Receptors & Clinical Investigation 2015; 2: e883. doi: 10.14800/rci.883; © 2015 by Vincent Goffin, *et al*.
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RESEARCH HIGHLIGHT

Structural motifs in the extracellular domain of the prolactin receptor govern fold and functionality

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Received: June 25, 2015
Published online: July 06, 2015

The prolactin receptor (PRLR) is an archetype cytokine receptor. It is a single-pass transmembrane receptor with limited complexity that is devoid of enzyme activity. Intracellular signaling involves various receptor-associated kinases including Jak2, Erk1/2, Src and Akt. As the PRLR is emerging as a relevant target in Oncology the understanding of the molecular basis of its activation is crucial. In the frame of an inter-disciplinary consortium involving biophysicists, structural biologists and cell biologists, we have successfully combined complementary approaches such as optical and nuclear magnetic resonance (NMR) spectroscopic analyses, X-ray crystallography, surface plasmon resonance and cell-based assays to start elucidate the structural features of ligand-receptor interaction. However, the features of the PRLR extracellular domain (ECD) that participate in the transmission of the hormonal message across the cell membrane and/or in selective activation of intracellular signaling cascades remained uncharacterized. In two recently published studies, we identified residues 146 and 170 as two key residues of the PRLR-ECD that control critical receptor properties including basal signaling activity, ligand sensitivity, species specificity, folding, stability and receptor turnover. These two residues are in close proximity of each other in the membrane proximal domain of the PRLR-ECD and participate in a network of interactions with other residues, in particular within a specific residue quartet. Strikingly, these residues are involved in, or close to, the receptor dimerization interface, suggesting that their mechanism of action may involve structural reorientation of the receptor chains that are necessary to (selectively) disseminate the signal from the ECD to the intracellular domain. The identification of such residues in this and other cytokine receptors should affect future structure-directed drug development strategies aimed at providing pathway-selective treatment approaches.

Keywords: prolactin receptor; cytokine receptor; gain-of-function; Ile146; Stat5; MAPK; cell proliferation; structure; nuclear magnetic resonance (NMR); cancer

To cite this article: Vincent Goffin, *et al*. Structural motifs in the extracellular domain of the prolactin receptor govern fold and functionality. Receptor Clin Invest 2015; 2: e883. doi: 10.14800/rci.883.

The PRLR as a novel target in cancer

The prolactin receptor (PRLR) is emerging as a relevant therapeutic target in Oncology [1]. The involvement of prolactin (PRL) in breast cancer has been supported since the 1970s by a wide array of experimental data mainly involving

immortalized cell lines and transgenic mouse models. Several arguments have accumulated supporting the role of PRL as a promoter of cell proliferation, survival, motility and chemoresistance [for reviews, 2, 3, 4]. The observation that systemic as well as mammary-specific expression of PRL transgenes in mouse models initiated mammary tumorigenesis [for a review, 5] echoes with the increased risk of estrogen receptor-positive breast cancer associated with circulating levels of PRL in the high-normal range in humans [6]. The precise role of PRL in breast tumorigenesis remains debated since activation of Stat5, which is one of the main intracellular signaling pathways used by the PRLR, was intriguingly shown to underlie good prognosis in breast cancer patients [7, 8]. Thus, the current working model suggests that PRL may participate in the initiation/growth of breast cancer while limiting its progression towards aggressive phenotypes [9].

Besides being a player in breast cancer, PRL has recently emerged as a driver of other cancers, including prostate cancer. Locally-produced (autocrine) rather than systemic (endocrine) PRL seems to be involved since prostate PRL expression and Stat5 activation in prostate cells have been correlated with high grade prostate cancer, suggesting a role in tumor progression [10,11]. At the cellular level, Stat5 signaling via the PRLR was shown to promote proliferation, survival and invasion of human prostate cancer cells [11,for reviews, 12,13]. Of interest, our recent animal studies have highlighted the capacity of autocrine PRL to regulate adult prostate stem cells, which have been proposed by others to act as tumor-initiating cells [14–16].

Database screening (e.g. http://cancergenome.broadinstitute.org/index.php) indicates that the *PRLR* gene is not prone to mutation in any cancer. Accordingly, most studies that investigated the breast oncogenome have been inconclusive regarding the existence of functional PRLR mutations or single nucleotide polymorphisms (SNPs). In 2008, we identified the first natural amino acid substitution that specifically altered PRLR properties [17]. This genetic variant involved the substitution of a leucine for the isoleucine naturally found at position 146 in the extracellular domain (ECD) of the receptor (Fig. 1A). When analyzed in reconstituted cell models (HEK293 fibroblasts, MCF-7 breast cancer cells, and Ba/F3 mouse lymphoid cells), $PRLR^{I146L}$ appeared to exhibit sub-maximal basal signaling activity, i.e. it triggered some level of Stat5 and MAPK phosphorylation in the absence of PRL stimulation. Furthermore, while PRLR-expressing Ba/F3 cells are totally dependent on the presence of PRL for survival and proliferation, PRL was dispensable to immortalize Ba/F3 cells expressing $PRLR^{I146L}$ [17–19]. The gain-of-function properties of $PRLR^{I146L}$ were also confirmed by another group using metabolic parameters as readouts [20]. Finally, $PRLR^{I146L}$ has so far been identified as a germinal (hereditary) SNP, as opposed to a somatic mutation (only identified in some tumors) [17,21] and its prevalence in the general population is in the range of a few percent.

Together, these data indicate that the PRLR, irrespective of any mutation, is a susceptibility gene for tumorigenesis. It is therefore increasingly important to understand the structural drivers of PRLR activity, not only to understand the molecular basis of $PRLR^{I146L}$ constitutive signaling, but also to help design potent PRLR inhibitors.

Structure of the PRLR

The PRLR represents the archetype of hematopoietic cytokine receptors [22]. It is the simplest type 1 cytokine receptor, with the functional unit made of two identical transmembrane chains. Its ECD exhibits minimal structural complexity as it includes a single cytokine receptor homology module consisting of two fibronectin type III (FNIII) domains, termed D1 (membrane-distal) and D2 (membrane-proximal) (Fig. 1A). Signaling by the wild type (WT) PRLR requires the formation of a ternary complex involving one molecule of PRL interacting with a PRLR homodimer via two asymmetrical binding sites, termed site 1 and site 2 (Fig. 1A) [23, 24].

The D2 domain contains several structural features that are assumed to play important roles in triggering PRLR signaling. First, it harbors the WS-motif which is a five-residue stretch conserved among cytokine receptors (Trp-Ser-X-Trp-Ser, X can be any amino acid). Recently, we identified this motif to act as a molecular switch in PRLR activation: in the 'off-state' (i.e. unbound) the tryptophans adopt a T-stack conformation, while in the 'on-state' (i.e. ligand bound), they engage in a ladder conformation with several basic residues (Fig. 1B) [25,26]. Second, several residues of the D2 domain are involved in the receptor-receptor dimerization interface, also called site 3 [23,24] (Fig. 1A). Last but not least, Ile146 is part of the D2 domain and is buried in the core of the domain (Fig. 1A,C). When substituted by leucine, the NMR properties of residues in the receptor dimerization interface were affected leading to a small, but significant shift in the monomer-dimer equilibrium suggesting this to be the underlying cause for its constitutive activity in cells [25]. Importantly, this study highlighted an interconnection between residues of the WS-motif, Ile146 and the receptor dimerization interface [25].

In two recently published studies, we manipulated residue 146 [27] and various interface residues [28] and characterized cognate PRLR variants using a panel of complementary

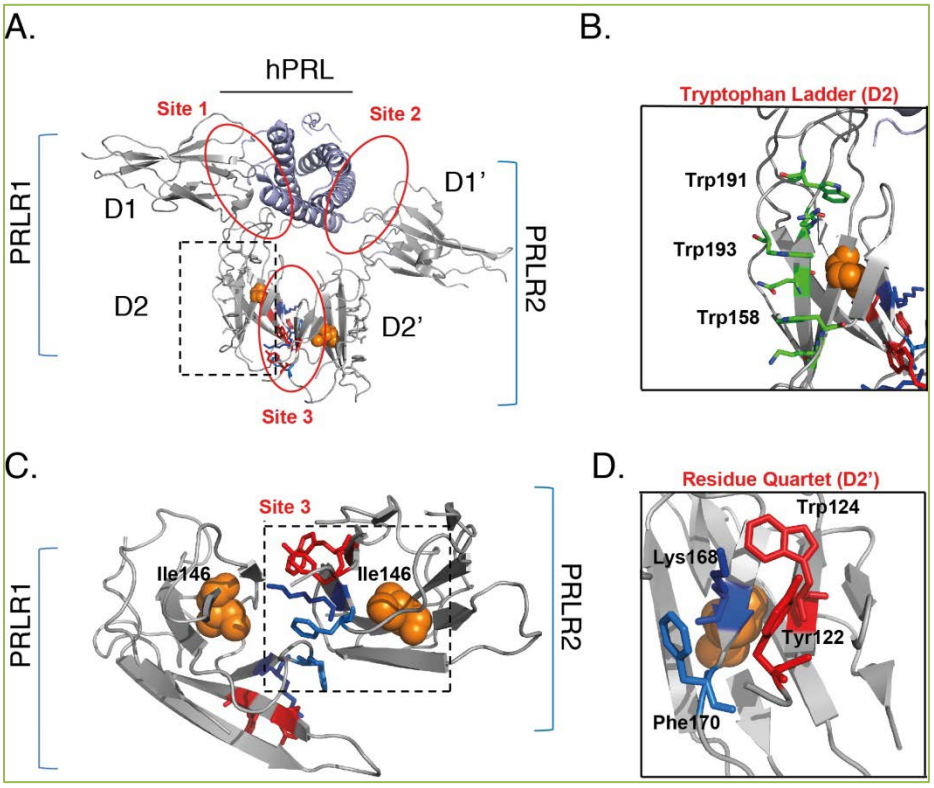

Figure 1. (A) Three-dimensional structure representation of the 2:1 rat PRLR/human PRL complex (PDB accession code 3EW3) [24]. The two receptors (PRLR1 and PRLR2) are represented in grey with their D1- and D2-domains indicated. PRL is shown in blue ribbon. The three inter-molecular interaction sites (sites 1 and 2 between PRL and each receptor, site 3 between the two receptors) are identified by red circles. Ile146 is shown in orange space filled atoms, Lys168 and Phe170 in blue sticks and Tyr122 and Trp124 in red sticks. The tryptophan ladder is shown in grey sticks (boxed by dashed lines for PRLR1) (B) Zoom on the tryptophan ladder from receptor PRLR1. (C) 90° backwards rotation of (A) allows a view into site 3 from the bottom with Ile146 highlighted in orange spheres. To guide the eye, Lys168 and Phe170 are in blue sticks, and Tyr122 and W124 in red sticks. To the right is shown a magnification of site 3 with similar coloring. (D) Zoom on the quartet residues Lys168, Phe170 (both in blue sticks) and Tyr122, and Trp124 (shown as red sticks). Ile146 is shown in orange spheres.

spectroscopic/NMR and cell-based assays. These studies revealed a new structural feature that together with Ile146 direct critical receptor properties.

$PRLR^{I146L}$ as a driver of PRLR folding, basal activity and ligand binding

In initial studies we aimed to produce recombinant $PRLR^{I146L}$–ECD in bacteria to determine its structure by X-ray crystallography as earlier performed for the $PRLR^{WT}$–ECD [23,24]. Unfortunately, the $PRLR^{I146L}$–ECD protein systematically precipitated upon refolding, suggesting structural disturbance despite the conservative nature of the Ile-to-Leu substitution. We were nevertheless successful to produce the recombinant D2 domain containing the I146L mutation ($D2^{I146L}$) as a soluble protein that was suitable for NMR analyses. These studies revealed that although the WS motif of $D2^{I146L}$ adopted the typical 'off-state' T-stack conformation, the mutation affected the D2-D2 dimerization interface constructively [25]. Together, these initial structural characterizations fitted well with the observations that in cell-based assays $PRLR^{I146L}$ exhibited basal activity that was higher compared to unstimulated $PRLR^{WT}$ but lower than PRL-stimulated $PRLR^{WT}$.

To address whether the gain-of-function properties of human $PRLR^{I146L}$ resulted from the specific loss of Ile^{146} or from the specific introduction of a Leu at this position, we manipulated position 146 by introducing various amino acids exhibiting different physicochemical properties (I146X, where X=Ala, Val, Gly, Asn, Asp or Arg). The basal activity and PRL responsiveness of the I146X variants were determined using our classical cell bioassays involving HEK293 and Ba/F3 cells stably expressing either of these PRLRs. We monitored ERK1/2 and Stat5 activation using immunoblotting and/or luciferase reporter gene, and Ba/F3

cell proliferation using colorimetric assays. Based on their bio-characteristics these *in vitro* assays revealed the existence of two categories of variants. $PRLR^{I146A}$ and $PRLR^{I146V}$ (group 1) were indistinguishable from $PRLR^{WT}$, meaning they had no detectable basal activity and responded similarly to PRL stimulation in dose-response experiments. In contrast, $PRLR^{I146N}$, $PRLR^{I146D}$ and $PRLR^{I146R}$ (group 2) behaved oppositely as they were unresponsive to PRL stimulation but displayed marked constitutive activity (higher than $PRLR^{I146L}$). $PRLR^{I146G}$ had intermediate properties. Far-UV circular dichroism (CD) spectroscopic analyses of recombinant $D2^{I146D}$, $D2^{I146N}$ and $D2^{I146G}$ variants showed a distinct β-strand signature with minima at 218 nm, indicating that they were not unfolded. However, [^{1}H,^{15}N]-HSQC NMR spectral and size-exclusion analyses of $D2^{I146D}$ and $D2^{I146N}$ concluded heterogeneity of the samples with predominant large size oligomers at the expense of correctly folded monomers. These findings suggested that the altered bio-characteristics of group 2 variants presumably resulted from strong structural perturbations. Whether their PRL insensitivity resulted from failure to bind the ligand and/or of receptor export to the cell surface remains to be determined. The $D2^{I146G}$ sample appeared structurally less affected than the other two variants as it exhibited a small population of monomeric folded protein. This again was in agreement with its intermediate behavior in functional assays.

Since the active PRLR complex involves a receptor homodimer (Fig.1A), we then asked whether such variants could functionally interact with the $PRLR^{WT}$. These experiments were conducted using HEK293 cells, in which endogenous PRLR expression is virtually undetectable. We co-expressed different ratios of $PRLR^{WT}$ and $PRLR^{I146D}$ taking advantage of a previously validated tagged version of $PRLR^{WT}$ that can be discriminated from the untagged receptor by SDS-PAGE [29]. These analyses showed that i) both receptors co-immunoprecipitated using an anti-tag antibody, suggesting the formation of heterodimers, ii) $PRLR^{WT}$ dose-dependently quenched the basal activity of $PRLR^{I146D}$, but iii) $PRLR^{I146D}$ did not inhibit the PRL-sensitivity of the $PRLR^{WT}$. Clearly, further studies are required to fully dissect the molecular mechanisms underlying these complex effects.

Finally, $PRLR^{I146D}$ (or $PRLR^{WT}$ as a control) was ectopically expressed in breast cancer cells. We used two classical cell lines representative of two different molecular sub-types: MCF-7 (a well-differentiated epithelial cell line) and MDA-MB231 (a poorly differentiated mesenchymal-like cell line). The differentiation status of these breast cancer cells as estimated by their morphology and expression of epithelial/mesenchymal markers was not markedly affected by $PRLR^{I146D}$ expression. Furthermore, the latter failed to promote cell proliferation, and could even decrease it under some experimental conditions. The absence of obvious effects in MDA-MB231 cells was tentatively explained by the very low levels of basal PRLR signaling that could be obtained in stably transfected cell clones, which may have been insufficient to produce measurable effects on cell properties. Regarding MCF-7, the expression of significant levels of endogenous $PRLR^{WT}$ and its possible heterodimerization with ectopic $PRLR^{I146D}$ variant may also affect the functional outcome, as observed in HEK293 cells (see above). These studies underline the complexity of interpreting the ultimate functional impact of PRLR mutants in human pathophysiology, as recently observed for a loss-of-function PRLR variant in familial hyperprolactinemia [30].

Taken together, these biological and structural characterizations demonstrate that position 146 is critical for maintaining the structural integrity of the D2 domain, which in turn is mandatory for stabilization of the receptor off-state and PRL-binding potency [27].

A residue quartet controls PRLR-induced ERK1/2 activation

In addition to binding circulating ligands, one function of cytokine receptor ECDs may be to participate in selective activation of intracellular signaling pathways. Indeed, several studies involving different receptors have shown that the same receptor can elicit different cellular responses depending on the nature of the stimulus (e.g. antibodies, small-molecule agonists, activating mutations intrinsic to the receptor, etc) [31–34]. These findings strongly suggest that cytokine receptors are able to discriminate between agonistic signals and to translate this information into different signaling capacities. However, the structural and mechanistic features of the ECD that may be involved in the regulation of this process are poorly understood.

Using oriented Surface Plasmon Resonance (SPR) to measure the stabilities of PRL/PRLR-ECD ternary complexes, we recently identified Gln164, Lys168 and Phe170 as hot-spot residues of the rat PRLR dimerization (site 3) (Fig 1A) [24]. We then extended these in vitro observations to signal transfer efficiency using cell-expressed full-length rat and human PRLR variants. Substitution of an Ala for Lys168 or Phe170 in the rat PRLR had no effect on Stat5 signaling while it significantly reduced ERK1/2 signaling. In the human PRLR, Lys168 is conserved but position 170 is occupied by a Leu. When a Phe (as found in the rat PRLR) was introduced at position 170 in human PRLR, this again had no effect on Stat5 signaling but markedly increased Erk1/2 signaling. These experiments revealed the chemical structure of the residue at position 170 to play a key role in regulating

intracellular signaling properties and hence explain why the maximal level of ERK1/2 signaling achieved by the rat PRLR (Phe170) is intrinsically higher compared to the human PRLR (Leu170). Using our established SPR setting, we could demonstrate that F170A substitution in the rat PRLR was detrimental to the formation of the ternary PRL/PRLR-ECD complex [24], while the L170F substitution in the human PRLR had the opposite effect [28]. These results strongly suggested that the level of ERK1/2 activation is related to the life-time of the ternary complex, which is directed by residue 170. We also showed that this very same position controlled PRLR turnover as well as receptor sensitivity to PRL of various origins (species-specificity).

In the context of the recombinant ECD or D2-domain we collected NMR and/or CD spectroscopy data on WT and variants, which suggested two routes to minimize the life-time of the ternary complex. The first involved a destabilization of the structural scaffold, severely noticed for the rat PRLR-ECD-F170A and more moderately for human PRLR-D2-L170A. The second and most dominant way was through modulation of the local structure of a residue quartet involving the residues Tyr122, Trp124, Lys168 and Leu/Phe170. Two different orientations of the aromatic ring of Tyr122 were elucidated from CD exciton couplings and NMR chemical shifts analyses. In one structure, and in analogy to the first route, the interaction between the two aromatic rings was destabilized, which correlated with decreased MAPK signaling. In contrast, in the other structure, the interaction between them was stabilized and correlated with increased MAPK signaling. Thus, the intrinsic structure formed by the quartet is intimately optimized in the individual species affecting the receptor-ligand complex life-time and the subsequent extent of MAPK signaling. Importantly, Trp124 only contributes to binding from the second receptor of the pair, and the regulatory potential may thus reside from here.

Conclusions

The two recent studies by Zhang and colleagues add to the current knowledge of the extracellular residues that govern seminal properties of the PRLR. As the key residues identified in these works are involved in, or close to, the receptor dimerization interface, we point to the ternary receptor lifetime as key to modulate signaling aided by local conformational changes whose dissemination towards the transmembrane domain is currently unknown. The identification of such residues in this and other cytokine receptors should affect future structure-directed drug development strategies aimed at providing pathway-selective treatment approaches.

Acknowledgements

We acknowledge the work, help and ideas of all the authors of our recent papers on PRLR biology including (in alphabetical order) Marie Bernadet, Roman L. Bogorad, Florence Boutillon, Jens Breinholt, Ibtissem Cherifi, Robert Dagil, Jacques-Emmanuel Guidotti, Gitte W. Haxholm, Sylviane Hoos, Maiken Jæger Knudsen, Mads Nygaard, Johan G. Olsen and Kaare Teilum.

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