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Impact of subdomain D1 of the short form S1b of the human prolactin receptor on its inhibitory action on the function of the long form of the receptor induced by prolactin ☆

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ABSTRACT

Background: Long-form (LF) homodimers of the human prolactin receptor (PRLR) mediate prolactin's diverse actions. Short form S1b inhibits the LF function through heterodimerization. Reduced S1b/LF-ratio in breast cancer could contribute to tumor development/progression. Current work defines the structural and functional relevance of the D1 domain of S1b on its inhibitory function on prolactin-induced LF function.
Methods: Studies were conducted using mutagenesis, promoter/signaling analyses, bioluminescence resonance energy transfer (BRET) and molecular modeling approaches.
Results: Mutation of E69 in D1 S1b or adjacent residues at the receptor surface near to the binding pocket (S) causes loss of its inhibitory effect while mutations away from this region (A) or in the D2 domain display inhibitory action as the wild-type. All S1b mutants preserved prolactin-induced Jak2 activation. BRET reveals an increased affinity in D1 mutated S1b (S) homodimers in transfected cells stably expressing LF. In contrast, affinity in S1b homodimers with either D1 (A) or D2 mutations remained unchanged. This favors LF mediated signaling induced by prolactin. Molecular dynamics simulations show that mutations (S) elicit major conformational changes that propagate downward to the D1/D2 interface and change their relative orientation in the dimers.
Conclusions: These findings demonstrate the essential role of D1 on the S1b structure and its inhibitory action on prolactin-induced LF-mediated function.
General significance: Major changes in receptor conformation and dimerization affinity are triggered by single mutations in critical regions of D1. Our structure–function/simulation studies provide a basis for modeling and design of small molecules to enhance inhibition of LF activation for potential use in breast cancer treatment.
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1. Introduction

The prolactin receptor (PRLR) is a member of the class I cytokine/lactogen receptor family which mediates the diverse cellular actions of prolactin in several tissues [1]. PRLRs are expressed in normal and neoplastic human breast tissue, and in most breast cancer cells. Prolactin (Prl) via its cognate receptor is a major factor in the proliferation of breast epithelium and is essential in the stimulation and maintenance of lactation. Moreover, there is increasing etiological [2] and experimental evidence [3] that Prl supports human breast tumor growth and promotes invasion.

The human PRLR (hPRLR) contains an extracellular (EC) hormone binding domain with fibronectin like type III structure (D1 and D2 subdomains), a single transmembrane module, and a cytoplasmic domain required for the activation of the Jak2–Stat5 signal transduction pathway by Prl which is essential for transcriptional activation of all known prolactin regulated genes. Other pathways are also activated via Prl/PRLR, including MAPK and PI3K [4]. In addition to the full-length long form of hPRLR (LF), variants resulting from alternative splicing of the PRLR gene have been identified [5–8]. Two short forms of hPRLR (S1a and S1b) with abbreviated cytoplasmic domain display inhibitory action on Prl-induced LF-mediated signaling [5]. Both LF and SF are transmembrane receptors which, as dimers, are capable of ligand binding and Jak2 activation (hormone-induced Jak2-phosphorylation), but the SFs lack downstream Stat5 activation due to the absence of extended cytoplasmic sequences. Our previous studies have demonstrated the presence of constitutive homodimers and heterodimers of LF and SFs in the absence of hormone and the action of Prl as a conformational modifier [9]. The dominant negative

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effect of the SF was found to occur through their heterodimerization with LF, with formation of an incomplete dimer and thus abolition of Stat5 signaling [9,10]. In addition, the SFs appear to have intrinsic functions since they display basal Jak2 phosphorylation which is further increased by Prl. In breast cancer the ratio of SF (inhibitory) to LF (activating) is reduced when compared to the adjacent tissue [11]. These findings suggest that the loss of inhibitory regulation of SF to LF may accelerate abnormal cell proliferation.

Our previous studies have shown that the conformation of the extracellular domain of the hPRLR S1b form, as stabilized by two intramolecular disulfide bonds located in the interior of the D1 subdomain, is required for its inhibitory action on Prl-induced LF-mediated signal transduction function [10]. However, S–S disruption in S1b abrogated Prl-induced Jak2 signaling of this short form [10]. This motivated us to explore the role of individual amino acids located on the surface of the D1 domain of S1b whose mutations would preserve the signaling function induced by Prl, and indicate their requirement to maintain the conformation of the receptor for its inhibitory effect on LF function. In the present study we have revealed region(s) of the extracellular domain involved in the inhibitory action of the short form S1b. Functional and biophysical analyses, as well as computer modeling were used to explore the effects of single mutations of several residues in the D1 and D2 subdomains of SF. From these studies we conclude that the D1 subdomain of SF has a significant effect on the inhibitory action of SF on Prl-induced LF function.

2. Materials and methods

2.1. Site-directed mutagenesis

Amino acids (aa) arginine R66, and glutamic acids E42, E67, E69, and E74 in the D1 domain of the human prolactin receptor (hPRLR) short form S1b [5] were individually substituted with glycine (G), alanine (A) or cysteine (C) using a QuikChange I Site-Directed Mutagenesis Kit (Agilent Technologies, Inc., CA, USA). Briefly, plasmids of hPRLR expressing N-terminus FLAG tag or C-terminus YFP or Renilla luciferase (RL) were site-specifically mutated under PCR reactions using the following primers for the indicated residues.

ACCAATTATTCACTGACTTACCACgGGGAAGGAGAGACACTCATG; R66G,
CTGACTTACCACAGGGAAGGAGgGACACTCATGCATGAATGTCC; E42G,
CACTGACTTACCACAGGGgAGGAGAGACACTCATGC; E67G,
CTGACTTACCAAGGGAAGGAGgGACACCATGCATGAATGTCC; E69G,
CTGACTTACCAAGGGAAGGAtgtACACCATGCATGAATGTCC; E69C,
CTGACTTACCAAGGGAAGGAGcGACACCATGCATGAATGTCC; E69A,
GAGACACTCATGCATGgATGTCCAGACTACATAAC; E74G,
GAGAAAGCAGCTGAGTGGGcGATCCATTTTGCTGGGCAG; E181A,
GAGATCCATTTTGCTGGGGcGCAAACAGAGTTTAAGATTCTC; Q187A

The lower case nucleotide(s) underlined was changed to generate the amino acid G or A or C. The sequences of all plasmids were verified.

2.2. BRET assay

BRET assays were carried out as previously reported [12]. Briefly, cells of 48 h transfection were washed twice with PBS and detached with 5 mM EDTA in PBS. Cells were then resuspended in BRET buffer (PBS containing 5.5 mM D-glucose, 0.9 mM $CaCl_2$, and 0.5 mM $MgCl_2$) and distributed in a 96-well microplate (50,000 cells per well). Luciferase substrate (Coelenterazine h) was added to a final concentration of 5 μM, and BRET readings were collected using a multi-detector plate reader, Mithra LB940 (Berthold Technologies, Bad Wildbad, Germany), allowing the sequential integration of the signals detected in the 485 nm and 530 nm wavelengths. The BRET ratio is defined as [(emission at 530 nm) − (emission 485 nm) Cf] / (emission at 485 nm), where Cf corresponds to (emission at 530 nm) / (emission at 485 nm) for the RL construct expressed alone in the same experiment. Total fluorescence (excitation at 485 nm and emission at 530 nm) and luminescence (emission at 400 nm) signals were also determined for all samples using Mithra LB940 (Berthold Technologies) to assess the total levels of expression of the YFP and RL fusion constructs. Saturation curves of nonlinear regression for one-site binding with 95% confidence interval were plotted with GraphPad Prism4 software (GraphPad Software, Inc., La Jolla, CA, USA).

2.3. Transient transfection assay

In addition to the normal HEK293 cells a stable HEK293 line stably expressing the long form of hPRLR [10] was also used in this study. These cells were transiently transfected with various plasmids using LIPOFECTAMINE 2000 (Invitrogen, NY, USA) according to the manufacture's protocol. Briefly, cells were seeded on a six-well plate at a density of 50,000 cells/mL and incubated overnight. Cells were then transfected with individual or combination of appropriate plasmids for 5 h followed by additional 48 hr incubation in a fresh 10% FBS RPMI medium. Cells were treated with human prolactin (hPrl) at a final concentration of 100 ng/mL for 30 min to analyze phosphorylation levels of Jak2 and Stat5 or for 16 h for β-casein promoter activity.

2.4. β-Casein gene promoter assay

Twenty four hours after transfection, cells were changed to a fresh DMEM medium without serum and treated with hPrl (100 ng/mL) for additional 24 h. Cells were washed twice with PBS and lysed with passive lysis buffer (Promega Madison, WI, USA). The lysate was cleared by centrifugation at 12,000 *g* and the supernatant was used for the measurement of luciferase and β-galactosidase activities using a luciferase reagent kit (Promega) with Mithras LB940 (Berthold Technologies).

2.5. Immunoblotting

Nitrocellulose membranes were blocked with 5% non-fat milk in wash buffer (135 mM NaCl, 10 mM Tris–HCl, pH 7.4, and 0.05% Tween 20) for 2 h and then incubated with rabbit anti-Jak2 (1:1000), rabbit anti-Stat5 (1:1000), anti-phospho(Tyr1007/1008)-Jak2 (1:1000), anti-phospho(Tyr694)-Stat5 (1:1000), rabbit anti-YFP (1:2000) (Cell Signaling Technology Inc., Danvers, MA, USA) or mouse anti-β-actin (1:5000) (Sigma-Aldrich, St. Louis, MO, USA) in wash buffer containing 3% non-fat milk overnight. The membranes were rinsed three times with wash buffer and then incubated with secondary horseradish peroxidase-conjugated goat anti-rabbit IgG (1:5000) (Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA), and goat anti-mouse IgG (1:5000) (Santa Cruz Biotechnology Inc.) in wash buffer containing 3% non-fat milk for 2 h. Membranes were rinsed three times with wash buffer and developed with the enhanced-chemiluminescence system (Pierce Chemical, Rockford, IL, USA).

2.6. Statistical analysis

The significance of the difference in the studies between groups of Prl-stimulated PRLR-mediated signaling and BRET saturation analyses of homo- and heterodimer formation between wild-type and mutated S1b variants was determined by Tukey's multiple-comparison test (one-way ANOVA).

2.7. Molecular modeling

The structure of the hPRLR monomer was obtained from the hPrl–hPRLR complex (PDB ID: 3d48). A number of residues near the C-terminus region of D2 which are missing in the crystal structure were modeled upon the D2 structure in the hGH–hPRLR complex

(1bp3) as described previously [10]. The template for the dimers is the rPRLR structure in the hPRL–rPRLR$_2$ complex (PDB ID: 3npz). Dimers were modeled by superimposing the D2 domains of two monomers upon the D2 domains of the template [10]. Point mutations were introduced directly into the sequence of the wild-type prior to the monomer simulations, and internal coordinates were generated with the CHARMM program. Molecular dynamics simulations were carried out on monomers and homodimers of SF. The simulations were performed at 37 °C and physiological pH. Friction coefficients of 50 1/ps were applied to all non-hydrogen atoms, and the SHAKE algorithm was used. To study long-range inter-domain electrostatic effects no cutoffs were used for the non-bonded interactions. The SCP solvent model [13] was used to represent electrostatic and hydrophobic effects of the aqueous solution. The system is described by the all-atom (param22) representation of the CHARMM force field [14] (version c36), with CMAP dihedral corrections [13,14]. The simulations of the monomers were extended up to 50 ns, and the dimers for 0.1 μs. These long simulations were needed for the system to undergo structural changes and reach stable structures.

3. Results

3.1. Functional importance of the short form S1b subdomain D1 for its inhibitory effect on prolactin induced transcription mediated by the long form of the receptor

To gain insights into the regions of the extracellular domain involved in the inhibitory action of the S1b, we explored the contribution of amino-acids residing close or away from the hormone binding pocket in the D1 and D2 domains of S1b to the inhibitory action of this SF variant. Our aim was to evaluate the impact of S1b mutants that while preserving their signaling function will cause loss of inhibitory activity on the function of LF induced by Prl. These included mutations suggested in previous simulation studies as potential inter-D1 and D2 hydrogen-bonded pairs that might contribute to S1b inhibitory function [10]. Residues in D1 were mutated to either glycine (G), alanine (A) or cysteine (C). Mutants R66G, E67G, E69G and E42G located at the surface of the hormone binding pocket abolished the normally observed inhibitory effect by the wild-type S1b on Prl-induced LF mediated action on β-casein gene promoter (Fig. 1). A similar effect was observed when E69 was mutated to either A or C. In contrast, the E74G mutant displayed a similar inhibitory activity as the wild type. This residue is also charged and exposed to the solvent, but is located on a β-sheet away from the surface of the Prl binding pocket (Fig. 1). Moreover, mutations of residues on D2 at the putative D2/D2 interface, either singly (E181A or Q187A) or combined (E181A/Q187A), displayed a similar inhibitory effect on the LF-mediated action as the wild type S1b. These results indicate the functional importance of the D1 domain of S1b inhibitory activity of the LF function induced by Prl. In contrast, a lack of involvement of the D2 domain was observed.

3.2. Effect of S1b D1 subdomain mutation on prolactin-stimulated Stat5 signaling mediated by LF

To learn about the impact of individual mutations in the D1 domain of S1b wild type or mutant on Stat5 phosphorylation mediated by LF/LF homodimer, that would directly affect the transcription of prolactin responsive genes, we evaluated Stat5 phosphorylation (pStat5) in HEK293 cells stably expressing LF with transiently co-transfected wild-type (Fig. 2A, lanes 3 & 4) or mutant [S1b(E69G)-. S1b(E42G)- or S1b(E74G)- or S1b(E181A)-YFP] (Fig. 2A, lanes 5–12). Nuclear Stat5 phosphorylation was highly induced by Prl in the controls expressing LF only (lane 2 vs lane 1). In cells co-transfected with wild-type S1b a marked reduction of pStat5 was observed upon Prl activation compared to the control group (Fig. 2A, lane 4 vs lane 2, Fig. 2B). However, a significant increase of pStat5 was observed upon cotransfection with S1b mutants (S1bE69G or E42G) over that of WT S1b (lanes 6 and 8 vs lane 4), and comparable to controls LF (Fig. 2A, lanes 6 and 8 vs lane 2, Fig. 2B). These findings are consistent with and reflective of our transcriptional studies (Fig. 1). The increase in Jak2/Stat5 phosphorylation after Prl treatment (Fig. 2A & B) supports the presence of active signaling and transcriptional activation and is consistent with the lack of inhibitory action observed by the indicated D1 mutants upon Prl-induced Stat5 dependent transcriptional activity mediated by LF (Fig. 1).

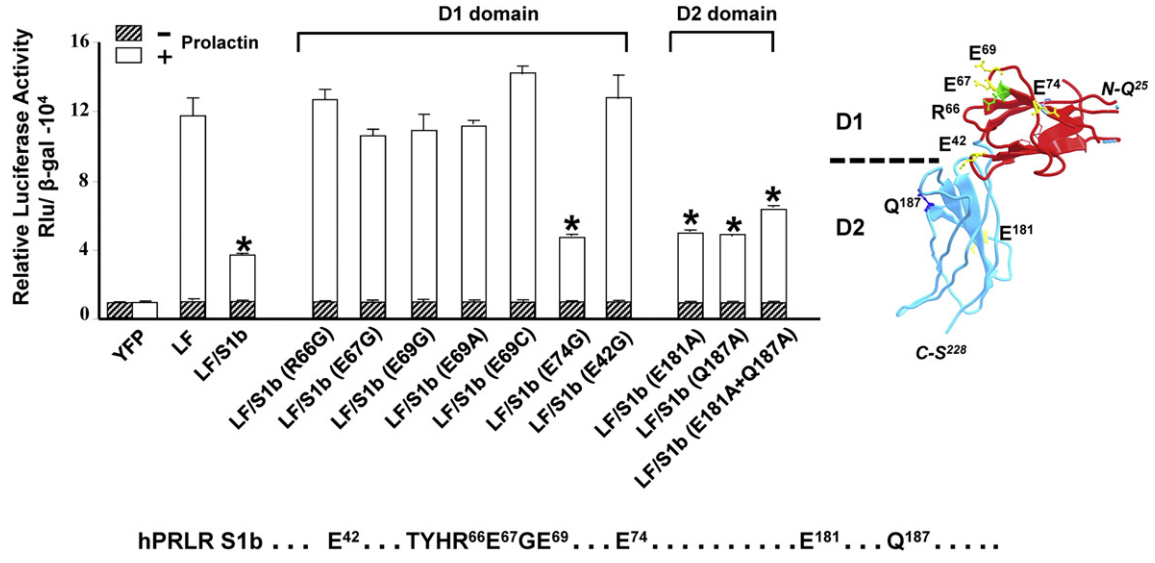


Fig. 1. Functional importance of the short form S1b subdomain D1 for its inhibitory effect on prolactin stimulated transcription mediated by the long form (LF) of the prolactin receptor. Luciferase reporter gene activity driven by β-casein promoter was measured in HEK293 cells cotransfected with hPRLR LF (0.1 μg) alone or alone or with short form (SF, 0.88 μg) wild type S1b or designated amino acid mutation in subdomain D1 or D2. β-Galactosidase plasmid (0.5 μg) was also included in the transfection and its activity was measured and used for normalization of the reporter activity. Cells were treated with human prolactin (100 ng) for 16 h before termination. Results are representative of at least three independent experiments, shown as a normalized relative luciferase activity (Rlu) in the presence (open bar) and absence (shaded bar) of prolactin. *: changes compared to the LF transfected group only with statistical significance ($p < 0.01$). Both LF and SFs are in frame with YFP. pEYFP-N1 empty vector was used for equalization of DNA transfection.

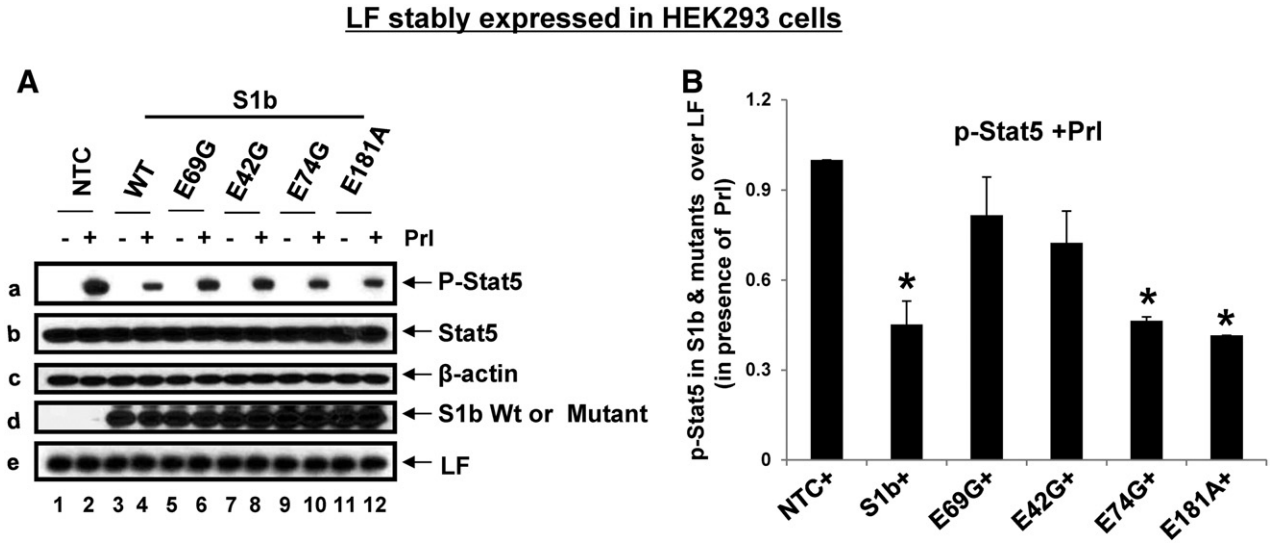


Fig. 2. Impact of S1b D1 subdomain mutation on prolactin stimulated LF mediated Stat5 signaling. HEK293 cells stably expressing LF were cotransfected with either YFP empty vector (as control group) or S1b wild type (S1b-YFP, 1 μg) or mutant (E42G, E69G, or E74G, or E181A 1 μg) and incubated in the presence (+) or absence (−) of prolactin (Prl) for 0.5 h. (A) Nuclear phospho-Stat5 (p-Stat5) and total Stat5 were determined by Western blot analyses. S1b and LF expression was determined using anti-YFP antibody. Results are representative of three independent experiments. (B) Western signals of p-Stat5 stimulated by prolactin were quantified and normalized by Stat5 from three independent experiments. The phosphorylated values are presented as fold-over the LF (control) induced by prolactin.

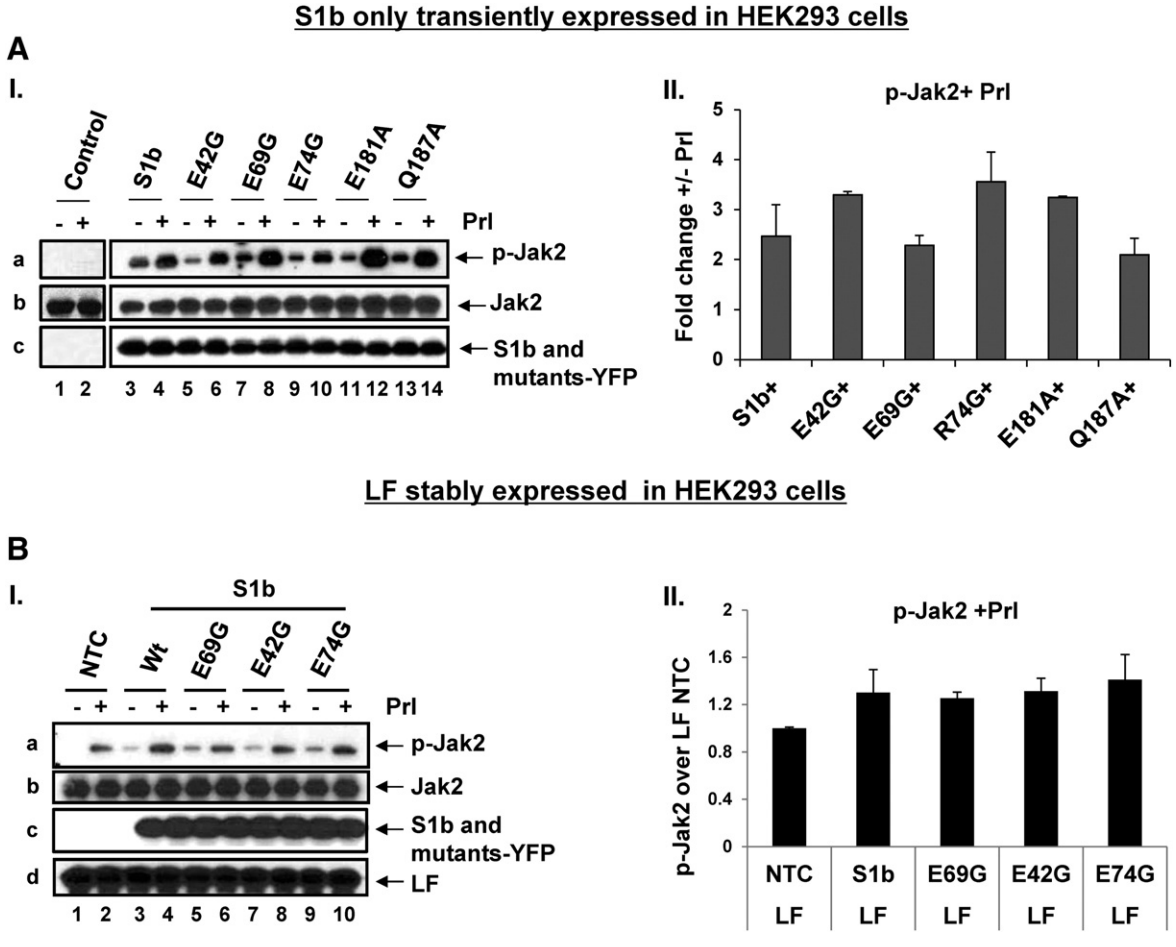


Fig. 3. Effect of S1b D1 subdomain mutation on prolactin-induced Jak2 signaling in HEK293 cells transiently expressing S1b and mutants (A) and in HEK293 cells stably expressing LF (B). Western blot analysis was performed in HEK293 cells only (A) or LF stably expressed HEK293 cells (B) cotransfected with wild type S1b or S1b mutant (1 μg) and incubated in the presence (+) or absence (−) of prolactin (Prl) for 0.5 h. NTC: non-S1b transfected cells transiently expressed with empty YFP vector Western analysis (A-I and B-I) were performed using anti-phospho-Jak2 for Jak2 phosphorylation (p-Jak2), anti-Jak2 for transfected Jak2 expression, and anti YFP for antibody for endogenous LF and transfected SF expression. Western signals of p-Jak2 stimulated by prolactin were quantified and normalized by Jak2 from three independent experiments. The phosphorylated level is presented as fold-change of prolactin stimulated cells over the control (A-II) or as fold-over the LF NTC by prolactin (B–II).

3.3. Basal and Prl-induced Jak2 signaling mediated by PRLR S1b and mutants transiently expressed in HEK293 cells and in HEK293 cells stably expressing LF

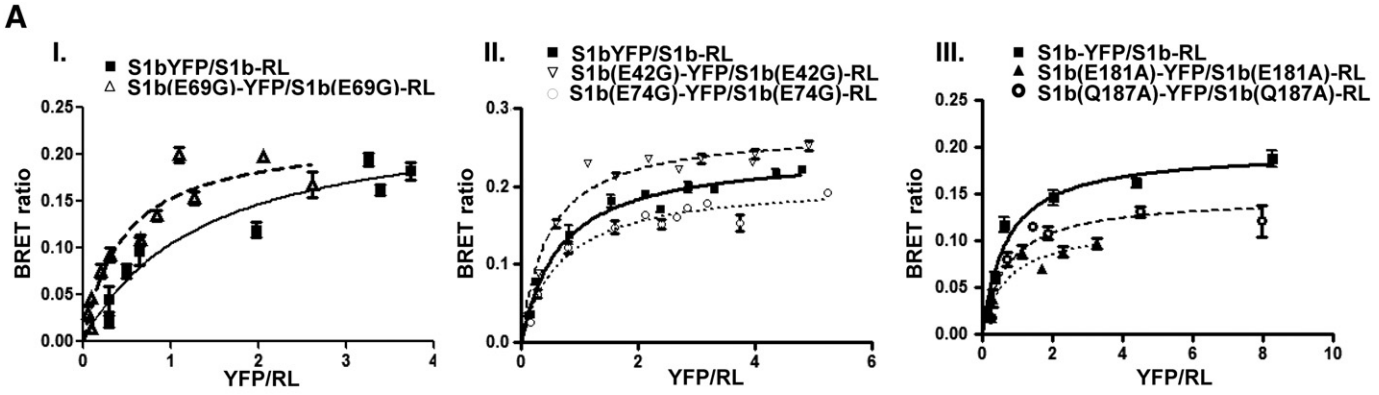
To elucidate whether the major reduction in Prl induced Stat5 phosphorylation caused by S1b resulted from changes in the upstream activation of Jak2 phosphorylation, cells either transiently expressing S1b (wild type or mutant) alone or cells stably expressing LF were examined. PRLR LF and SF are not expressed in HEK2 cells, thus this explains the lack of p-Jak2 in the control group of HEK293 cells either basally (Fig. 3A-I, lane 1) or in the presence of prolactin (Fig. 3A-I, lane 2). Constitutive Jak2 phosphorylation in the absence of Prl was observed in HEK293 cells, transiently expressing S1b wild type or mutant (Fig. 3A-I, lanes 3, 5, 7, 9, 11, 13). Similarly basal Jak2 phosphorylation levels were observed in HEK-293 cells stably expressing LF transiently transfected with S1b wild type and mutants (Fig. 3B-I lanes 3, 5, 7, 9). In contrast, basal Jak2 phosphorylation was absent in non-transfected cells (NTC, control) stably expressing LF (Fig. 3B, lanes 3, 5, 7, 9 vs lane 1). In HEK293 cells stably expressing LF (Fig. 3B) the degree of Prl-induced Jak2 phosphorylation was similar in cells transfected with wild-type S1b, mutants of S1b in the D1 domain (E42G, E69G, E74G), and in the D2 domain (E181A, E187A) (not shown) and in untransfected controls (LF only) (Fig. 3B, I &, II). Comparable results on Prl-induced Jak2 phosphorylation were also noted in cells transiently expressing S1b or mutant only (Fig. 3A, I &, II). This indicated that the D1 domain mutants which negated the S1b inhibitory activity (Fig. 1) preserved the signal transduction function induced by prolactin.

3.4. BRET saturation curve of dimerization of wild-type and mutated subdomain of S1b human prolactin receptor

Further studies were performed to investigate the molecular nature of the loss/reversal of the inhibitory function of the mutant associated with D1 domain. BRET saturation analyses were utilized to study physical parameters of homo-dimerization S1b-RL to S1b-YFP (Fig. 4), where S1b wild type was compared to the S1b mutants. The BRETmax was similar in S1b WT and E69G mutant in the homodimerization to its pair-RL (Fig. 4A-I and B-I). A lower value of $BRET_{50}$ was observed for homodimers of S1b(E69G) (Fig. 4A-I; and B-I) and S1b(E42G) (Fig. 4A-II; and B-II) but not the E74G mutant (Fig. 4A-II; and B-II) compared to wild type S1b. Analysis of the lysates from cells transfected with mutant S1b in non-denaturing gels, showed a significant increase in dimer formation of the mutant S1b E69G class compared to the WT S1b (not shown). Taken together these results indicated that the increased in affinity of S1 mutants favors their homodimerization and LF Prl-mediated action (Figs. 2 and 3). When similar experiments were performed with the D2 domain mutant, no significant change in the $BRET_{50}$ parameters of the wild-type S1b for its homodimerization was observed (Fig. 4A-III; and B-III). These results highlight the important functional role of the D1 subdomain of S1b on Prl-stimulated LF function.

3.5. Molecular modeling of the wild-type and mutants of hPRLR

Computer simulations were conducted to study the structural and dynamic effects of the mutations and to investigate the relationship



B

	Paired hPRLR	BRETmax	$BRET_{50}$
I.	S1b-YFP/S1b-RL	0.20 ± 0.01	0.93 ± 0.18[a]
	S1b(E69G)-YFP/S1b(E69G)-RL	0.22 ± 0.01	0.45 ± 0.08[a]
II.	S1b-YFP/S1b-RL	0.24 ± 0.007	0.66 ± 0.08[b]
	S1b(E42G)-YFP/S1b(E42G)-RL	0.27 ± 0.02	0.46 ± 0.05[b,c]
	S1b(E74G)-YFP/S1b(E74G)-RL	0.21 ± 0.007	0.67 ± 0.09[c]
III.	S1b-YFP/S1b-RL	0.20 ± 0.007	0.67 ± 0.09
	S1b(E181A)-YFP/S1b(E181A)-R	0.11 ± 0.013	0.68 ± 0.22
	S1b(Q187A)-YFP/S1b(Q187A)-RL	0.15 ± 0.011	0.70 ± 0.18

Fig. 4. BRET saturation curve of dimerization of wild-type and mutated D1/D2 domain of S1b human prolactin receptor. HEK293 cells were co-transfected with a constant wild type S1b-RL or mutated (E69G, or E42G, or E74G, or E181A or E187A) RL (0.2 μg) with increasing DNA concentrations of YFP corresponding fusion construct. BRET levels were plotted as a function of the ratio of the expression of the YFP construct (quantitated by the total fluorescence of the cells) over the RL construct (quantitated by the luminescence of the cells) (YFP/RL). A: Homodimer formation of short form of PRLR. Cells transiently expressing S1b wild-type (WT) or mutant (Mut) of E69G (I), E42G/E74G (II) and E181A/Q187A (III). B. Summary table of parameters derived from BRET saturation curves. The results are representative of three independent experiments carried out in triplicate. Identical index a or b indicates statistical significance between experimental groups ($p < 0.05$).

between the S1b receptor conformation and the functional role of individual amino acids on the inhibition of LF function. These included five mutations with effects in activity (R66G, E67G, E69C, E69G, and E42G) and three with WT-like activity (E74G, E181A and Q187A), as shown in Fig. 1. The major structural effects of mutations on the monomers are reorientations of D1 relative to D2, with only minor changes in the backbone conformations of these subdomains (C_α-rmsd ~1.5–2.5 Å with respect to the wild-type structure). Both subdomains are linked by a short unstructured segment, which is the weakest link in the EC domain of the receptor. Reorientations occur in various degrees depending on the specific position of the mutated residue (Fig. 5A), and originate in non-specific perturbations of the D1/D2 interface. Dimers formed upon the association of the restructured monomers undergo further conformational changes in the course of the simulations. Fig. 6A shows the snapshots of the WT and E69G structures at the end of the simulations, which are representative of the two major structural classes identified herein. The reorientations of the domains in these classes are illustrated schematically in Fig. 5B. At the start of each simulation the D1 subdomains are in close proximity in both cases, but they drift away from each other in the E69G dimer, while remain close to each other in the WT. Fig. 5C shows the angle ψ of the monomers in the course of the dynamics of the dimer, measured as orientation of the principal axis of inertia of D1 relative to the plane determined by the principal axes of the two D2 domains (see caption). In the WT-like class the angle changes in one direction to better align the D1 domains; in contrast, the movement of the angle in the E69G-like class is in the opposite direction and tends to position the D1 domains parallel to one another. The WT dimer seems to be stabilized electrostatically through a network of charged residues in the D1 subdomains and at the D1/D1 interface (Fig. 6B and C). Structural changes similar to those in the E69G mutant are observed in the R66G, E69C, and E42G mutants (not shown). Mutations of E181 and Q187 (both in D2) have little effects on the internal conformations of D2 (or D1) in the monomers, but they still affect the D1/D2 interface resulting in the reorientations of D1 relative to D2 (not shown). However, in contrast to mutations in D1, the simulations of these dimers show that the D1 subdomains remain in close proximity to one another, like in the WT. Finally, mutation of E74 (which unlike the other mutations in D1 is located away from the hormone binding pocket, hence from the network of inter-domain electrostatic interactions (Fig. 1). This mutation elicits the largest internal conformational changes in the D1 subdomain of the monomers, but the structure of the dimer was similar to that of the WT dimer, with both D1 subdomains remaining in close proximity over the course of the simulation .

To estimate the strength of the electrostatic interactions between monomers in the WT and mutant dimers dissociation energies were calculated [15] by gradual heating, using rigid-body Monte Carlo simulation [16] (Fig. 5D). The calculations show that interactions between WT-dimers are generally weaker than in the E69G-like dimers, although important differences in binding energies were observed within each of the groups. These calculations show that the EC domain of the receptor makes important contribution to the dimerization affinity and can be modified by introducing single mutations in critical regions of D1. The differences in dimerization strength revealed by this analysis are compatible with and may explain the data derived from BRET analysis.

4. Discussion

Our studies demonstrate the essential role of the D1 domain of the PRLR short form S1b inhibitory action on the LF-mediated function. A number of critical amino acids, including E42, T63, R66, E67 and E69 residing at the D1 surface close to the binding pocket were found to participate in the inhibitory effect of SF S1b. Individual amino acid

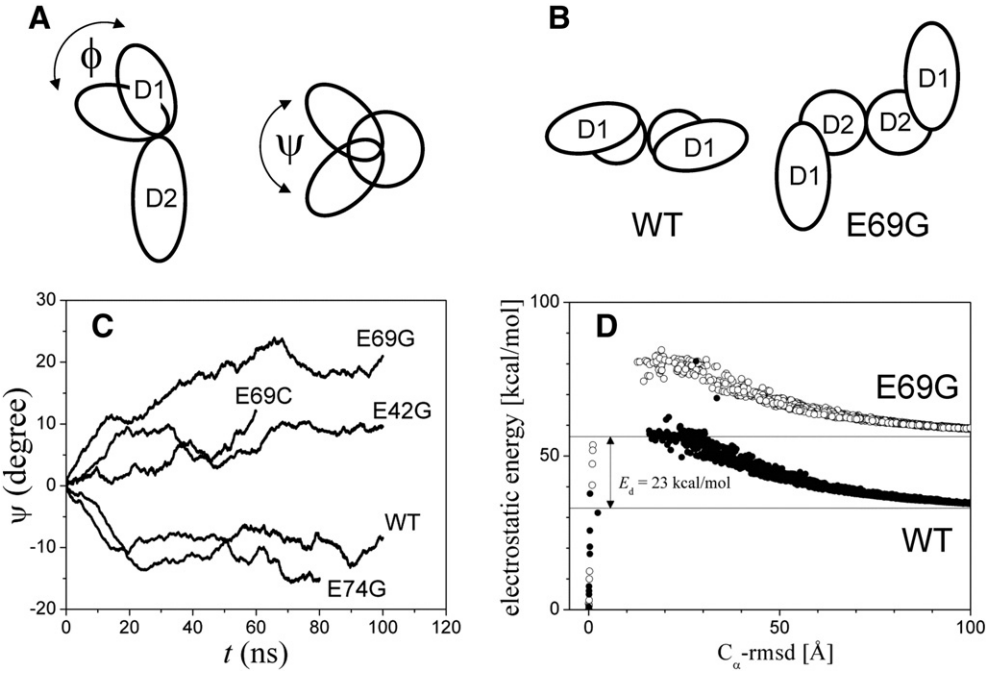


Fig. 5. Molecular modeling and dynamic simulations of hPRLR monomers and homodimers. (A) Schematic representation of changes in D1–D2 orientations upon mutations, as observed in molecular dynamics (MD) simulations of the monomers. Structural changes (indicated by arrows) are specific of each mutation and result from perturbations of the D1/D2 interface. (B) Schematic representation of the two major groups of homodimer conformations obtained upon the association of the restructured monomer, as observed in the molecular dynamics simulations. In one group (WT-like), the D1 subdomains become more aligned to one another, in the second group (E69G-like), the D1 domains adopt a more parallel conformation. (C) Time evolution of the angle ψ in the dimer dynamics. The angle is measured with respect to the D1 orientation at the beginning of the dimer simulation, and its sign is arbitrary to indicate the direction of movement. The angle is determined by the principal axis of inertia of D1 (indicated by a unit vector d_{11}, for one of the monomers, and d_{12} for the second monomer) and the principal axes of inertia of D2 in both monomers (indicated by unit vectors d_{21} and d_{22} in each of the two monomers). Conventional vector products $d_1 \times d_{21}$ and $d_{21} \times d_{22}$ are used to define the corresponding plane orientations. (D) Dissociation energy calculated by gradual heating, using rigid-body Monte Carlo simulations with the united-atom (param19) representation of the CHARMM force field and the SCP continuum solvent model [13]. The bound states are taken from the last snapshot of the simulations shown in Fig. 6A. The reaction coordinate is the C_α-rmsd with respect to the bound structure. The plot shows that dimerization energies can be affected by mutation in D1, mainly due to the conformational changes that propagates to the D2/D2 dimer interface.

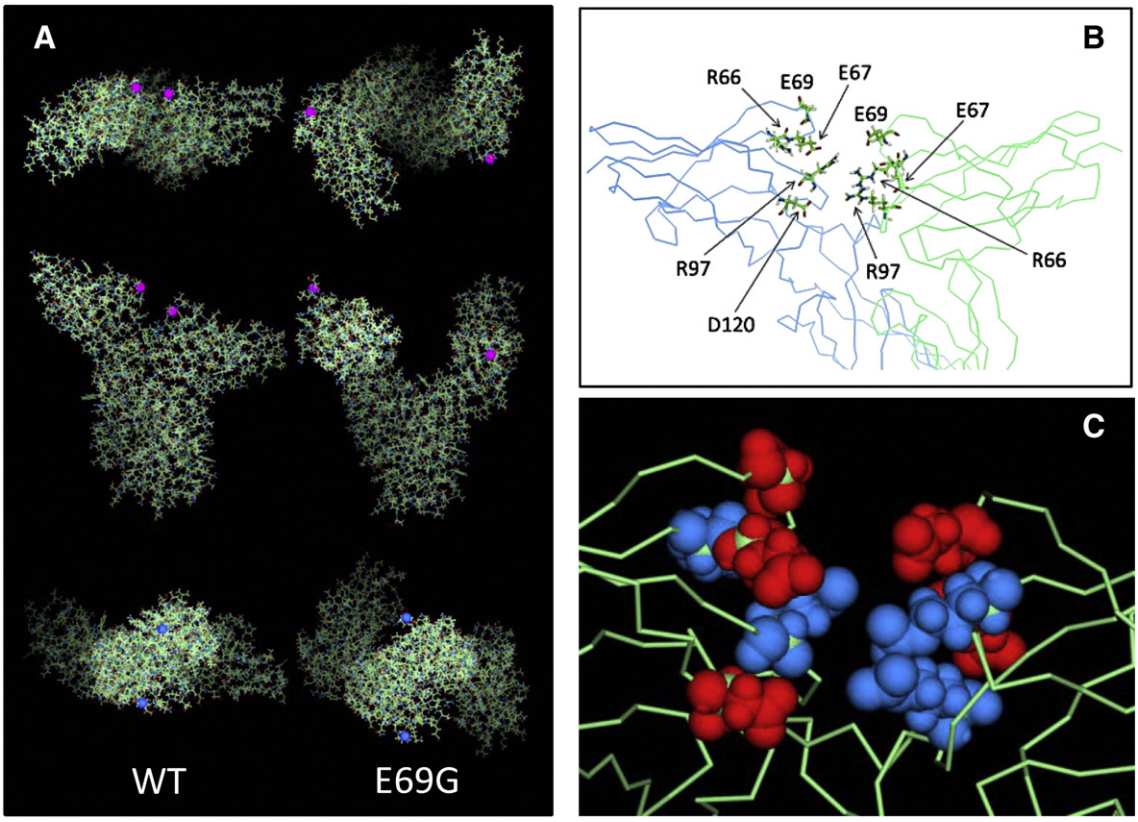


Fig. 6. (A) Atomistic representations (snapshots at the end of the simulations) of the two major structural groups, illustrated here for WT and the E69G mutant: view from the extracellular region (upper panel; purple spheres indicate C_α atoms of E69 and G69); side view (middle panel; cell membrane at the bottom, not shown); view from the membrane side (lower panel; blue spheres are C_α atoms of the C-terminal residue and illustrate the restructuring of the D2/D2 interface triggered by the D1 reorientations). (B) Network of charged amino acids at the D1/D1 interface in the WT homodimer taken from the same snapshot in (A). WT-like dimers appear to be stabilized by D1–D1 electrostatic interactions with no hydrogen are observed at the D1/D1 interface. Perturbation of this network upon mutation may explain the conformational changes of the E69G-like group. (C) Detail of the D1/D1 interface in (D) showing the network of charged residues (blue: positive; red: negative) in a van der Waals representation.

mutations abolished the inhibitory action S1b on LF function. This seems to result from an increase affinity of the mutated S1b favoring homodimerization (S1bX:S1bX) that would lead to Prl induced LF mediated signaling through LF:LF dimer formation. Computer simulations show that these mutations in D1 have a major impact on the structure of the dimer required for the inhibitory action of the short form. In contrast, mutation E74G preserves the SF inhibitory function. Based on the crystal structure of the hGH–hPRLR monomer complex (1bpr) all mutated residues in the present study are located close to the hormone binding pocket, except E74 which is on the D strand of a β-sheet, away from the Prl binding cleft. This led us to propose that a designated aa location in S1b adjacent to or away from the hormone binding pocket might impact differently on LF, and for reasons unrelated to the presence of the hormone. However, all of these S1b mutants revealed comparable prolactin-induced Jak2 signaling as the wild type, indicating preservation of a functional PRLR in all the mutants. The difference between E74G to other mutants must result from its structural configuration favoring heterodimerization (S1bX(E74G):LF), as is the case for S1b WT, (S1b WT:LF), see below. In the case of the D2 domain, mutation of either E181 or Q187 showed a similar inhibitory action to wild-type S1b. This domain of the receptor is known as the dimerization platform in the extracellular region of the receptor. WS motifs (215–219) in the D2 domain are required for the correct PRLR folding [17,18]. It is interesting to note that mutations of two residues (Gln 187 and Glu 181) at the D2/D2 interface previously predicted to form H-bonding between the D2 domains of the S1b homodimers [10] did not alter the inhibitory function in the present study. These results clearly portrait the vulnerability of the D1 but not D2 sub-domain in terms of maintaining the integrity of the S1b structure in its inhibitory activity on prolactin-stimulated LF function.

A constitutive expression of Jak2 phosphorylation is present in all the short forms expressed in HEK293 cells (Fig. 3A-I.) This is contrast with cells expressing LF only where JAK2 phosphorylation is not observed in the absence of Prl (Fig. 3B-I.). Both LF and SF forms contain the Box-1 region immediately adjacent to the membrane in the cytoplasmic domain (proline rich) which is the docking site for Jak2 association. We postulate that the differential basal Jak2 phosphorylation observed in our studies could be related to the length of cytoplasmic domain of the forms. The short-length of the cytoplasmic domain in S1b would facilitate basal Jak2 phosphorylation at Tyr residues in the activation loop via auto or transphosphorylation. Jak2 engagement with S1b could induce partial relaxation of the catalytic domain from its inhibition by the pseudokinase and result in basal activation. Further relaxation caused by the hormonal stimulus would cause additional Jak2 phosphorylation. In the case of LF it is conceivable that the extended length of the cytoplasm domain would impact the Box-1 conformation and prevent basal phosphorylation.

When we examined the downstream Stat5 signaling in the LF stably expressing cells cotransfected with S1b or its mutants, Prl-stimulated phosphorylated Stat5, essential for its transcriptional activation of a relevant Prl/PRLR responsive gene promoter (β casein), was significantly inhibited in cells cotransfected with wild-type S1b and E74G mutants but not others. This clearly supports the presence of active LF signaling and transcriptional activation upon cotransfection of the mutants residing at the surface of the pocket and consistent with their lack of inhibitory function upon prolactin-induced LF-mediated action. It is interesting to observe no change in the upstream activation Jak2 phosphorylation induced by Prl in any of S1b mutants when compared to the wild type. However Stat5-p level was clearly correlated with the change in the promoter activity differentially regulated by S1b mutants. Stat5-

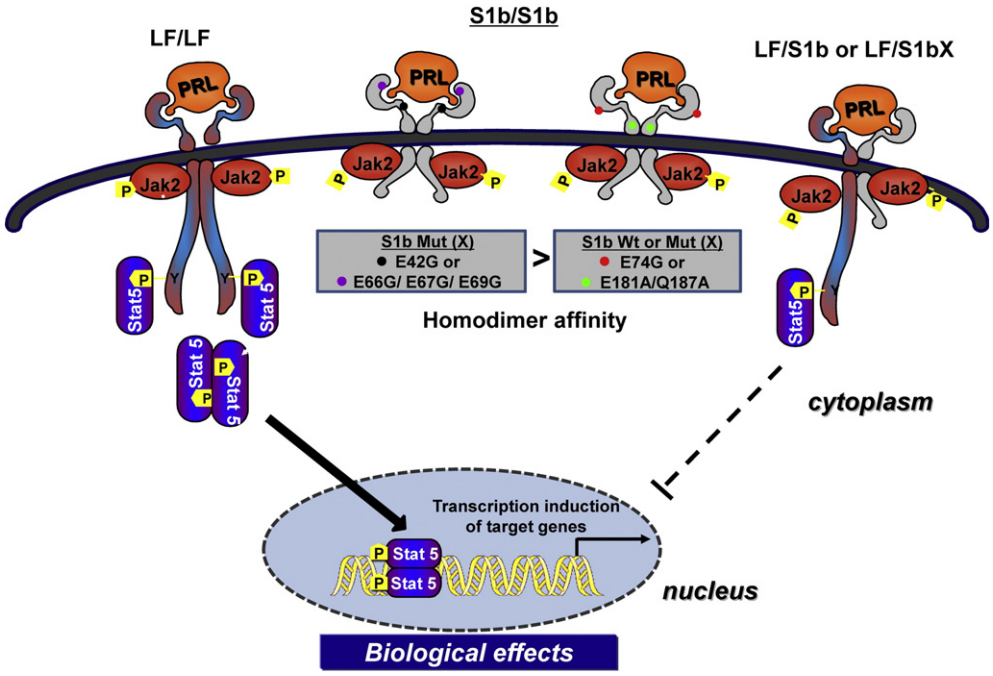


Fig. 7. Proposed model: Mechanism of inhibitory action of short form S1b PRLR on prolactin-induced long-from (LF) receptor signaling. Homodimer of the hPRLR long form (LF) mediates prolactin stimulated Jak2/Stat5 signaling required for transcription/expression Prl target genes which are essential for the various biological effects of the hormone. The inhibitory action of short form (SF) S1b on LF's function induced by Prl results from LF/SF heterodimer formation and marked reduction of LF/LF homodimers which are required by Stat5 activation. Prl induces Jak2 phosphorylation (Jak2-p) via LF/LF, LF/SF and SF/SF dimers due to the presence of Box 1 (anchor of Jak2) in all forms. For signaling to proceed, the distal activation of Stat5 (Stat5-p), essential for transcriptional induction of Prl responsive genes, LF/LF dimers are required. Mutation (X) of amino acids located on the D1 domain adjacent to the hormone binding domain (black and purple dots) reverses the inhibitory action of the wild type S1b while other mutants located away from hormone binding pocket (red dot) and in the D2 domain (green dot) retained the inhibitory function as the wild type S1b. The loss of the inhibitory S1bX results from differences in the affinity of homodimerization between the S1b wild type and single aa mutants residing close to binding pocket. Higher affinity observed in E42G/E66G/E67G/E69G compared to S1b wild type and E74G/E181A/Q187A leads to LF/LF homodimerization with intact Jak2/Stat5 signaling. The inhibitory mode of S1b [WT] or with mutations Mut (X) away from the binding pocket (E74G) and in the D2 domain (E181A/Q187A]; and the non-inhibitory mode of S1b (indicated MutX: E42G or E66G/E67G/E69G adjacent to the pocket) were found by modeling and simulation studies to relate to conformational changes that propagate to the D1/D2 interface and to the relative change of the orientation of the dimers.

phosphorylation is distal in the downstream cascade of Jak2 activation and the most C-terminal tyrosine at 580 aa in the cytoplasmic tail is required to phosphorylate Stat5 which is only present in LF. The tyrosine residue on both LF monomers of the dimerized PRLR is required for a fully functional PRLR receptor activation of Prl-responsive target genes [19]. LF/SF with only a single cytoplasmic tail does not provide the efficient docking site for Stat5 phosphorylation. However, the presence of box 1 for Jak2 docking/activation remains intact in homo LF/LF, LF/SF and SF/SF. This explains the observed constant level of Jak2-p and the reduced Stat5 in WT and certain mutants with asymmetrical dimerization.

We have previously demonstrated a ligand-independent homo- and heterodimerization of human PRLR [9]. Prolactin binds to the pre-existing dimers to activate tyrosine phosphorylation of the receptor associated Jak2 and signaling. Our results clearly present the evidence that the loss or recovery of inhibitory action for individual mutants is likely caused by the nature of the dimerization process among variants since the PRLR preserves the PRL induced signal transduction (Fig. 7). BRET analysis demonstrated that mutation of individual residues affects the affinity of dimer formation between PRLR forms. In contrast to E74G or S1b, the loss of the inhibitory action of E69G-like mutants results from the formation of high-affinity S1b homodimers favoring the formation of functional LF homodimers fully responsive to the hormonal stimulus. The inhibitory action of short form S1b on LF thus is caused from heterodimerization of LF:S1b which abolishes the LF mediated distal Stat5 signaling and PRLR mediated transcription induced by Prl (Figs. 1 & 2). This results from the lack of an extended cytoplasmic domain in the S1b partner of LF in the heterodimer.

Computer simulations were carried out to investigate the effects of mutations on the structure and dynamics of hPRLR monomers and dimers. By comparing the different simulations a general picture of the behavior of hPRLR upon mutation emerged, the orientation of D1 relative to D2 in the monomer changes in various degrees depending on the particular residue mutated, mainly as a result of perturbations of the D1/D2 interface. Upon association, the restructured monomers trigger specific structural changes in the dimer. For the mutations studied here the homodimer conformations can be grouped into two main classes: that with the D1 domains aligned and in relatively close proximity (WT-like mutants, Fig. 5B and C); and that with the D1 domains anti-parallel to each other (E69G-like mutants). These two classes are characterized by different dimerization affinities, which may lead to different populations of LF/LF, LF/SF and SF/SF in the cell.

In the WT dimer the D1/D1 interface is electrostatically stabilized by a network of charged residues that includes R66 and E69 (Fig. 6B and C) among other ionizable groups. Mutations of any of these residues by nonpolar side chains perturb the delicate balance of electrostatic forces, which appears to be the destabilizing factor of the D1 subdomains in the E69G-like dimers. Furthermore, the dimerization energies are affected by these mutations due to conformational changes that propagate downward to the D2/D2 dimer interface. Despite their close proximity in the WT-like dimers, the D1 domains are not H-bonded to one another. In the hPrl–rPRLR$_2$ complex (PDB ID: 3npz) a number of amino acids in the receptor interact with the hormone. In the hPRLR$_2$ model several of these residues are accessible to the solvent in both WT and mutants and thus readily available for interaction with sites 1 and 2 of the hormone. In the WT-like dimers binding requires a slight opening of the pocket, whereas in the E69G-like dimers requires closing. In either case the changes are likely induced by the hormone itself during association, possibly through an induced-fit mechanism [20,21]. The functional binding does not seem to be altered in either case, which is consistent with and demonstrated by the observation that Prl-induced Jack2 activation similarly in WT and mutants (Fig. 3).

In this study we have demonstrated the essential role of the D1 subdomain on the inhibitory action of the hPRLR short form S1b on Prl-induced function of the long form of the receptor. Our findings that the affinity of the prolactin receptor is highly sensitive to single mutations in D1 combined to the simulation results that major restructuring of the EC domain may be responsible for these changes, suggest that allostery may be exploited in rational structure-based drug-design strategies [22], in contrast to orthosteric-based inhibitory approaches most commonly used to date [23]. Because the targeted amino acids are accessible to the solvent, hence to small organic molecules, this approach may reveal critical sites of intervention for drug design and therapy. More specifically, design and screening of libraries of small molecules (peptides/non-peptides) that may perturb either the electrostatic interactions between the D1 domains directly or the orientation of D1 relative to D2 indirectly (by affecting the monomers D1/D2 interfaces) could change the dimerization energy and the relative populations of homo- and heterodimers in the cell. This could be conveniently detected by readouts of the end-points determined in these studies or using other approaches, such as assessment of cell apoptosis, growth rate, survival and migration. In particular, promoting heterodimerization could yield therapeutic agent(s) for use in treatment of advanced breast cancer. This could also be applicable to other tumors, such as in prostate's, where endogenous Prl-mediated PRLR/Stat 5 activation has been correlated with high tumor grade and aggressive course of the disease [24], and ovarian cancer, where prolactin-induced increases in survival and migration of ovarian cancer cells were effectively inhibited by S1b [25]. Such compounds could also be used in cases of resistance to endocrine therapies in breast cancer, where in an autocrine fashion endogenous prolactin in the absence of estrogen could contribute to fuel tumor growth and metastasis.

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