Proteasomes Mediate Prolactin-induced Receptor Down-regulation and Fragment Generation in Breast Cancer Cells*

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Prolactin regulates a variety of physiological processes, including mammary gland growth and differentiation, and recent findings support an important role in breast cancer development and progression. However, little is known about the trafficking of its receptor, a member of the cytokine receptor superfamily. In the present study, we examined the effect of ligand on the endogenous “long” isoform of the prolactin receptor in breast cancer cells. We found that prolactin caused rapid and prolonged down-regulation of this receptor. The prolactin-induced increase in degradation was blocked by inhibitors of both proteasomes and lysosomes. However, the ubiquitin-conjugating system was not required for internalization. Prolactin also resulted in the concomitant appearance of a cell-associated prolactin receptor fragment containing the extracellular domain. This latter process required proteasomal, but not metalloprotease, activity, distinguishing it from ectodomain “shedding” of other membrane receptors, which are secreted as binding proteins. The prolactin receptor fragment was labeled by surface biotinylation and independent of protein synthesis. Together, these data indicated that prolactin binding initiates limited proteasomal cleavage of its receptor, generating a cell-associated fragment containing the extracellular domain. Our findings described a new potential mediator of prolactin action and a novel mechanism whereby proteasomes modulate cellular processes.

The hormone/cytokine prolactin (PRL) is critical for development and differentiation of the mammary gland. It also participates in numerous other processes, including behavior, metabolism, immune function, and osteogenesis (for reviews, see Refs. 1 and 2). Although the kinase cascades and alterations in gene expression that are initiated by PRL binding to its receptors have received considerable study (for reviews, see Refs. 3 and 4), relatively little is known about the effect of ligand on subsequent receptor trafficking, an important determinant of cellular responsiveness. The actions of PRL are mediated by the PRL receptor (PRLR), a member of the class I cytokine receptor superfamily. Alternative splicing results in isoforms of differing cytoplasmic domains (5). However, the so-called “long” isoform (IPRLR), which transmits the most signals, is the best-studied. Ligand binding to the IPRLR initiates internalization (6–8), which is dynamin-dependent and is at least partially mediated by clathrin in a COS-7 cell model (7). Several cytoplasmic motifs distinct for the IPRLR (7) and short isoforms (7, 9) have been identified that are critical for this process. Although PRL has been observed to either up- or down-regulate its receptor in complex in vivo systems, exogenous PRL reduced receptor levels in more defined experimental systems in vitro, a process blocked by inhibitors of lysosomal function (for review, see Ref. 10). Recently, PRL was reported to increase PRLR interaction with SCFβ-TrCP E3 ubiquitin ligase, increasing PRLR ubiquitination and thereby destabilizing the receptor (11). However, the role of ubiquitination in ligand-stimulated PRLR endocytosis and proteasomes in receptor down-regulation was not examined.

For several membrane receptors, including the closely related growth hormone receptor (GHR), ligand binding has been shown to initiate proteolytic cleavage(s) of the receptor (for reviews, see Refs. 12–15). The resulting extracellular domain (ECD) is released into the extracellular milieu, where it competes for ligand binding to cell-associated receptors. In some cases, receptor fragments have been reported intracellularly, where they can potentially serve as signaling molecules (for reviews, see Refs. 13–15). Analogous events have not been reported for the PRLR, although PRL binding proteins in serum and milk have been observed (16–18), and a 62-kDa chromatin-associated protein that binds PRL was identified in Nb2 cells (19). The importance of these events in modulating signal transduction, surface receptor levels, and therefore cellular responsiveness, as well as generation of potential new mediators, underscores the need to understand these processes and their regulation and the basis of receptor/ligand/cell specificity.

In addition to its role in regulation of physiologic mammary function, PRL also plays an increasingly recognized role in the pathogenesis and progression of breast cancer (for reviews, see Refs. 4, 20, and 21). A majority of human tumors express the PRLR (22–24), and some studies have found higher PRLR levels in tumors than in adjacent normal tissue (25). Mammary epithelial cells are exposed to both circulating PRL as well as endogenously synthesized PRL, which is particularly prominent in primates (for review, see Ref. 4). This local synthesis complicates the study of ligand-induced responses in experimental in vitro systems and also increases PRLR expression (26), convoluting studies of membrane receptor trafficking subsequent to ligand binding. We have derived cell lines from MCF-7 breast cancer cells that are deficient in endogenous PRL production (27), permitting us to examine the effect of exogenous ligand on endogenously expressed IPRLR in clinically relevant target cells rather than transiently transfected model systems. Here we have demonstrated that proteasomal activity is critical for the PRLR-initiated dramatic IPRLR down-regulation and generation of a PRLR fragment containing the ECD, although the ubiquitin-conjugating system is not
required for internalization. This fragment was derived from surface iPRLR and was not dependent on translation, consistent with post-internalization processing of the iPRLR. Our findings showed the importance of the proteasomal system in mediating ligand effects on the iPRLR and suggest a novel mechanism for PRL signaling.

**EXPERIMENTAL PROCEDURES**

Reagents—Recombinant human PRL (lot AFP9042) was obtained through the National Hormone and Pituitary Program, NIDDK, National Institutes of Health, and Dr. Parlow. Bovine PRL (U. S. Department of Agriculture (USDA) bPRL B-1, AFP 5300) was obtained from the Animal Hormone Program of the USDA Reproduction Laboratory (Beltsville, MD), and recombinant bovine placental lactogen was a gift from the Monsanto Co. (St. Louis, MO). Protein G-Plus agarose was purchased from Santa Cruz Biotechnology, Inc., Santa Cruz, CA. Anti-MMS-101R) and anti-ubiquitin, Covance Research Products, Inc., Denver, PA); and anti-V5 (catalog number R9650-25, Invitrogen. Alexa Fluor-conjugated human transferrin, cholera toxin subunit B, and goat-anti-mouse antibody were purchased from Molecular Probes (Eugene, OR). Anti-GHR intracellular was a gift from Dr. S. J. Frank (28). The ubiquitinated protein enrichment kit was obtained from Calbiochem, and the kit for biotinylation was from Pierce. All inhibitors and culture media were purchased from Sigma with these exceptions: lactacystin and matrix metalloproteinases inhibitor GM6001 (Chemicon International, Temecula, CA).

Plasmids—The expression plasmid for the human long PRL isoform was a gift from C. Clevenger (29). MIT23 (HA-Ub) (30) was used to assess receptor-associated ubiquitination. The HA epitope, YPYDDVPIFY, was introduced between the signal peptide and the first amino acid of the mature bovine iPRLR as follows. First, 30 cycles of PCR were used to synthesize two fragments overlapping with the HA sequence: Fragment 1, starting from a unique 5′ EcoRI site through the signal peptide, ending with the HA tag and two oligonucleotides, 5′-TGGCG-TGGAATTCGGCGGGAATG-3′, 5′-GGCGTATTCGGCGCAGCTC-ATAGGGGATATTCAAGGGCTCAG-3′. Fragment 2, starting with the HA tag followed by the first amino acid of the mature PRLR to the unique BstEI site with two other oligonucleotides, 5′-TACCCCTATGACGATGCCGCATTACGCCAGTCCACCTCTGAAAAC-3′, 5′-GGATCGACGTCACATGCTGA3′. Second, five cycles of PCR supplied with dNTPs were used to anneal and fill the ends of the two fragments. Third, 25 cycles of PCR supplied with two outside oligonucleotides containing unique sites were used to amplify the whole fragment. The PCR product was digested with EcoRI and BstEI and then substituted into the wild-type PRLR with the same restriction enzymes.

**MCF-7 and NIH-3T3-F442A Cell Culture—**PRL-deficient cells derived from the human mammary adenocarcinoma cell line, MCF-7, were grown in RPMI 1640 containing 10% horse serum and 50 μM ganciclovir as reported previously (27). NIH-3T3-F442A cells were a gift from Dr. P. Bertsch; these cells were grown in Dulbecco’s modified Eagle’s medium F12 containing 10% fetal bovine serum. For some experiments, 10⁶ cells/60-mm plate were incubated in serum-free medium or serum-free medium supplemented with 0.5% bovine serum albumin (MCF-7 and 3T3-F442A, respectively), for 24–48 h prior to treatments. Cells were then pretreated with vehicle or inhibitor for 30 min or 1 h followed by the addition of vehicle, 4 nM PRL, or 1 μg/ml 4β-phorbol 12-myristate 13-acetate (PMA) for an additional incubation. In some experiments, media were harvested, floating debris were removed by centrifugation, and protein was precipitated by incubation with 3 volumes of acetone at −20 °C.

**Ubiquitination—**To examine ubiquitin associated with the PRLR, 3.9 × 10⁶ Chinese hamster ovary cells were transfected with the HA-Ub expression vector together with the human iPRLR expression plasmid using SuperFect reagent (Qiagen Inc., Santa Clarita, CA). Cells were grown in serum-free media overnight after the removal of transfection reagent and then treated with or without 4 nM PRL for 10 min and rinsed twice with PBS and scraped into immunoprecipitation assay buffer (10 mM Tris, pH 8, 150 mM NaCl, 1 mM EDTA, 1% Triton X-100, 0.5% sodium deoxycholate, 0.05% SDS, containing 1 mM Na3VO4, 1 mM phenylmethysulfonyl fluoride, 1 μg/ml leupeptin, 1 μg/ml aprotinin, and 5 mM N-ethylmaleimide). The cell extract was centrifuged at 4 °C, 14,000 rpm for 10 min, and 1 mg of the supernatant was precleared by incubating with 16 μl of Protein G-Plus agarose and 0.25 μg of normal mouse IgG at 4 °C for 1 h. Cell lysate was centrifuged at 2,500 rpm for 5 min at 4 °C, and the supernatant was collected and incubated with V5 antibody (1:1000) at 4 °C for 1 h, and 20 μl of Protein G-Plus-agarose was added and incubated overnight. The pellet was collected by centrifugation at 2,500 rpm for 5 min at 4 °C and washed once with ice-cold immunoprecipitation assay buffer and two times with ice-cold PBS and then resuspended in 30 μl of 2X sample buffer prior to examination by Western analysis as below. To investigate PRLR ubiquitination using another approach, we employed the GST-UIM kit (Calbiochem). 5 × 10⁶ PRL-deficient MCF-7 cells were serum-starved for 24 h and then treated with or without 4 nM PRL for 10 min. Cells were washed twice in PBS and harvested in cell lysis buffer (25 mM Tris, pH 8.0, 2 mM EDTA, pH 2.0, 10% glycerol, 1% Triton-X-100, 2 mM Na3VO4, 20 mM NaF, and 5 mM N-ethylmaleimide). 1 mg of total protein was incubated with either the UBA domain of yeast Rad23 protein conjugated to glutathione (31) or control glutathione alone, immobilized on agarose beads for 3 h at 4 °C. Samples were then centrifuged at 4 °C for 3 min at 1000 × g. Pellets were washed three times and resuspended in 2X sample buffer, boiled for 5 min, and analyzed by Westerns.

**Western Analysis—**Western analysis was performed as described previously (32). In brief, 30 μg of protein of cellular lysate, immunoprecipitated proteins, or the acetone-precipitated conditioned media was electrophoresed through standard Laemmli SDS-polyacrylamide gels (7–12% gels), transferred to polyvinylidene fluoride membranes, and then probed with appropriate antibodies. Membranes were blocked for 4 h in 0.25% gelatin in TBST (100 mM Tris-HCl, pH 7.5, 150 mM NaCl, and 0.1% Tween 20) and then incubated in primary antibody overnight at 4 °C (PRLR-ECD, 1:1000; V5, 1:500; ubiquitin, 1:1000; GHR intracellular, 1:2000). Membranes were washed three times with TBST and then incubated with secondary antibody in 5% milk in TBST at room temperature for 1–2 h. Membranes were washed three times with TBST, and then signals were visualized by enhanced chemiluminescence followed by autoradiography. For some experiments, signals were quantified by densitometry (ImageQuant software, v.4.2a, Amersham Biosciences).

**Indirect Immunofluorescence—**PRL-deficient MCF-7 cells were grown as subconfluent monolayer cultures on glass coverslips overnight. To examine internalization of the iPRLR, cells were transfected with the HA-iPRLR expression plasmid. After the removal of the transfection reagent, cells were grown in serum-free RPMI 1640 overnight. Cells were treated with inhibitors for 20 min and then cooled on ice for 15 min, prior to incubation with antibody HA.11 (1:500) together with the same inhibitor in the blocking media (RPMI 1640 with 1% bovine
and the internalized ligand were harvested as described previously (7). Specific binding in each fraction was determined as the difference between $^{125}$I ligand detected in the presence and in the absence of an excess of unlabeled hormone. The internalization ratio was expressed as a percentage of the specific internalized fraction with respect to total specific binding at 90 min as described (33).

**Biotinylation**—Cell surface proteins were biotinylated using a cell surface protein labeling kit (Pierce) with some adaptations. PRL-deficient MCF-7 cells were plated at a density of $5 \times 10^5$ in 100-mm Petri dishes and serum-starved overnight. Cells were washed twice in ice-cold PBS and incubated at $4^\circ$C in 200 mg/ml sulfo-NHS-SS biotin for 30 min. Following removal of unbound biotin with quenching solution and two washes with ice-cold PBS, cells were stimulated with 4 nM hPRL at $37^\circ$C for 2 h. Lysates were harvested from the buffer provided in the kit supplemented with 1 $\mu$g/ml leupeptin, 1 $\mu$g/ml aprotinin, and 1 mM Na$_2$VO$_4$. Lysates were cleared by centrifugation, and 1 mg of total protein was solubilized and subjected to SDS-PAGE and Western analysis with anti-PRLR-ECD (33). A loading control from the same blot is shown (lower panel). B, quantification of PRLR levels from 3 independent experiments. Each point represents the mean ± S.D. When not shown, S.D. bars are smaller than the symbol.

**RESULTS**

**PRL Down-regulates the PRLR in the PRL-deficient MCF-7 Cells**—Receptor down-regulation permits peptide ligands to regulate availability of their receptors on the cell surface. However, some target cells, including breast cancer cells, express PRL endogenously, making it difficult to observe the effect of circulating ligand on the PRLR. Using a genetic selection method, we derived cell lines from MCF-7 cells that are deficient in endogenous PRL production (27), which allowed us to examine the effect of exogenous PRL on the PRLR. PRLR is the predominant isoform expressed in this cell line; other isoforms are not detected using an antibody to the shared
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**FIGURE 3.** Involvement of proteasomes and lysosomes in PRL-induced down-regulation of PRLR. A, serum-starved PRL-deficient MCF-7 cells were pretreated with vehicle, 20 μM MG132 (M), 20 μM lactacystin (L), or 100 μM chloroquine (C) for 1 h at 37 °C and then were treated with 4 nM PRL for an additional 2 h at 37 °C. Results shown are representative of at least 3 independent experiments. Loading controls from the same blots are shown (lower panel). B, quantification of the effects of MG132 and chloroquine on PRLR down-regulation from 3 independent experiments. Each point represents the mean ± S.D. Asterisks denote significant differences between vehicle and PRL-treated samples using the Student’s t test, *p < 0.05. C, serum-starved MCF-7 cells were treated with 4 nM PRL for 10 min. Cell lysates were collected and incubated with either UIM-GST or GST alone conjugated to agarose beads, and complexes were examined by Western analysis with for the lPRLR (left). The blot was reprobed with anti-ubiquitin as a control (right). IB, immunoblot. D, ubiquitination of PRLR and/or associated proteins. Chinese hamster ovary cells were transfected with the V5-lPRLR and HA-Ub, serum-starved for 24 h, and then treated with or without 4 nM PRL for 10 min, prior to immunoprecipitation (IP) of lysates with anti-V5 followed by Western analysis with HA.11. the Western blot was reprobed with anti-V5 to identify the location of the IPRLR.

ECD (data not shown), consistent with previous reports of the parent cell line (34). As shown in Fig. 1, A and B, exogenous PRL rapidly down-regulated the lPRLR, which remained low through at least 24 h. This down-regulation was dependent on ligand concentration, as shown in Fig. 1C. However, lower concentrations of PRL were able to elicit substantial lPRLR down-regulation after longer incubations (Fig. 1, compare D with A).

The **lPRLR Undergoes Rapid Turnover**—Levels of receptor expression are determined by the balance between synthesis and degradation. To determine the effect of ligand on turnover of the lPRLR, we used cycloheximide to inhibit protein synthesis. In the absence of PRL, cycloheximide rapidly decreased cellular lPRLR levels with a half-life of about 100 min, indicating high turnover of the lPRLR even in the absence of ligand (Fig. 2). In the presence of PRL, the apparent half-life of the lPRLR fell to ~25 min. This rate of degradation of endogenous lPRLR is comparable with surface receptors in rabbit mammary explants (35) and in Chinese hamster ovary cells stably expressing the rabbit PRLR (6), suggesting that rapid turnover of the lPRLR is common across cell type and experimental system.

**Down-regulation of the lPRLR by PRL Is Mediated by Proteasomes and Lysosomes**—Both proteasomes and lysosomes are important mediators of degradation of cellular proteins. To determine their respective roles in PRL-induced down-regulation of the lPRLR, we used pharmacological inhibitors. Cells were pretreated with proteasomal inhibitors with distinct mechanisms of action (MG132 or lactacystin) or the lysosomal inhibitor, chloroquine, for 1 h followed by the addition of PRL or vehicle for an additional 2 h. As shown in Fig. 3, A and B, inhibition of both processes reduced PRL-induced down-regulation of the lPRLR.

Over this time frame, these inhibitors had little effect on IPRLR levels in the absence of ligand.

**The lPRLR and/or Associated Proteins Are Ubiquitinated**—The involvement of proteasomes in ligand-induced degradation of the lPRLR suggested ubiquitination of the receptor or associated proteins. Ubiquitination of the PRLR was initially demonstrated in a baculovirus-insect system (36), and more recently, in transiently transfected mammalian cells (11). To examine the endogenously expressed lPRLR in MCF-7 cells for this modification, we employed a GST-UIM fusion immobilized on agarose beads. As shown in Fig. 3C, no ubiquitination of the endogenously expressed lPRLR was evident with this approach; longer exposures did not reveal additional signals. However, this method favored capture of polyubiquitinated proteins, so monoubiquitinated receptor may not be detected. To examine the lPRLR for this modification in a more sensitive system, we transfected HA-tagged ubiquitin (HA-Ub) and V5-tagged lPRLR constructs into Chinese hamster ovary cells. As shown in Fig. 3D, ubiquitin associated with the lPRLR both in the presence and in the absence of ligand. These data did not discriminate between ubiquitination of the lPRLR itself and that of associated proteins.

**Ubiquitin-conjugating System Is Not Required for Internalization of the lPRLR**—The ubiquitin-conjugating system has been shown to be required for internalization of the closely related GHR (37), as well as other membrane receptors (38). The Chinese hamster lung cell line ts20 (39), which contains a thermolabile ubiquitin-activating enzyme E1, has proven useful for these studies (37, 40, 41). We generated a stable line expressing the lPRLR (ts20-lPRLR) and examined the role of the ubiqui-
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**Figure 4.** Ubiquitin-conjugating system and proteasomes are not involved in internalization of the PRLR. A, ts-20 cells stably expressing the PRLR (ts20-IPLR, left panel) or the rabbit GHR (ts20-rabGHR, right panel) were incubated at either the permissive temperature (30 °C) or the non-permissive temperature (42 °C). As shown in Fig. 4A (left panel), incubating ts20-IPLR cells at 42 °C, inactivating ubiquitin-activating enzyme E1, did not inhibit PRLR internalization. In contrast, these conditions inhibited GHR endocytosis in parallel experiments (Fig. 4A, right). Proteasomal function is also required for endocytosis of the GHR (33). To evaluate this requirement in ligand-induced internalization of the PRLR, we transiently transfected HA-IPLR into MCF-7 cells. Surface receptor was labeled by anti-HA on ice before permitting ligand-induced internalization at 37 °C (Fig. 4B, left panel). PRL-induced endocytosis of the PRLR was not blocked by MG132 treatment (Fig. 4B). Together, our findings indicated that ubiquitin-conjugating and proteasomal activities are not required for internalization of the PRLR, in contrast to the GHR.

**Figure 5.** PRL induces a proteolytic product generated from the PRLR. A, serum-starved PRL-deficient MCF-7 cells were treated with or without 4 nM hPRL for 0, 1, 2, 6, or 24 h. B, serum-starved PRL-deficient MCF-7 cells were pretreated with vehicle, 20 μM MG132 (M), 20 μM lactacystin (L), or 100 μM chloroquine (C) and then treated with 4 nM PRL for an additional 2 h at 37 °C. C, serum-starved PRL-deficient MCF-7 cells were pretreated with vehicle and 50 μg/ml cycloheximide (CHX) for 1 h at 37 °C and then were treated with 4 nM PRL for an additional 2 h at 37 °C. Cellular proteins were solubilized and subjected to SDS-PAGE and Western analysis with anti-PRLR ECD. Loading controls from the same blots are shown (lower panel). D, serum-starved PRL-deficient MCF-7 cells were labeled with 200 ng/ml sulfo-NHS-SS-biotin and then treated with or without 4 nM PRL for 2 h. 1 mg of total protein was incubated with immobilized streptavidin. Captured biotinylated proteins were subjected to SDS-PAGE and Western analysis with anti PRLR ECD (top, short exposure; bottom, longer exposure). The arrow highlights the position of the intact PRLR; the arrowhead marks the position of the proteolytic fragment. Blots shown are representative of at least 3 independent experiments.

**Figure 6.** Metalloproteases Do Not Play a Role in PRLR Fragment Generation. Nor is the PRLR Fragment Secreted into the Media. Because of the key role of metalloproteases in “shedding” of the ECD of multiple transmembrane proteins, including the GHR, interleukin-6 receptor, l-selectin, as well as others (for reviews, see Refs. 12 and 43–45), we examined components of this pathway to ascertain any role in generation of this PRLR fragment using several approaches. GM6001, a broad spectrum hydroxamic acid inhibitor of metalloproteinases, including tumor necrosis factor-α-converting enzyme (TACE, also known as “a disintegrin and metalloproteinase,” ADAM-17), which catalyzes the cleavage of these other membrane receptors, had no effect (Fig. 6A). Inhibition of the γ-secretase complex, which releases soluble intracellular domains of some of these same proteins, also failed to perturb this event (data not shown). 

Several methods were employed to examine the population of PRLR that undergoes this limited proteolysis. To confirm that it did not occur cotranslationally on polysomes, as reported for NF-κB (for review, see Ref. 42), cycloheximide was used to inhibit translation. As shown in Fig. 5C, although this compound reduced levels of full-length receptor, as expected from the short half-life shown in Fig. 2, it did not alter the ability of ligand to induce the fragment. To determine whether the fragment was generated from receptor that had been on the plasma membrane, surface proteins were labeled with biotin, purified, and then examined by Western analysis. As shown in Fig. 5D, the PRLR fragment was precipitated by streptavidin, consistent with this origin.
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FIGURE 6. The IPRlR ECD cleavage product is generated by mechanisms distinct from shedding of other membrane receptors, such as the GHR. A, serum-starved PRL-deficient MCF-7 cells were pretreated with vehicle or 1 μg/ml PMA, 20 μM GM6001, or 10 μM bisindolylmaleimide II (BisII) for 1 h followed by 1 nM hPRL for 2 h. Loading controls from the same blots are shown (lower panels). B, cells were treated ±4 nM hPRL in serum-free media for 24 h. Protein in the media was precipitated with acetone prior to Western analysis. Proteins were fractionated by SDS-PAGE, and the PRLR was visualized with anti-PRLR-ECD by Western analysis. Blots shown are representative of at least 3 independent experiments. The arrow marks the position of the intact IPRlR; the arrowhead marks the position of the proteolytic fragment. C, NIH 3T3 F442A cells were pretreated with vehicle, 20 μM GM6001 (GM), or 10 μM MG132 (M) for 30 min, prior to treatment with 1 μg/ml PMA for 30 min as described (28). Cellular lysates were subjected to Western analysis recognizing the cytoplasmic domain of the GHR. The open arrow marks the position of the intact GHR; the open arrowhead marks the position of the GHR remnant, containing the cytoplasmic domain.

shown). Activation of protein kinase C family members with PMA, which stimulates generation of a secreted ECD fragment from the GHR (for review, see Ref. 12), modestly reduced IPRlR levels in the absence of ligand. However, it did not induce this limited proteolysis in the absence of PRL, suggesting that protein kinase C modulated other aspects of receptor processing. Consistently, inhibition with bisindolylmaleimide II (GF109203X), which inhibits multiple protein kinase C isoforms (46), failed to block the appearance of this fragment. Epidermal growth factor, which initiates signals activating several of these cleavage enzymes, failed to induce detectable cleavage (data not shown). Furthermore, examination of the media for polypeptides containing the extracellular domain of the IPRlR revealed the accumulation of immunoreactive protein of the appropriate size but no regulation by PRL (Fig. 6B) or PMA (data not shown). Comparison of the levels of this fragment secreted in 24 h by 10⁶ cells to that associated with 10⁵ cells after 2 h of exposure to ligand (lanes 1 and 2 versus lanes 3 and 4) demonstrates that the relative amount of IPRlR “shed” by these cells into the media is very low. These findings demonstrated that the IPRlR cleavage event described here is quite different from metalloprotease-mediated release of the ectodomains of other receptors, such as the GHR. PMA-stimulated shedding of the extracellular domain of the GHR by ADAM-17-mediated proteolysis, which is inhibited by ligand (12), is reduced by GM6001, and the remaining cell-associated GHR “remnant” is stabilized by proteasomal inhibitors, such as MG132 (Fig. 6C) (28).

DISCUSSION

Binding of agonist to surface receptors initiates kinase cascades, modulating signaling pathways and gene expression within the target cell. However, ligand can also alter trafficking of the receptor, in ways specific to the ligand, receptor isoform, and cell type. For many membrane receptors, ligand binding is followed by increased rates of internalization and processing. The pathways and subsequent events have immediate consequences for signal transduction (for reviews, see Refs. 47–51). Our system, permitting study of endogenously expressed receptors rather than transiently transfected receptors, avoids complications that may be secondary to overexpression, such as a high proportion of improperly folded or mistransfected receptors (for review, see Ref. 52). Here we demonstrated that PRL dramatically down-regulates the endogenous IPRlR in these breast cancer cells. This fall in IPRlR levels was temporally linked to the appearance of a cell-associated ECD-containing receptor fragment, which is dependent on proteasomal, but not lysosomal or metalloprotease, activities. These findings demonstrated complex effects of ligand on IPRlR trafficking and pointed to a novel role for proteasomes in limited proteolysis resulting in generation of a stable IPRlR cleavage product.

Although a role for lysosomes in degradation of the PRLR has been established (for review, see Ref. 10), the current studies indicate a role for proteasomes in the post-ligand binding fate of the IPRlR as well. Our data were compatible with the emerging story of many receptor tyrosine kinases and G-protein coupled receptors, which employ ubiquitination to direct trafficking in response to ligand, terminating in degradation in lysosomes (for reviews, see Refs. 38 and 53–57). Many details remain to be worked out, including the identity of the ubiquitinated moiety(s), the nature and location of the ubiquitin chains, the PRL-initiated signal(s) leading to this modification, and the relationship between this event(s) and lysosomal degradation. SCFIRF/TrCP has been implicated in ubiquitination of the IPRlR (11). Whether this is the only E3 ubiquitin ligase involved in ubiquitin-mediated trafficking of the IPRlR remains to be determined. Other E3 ubiquitin ligases have been implicated in ubiquitination of cytokine receptors, including p33ϕE for the erythropoietin receptor (58), and several, including c-Cbl and Nedd4 family members, mediate trafficking of the epidermal growth factor receptor by direct ubiquitination of the receptor or associated proteins (for reviews, see Refs. 57 and 59). Unlike the closely related GHR and another cytokine receptor, the interferon-α receptor, the IPRlR does not require the ubiquitin-conjugating system for internalization (37, 60). Our current studies are consistent with our previous report of the requirement for multiple motifs within the cytoplasmic domain of the IPRlR for optimal internalization, as opposed to the single the Ub-dependent endocytosis motif of the GHR (7). Thus the PRLR appears to more closely resemble the erythropoietin receptor (61, 62) and interleukin-2 receptor β (41, 63), other members of the cytokine receptor family, in this regard.

Our studies also revealed that PRL-initiated proteasomal activity is critical for generation of a cell-associated 50-kDa PRLR fragment containing the ECD. The size of this fragment suggested that it results from a cleavage event(s) in the vicinity of the transmembrane domain. Although this PRLR-ECD fragment suggested a “shedding,” such as that reported for multiple other transmembrane receptors, including the cytokine receptors, GHR (12), the interleukin-6 receptor (64), ErbB4 (13), and Notch (65), the apparent regulation of this IPRlR proteolytic event is quite different. Cleavage of the IPRlR was insensitive to protein kinase C agonists and inhibitors, as well as inhibition of the metalloproteases implicated in secretion of these other receptor fragments. Interestingly, the etiology of this cell-associated PRLR-ECD fragment is strikingly similar to that reported for some membrane bound transcription factors (for reviews, see Refs. 42 and 66). Proteasomal cleavage of these
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transmembrane proteins occurs in polysomes or the endoplasmic reticulum. However, the origin of this IPR-LR fragment from surface receptor, and independence from concurrent translation, were not consistent with these locations. Our data suggested that the IPR-LR is cleaved after ligand-stimulated internalization, which would be a novel site for this limited proteasomal action. A proteasome-dependent fragment from the common B (Bc) subunit of hematopoietic cytokine receptors displays intriguing similarities, although few details are known, suggesting that the PRLR may not be alone in this respect (67). Clearly, the identity and function of this PRLR fragment require further study. Whether it is merely an intermediate in degradation of the PRLR or transmits additional signals, such as that proposed for other mitogenic receptor fragments (for reviews, see Refs. 13–15), is under investigation. Although early reports offered tantalizing suggestions of PRLR translocation into the nucleus (68), this has remained controversial (69). Our data suggested that this possibility should be reevaluated.

The mechanism whereby PRL initiates PRLR cleavage is currently under study. Inhibition of multiple kinase cascades did not block formation of the PRLR-ECD fragment. Whether PRL induces a conformational change in the PRLR, permitting cleavage, or initiates this process by signaling pathways not examined remains to be determined. Little is known about PRL-initiated signals that may result in serine/threonine phosphorylation of the IPR-LR and/or associated proteins. These modifications mediate many interactions important for post-ligand trafficking and facilitate ubiquitination of many membrane receptors (56, 57). Indeed, phosphorylation of Ser–349 was required for PRLR recognition by SCF escort in vivo (11).

In conclusion, we have provided evidence that proteasomes mediate ligand-stimulated generation of an ECD-containing fragment and IPR-LR degradation in breast cancer cells. Differences in PRL-activated signaling pathways (3, 4) and endocytic motifs among the alternatively spliced PRL-LR isoforms (7, 9) suggest that post-ligand trafficking of the short isoforms will be distinct from the IPR-LR. Differences in PRL-receptor interactions, and independence from concurrent translation, were not consistent with these locations. Our data suggested that the PRL-LR is cleaved after ligand-stimulated internalization, which would be a novel site for this limited proteasomal action. A proteasome-dependent fragment from the common B (Bc) subunit of hematopoietic cytokine receptors displays intriguing similarities, although few details are known, suggesting that the PRLR may not be alone in this respect (67).

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Clearly, the identity and function of this PRLR fragment require further study. Whether it is merely an intermediate in degradation of the PRLR or transmits additional signals, such as that proposed for other mitogenic receptor fragments (for reviews, see Refs. 13–15), is under investigation. Although early reports offered tantalizing suggestions of PRLR translocation into the nucleus (68), this has remained controversial (69). Our data suggested that this possibility should be reevaluated.

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