Localization of Disulfide Bonds in the Frizzled Module of Ror1 Receptor Tyrosine Kinase*

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The frizzled (FRZ) module is a novel module type that was first identified in G-protein-coupled receptors of the frizzled and smoothened families and has since been shown to be present in several secreted frizzled-related proteins, in some modular proteases, in collagen XVIII, and in various receptor tyrosine kinases of the Ror family. The FRZ modules constitute the extracellular ligand-binding region of frizzled receptors and are known to mediate signals of WNT family members through receptors. With an eye toward defining the structure of this important module family, we have expressed the FRZ domain of rat Ror1 receptor tyrosine kinase in Pichia pastoris. By proteolytic digestion and amino acid sequencing the disulfide bonds were found to connect the 10 conserved cysteines in a 1–5, 2–4, 3–8, 6–10, and 7–9 pattern. Circular dichroism and differential scanning calorimetry studies on the recombinant protein indicate that the disulfide-bonded FRZ module corresponds to a single, compact, and remarkably stable folding domain possessing both α-helices and β-strands.

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‡The abbreviations used are: FRZ, frizzled; MuSK, muscle-specific tyrosine kinase; SDS-PAGE, SDS-polyacrylamide gel electrophoresis; HPLC, high pressure liquid chromatography.

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The extracellular domains of the different members of the Ror family fulfill diverse biological functions. The muscle-specific tyrosine kinase (MuSK) has been shown to be indispensable for the formation of the neuromuscular junction because it is part of a receptor complex that mediates the action of agrin (8–10). The Drosophila proteins Dror and Dnk have been implicated in the development of the nervous system because during embryonic development expression of these proteins is restricted to neuronal tissues (11, 12). A more general role is suggested for the C. elegans Ror homologue, cam-1, which has been shown to guide migrating cells and orient the polarity of asymmetric cell divisions and axon outgrowth (13).

Recent studies suggest that vertebrate Ror1s and Ror2s may have distinct biological roles. Oishi et al. (14) have shown that during embryogenesis, expression of Ror1 is sustained in the nervous system and is also detected in non-neuronal tissues after birth. In contrast, the expression of Ror2 declines after birth. Takeuchi et al. (15) have demonstrated that mouse Ror2 receptor tyrosine kinase is primarily involved in heart development and limb formation: mice with a homozygous mutation in mouse Ror2 died just after birth, exhibiting dwarfism, severe cleft palate, and short limbs and tails. Consistent with these results, DeChiara et al. (16) have shown that Ror2 is required for cartilage and growth plate development. Disruption of mouse Ror2 leads to profound skeletal abnormalities, with essentially all endochondrally derived bones foreshortened. The important role of Ror2 in skeletal patterning is also supported by the results of Oldridge et al. (17). These authors have demonstrated that brachydyctaly type B in humans is caused by dominant mutations in the Ror2 gene. Recessive mutations of the gene encoding the human ROR2 tyrosine kinase were found to cause Robinow syndrome, a short-limbed dwarfism with cardiac malformations (18, 19).

The extracellular domains of the different members of the Ror family of receptor tyrosine kinases have quite different domain organizations. The extracellular regions of Ror1 and Ror2 receptor tyrosine kinases of vertebrates (20) and the C. elegans homologue, cam-1 (13), consist of an immunoglobulin-like domain at the amino terminus and a FRZ domain and kringle domain just amino-terminal to the transmembrane segment (Fig. 1). In the case of two Drosophila homologues, Dror and Dnk, both the FRZ and kringle domains are present, but they are devoid of immunoglobulin-like domains (11, 12). In the case of MuSK, the receptor tyrosine kinase involved in neuromuscular junction formation (21), the extracellular region contains three immunoglobulin-like domains and a FRZ domain, but no kringle domain (Fig. 1). It is thus noteworthy that the only domain type common to all members of the Ror family of receptor tyrosine kinases is the FRZ module, suggesting a critical functional role for this domain type.

There is experimental evidence consistent with the importance of the FRZ modules of various Ror-type receptor tyrosine kinases. Recently Zhou et al. (22) have carried out a systematic analysis of the contribution of distinct domains of MuSK to its ability to induce and associate with postsynaptic specializations. Their results indicate that deletion of the FRZ...
domain (consisting of a C6 box plus the Ig-IV region in the authors’ terminology) specifically eliminates MusK/Rapsyn;

The functional importance of the FRZ module may also explain the results of Forrester et al. (13). These authors have shown that the C. elegans Ror receptor tyrosine kinase cam-1 regulates cell motility and asymmetric cell division in the nematode; nonsense mutations lying within the FRZ domain of this receptor tyrosine kinase eliminate cam-1 function. The mutant phenotype is not due to loss of the activity of the downstream tyrosine kinase domain because mutations that lead only to loss of tyrosine kinase activity have only subtle effects on cell migrations (13). The observation of Afzal et al. (18) that nonsense mutations in the frizzled domain of Ror2 receptor tyrosine kinase can lead to Robinow syndrome indicates that this domain is essential for Ror2 function.

No ligands have been identified thus far for the Ror1 and Ror2 receptor tyrosine kinases. However, based on the presence of a FRZ module in the putative ligand-binding region, it is possible that WNT proteins might act as ligands for the FRZ modules of Rors (5, 6).

Despite the obvious biological importance of the FRZ module, nothing is known about its three-dimensional structure. In fact, there is still some controversy over whether the FRZ module corresponds to a single domain or is composed of two domains (5). This controversy stems primarily from the fact that in the middle of the FRZ module of Drosophila, there is a 55-amino acid insert between the fifth and sixth cysteines, separating the 10 conserved cysteines into two groups. Accordingly, the FRZ domain is sometimes subdivided into two regions, for example, the C6 box and the Ig-IV region in the case of MuSK (22).

To define the structure and function of this important module type, in the present work we have expressed the FRZ module of rat Ror1 in Pichia pastoris. Our structural studies on the recombinant protein indicate that the FRZ module corresponds to a single, compact, disulfide-bonded structural domain.

**EXPERIMENTAL PROCEDURES**

**Cloning the FRZ Domain of Rat Ror1 Receptor Tyrosine Kinase**—The DNA segment coding for the frizzled domain of rat Ror1 receptor tyrosine kinase (residues Gly168-Ala304) was amplified with the 5'-CGGCCGCGCAGATATGGGNTTYTGYCARCCNTA-3' (sense) and 5'-CGGATCCCTTTGCGCGCGGACATATGGGNTTYTGYCARCCNTA-3' (antisense) primers from a rat fetal brain cDNA library (CLONTECH). The amplified DNA sequence was digested with SmaI and HindIII restriction endonucleases and ligated into M13mp19 RI digested with the same enzymes. The sequence of the cloned DNA was determined by dideoxy sequencing on a LI-COR DNA sequencer.

**Expression of the FRZ Module in P. pastoris**—The FRZ domain of rat Ror1 receptor tyrosine kinase was expressed in the methylotrophic yeast *P. pastoris* by utilizing the Easy Select *P. pastoris* expression kit (Invirotekn, Carlsbad, CA). The DNA fragment encoding the FRZ domain was excised from M13mp19/CR16 with a restriction enzyme such as XbaI digestion and blunt-end-ligated into pPICZaA cut with EcoRI-XbaI. The sticky ends of the digested DNA fragment and the vector were filled with Klenow polymerase. The pPICZaA expression plasmid contains the zeocin resistance gene for easy selection of positive transformants, the inducible P<sub>AOX1</sub> promoter of the alcohol oxidase 1 gene of *P. pastoris*, and the yeast a-mating factor secretion signal sequence. At the 3' end, the inserted DNA fragment was in-frame-ligated to the sequence encoding the c-myc epitope and a hexahistidine His tag. The plasmid contains the 3' termination signal of the AOX1 gene. *Escherichia coli* JM 109 cells were transformed with the ligated mixture and plated on low salt LB medium (1% tryptone, 0.5% yeast extract, and 0.5% NaCl) containing 25 μg/ml zeocin. Zeocin-resistant colonies were screened for the presence of DNA encoding the FRZ domain by polymerase chain reaction using AOX1- and FRZ-specific primers and by restriction analysis. The plasmid
buffer (200 m M Tris and 50 m M imidazole, pH 7.9) was added. The
equilibrated with 0.1M ammonium bicarbonate, pH 8.0, and
was further purified by gel filtration on a Sephadex G-75 column,
deralysed, desalted by gel filtration on Sephadex G-25, equilibrated with
dialyzed against a 10-fold volume of distilled water. The pH of the
3























doglycosidase H (New England Biolabs, Beverly, MA). The recombinant
protein (1 mg/ml) was dissolved in 50 mM sodium citrate, pH 5.5, and
incubated with 500 New England Biolabs units of endoglycosidase H for
16 h at 37 °C. The digested protein was isolated by nickel-chelate
column chromatography.

Circular Dichroism Spectroscopy—CD spectra were measured over
the range of 190–250 nm by using a JASCO J-720 spectropolarimeter
thermostated with a Neslab RT-100 water bath. The measurements
were carried out in 1-mm pathlength cells and protein solutions of
0.1–0.3 mg/ml in 10 mM Tris-HCl buffer, pH 8.0. All spectra were
measured at 25°C with a 16 s time constant and a scan rate of 10
nm/min. The spectral slit width was 1.0 nm. All measurements repre-
sent the computer average of three scans. Secondary structure of re-
combining proteins was estimated from their CD spectra with the J-720
program for Windows Secondary Structure Estimation Ver.1.10.00,
JASCO. 3.

Differential Scanning Calorimetry—Calorimetric measurements
were carried out on a VP-DSC MicroCalorimeter at a heating rate of
1 °C/min and a solution concentration of 0.2–0.5 mg/ml. Experiments
were conducted at pH 8.0 in 20 mM Tris-HCl buffer. Buffer base lines
were obtained under the same conditions and subtracted from sample
tracings. The VP-DSC MicroCalorimeter was calibrated according to
the instructions of the manufacturer. Differential scanning calorimetry
data analysis was performed with the Microcal Origin Version 5.0
program.

Secondary Structure Prediction—Secondary structure prediction of
the FRZ module of rat Ror1 receptor tyrosine kinase, based on multiple
alignments of FRZ modules, was carried out with previously described
procedures (24–26) using the Profile Network Prediction Heidelberg
(PHD) mail server. The PHD server predicts secondary structural ele-
ments by evaluating the relative probabilities that a given segment can
be assigned to helix, strand, or loop. The estimated accuracy of this
multiple alignment-based method for the correct prediction of second-
ary elements is about 72%.

Protein Analyses—The composition of protein samples was analyzed
by SDS-PAGE using 11–22% linear polyacrylamide gradient slab gels
under both reducing and nonreducing conditions (27).

The concentration of the recombinant FRZ module of rat Ror1 recep-
tor tyrosine kinase was determined using the extinction coefficient 6700
m M−1 cm−1. The extinction coefficients were determined according to
a method described previously (28).

Localization of Disulfide Bonds—170-μg samples of recombinant
FRZ domain (in 70 μl of 1 mM HCl, pH 3) were digested with 1 μg of
pepsin (Sigma) at room temperature for 2 h. 100 μl of 0.1 M ammonium
acetate, pH 5.2, was added to the reaction mixture and digested with 8
μg of trypsin (Sigma) and 8 μg of chymotrypsin (Sigma) at 37 °C for
16 h. Samples were analyzed by reverse-phase HPLC on an Aquapore
OD 300 (220 × 2.1-mm) C18 column (PE Applied Biosystems Ltd.) in
0.1% (by volume) trifluoroacetic acid with a linear gradient of ace-toni-
trile. N-terminal sequencing was performed on an Applied Biosystems
RESULTS AND DISCUSSION

Cloning and Expression of the FRZ Module of Rat Ror1 Receptor Tyrosine Kinase—On the basis of the known sequences of human and mouse Ror1 receptor tyrosine kinases (GenBank™ accession numbers M97675 and AB010383), we have designed polymerase chain reaction primers for the amplification of the cDNA segment encoding the FRZ domain of rat Ror1 receptor tyrosine kinase. The amplified DNA fragment was cloned into M13 sequencing vector, and the nucleotide sequence was determined by dideoxy sequencing. Comparison of the nucleotide sequence of the FRZ module of rat Ror1 receptor tyrosine kinase with those of the human and mouse orthologues has revealed extensive sequence similarity (Fig. 2). The rat cDNA differs in 42 nucleotide positions from the human sequence and in 18 positions from the mouse sequence. The differences of nucleotide sequences are mainly in silent positions, resulting in only two and one amino acid substitutions relative to the human and mouse sequences, respectively (Fig. 2).

The cDNA encoding the FRZ domain of rat Ror1 receptor tyrosine kinase was subcloned into the pPICZαA P. pastoris expression vector. The cDNA was in-frame-ligated to the Saccharomyces cerevisiae preproα-mating factor secretion signal sequence. This fused the FRZ domain after the KEX2 (Lys-Arg) and two STE13 (Glu-Ala-Glu-Ala) cleavage sites. Analysis of the culture media of the induced Pichia culture revealed that a protein of ~35–37 kDa is secreted efficiently, corresponding to an expression level of 20–25 mg/liter. The N-terminal amino acid sequence of the secreted protein was Glu-Ala-Glu-Leu-Gly-His-Met-Gly-Phe-Cys-Gln, showing that the secretion signal peptide was cut off at the first STE13 cleavage site, leaving the second Glu-Ala repeat at the N terminus of the secreted protein. Note that the segment of the recombinant protein originating from rat Ror1 receptor tyrosine kinase starts with Gly-Phe-Cys-Gln and terminates with Ile-Gly-Ile-Pro-Met (cf. Fig. 2).

The protein purified from the culture media by nickel-agarose chromatography appeared as a diffuse band on SDS-PAGE gels and had a molecular mass of ~35–37 kDa (Fig. 3), whereas the molecular mass calculated for the amino acid sequence of the recombinant protein was only 18.3 kDa. The FRZ module of rat Ror1 receptor tyrosine kinase possesses a potential N-glycosylation site (Asn-Arg-Thr) at Asn184, so we assumed that the higher molecular mass and the heterogeneity seen on SDS-PAGE gels may be due to N-glycosylation at this position. To test this explanation, we have subjected the recombinant protein to endoglycosidase treatment. Because P. pastoris synthesizes N-linked glycoproteins with high-mannose-type oligosaccharide side chains (29), we treated the secreted protein with endoglycosidase H, an enzyme that cleaves high-mannose-type glycans. Endoglycosidase H treatment has reduced the molecular mass of the recombinant protein to ~18 kDa (Fig. 3). The endoglycosidase H-treated protein gave a compact, sharp band on SDS-PAGE gels, indicating that the higher molecular mass and size heterogeneity of the recombinant protein were caused by the oligosaccharide side chain.

**FIG. 4.** Far-ultraviolet circular dichroism spectra of the recombinant FRZ module of rat Ror1 receptor tyrosine kinase. The solid line indicates the spectrum of the glycosylated FRZ module, the dashed line indicates the spectrum of the deglycosylated FRZ module. Spectra were obtained in 10 mM Tris-HCl, pH 8.0, at 25 °C using 0.1 mg/ml protein.

**FIG. 5.** Structure of the recombinant FRZ module of rat Ror1 receptor tyrosine kinase (rRor1). A, amino acid sequence and predicted secondary structure of the FRZ module of rRor1; the 10 conserved cysteines are numbered and highlighted in bold. PHD indicates the secondary structural elements (H, α-helix; E, β-strand) predicted for FRZ of rRor1 with the PHD program. B, disulfide bond pattern of the FRZ module as derived from analysis of peptides shown in Table I.
Structural Characterization of the Recombinant FRZ Module of Rat Ror1 Receptor Tyrosine Kinase—

Samples of glycosylated and deglycosylated rat Ror1 FRZ module were first analyzed by circular dichroism spectroscopy at far-ultraviolet wavelengths (190–250 nm). The two species gave very similar CD spectra, indicating that the N-glycan is not necessary for structural integrity and does not have a major effect on the secondary structural elements of the FRZ fold (Fig. 4). Analysis of the CD spectra of the recombinant FRZ module of rat Ror1 receptor tyrosine kinase (Fig. 4) predicts 40% \( \beta \)-sheet and 23% \( \alpha \)-helix.

The presence of both \( \beta \)-sheets and \( \alpha \)-helices is consistent with the results of our secondary structure predictions for the FRZ module of rat Ror1 receptor tyrosine kinase (Fig. 5) and also seems to be generally valid for the large family of FRZ modules (6).

To further clarify structural characteristics of FRZ modules, we have determined the disulfide bond pattern of the recombinant FRZ domain of Rat Ror1 receptor tyrosine kinase. The peptides derived from the recombinant protein by digestion with pepsin, trypsin, and chymotrypsin were subjected to N-terminal amino acid sequence analysis. This analysis identified three disulphide-linked peptides (Fig. 6, peaks 20, 24, and 25) and allowed unambiguous assignment of disulphide linkages 1–5, 2–4, and 3–8 (Table I). Digestion with pepsin/trypsin/chymotrypsin did not cleave the peptide backbone between the sixth and seventh cysteines and between the ninth and tenth cysteines (fragment in peak 45; cf. Fig. 6 and Table I), therefore the fragment of peak 45 containing these cysteines was digested with V8 protease (Pierce; 2% by weight of peptide) in 0.1 M ammonium acetate, pH 5.2, at room temperature for 16 h. The reaction mixture was loaded onto the HPLC column, and the resulting peptides were sequenced. Sequence analysis has revealed a 7–9 and 6–10 disulfide bond pattern (Table I).

To further clarify whether the FRZ module consists of two domains or corresponds to a single domain, we have subjected the recombinant FRZ module to differential scanning calorimetry. The glycosylated protein had a \( T_{m} \) value of 103 °C (data not shown), and the deglycosylated domain had a \( T_{m} \) value of 84 °C (Fig. 7). Our observation that the N-glycan does not have a major effect on the secondary structure of the protein (Fig. 4) but increases its thermal stability is not unprecedented. Systematic comparison of glycosylated and deglycosylated forms of several proteins (30) have revealed that deglycosylation decreases thermal stability without a substantial effect on their conformation as indicated by the CD spectra in the ultraviolet range.

Thermal unfolding of the deglycosylated FRZ module was

![Fig. 6. HPLC separation of peptides derived from the recombinant FRZ module of rat Ror1 receptor tyrosine kinase by digestion with pepsin, trypsin, and chymotrypsin. Samples were analyzed by reverse-phase HPLC on an Aquapore OD 300 (220 × 2.1-mm) C18 column (PE Applied Biosystems Ltd.) in 0.1% (by volume) trifluoroacetic acid with a linear gradient of acetonitrile. Peptides identified by numbers in bold were used to define the disulfide pairings (cf. Table I). The solid line indicates the absorbance of the eluate at 220 nm.](image)

![Fig. 7. Differential scanning calorimetry of the deglycosylated recombinant FRZ module of rat Ror1 receptor tyrosine kinase.](image)

**Table I**

| Peptide no. | Peptides linked by disulfide bond | Cysteines connected |
|-------------|----------------------------------|---------------------|
| 20          | 2–4                              | AIPSLCHY IAACAR     |
| 24          | 1–5                              | EAEGLGIMGFCQPYR PVCDET |
| 25          | 3–8                              | VLCHTEY IGTSHLSDKCSQF |
| 45          | 6, 7, 9, 10                      | SSVPKPRDLCRDECVELEN |
| 45A         | 6–10                             | MRLKLPNCEDLQFPESPEAANCIR |
| 45B         | 7–9                              | MRLKLPNCE AANCIR CE |

![Thermal unfolding of the deglycosylated FRZ module was...](image)
fully reversible, the enthalpy change of the unfolding was $\Delta H_{\text{cal}} = 50.5 \pm 0.3$ kcal/mol. The fact that the FRZ module of rat Ror1 receptor tyrosine kinase collapses with a single $T_m$ value suggests that it corresponds to a single, compact fold, a finding that is not consistent with the suggestion that the FRZ domain could be subdivided into two distinct domains (5, 22).

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