (3.7)
	No. of unique/total reflections	22,544/85,823
Refinement statistics		
	Resolution (Å)	32.8–1.8
	No. reflections used	22,481
	No. reflections in working/test set	21,391/1090
	[b]R_{cryst}/R_{free} (%)	20.0/23.4
No. Atoms (*B factor: Å^2*)		
	Protein	1577 (23.75)
	Water	293 (35.49)
RMS deviations		
	Bond length (Å)	0.010
	Angle (°)	1.596
Ramachandran plot (%)		
	Preferred	187 (96.4%)
	Allowed	7 (3.6%)
	Outlier	0 (0.0%)

[a] R_{merge} (%) = 100 × $\Sigma|I - \langle I\rangle|/\Sigma I$, where I is the integrated intensity of a given reflection. Numbers in parentheses are statistics for the highest resolution shell.

[b] R_{cryst} (%) = 100 × $\Sigma|F_{obs} - F_{calc}|/\Sigma F_{obs}$, where the F_{obs} and F_{calc} are the observed and calculated structure factor amplitudes for all reflections in the working set. R_{free} was calculated as described for R_{cryst} but summed over the 5% of reflections that were not included in the refinement (Brunger, 1997).