RAPID COMMUNICATION

Prolactin stimulates ubiquitination, initial internalization, and degradation of its receptor via catalytic activation of Janus kinase 2

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Abstract

Prolactin (PRL) activates its receptor to initiate signal transduction pathways (including activation of Janus kinases, Jak) but also stimulates downregulation of this receptor to limit the magnitude and duration of signaling. Degradation of the long form of PRL receptor (PRLr) depends on its phosphorylation on Ser349 that is required to facilitate PRLr ubiquitination. Signaling events that mediate PRL-induced degradation of PRLr remain to be elucidated. Here, we investigated the role of Jak2 activity in ligand-triggered increase of PRLr phosphorylation on Ser349, PRLr ubiquitination, endocytosis, and degradation. Using Jak2

reconstitution in Jak2-null cells as well as pharmacologic approaches, we found that treatment with PRL (but not with PRLr antagonist) promotes phosphorylation of PRLr on Ser349 and accelerates endocytosis of PRLr. Furthermore, PRL-stimulated PRLr phosphorylation, endocytosis, and degradation in Jak2-null cells reconstituted with wild type but not with catalytically inactive Jak2. We discuss how Jak2-mediated signaling might be transduced into Ser349 phosphorylation of PRLr as well as its ubiquitination and endocytosis.

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Introduction

The prolactin receptor (PRLr) is a member of the cytokine receptor superfamily that transduces the signals of the pituitary hormone PRL, which is implicated in numerous biological functions (including mammary gland development and lactation) and plays an important role in mammary tumorigenesis (Bole-Feysot *et al.* 1998, Clevenger *et al.* 1998, 2003, Goffin *et al.* 2002, Vera-Lastra *et al.* 2002). The fine-tuning of PRL signaling is achieved by balancing/counter-balancing PRL-generated signals with concomitant down-modulation of PRLr, which limits availability of the receptor on the surface to bind PRL (Djiane *et al.* 1979, 1981). This mechanism plays a pivotal role in ligand-specific negative control of PRL signaling (Clevenger *et al.* 2003).

An existing paradigm for mechanisms that govern down-regulation of signaling receptors suggests that activated signaling leads to receptor ubiquitination that mediates efficient endocytosis and lysosomal degradation of the receptors (reviewed in Hicke (2001). Negative regulation of PRL signaling indeed involves phosphorylation of PRLr on Ser349 and recruitment of β -transducin repeats-containing protein (β Trcp)-containing E3 ubiquitin ligase that facilitates ubiquitination and subsequent degradation of PRLr (Li *et al.* 2004). This mode of negative regulation has also been observed for other cytokine receptors including erythropoietin receptor (EpoR; Meyer *et al.* 2007) and Type I interferon receptor subunit (IFNAR1; Kumar *et al.* 2003, 2004). Activation of Janus kinase 2 (Jak2) is a proximal event, which occurs following PRL stimulation and plays a vital role

in PRL-induced signaling (reviewed in (Clevenger & Kline 2001)). Besides activating canonical Jak–Stat pathway, PRL also activates other signaling kinases such as mitogen-activated protein kinase, Src, and phosphoinositol 3-kinase. However, role of these pathways in PRLr ubiquitination, endocytosis, and degradation is yet to be characterized.

In this study, we report that Jak2 catalytic activation is required for PRL-induced increase in PRLr phosphorylation on Ser349 and ensuing PRLr ubiquitination. We also found that kinase activity of Jak2 contributes to the regulation of PRLr endocytosis and degradation. Furthermore, data obtained using a PRLr that lacks intracellular tyrosine residues indicate that phosphorylation of these residues might play a role in transducing the signal from the ligand to PRLr phosphorylation on Ser349 and PRLr proteolysis.

Materials and Methods

Cell culture and plasmids

Human embryo kidney 293T cells, Jak2-null γ 2A cells (Kohlhuber *et al.* 1997), and their derivatives were maintained and transfected as described elsewhere (Li *et al.* 2004, Deng *et al.* 2007). Plasmids for expression of flag-tagged (Li *et al.* 2004) or V5-tagged (Miller *et al.* 2005) PRLr, as well as JAK2^{WT} or JAK2^{K882D} (Huang *et al.* 2001) were previously described. Human PRLr with a hemagglutinin (HA)-tag inserted after the signal sequence at the N-terminus between amino acids Q25 and L26 was constructed by the overlap PCR method using the following primers: 5'-GGCAGTACATCAATGGGCG-TGG-3', 5'-ACTCACGGGGATTCCAAGTCTCCAC-CC-3', 5'-ATAGTCAGGTACATCGTATGGGTACTGTCCATTCAGAAGGCAGG-3', 5'-CCATACGATGTACCTGACTATGCATTACCTCCTGGAAAACCTGAG-3', 5'-GATGCATGCTCGAGCGTGAAAGGAGTGTGTAA-AACATGCG-3', and 5'-CTGAGAATCTTAAACTCTGTTTGCTGCC-3'. The overlap PCR products were subjected to restriction enzyme digestion with KpnI and AgeI followed by sub-cloning into similarly digested pCDNA3-PRLr^{WT}. All the plasmid constructs were verified by sequencing.

Reagents, antibodies, and immunotechniques

Antibodies against FLAG tag (M2, Sigma), V5 tag (Invitrogen), β -actin (Sigma), ubiquitin (FK2, Biomol, Plymouth Meeting, PA, USA), pSTAT5, STAT5 (Cell Signaling, Danver, CA, USA), Jak2, anti-phospho-tyrosine (Upstate Biotech, Lake Placid, NY, USA), and PRLr (Zymed, San Francisco, CA, USA and Santa Cruz, Santa Cruz, CA, USA) were purchased. PhosphoSer349 PRLr antibody has been described previously (Li *et al.* 2006). Human PRL was purchased from the National Hormone and Peptide program (A F Parlow). PRLr antagonist PRL ^{Δ 1–9-G129R} was produced and purified as previously described (Bernichtein *et al.* 2003). AG490 and PP1 (Calbiochem, San Diego, CA, USA) were purchased. *In vivo* ubiquitination analysis, immunoprecipitation, and

immunoblotting were performed as described previously (Li *et al.* 2004). For PRLr degradation assay, γ 2A cells were serum starved overnight followed by stimulation with 200 ng/ml PRL in the presence of 50 μ g/ml cycloheximide (CHX chase).

Fluorescence-based internalization assay

This assay measures the loss of cell-surface immunoreactivity of epitope tagged or endogenous receptors using an ELISA assay as described previously (Barriere *et al.* 2006) with the following modifications. Briefly, 293T or γ 2A cells transfected with HA-PRLr and other constructs as indicated were serum starved and chilled on ice. Internalization was initiated by incubation of cells with serum-free DMEM for indicated time periods at 37 °C and terminated by placing the plate on ice. Levels of cell-surface HA-PRLr were analyzed by ELISA using anti-HA primary antibody and horseradish peroxidase-conjugated goat anti-mouse secondary antibody (Molecular Probes, Eugene, OR, USA) followed by incubation with AmplexRed Ultra Reagent (Molecular Probes). Resulting fluorescence was measured (530 nm for excitation and 590 nm for emission) and expressed after subtracting the value obtained with mock-transfected cells as percentage of fluorescence registered prior to internalization. Average results of three independent experiments (each in quadruplicate $\pm$ S.E.M.) are depicted.

Surface biotinylation-based internalization assay

Cell-surface biotinylation that uses immunoblotting analysis to detect biotinylated proteins that were protected from de-biotinylation due to their internalization was carried out as described elsewhere (Hammond *et al.* 2003). Briefly, surface of cells that stably express PRLr-Flag and either wild-type or kinase-deficient Jak2 was biotinylated on ice. After that, cells were either kept on ice (time point '0') or incubated at 37 °C with PRL for 7.5 min. Then, cells were returned to ice and either left untreated (point '0*', 100% of biotinylation) or subjected to selective removal of cell-surface biotin. After numerous washes, cells were lysed and biotinylated proteins were recovered by incubating with immobilized NeutrAvidin (Pierce, Rockford, IL, USA) overnight, washed, and analyzed by immunoblotting with anti-Flag M2 antibody.

Results and discussion

Phosphorylation of PRLr on Ser349 is a key regulator of its ability to recruit the β Tcrp E3 ubiquitin ligase and to undergo ubiquitination-mediated degradation (Li *et al.* 2004). In human embryo kidney 293T cells, which respond to PRL while displaying relatively low basal levels of PRLr phosphorylation on Ser349 as well as PRLr levels (Li *et al.* 2004), we observed that treatment of these cells with PRL (but not with signaling-deficient PRL ^{Δ 1–9-G129R} mutant (Goffin *et al.* 2005)) increased the extent of phosphorylation of PRLr on Ser349 (Fig. 1A). The extent of this

phosphorylation was reduced by pretreatment of cells with Jak inhibitor (AG490, Fig. 1B) indicating that activity of Jak plays an important role in this pathway.

Although PRL is capable of activating other Jak including Jak1 (Neilson *et al.* 2007), Jak2 is the major Jak associated with PRLr (Rui *et al.* 1994). To delineate the role of Jak2 in PRLr serine phosphorylation, we employed a reconstitution system by stably expressing FLAG-tagged PRLr and either wild-type Jak2 (Jak2^{WT}) or kinase-deficient Jak2 mutant Jak2^{K882D} (Jak2^{KD}) in Jak2-null γ 2A fibrosarcoma cells (Kohlhuber *et al.* 1997). Clones with comparable expression of transfected proteins (Fig. 1C) were chosen for analysis. As

expected, cells expressing Jak2^{WT} (but not Jak2^{KD}) responded to PRL treatment with a robust increase in tyrosine phosphorylation of Stat5 (Fig. 1D) and PRLr (Fig. 1E). A modest but consistent induction of Ser349 phosphorylation (Fig. 1E) and PRLr ubiquitination (Fig. 1F) was observed only in the cells that expressed catalytically active Jak2. These data provide genetic evidence in support of a role for Jak2 kinase activity in PRLr Ser349 phosphorylation and PRLr ubiquitination.

Ubiquitination of cell-surface proteins can serve as an efficient endocytosis signal (Hicke 2001). We next analyzed the rate of PRLr endocytosis using a highly sensitive

fluorescence-based endocytosis assay that allows for the analysis of internalization of receptor itself (see Materials and Methods). PRL significantly increased the initial internalization rate of its receptor in 293T cells. PRLr antagonist PRL $^{\Delta 1-9, G129R}$ had no effect alone but reversed the

stimulation of PRLr internalization by wild-type PRL (Fig. 2A). These results indicate that PRL-induced signaling is likely to promote receptor internalization.

To determine the role of Jak2 kinase activity on PRLr endocytosis, we have pretreated 293T cells with either Jak

Figure 2 PRL signaling via Jak2 regulates initial internalization of PRLr. (A) Effect of PRL (open squares and open triangles) or PRLr antagonist (PRL $^{\Delta 1-9, G129R}$, closed and open triangles) on the initial rate of internalization of amino-terminally HA-tagged PRLr expressed in 293T cells measured by a fluorescent assay as described in Materials and Methods. (B) Effect of Jak inhibitor AG490 (50 μ M, diamonds) or Src inhibitor PP1 (10 μ M, squares) on the initial rate of internalization of PRLr in 293T cells in the presence (closed symbols) or absence (open symbols) of PRL (50 ng/ml) measured by a fluorescent assay. Inhibitors were added to cells 40 min before the internalization start. Controls are represented by treatment of cells with vehicle (DMSO, closed or open circles). (C) Effect of transient expression of Jak2^{WT} (circles) or Jak2^{KD} (squares) on the initial rate of internalization of PRLr in Y2A cells in the presence (closed symbols) or absence (open symbols) of PRL (50 ng/ml) measured by a fluorescent assay. Controls are represented by internalization of PRLr in cells that did not receive any Jak2 (closed or open diamonds circles). Right panel depicts immunoblotting analysis of Jak2 expression in these cells. (D) Internalization of Flag-tagged PRLr expressed in Y2A that harbor indicated Jak2 species was measured by reversible surface biotinylation assay. Neutravidin beads pull downs were analyzed by immunoblotting using anti-Flag antibody. Appearance of Flag-tagged PRLr at time points after zero indicates the levels of internalized PRLr species that were protected from removal of biotin label from cell surface. '0*' denotes samples that did not undergo biotin removal ('100% biotinylation'). 'NS', nonspecific band.

inhibitor AG490 or potent Src inhibitor PP1 and then measured PRLr internalization in the presence or absence of the ligand. While neither of inhibitors affected basal PRLr internalization, the ligand-stimulated endocytosis of PRLr was impaired in cells treated with AG490 but not with Src inhibitor (Fig. 2B). Furthermore, analysis of PRLr internalization in γ 2A-derived cells revealed that, while re-expression of wild-type kinase significantly increased PRLr internalization, this effect was not observed in cells expressing inactive Jak2 (Fig. 2C). Given that inactive Jak2 is yet implicated in maturation and cell-surface delivery of PRLr and EpoR (Huang *et al.* 2001), these data also suggest that, unlike for receptor maturation, Jak2 role in internalization does require its protein kinase activity. Similar results were seen in stable clones using reversible surface biotinylation assay (Fig. 2D). Together, these data suggest that catalytic activity of Jak2 is required for ligand-facilitated acceleration of initial rate of PRLr internalization.

Given that endocytosis of PRLr might ultimately lead to its degradation, we assessed the rate of PRLr proteolysis using a CHX chase assay in the presence of PRL. This assay was validated by comparing the rates of degradation between wild-type PRLr and its stabilized mutant that lacks Ser349 and thus is unable to recruit β Trcp and to undergo efficient ubiquitination (Li *et al.* 2004). As seen in Fig. 3A, PRLr^{S349A} mutant exhibited much greater stability than PRLr^{WT} in γ 2A cells that express catalytically active Jak2. Remarkably, degradation of PRLr^{WT} was noticeably delayed in Jak2^{KD} cells. Together, these data suggest that Jak2 catalytic activity is required for efficient ubiquitination, internalization, and degradation of PRLr.

Jak2 activation in response to PRL results in tyrosine phosphorylation of PRLr. To investigate whether tyrosine phosphorylation of PRLr by Jak2 affects Ser349 phosphorylation of PRLr and its degradation we established γ 2A cell lines stably expressing Jak2^{WT} and either wild-type PRLr

Figure 3 Rapid degradation of PRLr in response to PRL requires Jak2 catalytic activity and PRLr tyrosine residues. (A) Degradation of Flag-PRLr (wild type, closed symbols, or S349A mutant, open circle) in γ 2A cells expressing either wild type (circles) or catalytically inactive (squares) Jak2 species in the presence of PRL (200 ng/ml) was analyzed by cycloheximide chase followed by immunoblotting analysis using anti-Flag (upper panels) antibody to detect exogenous PRLr or using anti- β -actin antibody (lower panels) as a loading control. Symbols correspond to graphic depiction of the results (shown in the right panel) that reflects the quantified PRLr levels calculated as a % of receptor remaining compared with time point '0' (100%). A representative of three independent experiments is shown. (B) Phosphorylation of PRLr on Ser349 (upper panel) or on Tyr residues (middle panel) in γ 2A cells expressing Jak2^{WT} and PRLr as indicated. Cells were treated with PRL (200 ng/ml for 25 min) before harvesting. Lysates were analyzed by immunoprecipitation of V5-tagged PRLr using anti-V5 antibody followed by immunoblotting using indicated phospho-specific antibodies. Levels of Jak2 in whole cell extracts ('WCE') were also analyzed by immunoblotting (lower panel). (C) Degradation of PRLr and its mutants was assessed by cycloheximide chase (as in panel A) followed by immunoblotting analyses of PRLr using anti-V5 antibody (upper panels) and of actin (as a loading control, lower panels) as indicated. Symbols correspond to the graphical depiction of the results shown in the right panel (calculated as in panel A). A representative of three independent experiments is shown.

(PRLr^{WT}) or PRLr mutants that harbor Tyr to Phe substitutions at all intracellular tyrosine positions (PRLr^{YF}). These proteins were immunopurified from PRL-treated cells and analyzed by anti-phosphotyrosine and anti-Ser349 antibodies. Remarkably, not only Tyr phosphorylation but also Ser349 phosphorylation was nearly abolished in PRLr^{YF} (Fig. 3B). Furthermore, this mutant exhibited a longer half life in CHX chase assay (Fig. 3C). These results suggest that tyrosine phosphorylation of intracellular domain of PRLr plays an important role in controlling the rate of its Ser349 phosphorylation and degradation.

The current study is the first to identify Jak2 tyrosine kinase as an important modulator of PRLr stability. The pharmacologic and genetic evidence suggests that PRL-induced activation of Jak2 promotes phosphorylation of Ser349 within the phosphodegron of PRLr as well as PRLr ubiquitination, endocytosis, and degradation. These data are in line with a model for putative mechanisms by which PRL stimulates downregulation of its receptor. This model suggests that ligand-induced activation of Jak2 (and, perhaps, ensuing tyrosine phosphorylation of the intracellular tail of PRLr) facilitate the recruitment and/or activation of factors contributing to Ser349 phosphorylation such as a yet to be identified serine kinase (although Tyr-independent mechanisms of such kinase activation cannot be ruled out). A hypothetical serine kinase that is capable of phosphorylating PRLr on Ser349 should conceptually be characterized by its ability to be activated/recruited to PRLr in a Jak2-dependent manner. An expected outcome of such event would be an increase in PRLr Ser349 phosphorylation, which enables PRLr ubiquitination followed by acceleration of endocytosis and degradation of PRLr.

Our hypothesis is consistent with activation of Jak2 being perhaps the most immediate segment of PRLr signaling that is important for both mediating downstream signaling effects (including an activation of Stat proteins) and in restricting the magnitude and duration of this downstream signaling via accelerating the degradation of the receptor. It is plausible that such a mechanism is common for a number of cytokine and protein hormone receptors. Indeed, recent studies suggest that, in its requirement for Jak activation, PRLr is similar to several other receptors, whose signaling promotes their own degradation through associated Jak. In addition to Jak2 being a key player in the degradation of EpoR (Walrafen *et al.* 2005) and growth hormone receptor (Deng *et al.* 2007), activation of other associated Jak was shown to regulate ubiquitination and turnover of IFNAR1 and IL-5R (Marijanovic *et al.* 2006, Martinez-Moczygemba *et al.* 2007).

Given that Jak2 activity is required for maximal efficiency of both ubiquitination and endocytosis of PRLr, a detailed investigation of the role of PRLr ubiquitination in internalization and post-internalization sorting is warranted. However, considering a well-established role of SOCS proteins in ubiquitination and degradation of Jak (including Jak2 (Ali *et al.* 2003)), it might be also important to determine whether ubiquitination- or proteasome-dependent degradation of Jak2

might indirectly affect internalization (and, thus, lysosomal degradation) of PRLr. Indeed, recently the role of SOCS3 in lysosomal turnover of the G-CSF receptor has been demonstrated (Irlandoust *et al.* 2007). Furthermore, an additional role of other PRL-induced pathways in PRLr endocytosis cannot be ruled out given that Src inhibitor impeded endocytosis of radiolabeled PRL in COS cells overexpressing bovine PRLr (Lu *et al.* 2002). It is plausible that a second, Src-dependent pathway might be responsible for downregulation of PRLr in Ser349 phosphorylation- and ubiquitination-independent manner. In this regard, it is worth noting that c-Src can be activated by PRL independent of Jak2 in chicken embryo fibroblasts (Fresno Vara *et al.* 2000). These pathways might either synergize to facilitate optimal/efficient PRLr downregulation or might differentially contribute to PRLr endocytosis and degradation in a cell-type-specific manner. Future studies are underway to characterize both pathways and determination of the role of PRLr ubiquitination in these mechanisms.

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