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Residue 146 regulates prolactin receptor folding, basal activity and ligand-responsiveness: Potential implications in breast tumorigenesis

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ABSTRACT

PRLR^{I146L} is the first identified gain-of-function variant of the prolactin receptor (PRLR) that was proposed to be associated with benign breast tumorigenesis. Structural investigations suggested this hydrophobic core position in the extracellular D2 domain to be linked to receptor dimerization. Here, we used a mutational approach to address how the conservative I-to-L substitution induced constitutive activity. Using cell-based assays of different I146-PRLR variants in combination with spectroscopic/nuclear magnetic resonance analyses we found that chemical manipulation of position 146 profoundly altered folding, PRL-responsiveness, and ligand-independent activity of the receptor in a mutation-specific manner. Together, these data further add to the critical role of position 146, showing it to also be crucial to structural integrity thereby imposing on the biological PRLR properties. When stably introduced in MCF-7 (luminal) and MDA-MB231 (mesenchymal) breast cancer cells, the most potent of the PRL-insensitive mutants (PRLR^{I146D}) had minimal impact on cell proliferation and cell differentiation status.

1. Introduction

The involvement of prolactin (PRL) in breast cancer is supported by a wide array of epidemiological and experimental data (for a review, Clevenger et al., 2003). For example, high–normal circulating levels of PRL have been shown to increase estrogen-receptor positive (ER+) breast cancer risk (Tworoger et al., 2013), while the pro-tumor potency of PRL signaling has been demonstrated in various transgenic mouse models expressing PRL locally or systemically (Manhes et al., 2006; Rose-Hellekant et al., 2003; Wennbo et al., 1997). However, the actual role of PRL signaling in mammary tumor initiation *versus* progression remains debated, as recent data have suggested that PRL signaling through signal transducer and activator of transcription 5 (STAT5) pathway could eventually protect breast cancer patients from disease progression (Peck et al., 2011). The underlying mechanism that has been proposed suggests that one of the outcomes of PRL/STAT5 signaling is to maintain the differentiation status of breast cancer cells, reminiscent of the physiological role of STAT5 during mammopoiesis (Hennighausen and Robinson, 2008). Accordingly, PRL was shown to promote breast cancer cell adhesion at the expense of epithelial–mesenchymal transition (EMT), which is classically associated with cancer progression and metastasis (Nouhi et al., 2006; Sultan et al., 2005, 2008). Prolactin signaling may thus exert a dual role in breast cancer by favoring cancer initiation while limiting cancer progression (for a review, Wagner and Rui, 2008).

Attempts to identify PRL receptor (PRLR) mutations in breast cancer have failed. At best, single nucleotide polymorphism (SNPs) – most often in non-coding regions – have been proposed to be associated with the disease, but neither functional data nor potential molecular mechanisms were provided to support the pathophysiological relevance of

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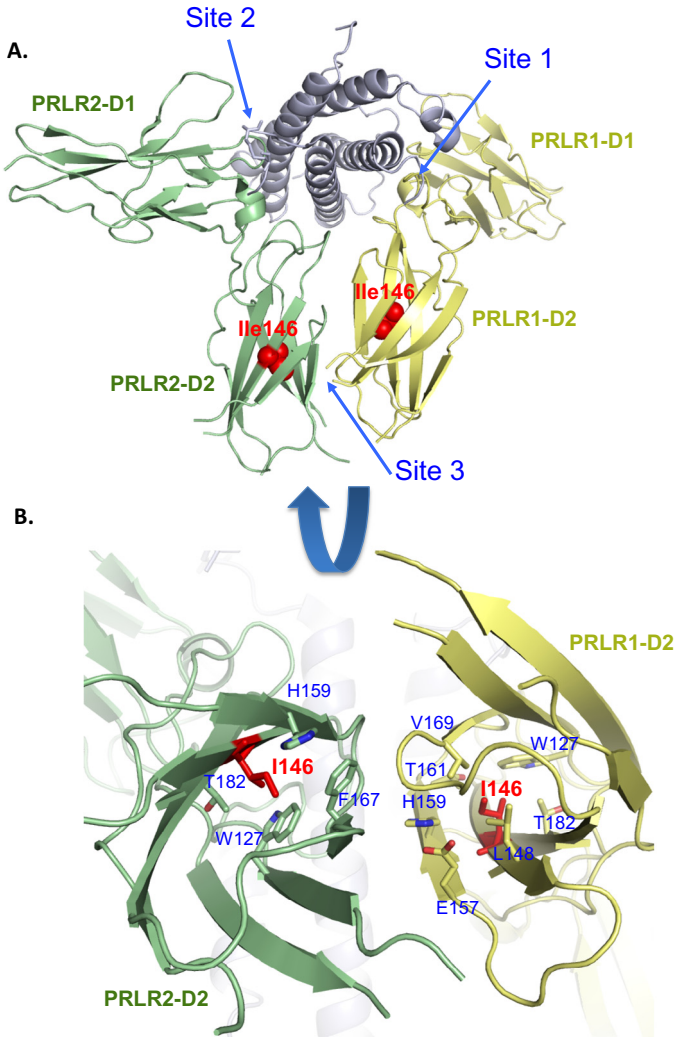

Fig. 1. Ile146 is near the PRLR homodimer interface (site 3). (A) Three-dimensional structure representation of the 2:1 rPRLR/hPRL complex (PDB accession code 3EW3) (Van Agthoven et al., 2010). The two receptors (PRLR1 and PRLR2) are represented in yellow and green, respectively, and their D1- and D2-domains are indicated. The three inter-molecular interaction sites (sites 1 and 2 between PRL and each receptor, site 3 between the two receptors) are indicated. Ile146 is represented in red space filling atoms. (B) Perpendicular orientation (viewed from the membrane) of the receptor–receptor interface zoomed in on the D2 domain highlighting the position of Ile146. Residues located less than 4 Å from I146 are represented in sticks and labeled. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

such variants (Canbay et al., 2004; Glasow et al., 2001; Lee et al., 2007; Mong et al., 2011; Nyante et al., 2011; Vaclavicek et al., 2006). In fact, database screening (*e.g.* http://cancergenome.broadinstitute.org/index.php) suggests that the PRLR gene is not prone to mutation in any cancer.

In the course of a study focused on benign breast diseases (BBDs), we recently identified a PRLR genetic variant that appeared to be slightly more frequent in BBD patients than in the population of control subjects (Bogorad et al., 2008). This germinal variant involved substitution of a leucine for the isoleucine naturally found at position 146, which is located within the extracellular domain (ECD) of the receptor (Fig. 1). Although the presence of the PRLR^{I146L} allele failed to stratify the cohort with respect to patient biological profiles or BBD characteristics, the potential relevance of this PRLR variant in breast tumorigenesis came from its elevated basal activity that was identified in reconstituted cell models (Bogorad et al., 2008; Courtillot et al., 2010). Indeed, PRLR^{I146L} appeared to trigger downstream signaling (STAT5 and Erk1/2) in the absence of PRL stimulus in stably transfected HEK293 fibroblasts, MCF-7 breast cancer cells, and Ba/F3 mouse lymphoid cells. Furthermore, PRLR^{I146L} was able to immortalize Ba/F3 cells, which are totally dependent on cytokine signaling for survival and proliferation, supporting its potential oncogenic potency (Bogorad et al., 2008; Courtillot et al., 2010; Goffin et al., 2010). The gain-of-function properties of this variant were further confirmed by another group who identified its ability to relieve pyruvate kinase M2 inhibition and the resulting lactate accumulation, irrespective of PRL stimulation; these findings further suggested the potential role of PRLR^{I146L} in mammary cell transformation as a metabolic regulator (Varghese et al., 2010).

Although the PRLR is encoded by a single gene, multiple isoforms resulting from alternative splicing have been identified in various species including humans (Hu et al., 2001). Together with the growth hormone receptor (GHR), the PRLR represents an archetype of class I cytokine receptors. Although these receptors were initially referred to as 'homodimeric' receptors (O'Sullivan et al., 2007), subsequent studies showed that various isoforms could actually heterodimerize (Ross et al., 1997; Xie et al., 2009) and even antagonize (Trott et al., 2003). These receptors exhibit minimal structural complexity compared to other family members (Bazan, 1990). Their ECDs contain a single cytokine receptor homology (CRH) module consisting of two fibronectin type III domains called D1 and D2 (Fig. 1A). In addition to residue I146, the D2 contains the conserved Trp-Ser repeat (WS motif), a hallmark of cytokine receptors whose structural alteration upon receptor activation has been recently elucidated by our group (Dagil et al., 2012) and reviewed (Olsen and Kragelund, 2014). In the absence of ligand, these receptors are pre-dimerized at the cell membrane in a structural conformation that prevents intracellular signaling (Brown et al., 2005; Gadd and Clevenger, 2006; Qazi et al., 2006; Tallet et al., 2011; Tan et al., 2005). Under physiological conditions, intracellular signaling is triggered upon binding of one ligand molecule to preformed receptor homodimers. Crystallographic analyses involving growth hormone (GH) or PRL bound to their cognate homodimerized receptor ECDs have shown that the ternary complexes are overall very similar, although they differ in many atomic details (Broutin et al., 2010; De Vos et al., 1992). The molecular mechanism of membrane-bound GHR activation has been recently elucidated (Brooks et al., 2014; Brown et al., 2005). Ligand binding induces relative rotation and translation of both GHR chains, leading to a shift in the transmembrane α-helix conformation from a parallel to a left-handed crossover arrangement; this results in the separation of GHR intracellular domains and pairing of the kinases domains of the receptor-associated Janus kinase 2 (Jak2), which facilitates their transphosphorylation (Brooks et al., 2014). Although this mechanism could be generalized to class I cytokine receptors, mutational studies of juxta-transmembrane sequences failed to support at least the first rotational model for the PRLR (Liu and Brooks, 2011). Thus, the actual mechanism of PRLR activation, and in particular the role of D2 domain in this process, still remains poorly understood (for a review, Brooks, 2012).

To address the mechanism by which the I-to-L substitution shifts the PRLR to a partially activated state, we initially aimed to use a structural approach to compare the 3D structure of free PRLR^{I146L}–ECD to that of ligand-bound PRLR^{WT}–ECD (Broutin et al., 2010; Van Agthoven et al., 2010). Unfortunately, attempts to produce recombinant PRLR^{I146L}–ECD were unsuccessful as the protein precipitated upon refolding, suggesting structural disturbance despite the conservative nature of the substitution. However, it was possible to produce soluble D2^{I146L} to perform nuclear magnetic resonance (NMR) studies. These analyses revealed that despite the fact that