The NMR Solution Structure of the Relaxin (RXFP1) Receptor Lipoprotein Receptor Class A Module and Identification of Key Residues in the N-terminal Region of the Module That Mediate Receptor Activation*

Received for publication, October 10, 2006, and in revised form, November 28, 2006 · Published, JBC Papers in Press, December 4, 2006, DOI 10.1074/jbc.M609526200

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The receptors for the peptide hormones relaxin and insulin-like peptide 3 (INSL3) are the leucine-rich repeat-containing G-protein-coupled receptors LGR7 and LGR8 recently renamed as the relaxin family peptide (RXFP) receptors, RXFP1 and RXFP2, respectively. These receptors differ from other LGRs by the addition of an N-terminal low density lipoprotein receptor class A (LDLa) module and are the only human G-protein-coupled receptors to contain such a domain. Recently it was shown that the LDLa module of the RXFP1 and RXFP2 receptors is essential for ligand-stimulated cAMP signaling. The mechanism by which the LDLa module modulates receptor signaling is unknown; however, it represents a unique paradigm in understanding G-protein-coupled receptor signaling. Here we present the structure of the RXFP1 receptor LDLa module determined by solution NMR spectroscopy. The structure is similar to other LDLa modules but shows small differences in side chain orientations and inter-residue packing. Interchange of the module with the second ligand binding domain of the LDL receptor, LB2, results in a receptor that binds relaxin with full affinity but is unable to signal. Furthermore, we demonstrate via structural studies on mutated LDLa modules and functional studies on mutated full-length receptors that a hydrophobic surface within the N-terminal region of the module is essential for activation of RXFP1 receptor signal in response to relaxin stimulation. This study has highlighted the necessity to understand the structural effects of single amino acid mutations on the LDLa module to fully interpret the effects of these mutations on receptor activity.

Relaxin and INSL3 (insulin-like peptide 3) are small two-chain peptide hormones that belong to the relaxin-insulin superfamily. Relaxin was observed in the 1920s to induce the widening of the birth canal (1) and subsequently has been studied for the roles that it plays during pregnancy. More recently it has become apparent that relaxin is a pleiotropic hormone involved in numerous nonreproductive processes such as vasodilatation and neoangiogenesis, embryo implantation (2), collagen turnover (3, 4), and cancer progression (5). INSL3 was more recently discovered in the 1990s (6–8), and although understanding of this hormone is still ongoing, in rodents it is clearly involved in testis descent (9, 10) and involved in oocyte maturation and male germ cell survival (11).

The receptors for relaxin and INSL3 are LGR7 and LGR8, recently renamed the relaxin family peptide 1 (RXFP1) and the relaxin family peptide 2 (RXFP2) receptors, respectively (12–14). These receptors are leucine-rich repeat-containing G-protein-coupled receptors (LGR)3 and thus belong to the LGR family. The discovery of the RXFP1 and RXFP2 receptors resulted in the addition of a third class of LGRs (15), and at present there are three subclasses of LGRs, type A, B, and C. Type A LGRs include receptors for the glycoprotein hormones follicle-stimulating hormone, thyrotropin, and luteinizing hormone and consist of a rhodopsin-like seven transmembrane domain, and the hallmark large α/β ectodomain between 5 and 7 leucine-rich repeats (LRRs). The three type B LGRs are currently orphan receptors. Apart from containing between 11 and 17 LRRs, they are of similar structural architecture to the type A receptors. The RXFP1 (LGR7) and RXFP2 (LGR8) receptors differ from type A and type B LGRs by having 10 LRRs and the addition of an N-terminal low density lipoprotein receptor class A (LDLa) module. RXFP1 and RXFP2 receptor orthologs are found in all mammals and are the only GPCRs with LDLa modules. Homologous LGR type C receptors have been identified in the fly (16) and snail (17), although there are no relaxin peptides in these species. Intriguingly, the pond snail LGR contains 12...
LDLa modules (17); however, the function of this receptor is unknown.

The LDLa modules of the RXFP1 and RXFP2 receptors are homologous to the ligand binding domains found in the LDL receptor and members of the LDL receptor family, thus acquiring their namesake. The structures of several of these LDLa modules have been solved by NMR spectroscopy (18–24) and x-ray crystallography (25), revealing a number of conserved features. They are all small (~4 kDa) and have a topology dominated by the three conserved disulfide bonds and the organization of the C-terminal region around a calcium ion ligated by a motif of acidic residues, Asp-X-X-Asp-X-X-Asp-X-X-Asp-Glu. All LDLa modules structurally characterized to date, including the RXFP1 receptor module (26), have displayed a distinct interdependence on the presence of both the disulfide bonds and a calcium ion to maintain a globular structure.

The discovery of the RXFP1 and RXFP2 receptors has significantly propelled understanding of relaxin signaling. Simplistically, ligand binding stimulates cAMP production through the activity of the RXFP1 receptor and members of the RXFP2 receptor family, thus acquiring their namesake. The structures of several of these LDLa modules have been solved by NMR spectroscopy (18–24) and x-ray crystallography (25), revealing a number of conserved features. They are all small (~4 kDa) and have a topology dominated by the three conserved disulfide bonds and the organization of the C-terminal region around a calcium ion ligated by a motif of acidic residues, Asp-X-X-Asp-X-X-Asp-X-X-Asp-Glu. All LDLa modules structurally characterized to date, including the RXFP1 receptor module (26), have displayed a distinct interdependence on the presence of both the disulfide bonds and a calcium ion to maintain a globular structure.

The discovery of the RXFP1 and RXFP2 receptors has significantly propelled understanding of relaxin signaling. Simplistically, ligand binding stimulates cAMP production through the activity of the RXFP1 receptor module (26). RXFP1 and RXFP2 receptors are the only human GPCRs to contain an LDLa module, this system represents a paradigm in our understanding of GPCR activation.

In this study we aim to understand how the LDLa module mediates RXFP1 receptor activation. We have solved to high resolution the solution structure of the module by NMR spectroscopy, and we demonstrate that the activity of the module is not interchangeable with another LDLa module of high sequence identity. We describe the effect of several side chain mutations on the structure of the module and signaling function of the RXFP1 receptor. Results of this work highlight that signaling of the RXFP1 receptor is mediated by specific residues of the LDLa module.

**EXPERIMENTAL PROCEDURES**

**Cloning, Expression, and Purification of the RXFP1 LDLa Module—**The method for sample preparation is essentially as reported elsewhere (26). Briefly, the RXFP1 LDLa module was cloned from the pcDNA3.1/Zeo plasmid containing the human RXFP1 sequence without the signal peptide (a kind gift from Dr. Aaron Hsu). The DNA fragment encoding amino acid residues Gln-1 to Gly-41 of the mature receptor was amplified by PCR and ligated via the BamHI and XhoI restriction sites into the pGEX2 vector, a thrombin-cleavable GB1 fusion expression vector (the kind gift from Dr. A. Gronenborn, National Institutes of Health). The resulting plasmid pGEX-LDLa was transformed into the T7 bacterial expression strain BL21(DE3)/trxB (Novagen) for the expression of the GB1-LDLa fusion protein. Freshly transformed cells were used for all protein expression cultures. All cultures were grown and protein expression was induced at 37 °C. Proteins were 15N- or 13C,15N-labeled by growing cultures in a 2-liter Braun Biostat fermenter containing 1 liter of minimal media with 15NH4Cl and D-[13C]glucose as the sole nitrogen and carbon sources. Fermentation was conducted as described by Cai et al. (29). Protein expression was induced by the addition of 1 mM isopropyl 1-thio-β-d-galactopyranoside for 2.5 h after which cells were harvested, pelleted, and stored at ~20 °C.

Fusion protein was purified using IgG-Sepharose (GE Healthcare) via the manufacturer’s instructions. The eluted protein was buffer-exchanged into 50 mM Tris-HCl, 150 mM NaCl, pH 8.5, and concentrated to ~100 μg/μl using a 3-kDa cutoff Vivaspin centrifugal concentrator (Sartorius). Protein concentration was adjusted to 100–300 μg/ml in refolding buffer (3 mM GSH, 0.3 mM GSSG, 50 mM Tris-HCl, 150 mM NaCl, 2.5 mM CaCl2, pH 8.5). The sample was incubated overnight at 4 °C with stirring to allow the complete formation of disulfide bonds. Oxidation was monitored by reversed phase (RP)-HPLC using a C18 analytical column (Vydac) on an Akta Explorer system (GE Healthcare) and a 20 (Buffer A) to 70% (Buffer B) gradient, where Buffer A consisted of 0.1% trifluoroacetic acid, and Buffer B consisted of 80% acetonitrile and 0.1% trifluoroacetic acid. Postoxidation, the GB1 fusion protein was cleaved from the LDLa module overnight by incubating with 1 unit of thrombin protease (GE Healthcare) per mg of fusion protein. The cleaved GB1 was separated from the LDLa module by passing the sample over IgG-Sepharose. The unbound LDLa module was further purified by RP-HPLC as described above but using a Jupiter Proteo 4-μm 90-Å column. The eluted protein was lyophilized and stored at ~20 °C.

**Expression and Purification of GB1-LDLa Mutants—**Protein samples of GB1-LDLa mutants were expressed and purified as described above for wild type protein; however, expression of 15N-labeled protein was achieved using the method described in Marley et al. (30). Briefly, 500-ml cultures were grown on LB media to an A600 ~0.7 at 37 °C with shaking. The culture was pelleted and the supernatant decanted, and cells were transferred to 125 ml of minimal medium containing 15NH4Cl as the sole nitrogen source. The culture was equilibrated for 1 h at 16 °C with shaking, after which protein expression was induced overnight at 16 °C by the addition of 1 mM isopropyl 1-thio-β-d-galactopyranoside.

**Site-directed Mutagenesis—**Site-specific mutants of the LDLa domain and the full-length receptor were prepared using the QuickChange PCR mutagenesis kit (Stratagene) following the manufacturer’s instructions. Complementary forward and reverse primers were designed to introduce the minimum number of base pair changes necessary to alter the codon. Primer sequences were as follows: C7S_FWD, CAGGATGTCAAGAGC-TCCCTTGGCTATTTCCC; C7S_RVSE, GGCGAATAGCCA-

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FEBRUARY 9, 2007•VOLUME 282•NUMBER 6

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**JOURNAL OF BIOLOGICAL CHEMISTRY**

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**VOLUME 282 • NUMBER 6**

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AGGGAGCTTGTGACATCCGT; L9A_FWD, GTCAAGTGCTCAGCTGTGATGGCAAGC; L11A_FWD, CCACAGGGGAAAGCCTGAGGAGCTCTTGACATCG; L25A_FWD, GCTTGGCCTAGCTGCGAGCTGAGGCAAGC; L24A_FWD, TTGTGATGTTCCCACAGGGGAAAGCGCCCTGAGGCAAGC; L20A_FWD, TCCGCTGGCTATTTCCCCACAGGGGAAAGCGCCCTGAGGCAAGC; L18A_FWD, GACGGAGCTCTTGACATCCTG; L9A_FWD, GCTTGGCCTAGCTGCGAGCTGAGGCAAGC; L10A_FWD, TCCGCTGGCTATTTCCCCACAGGGGAAAGCGCCCTGAGGCAAGC; L25A_FWD, GCTTGGCCTAGCTGCGAGCTGAGGCAAGC; L24A_FWD, TTGTGATGTTCCCACAGGGGAAAGCGCCCTGAGGCAAGC; L20A_FWD, TCCGCTGGCTATTTCCCCACAGGGGAAAGCGCCCTGAGGCAAGC.

Mutations of the recombinant LDLa module used the pGEV-LDLa plasmid. For the preparation of full-length receptor mutants, the pcDNA3.1-RXFP1 plasmid was used. The sequences of all plasmids generated from the mutagenesis reaction 1.0.7 were verified by sequencing.

NMR Spectroscopy—For 1H, 15N, and 13C assignments, NMR data were collected on 1.0 mm 15N-labeled and 1.5 mm 13C,15N-labeled RXFP1 LDLa in 50 mm acetic acid, pH 5.5, in the presence of 10 mm CaCl₂ at 30 °C on a Varian Inova 500-MHz spectrometer equipped with a room temperature triple resonance probe. Assignments were made from CBCA(CO)NH, 13C-NOESY-HSQC (150 ms mixing time), HBBH(CO)NH, HC(CO)NH, HCHC-TOCSY, HNCA, HNCO, HNHA, HNHB, 13C-HSQC-NOESY (150 ms mixing time), and 15N-NOESY-HSQC (150 ms mixing time) three-dimensional experiments. All spectra were processed using NMRPipe (31) and typically Fourier-transformed after applying Lorentz-to-Gauss window functions in the direct dimension and cosine bells in the indirect dimensions. Data were analyzed using SPARKY (32) or NMRView (33).

LDLa mutant samples ~0.3–0.6 mM were prepared from lyophilized 15N-labeled protein in 50 mM imidazole, pH 6.0, and 10 mM CaCl₂. Two-dimensional 15N,1H-HSQC data were routinely collected at 25 °C on a 600-MHz Varian Inova spectrometer equipped with a triple resonance cold probe.

Structure Calculations—Distance constraints were obtained from three-dimensional 15N-edited NOESY-HSQC and 13C-edited HSQC-NOESY spectra. A total of 29 backbone φ and ψ angle constraints were generated from TALOS, and 19 χ₁ angles were derived from inspecting peak intensities of three-dimensional HACAHB and HNHB spectra. NOE data were assigned using the CANDID (34) module of CYANA (35) (version 1.0.7) with rounds of both manual and automatic assignment. The NOE constraints were further added to and refined with calculations using XPLOR-NIH (36) (version 2.9.9) using a simulated annealing protocol with molecular dynamics in both torsion and Cartesian space. The final force constants used were 50 kcal mol⁻¹ for experimental distance constraints, 200 kcal mol⁻¹ rad⁻² for dihedral angle constraints, and 1.0 kcal mol⁻¹ for the Ramachandran data base potential of mean force (37, 38). The quality of structures was validated using PROCHECK-NMR (39), and MOLMOL (40) was used to inspect structures, calculate ring current shifts, and produce figures.

Chemical Shift Mapping—Assignment of spectra of the mutant proteins was guided by the wild type LDLa module chemical shifts (26). Yields of folded Y9A and F10A were poor reducing the sensitivity of the spectra; therefore, spectra of the fusion proteins GB1-LDLa Y9A and GB1-LDLa F10A were compared with GB1-LDLa. Chemical shift differences were scaled according to Equation 1 (41),

\[
\Delta \delta_{\text{total}} = ((\Delta \delta_{\text{nm}})^2 + (\Delta \delta_{\text{n}} \times 0.154)^2)^{1/2}
\]

where \(\Delta \delta_{\text{nm}}\) and \(\Delta \delta_{\text{n}}\) are the chemical shift differences for the NH and 15N resonances of the mutant and wild type proteins. Errors were calculated as a ratio of peak line width to signal to noise and scaled according to Equation 1.

Cloning of LB-RXFP1 Chimera—The LB2-RXFP1 chimera was created by amplifying the DNA segment encoding the LB2, bp 198–312 of the LDL receptor, from the pGEX LB1-LB2 plasmid (kind gift of Ross Smith, the University of Queensland) with EcoRI sites at both the 5’ and 3’ ends. The primers designed were as follows: LB2EcoRI Fwd, CATCATGAATTCTGCACTTGCAGCTGCGAGCTGAGGCAAGC; and LB2A_FWD, GCTTGGCCTAGCTGCGAGCTGAGGCAAGC. The pcDNA3.1/Zeo plasmid containing the LG7 short construct (28), missing the LDLa module, retained an EcoRI site following the bovine prolactin signal sequence and FLAG epitope, prior to the beginning of the LG7 sequence. The LB2 insert and the pcDNA3.1/ZeR7 short plasmid were therefore simultaneously digested with EcoRI and ligated together to create the LB2-RXFP1 chimeric receptor. The correct orientation of insertion and sequence was verified by sequencing.

33P-Labeled H2 Relaxin Binding Assays—Human embryonic kidney (HEK) 293T cells grown in RPMI 1640 media (Sigma) supplemented with 10% fetal bovine serum, 100 μg/ml penicillin, 100 μg/ml streptomycin, and 2 mM l-glutamine were plated into 24-well poly-l-lysine-coated plates for whole cell binding assays. Upon reaching 80% confluency, cells were washed with 1 ml of PBS before incubation with 1 μg of pcDNA3.1/Zeo plasmid containing the receptor of interest in Lipofectamine 2000 (Invitrogen) and Opti-MEM serum-free media (Invitrogen). 5 h after transfection, 0.5 ml of RPMI 1640 media supplemented with 10% fetal bovine serum and 2 mM l-glutamine was added to each well and further incubated for 16 h. Competition binding assays were performed with 100 μM of 33P-labeled H2 relaxin in the presence or absence of increasing concentrations of unlabeled H2 relaxin. Data were collected as triplicate points in three independent experiments and fitted to one-site binding curves in Graphpad Prism 4.

Anti-FLAG Cell Surface Expression Assay—HEK293T cells transfected with pcDNA3.1/Zeo plasmids containing the FLAG-tagged receptor of interest were plated into 6-well poly-l-lysine coated plates (1,000,000 transfected cells per well). 24 h later, cells were washed with 1 ml of PBS before incubation with 1 ml of a 5% BSA/PBS solution containing 5 μg/ml anti-FLAG M2 antibody (Sigma) for 1 h at room temperature on a gently tilting platform. Cells were then lifted from wells via washing and resuspension to 1.5 ml in the above solution and centrifuged for 2 min at 14,000 rpm in a Heraeus Biofuge 13 (Sepatech). The antibody solution was aspirated, and the cell pellet was washed twice in ice-cold PBS prior to resuspension in 200 μl of ice-cold solution of 5% BSA/PBS containing 2 × 10⁶
cpm/ml of $^{125}$I-labeled anti-mouse IgG sheep antibody (Amer-
sham Biosciences). The cell suspension was incubated on ice for
2 h and then subjected to two cycles of centrifugation, aspira-
tion, and washing with 1 ml of 1% BSA/PBS. The final pellet was
counted in a Cobra Auto-Gamma liquid scintillation analyzer
(Packard Instrument Co.). All points were collected in triplicate in
three independent assays. The level of nonspecific binding
was determined using cells transfected with an empty vector.

**cAMP Enzyme-linked Immunosorbent Assay**—HEK293T
cells in 48-well plates were transiently transfected upon reach-
ing ~80% confluency with 0.5 μg of pcDNA3.1/Zeo plasmid
containing the receptor of interest in Lipofectamine 2000 (In-
vitrogen) and Opti-MEM serum-free media (Invitrogen).

The transfection media were aspirated after ~24 h and replaced
with RPMI 1640 media containing 0.25 mM 3-isobutyl-1-methyl-
exanthine and the appropriate concentration of H2 relaxin and
incubated for 30 min at 37 °C. The maximum response capacity
for cAMP production of the cells was determined by stimulat-
ing cells with 5 μM forskolin in the presence of 0.25 mM 3-isobu-
tyl-1-methylxanthine. Upon stimulation the media containing
treatments were aspirated. Cell lysis and measurement of intra-
cellular cAMP produced in response to treatments were per-
formed using a cAMP Biotrak enzyme immunoassay kit (GE
Healthcare) according to the manufacturer’s protocol. Each
treatment point was carried out in triplicate in three independ-
ent experiments. Data were analyzed using Graphpad Prism 4
and represented as the mean ± S.E. Significance was deter-
mimed using a one-way analysis of variance and Bonferroni
post-test comparison.

**Isothermal Calorimetry**—Protein samples purified by
RP-HPLC to remove residual calcium were reconstituted in 100
mM Tris-HCl, pH 7.4, 100 mM NaCl2 buffer treated with
Chelex. Experiments were conducted at 30 °C on a Microcal
MCS ITC unit (Microcal Inc.) based on methods described pre-
nviously (23, 42). Typically, 2–μl injections of 2–10 mM CaCl2
were made into 5 or 10 μM samples of wild type protein and
GB1-LDLα L7A and 50 μM samples of GB1-LDLα F10A or GB1-
LDLα Y9A with 180-s delays between injection to allow suffi-
cient recovery. The peaks generated by heat of binding were
integrated and data-fitted using the program MicroCal Ori-
gin version 2.9 assuming a single site model and fixing the
value of N to 1 to generate the best fit. The reported apparent
$K_d$ values are the means ± S.E. averaged from three inde-
pendent experiments.

**RESULTS**

**NMR Solution Structure of the RXFP1 LDLα Module**—The
NMR solution structure was calculated based on distance con-
straints from three-dimensional $^{15}$N- and $^{13}$C-edited NOESY
spectra. A total of 257 distance constraints and 48 dihedral
angles was used to calculate a good quality structure (Table 1).
The ensemble of 24 best structures presented in Fig. 1 indicate
that the structure is well defined between residues 5 and 38 with
a backbone r.m.s.d. value of 0.46 Å. The first two residues,
Gly(−1) and Ser(−2), which are cloning artifacts, show no NOE
data and are therefore unstructured. The next four residues are
fully assigned, but equivalent side chain groups typically show
degeneracy, for example Val-3 shows only one methyl peak in
both $^1$H and $^13$C dimensions. Little NOE data are observed for
these residues, consistent with structural disorder, and most
likely reflect the state of these residues in the intact receptor.
The remainder of the module shows a general topology consis-
tent with other LDLα modules in the Protein Data Bank. The
structure of the module can be divided into two regions as fol-
loows: an N-terminal region consisting of a short anti-parallel
β-sheet (strand 1, residues 9–11, and strand 2, residues 18–20)
immediately followed by a 3$_{10}$ helix (residues 21–23); and a
C-terminal region that is predominantly organized around a
calcium ion (Fig. 1). NOE data, particularly $\alpha_{H7} + \alpha_{C7}$
NOEs, suggest that residues 33–37 are organized into a 3$_{10}$
helix, consistent with the topology of the LB5 crystal structure
(25), but this element of secondary structure is not represented
by MOLMOL (Fig. 1). Based on sequence alignment of other
LDLα modules and analysis of chemical shift changes during
calcium titrations, the residues involved in calcium ligation are
Asn-26, Asp-30, Asp-36, and Glu-37 (26). The $\chi_1$ angles of Asn-
26, Asp-36, and Glu-37 are well defined consistent with limited
rotation of these side chains. As the Hβ protons of Asp-30 are
degenerate, we could not determine its $\chi_1$. The orientation of
these side chains in structures calculated in the absence of cal-
cium were consistent with their role in calcium ligation (Fig. 1),
and the addition of calcium in final structure calculations did
not produce any violations of the experimental data and only a
minor improvement in r.m.s.d. (from 0.51 to 0.46 Å for the
backbone atoms of residues 5–38).

The N- and C-terminal regions pack against each other
through modest hydrophobic contacts, consistent with the
general structural features of other LDLα modules (43). The
side chain of Phe-10, which is in the center of β-strand 1, packs
against the imidazole ring of His-24, which is located at the
C-terminal end of the first 3$_{10}$ helix and prior to Cys-25. This is
consistent with the x-ray structure of LB5 (25) (Fig. 1) and the

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**TABLE 1**

NMR and refinement statistics for the LDLα module of RXFP1

| Protein          | NMR distance and dihedral constraints |         |
|------------------|---------------------------------------|---------|
|                  | Distance constraints                    | 257     |
|                  | Total NOE                               | 20      |
|                  | Intra-residue                           | 104     |
|                  | Inter-residue                           | 64      |
|                  | Sequential ($i - j = 1$)               | 48      |
|                  | Medium range ($i - j < 4$)             | 21      |
|                  | Long range ($i - j > 5$)               |         |
|                  | Calcium-protein                         |         |
|                  | Total dihedral angle restraints         | 48      |
|                  | $\phi$                                  | 15      |
|                  | $\psi$                                 | 14      |
|                  | $\chi$                                 | 19      |

Structure statistics

- Violations (mean ± S.D.)
- Distance constraints (Å) 0.0239 ± 0.0040
- Dihedral angle constraints (°) 0.3414 ± 0.0934
- Maximum distance constraint violation (Å) 0.27
- Maximum dihedral angle violation (°) 2.7
- Deviations from idealized geometry
- Bond lengths (Å) 0.0061 ± 0.0000
- Bond angles (°) 0.9391 ± 0.0012
- Improper (°) 2.8859 ± 0.0023
- Lennard-Jones (kcal mol$^{-1}$) −88.43 ± 10.53
- Average pairwise r.m.s.d. (residue 5–38) (Å)
  - Heavy 0.72
  - Backbone 0.46

* Data for the ensemble of the 24 lowest energy structures are shown.
NMR structures of LB1 (19) and LB2 (18) in which a Phe at the equivalent position packs against an unconserved residue (for example, Val and Arg) also prior to a cysteine. Of interest, Phe-10 in RXFP1 LDLa adopts a $\chi_1$ of $+60^\circ$, whereas in other LDLa modules it is $+180^\circ$. Signal intensities in HNHB (strong and weak) and HACAHB (weak and weak) are consistent with this orientation. Furthermore, no peak is observed in $^{15}$N-$^1$H spin-echo difference $^{1}$$^1$H, $^{15}$N HSQC experiment (44) confirming that the $\chi_1$ of Phe-10 is not $180^\circ$. A hydrophobic patch near Phe-10 and His-24 that extends to the surface includes Leu-19 and Pro-20 ($\beta$-strand 2) and Leu-22 and Leu-23 (first $3_10$ helix). The $C^\alpha H_2$ of Leu-19 shows weak NOEs to the aromatic ring of Phe-10. The hydrophobic patch continues toward the N terminus, including Leu-7 and Tyr-9, and the latter is in $\beta$-strand 1, packs against Pro-20, and is oriented in the opposite direction to Phe-10.

The surface charge of the module is predominantly acidic within the C-terminal region. However, most of these residues (Asp-30, Asp-36, and Glu-37) are involved in calcium binding. The remaining two acidic residues Asp-29 and Asp-38 are surface-exposed but distant from each other. At the N-terminal end of the protein are three basic residues Lys-4, Lys-17, and Tyr-9. Lys-4 is positioned among the largely disordered region prior to Cys-5, and therefore the orientation of Lys-4 is difficult to determine; however, structures suggest that it could cluster with Lys-17 to form a basic surface. Tyr-9 is not a part of this basic patch and packs against Pro-20 as a part of a largely hydrophobic surface patch. Its side chain OH, however, is oriented into the solvent, and there is no evidence of it forming a hydrogen bond as suggested for the equivalent residue in other LDLa modules (43).

Effects of Mutation to Leu-7, Tyr-9, Phe-10, Leu-22, Leu-23, and Cys-5/18 to the RXFP1 LDLa Module Refolding and Structure—Structure and sequence alignments of the LDLa module of RXFP1 with other LDLa modules highlights several residues and regions that may significantly contribute to the structure or function of the module. Of particular interest is a hydrophobic patch in the N-terminal region of the module consisting of residues Leu-7, Tyr-9, Leu-19, Pro-20, Leu-22, and Leu-23 (Fig. 2). Potentially this hydrophobic surface may be involved in a protein interaction that drives activation of the receptor. Leu-7, Tyr-9, and Leu-22 are significantly solvent-exposed (>30%) and were therefore mutated to alanine to test the hypothesis that these residues are involved in the unknown interaction that leads to receptor activation. Leu-19 (10%), Pro-20 (15%), and Leu-23 (18%) are all buried; however, Leu-23 was also mutated to alanine to test whether it has a role in recognition or is essential for structure. Phe-10, which is highly conserved in LDLa modules, is located on the opposite side of the module to the hydrophobic surface. It is largely buried and links the N- and C-terminal regions and was therefore mutated to alanine to test its structural and functional importance. In addition, the two cysteine residues, Cys-5 and Cys-18, which form the first disulfide bond and tether the structure of the N-terminal region, were mutated to serine. A similar mutation in LB5 of the LDL receptor did not disrupt the oxidation of the
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The orientation of the side chains of residues Leu-7, Tyr-9, Leu-22, and Leu-23 suggest these residues cluster to create a hydrophobic surface on the molecule. N-Linked glycosylation of Asn-14 has been reported recently not to be essential for receptor activity (28), suggesting that this region is not involved in the primary activation of RXFP1 receptor signaling.

remaining two disulfide bonds, and ligation of the calcium ion was retained whereas the N-terminal region became disordered (25). If such a mutation could be made in RXFP1, the functional importance of both the N- and C-terminal regions could be tested.

All mutations were made to the pGEV-LDLa vector for the recombinant expression of the LDLa module as a fusion to GB1 harboring the desired mutation. The ability for these mutated proteins to refold and maintain structure under the same conditions as the wild type protein was assessed via RP-HPLC and NMR spectroscopy. RP-HPLC traces show that the RXFP1 protein elutes as several broad peaks representing chemical shifts, which in most cases are similar to those of the wild type protein (26) (Fig. 3). Each eluted HPLC peak was collected, and two-dimensional 1H,15N HSQC spectra were acquired on the fractions in the presence of calcium (26) refolds to a single native structure that elutes as a single peak. In contrast, if the LDLa module is oxidized in the presence of calcium, the protein elutes as several broad peaks representing the formation of several disulfide-bonded isomers of the protein. When correctly folded, the LDLa module shows well dispersed two-dimensional 15N,1H-HSQC spectra consistent with a globular structure (26).

The mutation of either Leu-7 or Leu-22 does not affect the folding of the protein to a single native-like species in the presence of calcium. Both proteins, GB1-LDLa L7A and GB1-LDLa L22A, when oxidized in the presence or absence of calcium produce RP-HPLC elution profiles similar to the wild type protein (Fig. 3). Both proteins give two-dimensional 15N,1H HSQC spectra with chemical shifts similar to those of the wild type LDLa module (Fig. 4). The differences in the chemical shift of the peptide 15N and NH of the LDLa mutants were compared with the wild type protein to identify structural perturbations caused by the mutation. Several mutants were studied as GB1 fusions, and in these cases chemical shifts were compared with wild type also as a GB1 fusion. As expected, the mutations cause a local perturbation that is most likely because of the change of the side chain group to an alanine. Other than this local effect, the chemical shift mapping of the LDLa L7A and LDLa L22A resonances compared with the wild type protein revealed that neither mutation inflicted large perturbations to the structure of the LDLa module distant from the site of mutation (Fig. 5). To determine whether calcium binding has been affected, we have also analyzed apparent calcium affinities by ITC. The apparent Kd values of calcium binding to wild type was estimated to be 20 ± 0.38 μM (Table 2) and is within the range of Kd values (0.5–40 μM) reported for other LDLa modules (23, 42, 45–50). The apparent Kd value for the mutant GB1-LDLa L7A is 13.5 ± 0.8 μM, which is also similar to wild type. As there is no evidence to suggest mutation of Leu-22 affects module structure or its activity (see below), the affinity of calcium binding of GB1-LDLa L22A was not determined. Collectively, characterization of LDLa L7A and LDLa L22A demonstrated that neither mutation disrupts refolding or stability of the module.

Mutation of Leu-23 to alanine disrupts the ability of the LDLa module to refold to a single isomer, and consequently GB1-LDLa L23A elutes as several broad peaks on RP-HPLC (Fig. 3). Each eluted HPLC peak was collected, and two-dimensional 15N,1H HSQC spectra were acquired on the fractions in the presence of calcium. Only one fraction gave spectra of dispersed chemical shifts, which in most cases are similar to those in spectra of the wild type protein (26) (Fig. 4) suggesting that this fraction is correctly folded. Two-dimensional 1H,15N HSQC spectra collected for the other fractions showed resonances clustered in the random coil region indicating the protein in these fractions could not adopt a globular structure. Comparison of NH and 15N resonances of GB1-LDLa F10A in the spectra of the fraction that gave dispersed resonances compared with those of wild type GB1-LDLa showed that the largest chemical shift differences (>0.1 ppm) occur for Lys-17, Asn-26, Val-28, Asp-30, Gln-34, Asp-36, and Glu-37. These residues, except for Lys-17, are from the C-terminal region, and the carboxylate-containing residues are all involved in the ligation of the calcium ion. Further inspection of spectra collected...
on $^{15}$N GB1-LDLa F10A indicated that the intensity of the peaks assigned to the LDLa F10A module are typically weaker than those of the GB1 protein. Measurement of calcium affinity by ITC of this sample yields an apparent $K_d$ of 150 $\mu$M ± 38 $\mu$M (Table 2), which is much higher than the $K_d$ of 20 $\mu$M for wild type protein. Two factors may contribute to this higher $K_d$ value as follows: the possibility that the peak was not fully separated from other isomers of the protein via HPLC and the perturbation of the side chains of those residues that bind calcium.

Tyr-9 is positioned on the opposite side of the $\beta$-sheet to Phe-10 and forms a part of the hydrophobic surface (Fig. 2). Mutation of Tyr-9 to alanine also reduces the capacity of the LDLa module to refold to a single isomer. The GB1-LDLa Y9A oxidized in the presence of calcium elutes as several broad peaks with a similar profile to GB1-LDLa F10A (Fig. 3). Two-dimensional $^{15}$N, $^1$H HSQC spectra were acquired for each fraction resolved on RP-HPLC, but only one generated spectra that resembled those of the wild type protein. Chemical shift mapping of $^{15}$N GB1-LDLa Y9A compared with wild type protein suggests that the mutation has not significantly affected the structure of the protein (Fig. 5). However, similar to GB1-LDLa Y9A, resonances that were assigned to the LDLa Y9A module were of much lower signal intensity than those of the GB1 fusion protein. An apparent $K_d$ value for calcium binding of $\sim$350 $\mu$M was determined for the fractions corresponding to folded protein, which is much higher than the $K_d$ values observed for wild type and GB1-LDLa F10A. This infers that the reduced binding affinity of GB1-LDLa Y9A for calcium is contributed by residual unfolded protein and/or as suggested by the RP-HPLC traces reflects a role of Tyr-9 in the stabilization of the structure upon calcium binding.

GB1-LDLa C5/18S oxidized in the presence or absence of calcium elutes predominantly as a single peak on RP-HPLC (Fig. 3). Two-dimensional $^{15}$N, $^1$H HSQC spectra acquired on the various fractions as either GB1 fusions or isolated LDLa modules and in the presence of calcium show that only the major peak has significant amounts of protein. However, the chemical shifts were clustered in the region of 8 – 8.5 ppm indicative of disordered structure (supplemental Fig. S1). As the resonances in the spectra of this species of LDLa C5S/C18S could not be assigned, it was not possible to assess the global
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The effect of the mutation using chemical shift difference mapping. These results suggest that in the absence of the first disulfide bond the protein is unstructured, and this differs with similar mutations of the fifth module of the LDL receptor that showed the C-terminal region could independently refold and retain its capacity to bind calcium, albeit lower (25).

Replacement of the RXFP1 Receptor LDLa Module with the Second Ligand Binding Domain (LB2) of the LDL Receptor—The LDLa module of RXFP1 is essential for receptor activation (28). To demonstrate that the function of the RXFP1 LDLa module depends on specific side chain interactions, we replaced it with the second ligand binding domain (LB2) of the LDL receptor producing the chimera LB2-RXFP1. LB2 was chosen as it has a high level of sequence identity (48%) with the RXFP1 receptor LDLa module, and the structure solved by NMR (18) is also a typical LDLa fold (Fig. 6). The LB2-RXFP1 receptor was transfected into HEK293T cells and tested for cell surface expression and affinity for relaxin. These assays confirmed that replacement of the LDLa module with LB2 had no effect on trafficking of the receptor to the cell surface or the binding of H2 relaxin (Fig. 6). To determine the potential of LB2 to mediate RXFP1 signaling, the receptor chimera was stimulated with H2 relaxin in a dose-dependent manner. Compared with wild type RXFP1, the LB2-RXFP1 chimera failed to stimulate the production of intracellular cAMP (Fig. 6). These data support the concept that specific residues in the LDLa module of the RXFP1 receptor are required for activation.

Cell Surface Expression of RXFP1 Receptor Mutants L7A, Y9A, F10A, L22A, L23A, and C5S/C18S—To further investigate which residues or regions are important in receptor activation, HEK293T cells were transfected with a pcDNA3.1 vector expressing the RXFP1 receptor with the mutations described above (L7A, Y9A, F10A, L22A, L23A, and C5S/C18S). Initially, the effect of bearing a potentially unstructured LDLa module on cell surface expression was determined. All the mutant receptors produced in this study were expressed on the cell surface at levels equal to the wild type receptor (Fig. 7), providing confidence that any loss of relaxin binding or signaling phenotypes was not because of low expression of the receptor at the cell surface. As in vitro GB1-LDLa C5S/C18S is unstructured, these data demonstrate that expression of an unstructured LDLa module does not reduce trafficking of the receptor to the cell surface.

Relaxin Binding to RXFP1 Receptor LDLa Module Mutants L7A, Y9A, F10A, L22A, L23A, and C5S/C18S—The affinity of the mutant receptors for H2 relaxin was next determined using a 3p-labeled H2 relaxin competition assay (Table 3). The results of these assays indicate that binding of relaxin is not altered by any of the mutations introduced in this study (supplemental Fig. S2), and correlate with previous reports that the LDLa module is not involved in primary binding of relaxin, because an RXFP1 receptor lacking the LDLa module binds relaxin with the same affinity as the full-length receptor (28).

Relaxin-induced cAMP Levels Generated by RXFP1 Receptor LDLa Module Mutants L7A, Y9A, F10A, L22A, L23A, and C5S/C18S—To determine the signaling potential of the mutant RXFP1 receptors, transfected HEK293T cells were dose-dependently stimulated with increasing amounts of H2 relaxin, and cAMP produced intracellularly was measured using an enzyme immunoassay (Fig. 8). As expected the mutation C5S/C18S of RXFP1 showed no activation. The in vitro refolding experiments described above indicate that the LDLa module missing the first disulfide will not fold and consequently should not activate.

The mutations of Leu-7 and Leu-22 elicited responses of increasing cAMP production (Fig. 8). However, the dose-response curves of both mutant receptors appear to be right-shifted compared with those of the wild type receptor. The pEC50 of RXFP1 L22A of 9.980 ± 0.33 does not statistically differ from the wild type receptor of 10.24 ± 0.32 (p > 0.05), whereas the pEC50 of RXFP1 L7A is higher at 9.337 ± 0.17 (Table 3) and represents a significant lower efficacy than the wild type receptor (p < 0.001). As the in vitro calcium-induced refolding and the NMR spectra analysis of the L7A mutant show minimal perturbation to the structure, we conclude that the decrease in RXFP1 receptor signaling is a direct perturbation of a protein-protein interaction that leads to receptor activation.

The in vitro refolding experiments described for the mutations Y9A, F10A, and L23A suggest that in vivo there could be multiple disulfide isomers or a significant population of...
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 unfolded protein despite high concentrations of extracellular calcium; therefore, it may not be simple to interpret signaling experiments for these mutations. Nevertheless, both RXFP1 Y9A and RXFP1 L23A receptors retain the capacity to activate signaling; however, dose-response curves were significantly right-shifted with an pEC\textsubscript{50} of 8.836 ± 0.24 (\(p < 0.01\)) and 9.116 ± 0.20 (\(p < 0.001\)), respectively, whereas RXFP1 F10A fails to signal (Fig. 8; Table 3). These data combined with the chemical shift mapping suggest that the inability of the RXFP1 F10A receptor to signal cAMP production could be a direct consequence of a perturbation to the C-terminal region. However, the lack of signaling may be due to few receptors at the cell surface containing folded LDL\textalpha{} modules.

The signaling experiments demonstrate that RXFP1 Y9A and RXFP1 L23A receptors can express folded and functional LDL\textalpha{} modules at the cell surface. The dose-response curve of the RXFP1 L23A receptor shows that the maximum levels of cAMP production are equivalent to the wild type receptor, as is the case for RXFP1 L7A and RXFP1 L22A. Hence, it is likely that the RXFP1 L23A receptor has a fully folded LDL\textalpha{} module at the cell surface and that Leu-23 is involved in a specific inter-

![Comparison of NH and ¹⁵N chemical shifts of wild type and mutant RXFP1 LDL\textalpha{} modules.](image)

**TABLE 2**

| Protein            | Approximate K\textsubscript{d} of binding | S.E. |
|--------------------|------------------------------------------|------|
| GB1 LDL\textalpha{}          | 20 ± 0.38                              |      |
| GB1 LDL\textalpha{} L7A      | 13.5 ± 0.8                              |      |
| GB1 LDL\textalpha{} Y9A      | 350 ± 140                               |      |
| GB1 LDL\textalpha{} F10A     | 150 ± 38                                |      |

![Sequence alignment of the second LDL\textalpha{} module (LB2) of the human LDL receptor to the human RXFP1 receptor LDL\textalpha{} module.](image)

![Overlay of the RXFP1 receptor LDL\textalpha{} NMR structure onto the NMR structure of LB2.](image)

![Cell surface expression of the LB2-RXFP1 receptor in comparison with the wild type receptor.](image)

![Specific binding of H2 relaxin to the LB2-RXFP1 receptor compared with the RXFP1 receptor.](image)

FIGURE 6. LB2-RXFP1 chimera. A, sequence alignment of the second LDL\textalpha{} module (LB2) of the human LDL receptor to the human RXFP1 receptor LDL\textalpha{} module. The two modules share 48% identity. B, overlay of the RXFP1 receptor LDL\textalpha{} NMR structure onto the NMR structure of LB2 (18). C, cell surface expression of the LB2-RXFP1 receptor in comparison with the wild type receptor. The replacement of the LDL\textalpha{} module has no detrimental effect on the trafficking of the receptor to the cell surface. D, specific binding of H2 relaxin to the LB2-RXFP1 receptor compared with the RXFP1 receptor. The receptors bind relaxin with a similar affinity. E, relaxin induced cAMP signaling of the LB2-RXFP1 and RXFP1 receptors. The LB2-RXFP1 receptor is unable to elicit cAMP production in response to relaxin.
The wild type receptor, and any pEC50 changes would reflect a LDLa module would have the same maximal cAMP response as protein if it can fold correctly. If this is the case, we would the mutation does not significantly perturb the structure of the proportion of unfolded LDLa module in the mutated receptor. of receptors expressed on the surface of the HEK293T cells, indicating that the receptor is trafficked to the cell surface even in the presence of an unstructured LDLa module. The LDLa modules of the RXFP1 and RXFP2 receptors are critical for eliciting the stimulation of intracellular cAMP production upon ligand binding (28). The molecular details of the mechanism by which the LDLa module elicits cAMP signaling are unknown. The elucidation of this mechanism, however, would present a paradigm in the understanding of GPCR activation. In an effort to gain insight into the mechanism by which the RXFP1 receptor LDLa module mediates receptor signaling, the structure of the module was first determined via solution NMR spectroscopy. This was followed by analysis of LDLa mutants in both recombinant proteins produced in bacteria and in the full-length receptors expressed in mammalian cells. To comprehensively understand the local or global effect of mutation and the loss of function of the RXFP1 receptor, we tested calcium-dependent in vitro refolding, compared chemical shift data in NMR spectra, and determined calcium binding affinity of the recombinant proteins while in parallel assessing receptor cell surface expression, relaxin binding, and relaxin-induced cAMP signaling in full-length receptor LDLa mutants. The NMR structure of the RXFP1 receptor LDLa module has retained many of the features common to these domains, such as the general fold that is dominated by six conserved cysteine residues and the ligation of a calcium ion by a motif of acidic residues also largely conserved. LDLa modules are well defined for their function in lipoprotein binding and metabolism as repeating domains in members of the LDL receptor family. Apart from the Tva receptor LDLa module (24, 51), the structure of the RXFP1 receptor LDLa module is the only other LDLa module that is not associated with the LDL receptor family to be structurally characterized to date. The solution structure of the RXFP1 receptor LDLa module can be considered as divided into N- and C-terminal regions. The N-terminal region of the structure includes a short anti-parallel β-sheet consisting of residues 9–11 and 18–20. The loop between these strands contains an N-linked glycosylation site at Asn-14 (28). Immediately following the β-sheet is a short 310 helix (residues 21–23). The C-terminal region contains the conserved acidic motif responsible for the ligation of the calcium ion. Because of the high level of sequence conservation over this region of the molecule, between all LDLa modules the C-terminal region is most structurally similar to the other modules characterized to date, including LB2 (18) and LB5 (25).

Despite the high level of sequence and structural similarity shared between the RXFP1 receptor LDLa module and the second ligand binding domain of the LDL receptor, LB2, the modules were not interchangeable. The LB2-RXFP1 receptor chimera bound relaxin with full affinity in comparison with the wild type receptor, but it was unable to elicit intracellular cAMP production. This observation implies that there are specific residues in the RXFP1 receptor LDLa module essential for receptor activation. Sequence and structural alignment of the two modules highlights that the majority of differences between the two are in the N-terminal region of the modules. From here it was hypothesized that should the acidic C-terminal region
organized around the calcium ion in fact be the region of the module that directs signal activation, then the modules should have been interchangeable to retain at least a modest level of receptor efficacy. As the LB2-RXFP1 receptor was inactive, this suggests that the N-terminal region of the LDLa module is primarily involved in the unknown interaction that leads to the activation of signaling. Further inspection of the structure of the LDLa module shows a clustering of hydrophobic residues, including Leu-7, Tyr-9, Leu-22, and Leu-23. The prominence of this area suggests a role for this region in signal activation. Importantly, most of these residues are conserved in RXFP1 sequences from other mammalian species (16). The converse side to the hydrophobic patch includes the loop between the two β-strands, which includes the N-linked glycosylation site at Asn-14. Because the absence of glycosylation at this site has been reported to not reduce receptor efficacy (28), it seems unlikely that this region of the module is directly involved in the interaction that leads to receptor activation. We therefore performed a series of mutations within the N-terminal region to assess their effects on LDLa structure and function.

The disulfide bond between Cys-5 and Cys-18 that tethers the N-terminal region of the structure was removed via mutation of these residues to serine in order to probe the importance of global structure in this region for signal activation. The equivalent mutation in the LB5 module did not disrupt the oxidation of the remaining two disulfide bonds, and the C-terminal region retained calcium ion ligation (25). However, in the presence of this mutation the recombinant GB1-LDLa protein was unable to refold or bind calcium. In two-dimensional 15N, 1H HSQC spectra of this protein, acquired in the presence of calcium, the 1H resonances were unassignable as they clustered between 8 and 8.5 ppm, implying LDLa C5S/C18S is unable to adopt a defined globular structure that may reflect the lowered affinity of calcium binding that the RXFP1 receptor has (~20 μM) in comparison with LB5 (0.5 μM) (23). Despite this fact, RXFP1 C5S/C18S receptors, assumed to express with unfolded LDLa modules, are trafficked to the cell surface at levels equivalent to the wild type receptor with folded modules.

Mutation of Leu-7 and Leu-22 does not impact the refolding of the LDLa module in vitro. Additionally, both the RXFP1 L7A and the RXFP1 L22A receptors elicit a maximum cAMP response similar to the wild type RXFP1 receptor, highlighting that they have fully folded LDLa modules at the cell surface. The dose-response curve of both receptors is right-shifted in comparison with the wild type receptor; however, only the shift of the RXFP1 L7A receptor dose-response curve was significant. These data demonstrate the L22A does not make a significant contribution to either structural integrity or receptor function while supporting the hypothesis that Leu-7 is involved in a specific interaction that leads to cAMP signaling.

Tyr-9 and Leu-23 are structurally close to Leu-7 and Leu-22, but the results from their mutation are not simply interpreted. The refolding profile of the GB1-LDLa fusions of both mutants demonstrates that Tyr-9 and Leu-23 are essential for stabilizing the structure during in vitro calcium-dependent refolding. GB1-LDLa Y9A is unable to refold to a single disulfide isoform; however, the RXFP1 Y9A cell surface receptor retains the capacity to direct signal activation in response to relaxin stimulation. The dose-response curve of the RXFP1 Y9A receptor shows that the receptor is only capable of eliciting a response that is equivalent to 40% of the maximum RXFP1 receptor

![FIGURE 8. Relaxin induced cAMP signaling of RXFP1 receptor mutants.](image)
response to relaxin. This would support that the receptors are expressed on the cell surface in a mixed population of those with folded LDLa modules and those with unfolded LDLa modules. Results of the RXFP1 C55/C18S receptor mutation show that the presence of the unfolded LDLa module does not affect receptor cell surface expression (Fig. 7) or binding of relaxin (supplemental Fig. S2). Therefore, in this situation where a population of receptors with folded and unfolded LDLa modules exists, those receptors with unfolded LDLa modules will bind relaxin but be unable to signal cAMP production, resulting in a lowered and right-shifted cAMP response. Chemical shift difference mapping indicates that for correctly folded modules the Y9A mutation does not introduce significant perturbations to the structure. The observation that the RXFP1 Y9A receptor retains 40% of the maximal cAMP response while having only 3.8% of the wild type RXFP1 receptor efficacy is further evidence that a specific interaction involving Tyr-9 in the population of receptors with correctly folded modules has been disrupted. In the RXFP1 receptors of most mammalian species, tyrosine is conserved in this position except for mouse and rat where a serine residue is observed (S2). The presence of a serine residue at this position would reduce the size of the side chain but not the overall polarity.

In contrast to the RXFP1 Y9A receptor the dose-response curve of the RXFP1 L23A receptor reveals that it is capable of stimulating the production of cAMP to levels equivalent to wild type. From the RP-HPLC elution profile of the recombinant GB1-LDLa L23A mutant that demonstrates that the protein is unable to fold to a single isomer and the tendency of the protein to precipitate, we expected that the RXFP1 L23A receptor would have lowered signaling capacity. So it appears that despite this in vitro instability, RXFP1 L23A receptors at the cell surface express correctly folded LDLa modules. However, the dose-response curve is significantly right-shifted in comparison with the wild type receptor thus questioning whether the reduction in receptor efficacy is the consequence of the specific loss of the Leu-23 side chain or if mutation of Leu-23 has perturbed other residues. In the absence of further structural and folding studies, it is not possible to determine the conformational effects of this mutation on the LDLa module.

Phe-10 is conserved at the equivalent position in almost all LDLa modules and is absolutely conserved in all mammalian RXFP1 sequences, suggesting a crucial structural or functional role. Notably, the orientation of Phe-10 in the RXFP1 receptor differs from other modules studied previously. The significance of this may lie in the observation that in the presence of the F10A mutation the RXFP1 receptor is unable to elicit cAMP signaling. Although in vitro, the recombinant LDLa F10A module is able to refold to a conformation similar to the wild type protein, it loses the ability to fold only to a single correct isoform. Chemical shift mapping of the species that does fold to a globular structure indicates that in comparison with the wild type protein residues in the C-terminal region, primarily those of the calcium binding motif, are significantly perturbed by the mutation of Phe-10. Chemical shift perturbations are not simply because of loss of ring current effects. For example, calculation of ring current effects shows only small contributions for Asp-30 and Glu-37, which show large chemical shift changes in LDLa F10A compared with the wild type LDLa module. Asp-36 also shows significant chemical shift differences, but these may be due to the loss of ring current effects for the mutant. Asp-30, Asp-36, and Glu-37 are all calcium ligands; therefore, the mutation F10A may have perturbed the structure of this region. These differences suggest that Phe-10 plays a critical role in the arrangement of the residues that bind calcium and therefore the overall stability of the structure.

The RXFP1 and RXFP2 receptors are novel GPCRs, and their exclusive activation by the N-terminal LDLa modules represents a paradigm in understanding of GPCR activation. It also presents a unique opportunity to develop highly specific agonist/antagonists to the receptors by mimicking or blocking LDLa module function. Our studies have highlighted the key role that specific residues in the N-terminal region of the LDLa module play in receptor activation. Furthermore, we have demonstrated the necessity to study the structural effects of single amino acid mutations on the module to fully interpret their effects on receptor activity.

Acknowledgments—We thank Haydyn Mertens and Ben Atcliffe for their technical assistance with ITC experiments. We also thank Andrew Perry and Daniel Scott for helpful discussions throughout this study.

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