Mutational Analysis of Norrin-Frizzled4 Recognition

Received for publication, October 12, 2006, and in revised form, December 4, 2006. Published, JBC Papers in Press, December 6, 2006, DOI 10.1074/jbc.M609618200

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Norrin and Frizzled4 (Fz4) function as a ligand-receptor pair to control vascular development in the retina and inner ear. In mice and humans, mutations in either of the corresponding genes lead to defects in vascular development. The present work is aimed at defining the sequence determinants of binding specificity between Norrin and the Fz4 amino-terminal ligand-binding domain (the “cysteine-rich domain” (CRD)). The principal conclusions are as follows: 1) Norrin binds to the Fz4 CRD and does not detectably bind to the 14 other mammalian Frizzled and secreted Frizzled-related protein CRDs; 2) Norrin and Xenopus Wnt8 recognize largely overlapping regions of the Fz4 CRD; 3) surface determinants on the Fz4 and Fz8 CRDs that allow Norrin to distinguish between these two CRDs reside within several small regions on one face of the CRD; 4) Norrin function depends critically on three pairs of cysteines that form the highly conserved trio of disulfide bonds shared among all cystine knot proteins, but the remaining two putative disulfide bonds are less important; 5) Norrin-CRD binding depends on a largely contiguous group of amino acids in the extended β-sheet domain of Norrin that are predicted to face away from the interface between the two monomers in the Norrin homodimer; 6) Norrin-CRBD binding is strongly modulated by interactions involving charged amino acid side chains; and 7) Norrin-CRBD binding is enhanced ~10-fold by the addition of heparin. These observations are discussed in the context of Frizzled signaling and the structure and function of other cystine knot proteins.

The Frizzled family of cell surface receptors is present throughout the animal kingdom, with 10, four, and three family members encoded in the genomes of mammals, Drosophila, and Caenorhabditis elegans, respectively. The Frizzleds play an essential role in processes as diverse as embryonic segment polarity, midgut development, and bristle orientation in Drosophila, and axon guidance, retinal vascular development, and hair follicle orientation in mice (1–7). At present, the biochemically best understood aspect of Frizzled receptor function is the binding of Wnt ligands and the resulting activation of a “canonical” signaling pathway in conjunction with the coreceptors Lrp5 or Lrp6 (8). Wnts bind to a compact domain at the Frizzled NH2 terminus referred to as the cysteine-rich domain (CRD)2 (9, 10), for which a high resolution crystallographic structure has been obtained (11). In mammals, closely related CRDs are present in a family of five secreted Frizzled-related proteins (sFRPs), which probably function as competitive inhibitors of Wnt-Frizzled signaling (12, 13).

Recently, Norrin, a protein that is not a member of the Wnt family, has been identified as a ligand that binds the Fz4 CRD with high affinity and potently activates the canonical signaling pathway (7). Norrin is a small, cysteine-rich, secreted protein with weak homology to the transforming growth factor (TGF)-β family of ligands (14–17). In humans, mutations in the corresponding gene (NDP) cause Norrie disease, an X-linked disorder characterized by hypovascularization of the retina and a severe loss of visual function (18). A milder retinal hypovascularization (familial exudative vitreoretinopathy) is seen in humans heterozygous for mutations in the genes coding for Fz4 (19, 20) or the coreceptor Lrp5 (21), and severe hypovascularization is seen in humans and mice carrying a homozygous loss of function mutations in Lrp5 (22, 23). Mice carrying a targeted deletion of the Ndp gene (hemizygous mutant males or homozygous mutant females) or of both copies of the Fz4 gene exhibit nearly identical retinal hypovascularization phenotypes, as well as a progressive enlargement and subsequent loss of blood vessels in the stria vascularis, the specialized epithelium that generates the endolymphatic fluid within the inner ear (7, 24–26). Norrin binds with nanomolar affinity to the Fz4 CRD but not to several other Frizzled CRDs. Like Wnts, Norrin associates with the extracellular matrix, which limits its range of action to those target cells immediately surrounding its site of synthesis. Thus, Norrin appears to function in many respects like a Wnt despite a complete absence of primary sequence homology with the Wnt family.

In this paper, we have addressed several questions raised by the discovery that Norrin and Fz4 constitute a ligand-receptor pair. First, how selective is the binding of Norrin to the Fz4 CRD as compared with other Frizzled and sFRP CRDs? Second, which regions of the Fz4 CRD are responsible for Norrin binding, and what is the relationship of these regions to those responsible for Wnt binding? Finally, which regions of Norrin are involved in Fz4 binding and canonical pathway activation?

2 The abbreviations used are: CRD, cysteine-rich domain; sFRP, secreted Frizzled-related proteins; TGF, transforming growth factor; PBS, phosphate-buffered saline; WT, wild type; AP, alkaline phosphatase; Xwnt8, Xenopus Wnt8; GPI, glycosylphosphatidylinositol.

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As described below, Norrin has the ability to discriminate between the Fz4 CRD and the 14 other mammalian Frizzled and sFRP CRDs, and by site-directed mutagenesis we delimit the regions on both Norrin and Fz4 responsible for this recognition. These results have implications for other ligand-receptor families in which closely related proteins exhibit differential recognition of potential binding partners.

**MATERIALS AND METHODS**

**Site-directed Mutagenesis**—Mutations were constructed by tandem PCR. All DNA segments derived from PCR were sequenced to confirm the presence of the desired mutation and to rule out spurious mutations.

**Production of Xenopus Wnt8 (Xwnt8), Norrin, and Fz4 CRD Fusion Proteins**—AP-3Myc-Norrin was secreted from transiently transfected 293 cells using a vector with a cytomegalovirus immediate gene enhancer and promoter; the AP-3Myc-Norrin was collected in Dulbecco’s modified Eagle’s medium/F-12 medium containing penicillin/streptomycin and 10% calf serum and stored at 4 °C. Xwnt-8Myc-AP was produced under the control of a metallothionein promoter in stably transfected hygromycin-resistant Drosophila S2 cells. The secreted fusion protein was collected in Schneider Drosophila medium (Invitrogen) supplemented with penicillin/streptomycin, 10% calf serum, 50 μg/ml hygromycin, and 0.5 mM CuSO4 and stored at 4 °C. To produce Fz CRD-IgG fusion proteins, the CRD (i.e. the 114-amino acid region extending from the first to the tenth conserved CRD cysteine) was inserted between MluI and Apal sites in a vector that contains a cytomegalovirus immediate early gene enhancer and promoter, followed by DNA coding for the mouse Fz8 signal peptide, the site for CRD insertion, 25 amino acids of Fz8 “linker” sequence immediately COOH-terminal to the Fz8 CRD, and the constant region of human IgG. CRD-IgG fusion proteins were secreted from transiently transfected 293 cells, collected in serum-free Dulbecco’s modified Eagle’s medium/F-12 containing penicillin/streptomycin, and stored in aliquots at −80 °C.

**Binding to Cell Surface CRD-Myc-GPI**—To display CRDs at the cell surface, each CRD was inserted between MluI and Apal sites in a vector that contains a cytomegalovirus immediate early gene enhancer and promoter, followed by DNA coding for the mouse Fz8 signal peptide, the site for CRD insertion, 25 amino acids of Fz8 “linker” sequence immediately COOH-terminal to the Fz8 CRD, and the constant region of human IgG. CRD-IgG fusion proteins were secreted from transiently transfected 293 cells, collected in serum-free Dulbecco’s modified Eagle’s medium/F-12 containing penicillin/streptomycin, and stored in aliquots at −80 °C.

**Luciferase Assays**—For a typical luciferase assay, a G418-resistant stable 293 cell line (STF cells) (7) carrying the Super Top Flash firefly luciferase reporter of canonical Wnt signaling (a construct with seven tandem LEF/TCF binding sites) was transfected in triplicate in a 24-well tray using Fugene 6 with the following quantities of expression plasmid DNA per well:

- 0.25 ml of plasmid DNA
- 0.5 ml of plasmid DNA
- 1 ml of plasmid DNA

The transfected cells were incubated in 1 ml of serum-free Dulbecco’s modified Eagle’s medium (described above) at room temperature for 3 h with gentle end-over-end rotation. The resin was transferred to a column and washed with PBS/calcium/magnesium, and the AP-3Myc-Norrin was eluted with PBS/calcium/magnesium containing 1 M NaCl. The partially purified AP-3Myc-Norrin was diluted with 7 volumes 10 mM NaPO4, pH 7.2, calcium/magnesium supplemented with 0.01% bovine serum albumin (to reduce the final NaCl concentration to 150 mM) and stored at 4 °C. Binding to Fz4 CRD-IgG that had been captured onto protein A microwells was performed in the presence of various concentrations of porcine intestinal heparin (Sigma).
Norrin, 50 ng; Fz4, 50 ng; Lrp6, 50 ng; *Renilla* luciferase, 1 ng. Two days after transfection, cells were washed with PBS and assayed using the Promega dual luciferase assay reagents. The firefly luciferase activity was normalized to the co-expressed *Renilla* luciferase activity, and the average of the triplicate samples was determined.

**Modeling Fz4 CRD and Norrin Structures**—The locations of different amino acid side chains on the Fz4 CRD surface were modeled by highlighting the corresponding residues in the high resolution Fz8 CRD crystal structure (11) using the program PyMol (available on the World Wide Web at pymol.sourceforge.net). The amino acid sequences of the Fz4 and Fz8 CRDs align without recourse to insertion or deletion. To model the three-dimensional structure of Norrin, we first superimposed the structures of TGF-β family members TGF-β2 (Protein Data Bank code 2TGI) (27), TGF-β3 (Protein Data Bank code 1TGI) (28), BMP7 (Protein Data Bank code 1BMP) (29), and BMP2 (Protein Data Bank code 2GOO) (30) using the program CCP4mg (31). This superposition was then used to align the amino acid sequences of these proteins. The subunits of TGF-β family dimers consist of two extended β-hairpins separated by an α-helical region. Each of the four β strands that make up the two hairpins contains cysteines that are conserved between TGF-β family members and Norrin and anchor alignment of the β-strand regions of Norrin with the TGF-β/BMP sequences. Branching out from these cysteines, a conserved pattern of hydrophilic and hydrophobic residues is evident between Norrin and the TGF-β family members in conserved secondary structure elements, which enabled reliable alignment of these regions of Norrin and TGF-β sequences (see Fig. 6). This sequence alignment was then used to identify sites on the BMP2 structure homologous to positions in the Norrin amino acid sequence. Since no TGF-β family member stands out as more related to Norrin, BMP2 was chosen for modeling because there is a relatively high resolution structure (2.2 Å), and a structure of BMP2 complexed with both types of TGF-β receptor allows comparison of these sites with interaction sites identified on Norrin.

**RESULTS**

**Norrin Binds Only to the Fz4 CRD**—To assess Norrin and Wnt binding to various CRD targets, we employed AP fusion proteins as probes. For Xwnt8, a fusion with a Myc epitope and AP at the COOH terminus was produced in soluble form from transiently transfected *Drosophila* S2 cells (10); for human Norrin, a fusion with AP and three Myc epitopes at the NH2 terminus was produced in soluble form from transiently transfected COS cells (Fig. 1A). Both AP fusion proteins were collected in medium containing 10% serum and, unless otherwise noted, were used for binding assays without further purification. Signaling assays were performed with a β-catenin-responsive luciferase reporter in stably transfected 293 cells transiently expressing Fz4, Lrp6, and human Norrin carrying a COOH-terminal rhodopsin tag (Fig. 1A).

For binding assays, the CRD targets were presented in two formats (Fig. 1). In the first format, the CRD was displayed as a Myc-tagged and GPI-anchored protein on the surface of transfected COS cells, and binding was performed with live cells. Cell surface localization and accessibility of the CRD was confirmed for each CRD construct by incubating live cells with an anti-Myc monoclonal antibody. In this format, the assay measures binding in the context of plasma membrane lipids, glycoconjugates, and cell-associated extracellular matrix molecules. Cell surface binding was scored qualitatively based on the average AP intensity per cell (Fig. 1B). In the second format, the CRD was expressed as a fusion to the constant region of human IgG, which was then immobilized in protein A-coated microwells. Binding in this context occurs free of cell-associated molecules and was scored quantitatively (Fig. 1, C and D).

To systematically assess the specificity of Norrin-Fz4 binding, each of the 10 Frizzled and five sFRP CRDs encoded in the mouse genome was displayed on the surface of transfected COS cells and probed with anti-Myc, AP-3Myc-Norrin, or Xwnt8-Myc-AP. As seen in Fig. 2 and supplemental Table 1, all 15 CRDs accumulate at the cell surface, and four of them (from Fz4, Fz5, Fz7, and Fz8) efficiently bind Xwnt8-Myc-AP. In previous work, we had assayed the CRDs of Fz2–Fz8 for AP-Myc-Norrin binding and observed binding only to the Fz4 CRD (7). Here we extend this analysis to the complete set of mammalian CRDs and observe that only the Fz4 CRD shows detectable AP-3Myc-Norrin binding. A dendrogram showing the related-
A

Electronic Supplemental Data

B

The terminal region from Fz8 or the reverse; Fz4/Fz8 chimeras or Fz8/Fz4 chimeras, respectively; Fig. 3A). In all of these strategies, the cysteines were left unaltered. Because both Fz4 and Fz8 CRDs bind to Xwnt8-Myc-AP but only the Fz4 CRD binds to AP-3Myc-Norrin, we would predict that all correctly folded CRD constructs generated with the second and third strategies would retain Xwnt8-Myc-AP binding, and, among these, differences in AP-3Myc-Norrin binding would reflect sequence differences between Fz4 and Fz8 that are relevant to the specific recognition of Fz4 by Norrin.

CRDs generated with each of the three strategies were expressed as Myc-GPI-anchored proteins and tested for binding to anti-Myc, Xwnt8-Myc-AP, and AP-3Myc-Norrin (Table 1 and Fig. 3E). All of the CRD constructs accumulate at the cell surface, and approximately half show binding to both Xwnt8-Myc-AP and AP-3Myc-Norrin that is comparable with the WT Fz4 CRD. For this subset of mutants, we can conclude that the CRD tertiary structure is largely unperturbed and that the mutated surface region is unlikely to be critically involved in AP-3Myc-Norrin or Xwnt8-Myc-AP binding. For the remaining CRD mutants that are variably defective in binding, the defects in Xwnt8-Myc-AP and AP-3Myc-Norrin binding are closely correlated (Table 1 and Fig. 3E). Although these data cannot distinguish between binding defects due to a distortion of tertiary structure and those due to defects in residues that have direct contact with the ligand, the surface locations of the alanine scanning and Fz8 block substitutions argue for the latter as the more likely explanation. We infer, therefore, that AP-3Myc-Norrin and Xwnt8-Myc-AP bind to largely overlapping sites on the CRD. The surface regions defined here for Xwnt8-Myc-AP binding are in good agreement with the regions defined by earlier mutagenesis studies using the Fz8 and Drosophila Fz2 CRDs (10, 11).

As a technical point, we note that making chimeras with a single junction between the Fz4 and Fz8 CRDs (Fig. 3A), a commonly used approach that does not rely on structural information to define surface residues for mutagenesis, was minimally informative, with only 2 of 9 chimeras retaining Xwnt8-Myc-AP binding (Table 1). Presumably, packing and/or other interactions between amino acid side chains is sufficiently altered in the remaining seven chimeras that Xwnt8-Myc-AP binding is disrupted.

Although none of the Fz8 block substitution mutants shown in Table 1 exhibited an all-or-none difference in Xwnt8-Myc-AP versus AP-3Myc-Norrin binding, block substitution G showed a clear reduction in AP-3Myc-Norrin binding with little or no effect on Xwnt8-Myc-AP binding. Inferring that the region encompassed by block G might contain a subset of the residues involved in the Fz8 versus Fz4 difference in AP-3Myc-Norrin affinity, we constructed a complete set of double Fz8 block substitutions using all pairwise combinations with block G (i.e. blocks A and G, blocks B and G, blocks C and G, etc.) and tested each for binding to anti-Myc, Xwnt8-AP, and AP-3Myc-Norrin. As seen in Table 2 and Fig. 4, A and B, substitution of blocks B and G or of blocks G and H reduced AP-3Myc-Norrin binding to background levels in both the cell-based and the CRD- IgG binding assays. By contrast, although Xwnt8-Myc-AP binding was reduced, it was still readily detect-
FIGURE 3. Fz4 CRD block substitutions and chimeras. A, alignment of Fz4 and Fz8 CRD sequences with the Fz4 sequence color-coded with aspartate and glutamate residues in green and the 10 cysteines in yellow. The disulfide bond arrangement (11) is shown below the alignment. The 12-alanine scan and 12 Fz8 block substitutions are color-coded and indicated below the CRD sequence alignments. The locations of cross-over points for nine Fz4:Fz8 chimeric CRDs are shown below the block substitutions, with the four amino acids straddling the single cross-over point of each chimera indicated above the divergent arrows. None of the constructs altered any cysteine residues or introduced any insertions or deletions. B, the backbone of the atomic resolution Fz8 CRD structure (11) in two views that differ by a 180° rotation about a vertical axis. The amino terminus (N) of the CRD is seen at the top; the COOH terminus is seen toward the right in the front view. Disulfides are shown in yellow, α helices in blue, and β-sheet in red. Each of the surface images of the CRD shown in Figs. 3–5 represents this pair of views. The CRD faces on the left and right of the panel are arbitrarily designated as the “front” and “back” of the CRD, respectively. To avoid uncertainties associated with modeling the Fz4 side chains onto the Fz8 CRD structure, the surface images are of the Fz8 CRD with the numerically correct residues colored. Fz4 and Fz8 CRDs align without insertion or deletion, but differences between Fz4 and Fz8 CRDs with respect to the exact location and surface exposure of side chains are unknown. C, the locations of Fz8 block substitutions in Fz4; substitutions are color-coded and labeled as in A. D, the locations of alanine scanning substitutions on the CRD surface; substitutions are color-coded and labeled as in A. E, the effect of alanine scanning block substitutions in the Fz4 CRD on the binding of AP-3Myc-Norrin. Qualitative assessment of binding to Fz4-Myc-GPI fusion proteins displayed on living COS cells was performed as in Fig. 1B (Table 1); red, little or no binding; yellow, intermediate binding; green, binding comparable with WT.
able in both assays. In the CRD-IgG assay, Xwnt8-Myc-AP binding to block substitutions B and G and to G and H was reduced to ~25% and ~10%, respectively, of the level seen with WT Fz4 CRD-IgG (Fig. 4B). These blocks partially overlap the regions implicated in both Xwnt8-Myc-AP and AP-3Myc-Norrin binding based on the alanine scanning series (Figs. 3, D and E, and 4C), and they either reside adjacent to or encompass, respectively, M105V and M157V, the two Fz4 CRD substitutions identified by Xu et al. (7) in families with familial exudative vitreoretinopathy.

In a separate analysis of the sequence requirements for Xwnt8-Myc-AP and AP-3Myc-Norrin binding to the Fz4 CRD, we examined the properties of a set of single amino acid substi-

![Figure 4](image_url)

**TABLE 1**

| Target                | AP-3Myc-Norrin | Xwnt8-AP | Anti-Myc |
|-----------------------|----------------|----------|----------|
| Fz4 WT                | +++            | +++      | +++      |
| Fz4 alanine scanning A| +++            | +++      | +++      |
| Fz4 alanine scanning B| –              | –        | +++      |
| Fz4 alanine scanning C| +/–            | +/–      | +++      |
| Fz4 alanine scanning D| +++            | +++      | +++      |
| Fz4 alanine scanning E| +++            | +++      | +++      |
| Fz4 alanine scanning F| +/–            | +/–      | +++      |
| Fz4 alanine scanning G| +/–            | +/–      | +++      |
| Fz4 alanine scanning H| +/–            | +/–      | +++      |
| Fz4 alanine scanning I| +/–            | +/–      | +++      |
| Fz4 alanine scanning J| +/–            | +/–      | +++      |
| Fz4 alanine scanning K| +/–            | +/–      | +++      |
| Fz4 alanine scanning L| –              | –        | +++      |
| Fz8 block A in Fz4    | +++            | +++      | +++      |
| Fz8 block B in Fz4    | +++/-          | +++      | +++      |
| Fz8 block C in Fz4    | +++            | +++      | +++      |
| Fz8 block D in Fz4    | +++            | +++      | +++      |
| Fz8 block E in Fz4    | +++/-          | +++      | +++      |
| Fz8 block F in Fz4    | +++            | +++      | +++      |
| Fz8 block G in Fz4    | +++/-          | +++      | +++      |
| Fz8 block H in Fz4    | +/–            | +/–      | +++      |
| Fz8 block I in Fz4    | +/–            | +/–      | +++      |
| Fz8 block J in Fz4    | +/–            | +/–      | +++      |
| Fz8 block K in Fz4    | +/–            | +/–      | +++      |
| Fz8 block L in Fz4    | +/–            | +/–      | +++      |
| Fz8/Fz8 chimera MP/NQ | +++/-          | +++      | +++      |
| Fz8/Fz8 chimera AE/LE | –              | –        | +++      |
| Fz8/Fz8 chimera CS/PD | –              | –        | +++      |
| Fz8/Fz8 chimera CT/ED | –              | –        | +++      |
| Fz8/Fz8 chimera RR/CA | –              | –        | +++      |
| Fz8/Fz8 chimera AG/LQ | +/–            | +/–      | +++      |
| Fz8/Fz8 chimera CS/PD | –              | –        | +++      |
| Fz8/Fz8 chimera CL/ER | –              | –        | +++      |
| Fz8/Fz8 chimera AG/CE | –              | –        | +++      |

**TABLE 2**

| Target                | AP-3Myc-Norrin | Xwnt8-AP | Anti-Myc |
|-----------------------|----------------|----------|----------|
| Fz8 blocks A + G in Fz4| +             | +/+/++   | +++      |
| Fz8 blocks B + G in Fz4| +/–           | +/+/++   | +++      |
| Fz8 blocks C + G in Fz4| +             | +/+/++   | +++      |
| Fz8 blocks D + G in Fz4| +             | +/+/++   | +++      |
| Fz8 blocks E + G in Fz4| +             | +/+/++   | +++      |
| Fz8 blocks F + G in Fz4| +/+/++        | ++       | +++      |
| Fz8 blocks G + H in Fz4| –             | –/++     | +++      |
| Fz8 blocks G + 1 in Fz4| +             | ++       | +++      |
| Fz8 blocks G + J in Fz4| –             | –/++     | +++      |
| Fz8 blocks G + K in Fz4| +             | ++/-     | +++      |
| Fz8 blocks G + L in Fz4| +++/-         | ++       | +++      |

**FIGURE 4.** Substitution of Fz8 blocks B and G or blocks G and H into the Fz4 CRD diminishes Xwnt8-Myc-AP binding far less than AP-3Myc-Norrin binding. A, anti-Myc monoclonal antibody (left column; visualized with AP-conjugated anti-mouse secondary antibody), AP-3Myc-Norrin (center column), or Xwnt8-Myc-AP (right column) were incubated with live COS cells displaying the indicated Fz4 CRD-Myc-GPI fusion proteins, and the specifically bound AP activity was visualized with nitro blue tetrazolium/5-bromo-4-chloro-3-indolyl phosphate. Under these conditions, Xwnt8-Myc-AP and AP-3Myc-Norrin give roughly equal levels of bound AP with WT Fz4-CRD-Myc-GPI (Fig. 2). B, quantitation of AP-3Myc-Norrin and Xwnt8-Myc-AP binding to WT or the two block substitution mutants of Fz4-CRD-IgG immobilized in protein A-coated microwells as in Fig. 1C. C, locations of Fz8 block substitutions B, G, and H on the CRD surface; front and back views are shown, and the blocks are colored as in Fig. 3A.
family in the cystine knot fold (three disulfide bonds) and 2) the cysteine that mediates disulfide linkage of two monomers into the active dimer (Fig. 6A). By threading the Norrin sequence onto the x-ray structure of TGF-β, Meitinger et al. (17) found that the four additional Norrin cysteines clustered into two pairs in the tertiary structure, suggesting that they would form two additional intrachain disulfides (Fig. 6A). Since this initial report, additional x-ray structures of TGF-β family members have been determined. In Fig. 6A, the Norrin sequence is shown aligned to four of these (TGF-β2, TGF-β3, BMP2, and BMP7), and in Fig. 6, C and D, the BMP2 α-carbon backbone is used as a scaffold for modeling the three-dimensional structure of Norrin. Because of the limited primary sequence homology between Norrin and the TGF-β family members (including BMP2), especially in those regions predicted to form loops, we have not attempted to refine this model further.

Our mutational analysis of Norrin had three aims: 1) to explore the role of the predicted disulfide bonds in the structure and function of Norrin by substituting individual or pairwise combinations of cysteine(s) with alanine(s); 2) to define the contribution of positive charges (most of which are predicted to be on the surface) to Norrin binding and signaling by substituting glutamate in place of each arginine and lysine; and 3) to systematically map those regions of Norrin involved in binding and signaling by substituting blocks of alanine for all amino acids other than cysteine and glycine between the second residue and the third to last residue in the mature Norrin polypeptide. For each of these Norrin mutants, we measured the binding of the corresponding AP-3Myc-Norrin fusion protein to immobilized Fz4 CRD-IgG relative to that of WT AP-3Myc-Norrin. Each Norrin mutant was also assayed for canonical Wnt signaling activity in a reporter cell line in the presence of co-expressed Fz4 and Lrp6. In Fig. 6B, the binding affinities and signaling activities are displayed as a fraction of the value for the WT Norrin control. Below, each of the three sets of mutants will be described in turn.

The collection of 11 single cysteine-to-alanine substitution mutants reveals a striking relationship between the severity of the binding and/or signaling defect and the evolutionary conservation of the different cysteines with the TGF-β family; those cysteines that form the three evolutionarily conserved intrachain disulfides (Cys$^{39}$, Cys$^{65}$, Cys$^{69}$, Cys$^{96}$, Cys$^{126}$, and Cys$^{128}$) are the only ones for which alanine substitution severely compromises Fz4 CRD-IgG binding and signaling. By contrast, CRD binding and/or signaling are generally either not decreased or are decreased to only a modest extent by mutation of the Norrin-specific cysteines (Cys$^{55}$, Cys$^{93}$, Cys$^{110}$, and Cys$^{131}$) or to Cys$^{96}$, the cysteine involved in dimerization. In particular, mutation of either Cys$^{93}$ or Cys$^{131}$, which are predicted to form a Norrin-specific disulfide bond, appears to have little deleterious effect. A partial exception to this pattern is seen with C55A, which exhibits substantially reduced binding but retains ~40% of WT Norrin signaling activity.

If the hypothesized Norrin-specific disulfide bonds between Cys$^{55}$ and Cys$^{110}$ and between Cys$^{93}$ and Cys$^{131}$ exist, then one might predict that, within a given disulfide-bonded pair, mutation of both cysteines would cause less of a disruption to protein structure and function than would mutation of a single cys-
Norrin and Frizzled4

A

Norrin

TDSFIDSDPQKCIYVDISRSPLLKCSSGIVLLRREEGHQSAQSEPPLVS3VTKQPFQSSHCPSQGPQSKLRLSGGMLRTTATRYYLSCHKEENS

TGFβ2

ALDAAYCFNNVQQNCGLCPFDKLQIKWPFKGNANFCAGACPYY---SDTQH3RHLSTLYNTIPNPE-ASASCPICGVSQDLIEPLTILYI-GKTPKEIIQLSNMVKSCKCS

TGFβ3

ALDNYCFRNLEENCVRPLYDFDPDLGKNWKHPGKYAYACGCQPLR---SATDTHS3VTLGLYNTLNP--ASASCPICGVSQDLIEPLTILYI-GKTPKEIIQLSNMVKSCKCS

BMP2

ANVAENSSQDGQFKCHLWYFSDRLQDDQQIIIPAEGYAYYECGEACPFLSMYNTHAIVQVIIIQH4IFIP-VTPKPCIAP2TILNISILYFDGSSWILKHYRMVRAGCH

BMP2

QAKHQQKQRLKSSCKRRPLYDFFSVGNNIWVAPPGYHAFYGEQCPFPLHDILNTHAIVQVTILVNSKIPKACVFTPELSILMYLDEKEVVLKNYQDMV66GGR

Conserved cysteines that form intrachain and interchain disulfides in the TGF-beta superfamily

B

AP-3myc-Norrin binding to Fz4-CRD-CDG (as a fraction of WT AP-3myc-Norrin binding)

Luciferase activity (as a fraction of WT Norrin activity)

C

Norrin threaded onto a BMP2 monomer with cysteine, lysine, and arginine indicated

D

Norrin threaded onto BMP2 dimer with binding and nonbinding alanine scan mutants indicated on one subunit
teine, because in the latter case, the free thiol could potentially participate in aberrant disulfide bond formation. To test this idea, we generated double mutants C55A/C110A, C93A/C131A, and C110A/C131A. Double mutant C55A/C110A appears to bear out the prediction by exhibiting greater binding and signaling activity than either C55A or C110A alone; double mutant C93A/C131A has roughly the same activity as C93A or C131A alone (which, as noted above, are within a factor of 2 of WT Norrin activity); and the double mutant C110A/C131A, predicted to affect cysteines involved in two different disulfides, is at least as defective as either C110A or C131A alone. Surprisingly, mutation of Cys95 (the cysteine predicted to mediate dimerization) produces less than a 2-fold decrement in signaling and a ~2-fold increase in CRD binding, and simultaneous mutation of Cys95 and Cys131 produces a ~5-fold increase in CRD binding. At present, these two observations are unexplained.

The mutational analysis of the lysine and arginine residues of Norrin reveals two residues that appear to be important for Fz4 CRD-IgG binding: Arg41 and Arg64 (Fig. 6, A–C). Curiously, the ~10-fold reduction in CRD binding exhibited by the R64E mutant is accompanied by canonical signaling activity that is slightly higher than the WT control, indicating that this mutant interacts with Fz4 and/or Lrp6 to activate signaling in a manner that compensates for a lower CRD binding affinity. A second interesting feature of the lysine and arginine mutants is the large number that show enhanced CRD binding unaccompanied by enhanced signaling. Taken together, these mutants suggest that binding between Norrin and the Fz4 CRD involves an ionic interaction involving multiple arginines and lysines and that this interaction is favored by the presence of one or more anionic groups (in this case, the glutamate introduced in place of lysine or arginine). As described below, these mutants may be partially mimicking the effect of heparin binding.

The 31 Norrin alanine scanning mutants show a generally good correlation between binding and signaling activities, with alanine scanning mutants 5, 6, 8, 11, 17, 25, and 29 showing a 10-fold or greater decrement in binding and a greater than 3-fold decrement in canonical signaling activity (Fig. 6, A, B, and D). As noted above for the R64E mutant, several alanine scanning mutants (mutants 7 and 30 and, to a lesser extent, mutant 12) show a substantially greater decrement in CRD binding compared with signaling. Modeling the locations of the subset of alanine scanning mutants with a 4-fold or greater decrement in Fz4 CRD binding (shown in red in Fig. 6D) shows that most of them cluster along a broad surface on the outer face of the presumptive dimer. This region may directly contact the Fz4 CRD.

**Heparin Is a Cofactor for Norrin-Fz4 CRD Binding**—The high PI of Norrin and the substantial effects of arginine and lysine mutations on Norrin CRD binding and signaling activities suggest that polyanions, such as heparin or heparan sulfate, may play a role in Norrin-CRD interactions. To explore this idea, we asked whether AP-3Myc-Norrin could bind to heparin under physiologic conditions. We observed that AP-3Myc-Norrin (in conditioned medium with 10% calf serum) quantitatively binds to heparin-Sepharose in 0.15 M NaCl and quantitatively elutes in 1 M NaCl. Under the same conditions, AP alone does not bind to heparin-Sepharose. The AP-3Myc-Norrin that was eluted from heparin Sepharose (and was presumably purified away from soluble serum-derived heparin) was tested for Fz4 CRD-IgG binding (Fig. 7). Without any further additions, the AP-3Myc-Norrin binding activity was roughly 10% of the level observed for the same amount of AP-3Myc-Norrin in conditioned medium containing 10% serum. Interestingly, the binding activity of this heparin-purified AP-3Myc-Norrin could be fully restored upon the addition of 100 µg/ml heparin, with a half-maximal effect at ~20 µg/ml heparin. These data implicate heparin or related polyanions as important co-factors in Norrin-Fz4 binding.

**DISCUSSION**

The present work represents an initial step in defining the sequence determinants of Norrin-Fz4 CRD binding specificity. The principal conclusions are as follows: 1) Norrin binds to the Fz4 CRD and does not detectably bind to the 14 other mammalian Fz and sFRP CRDs; 2) Norrin and Xwnt8 recognize largely overlapping regions of the Fz4 CRD; 3) surface determinants on the Fz4 and Fz8 CRDs that allow Norrin to distinguish between them reside within several small regions on one face of the CRD; 4) Norrin function depends critically on the three pairs of cysteines that form the highly conserved trio of disulfide bonds shared among all cystine knot proteins, but the other two putative disulfide bonds are less important; 5) Norrin-CRD binding depends on a largely contiguous group of amino acids in the extended β-sheet domain of Norrin that are predicted to face away from the dimer interface; 6) Norrin-CRD binding is strongly modulated by interactions involving charged amino acid side chains; and 7) Norrin-CRD binding is enhanced ~10-fold by the addition of heparin. Below, we discuss these obser-
The presence of multiple Frizzleds and Wnts in all metazoans thus far examined, together with clear evidence for Wnt-Fz specificity (as seen in the 10-fold affinity difference of *Drosophila* Wingless for *Drosophila* Fz versus Fz2 (32) and the ability of Xwnt8 to distinguish between mammalian Fz and sFRP CRDs (10) (this study)), indicates that differential ligand-receptor affinities are an ancient feature of this signaling system. In this context, the extreme specificity of Norrin is unusual, and it raises the question of whether Frizzled receptors other than Fz4 might also have “private” ligands. At present, there is no data on either the secondary or tertiary structure of Wnt proteins, and therefore no way to distinguish between a Noggin dimer and a BMP7 dimer, both cystine knot proteins, including Norrin (38), appear to function as either homo- or heterodimers, with most but not all dimers held together by a disulfide bond.

Within the context of the shared topological arrangement of three disulfide bonds that represent the defining feature of cysteine knot proteins, there is wide variation in the spatial arrangement of monomers within the dimer and in the surfaces that interact with receptors or other binding proteins. For example, complexes between BMP2 and the BMP type IA receptor, between TGF-β3 and the TGF-β type II receptor, and between activin A and the activin type IIB receptor reveal substantially different arrangements of ligand subunits and points of interaction with the receptors (30, 39–41). Additional variations in protein-protein contacts are seen in the complex between a Noggin dimer and a BMP7 dimer, both cystine knot proteins (42). The present work implicates one face of Norrin in CRD binding, but a definitive analysis will require crystallization of the complex.

With respect to the disulfide-bonded structure of Norrin, the properties of single and double cysteine mutants described here are similar to the properties of an analogous set of mutants in the α subunit of the gonadotropins (43, 44). Despite the near absence of sequence homology between Norrin and gonadotropin α (aside from the six cysteines that form the cysteine knot), in both proteins, 1) mutation of the cystine knot cysteines leads to more severe defects than mutation of cysteines involved in either of two less conserved disulfide bonds, 2) mutation of both members of a disulfide-bonded cysteine pair can produce a more modest defect than mutation of only a single member, and 3) mutation of some amino acids (e.g. Cys38 and Cys47 in the α subunit of gonadotropin and Cys55, Arg58, and several of the alanine scanning mutants in Norrin) paradoxically lead to a large reduction in receptor binding with little or no decrement in signaling.

Heparin and heparan sulfate binding are seen in a wide variety of extracellular ligands, including approximately one-fourth of the members of the 30 TGF-β family, many members of the fibroblast growth factor family, *Drosophila* Wingless, and the sequence to the CRD, can functionally substitute for a Frizzled CRD and support Wnt-dependent signaling (34). If this alternate model applies to Norrin signaling, then it should also be possible to activate canonical signaling by artificially tethering Norrin to the receptor complex independently of the CRD.

Comparison of Norrin with Other Cystine Knot and Heparin Binding Proteins—The cystine knot structure is found in a wide variety of extracellular proteins, including many involved in signaling. These include the COOH-terminal globular domains of mucins, the large and diverse TGF-β family, nerve growth factor, platelet-derived growth factor, vascular endothelial growth factor, Noggin, prepro-von Willebrand factor, and the common α-subunit of the gonadotropin hormones (chorionic gonadotropin, leutinizing hormone, follicle-stimulating hormone, and thyroid-stimulating hormone). Several of these cysteine knot proteins have additional cysteines within the knot domain that have been demonstrated crystallographically and/or chemically (e.g. the α subunit of the gonadotropins) (35, 36) or are inferred by mutagenesis (the mucins) (37) to form disulfide bonds. All of the cysteine knot proteins, including Norrin (38), appear to function as either homo- or heterodimers, with most but not all dimers held together by a disulfide bond.
cystine knot BMP2 antagonist Noggin (45–48). For Wingless and the Drosophila TGF-β family member Dpp (Decapentaplegic), heparan sulfate proteoglycans have been shown genetically to control the diffusion of the ligands in vivo (49–53). For basic fibroblast growth factor, heparin plays a critical role in receptor binding by functioning as a co-ligand (47, 54–57). A role for heparin as a co-ligand has recently been demonstrated biochemically and crystallographically in a second signaling system; high affinity binding of Hedgehog to its transmembrane binding partner Ihog requires the formation of a ternary complex with heparin (58). The observation that heparin strongly enhances Norrin-CRD binding indicates that Norrin-extracellular matrix interactions not only control the spatial localization of Norrin but also play an intimate part in the Norrin-Fz4 signaling complex.

Acknowledgment—We thank Amir Rattner for helpful comments on the manuscript.

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