Constitutive Activation of the Prolactin Receptor Results in the Induction of Growth Factor-independent Proliferation and Constitutive Activation of Signaling Molecules*

(Received for publication, December 30, 1998)

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The ability to induce the oncogenic activation of the human prolactin receptor (PRLR) was examined by deleting 178 amino acids of the extracellular ligand-binding domain. Expression of this deletion mutant in the interleukin-3 (IL-3)-dependent murine myeloid cell line 32Dc13 resulted in the induction of growth factor-independent proliferation. Parental 32Dc13 cells proliferated only in the presence of exogenous murine IL-3 (mIL-3), while 32Dc13 cells transfected with the long form of the human PRLR were able to proliferate in response to mIL-3, ovine prolactin, or human PRL. Cells expressing the Δ178 deletion mutant contained numerous phosphotyrosine-containing proteins in the absence of stimulation with either mIL-3 or ovine prolactin. Growth factor stimulation increased the number of proteins phosphorylated and the intensity of phosphorylation. These proteins included constitutively phosphorylated Janus kinase 2, signal transducer and activator of transcription 5, and SHC. Activated extracellular signal-regulated kinases 1 and 2 (ERK1 and ERK2) were observed in unstimulated 32Dc13 cells expressing the Δ178 mutant. Likewise, transfection of Nb2 cells with the Δ178 deletion mutant induced growth factor-independent proliferation and constitutive activation of Janus kinase 2, ERK1, and ERK2. In addition to the induction of a growth factor-independent state, the expression of the Δ178 deletion mutant also suppressed the apoptosis that occurs when 32Dc13 cells are cultured in the absence of growth factors such as IL-3. These data suggest that the constitutive activation of the PRLR can be achieved by deletion of the ligand binding domain and that this mutation leads to the oncogenic activation of the receptor as determined by the ability of the receptor to induce growth factor-independent proliferation of factor-dependent hematopoietic cells.

Ligand-induced oligomerization of growth factor receptors is critical in regulating the proliferation and differentiation of cells. Dimerization of transmembrane receptor tyrosine kinases leads to the activation of their intrinsic kinases, phosphorylation of the receptor molecules themselves, and the subsequent activation of secondary signaling molecules. Members of the cytokine receptor superfamily lack intrinsic kinase activity; however, several different tyrosine kinases become activated following ligand binding. These kinases phosphorylate the receptors as well as numerous other substrates, leading to the activation of many of the same signaling pathways that lie downstream of receptor tyrosine kinases. Cytokine receptors generally activate two classes of tyrosine kinases: one or more members of the Janus family of tyrosine kinases as well as one or more members of the src family of tyrosine kinases. Janus kinases are directly responsible for the phosphorylation and activation of a class of transcription factors referred to as STATs (1). It is not clear, however, which family of tyrosine kinases are required for the activation of the Ras/Raf/MAP kinase cascade or phosphatidylinositol 3-kinase.

Given the critical role of these receptors in regulating cellular proliferation, it is not surprising that mutations that result in their constitutive activation are also oncogenic. At least four constitutively activated transmembrane receptor tyrosine kinases have been transduced by oncogenic retroviruses: v-Erb B, v-Fms, v-Kit, and v-Ros. Erb-B, c-Fms, and c-Kit represent the receptors for epidermal growth factor, colony-stimulating factor 1, and stem cell factor, respectively (2–6). These receptors can be activated by point mutations in the ligand binding domain, the transmembrane domain, and/or the intracellular domain and by deletion of the majority of the extracellular domain (2, 7–12).

To date, only a single cytokine receptor has been discovered to be transduced by an oncogenic retrovirus; the v-mpl oncogene in the murine myeloproliferative leukemia virus is a truncated version of the receptor for thrombopoietin (13–15). Activating mutations have also been described in two other members of the cytokine receptor superfamily: the erythropoietin receptor and the βc subunit (β common subunit) of the receptors for IL-3, IL-5, and granulocyte-macrophage colony-stimulating factor (16–21).

The current study was undertaken to determine whether deletion of the extracellular domain of the prolactin receptor (PRLR) would lead to its constitutive activation and the ability to induce growth factor-independent proliferation of factor-deleted cells. The abbreviations used are: STAT, signal transducer and activated transcription; STAT5, signal transducer and activated transcription; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; ERK, extracellular signal-regulated kinase; ERK1 and ERK2, extracellular signal-regulated kinase 1 and 2, respectively; PRL, prolactin; PRLR, prolactin receptor; hPRL, human prolactin; oPRL, ovine prolactin; hPRL, human prolactin receptor; rPRL, rat prolactin; rhPRL, recombinant human prolactin; IL, interleukin; mIL-, murine interleukin; JAK2, Janus kinase 2; MAP, mitogen-activated protein.

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pendent hematopoietic cell lines. In this report, we demonstrate that the deletion of a 178-amino acid region of the extracellular domain of the PRLR resulted in the constitutive activation and the induction of growth factor-independent proliferation. The constitutive phosphorylation of multiple proteins including Jak2, Stat5, and Shc was observed in addition to growth factor-independent activation of MAP kinase. Although previous studies by Gourdou et al. (22) have demonstrated that deletion of a region of the extracellular domain of the rabbit PRLR resulted in the constitutive activation of the receptor as determined by the PRL-independent activation of β-casein transcription, these investigators did not demonstrate the activation of any signaling molecules, the induction of growth factor-independent proliferation, or the suppression of apoptosis. Our results are discussed in the context of signal transduction by the PRLR and its role in oncogenesis.

MATERIALS AND METHODS

Cell Culture—The 32D clone 3 (32Dcl3) cell line was obtained from Dr. Joel Greenberger (University of Pittsburgh, Pittsburgh, PA) (23), and it was maintained as described by Anderson and Jorgensen (24). The clone 3 was obtained from Sigma (St. Louis, MO). Recombinant human PRL (rhPRL) was purchased from Genzyme (Cambridge, MA) or R&D Systems (Lake Placid, NY). Unconjugated monoclonal anti-phosphotyrosine antibody 4G10, rabbit anti-JAK2, and rabbit anti-ERK1/2 were obtained from Upstate Biotechnology, Inc. (Lake Placid, NY). Anti-ERK2 antibody was from Transduction Laboratories (Lexington, KY). Anti-ACTIVE MAP kinase, a polyclonal antibody directed against the activated version of ERK2 that shows some cross-reaction with activated ERK1, was obtained from Promega (Madison, WI). Rabbit anti-STAT5 antibody was a gift of Andrew Larner (National Institutes of Health, Bethesda, MD), and a monoclonal antibody directed against STAT5 was obtained from Transduction Laboratories (Schleicher and Schuell), and the RNAs were cross-linked to the membrane with a Stratagene Stratallinker (La Jolla, CA). 32P-Labeled probes to the hPRLR and to GAPDH were prepared by random priming in the presence of [γ-32P]ATP (Amersham Pharmacia Biotech; 800 Ci/mmol). The rat GAPDH cDNA clone was obtained from Dr. Kenneth Marcu (State University of New York at Stony Brook).

Immunoprecipitation and Immunoblotting—Immunoprecipitation and immunoblotting were performed as described previously (24, 26). Cells to be immunoprecipitated were cultured overnight in McCoy’s 5 A medium supplemented with 5% charcoal-stripped serum (HyClone; Logan, UT) to reduce the background level of tyrosine-phosphorylated proteins. Cells were then resuspended in media containing 100 units/ml recombinant murine IL-3, 100 ng/ml (2.23 μg/ml) ovine PRL, or 0.5–1.0 μg/ml rhPRL for the indicated periods of time. Cells were lysed in either radioimmunoprecipitation buffer (150 mM NaCl, 50 mM Tris (pH 7.4), 1 mM EDTA, 1% Triton X-100, 1 mM sodium orthovanadate) or extraction buffer (50 mM NaCl, 10 mM Tris (pH 7.4), 5 mM EDTA, 50 mM NaF, 1% Triton X-100, 1 mM sodium orthovanadate). Both lysates buffers were supplemented with 100 units/ml aprotinin (Calbiochem). Agarose-conjugated monoclonal anti-phosphotyrosine antibody 4G10, rabbit anti-JAK2, and rabbit anti-ERK1/2 were obtained from Upstate Biotechnology, Inc. (Lake Placid, NY). Unconjugated monoclonal anti-phosphotyrosine antibody 4G10 was the generous gift of Brian Druker (Oregon Health Sciences University, Portland, OR). Rabbit anti-JAK2 (catalogue no. S14630) and monoclonal antibody directed against SHC (catalogue no. S14620) were obtained from Transduction Laboratories (Lexington, KY). Anti-ACTIVE MAP kinase, a polyclonal antibody directed against the activated version of ERK2 that shows some cross-reaction with activated ERK1, was obtained from Promega (Madison, WI). Rabbit anti-STAT5 antiserum was the gift of Andrew Larner (National Institutes of Health, Bethesda, MD), and a monoclonal antibody directed against STAT5 was obtained from Transduction Laboratories (Lexington, KY). Preparations of monoclonal antibody directed against the β-subunit of the murine IL-3 receptor has been described (27). Immunoprecipitated proteins were resolved on 8 or 10% SDS-polyacrylamide gels and electrotransferred to Immobilon membranes (Millipore Corp., Bedford, MA). Immunoblotting was conducted as described by Anderson et al. (26) with the ECL system according to the manufacturer’s recommendations (Amersham Pharmacia Biotech).

Proliferation Assays—Cells (32Dcl3, 32Dcl178, and 32D/hPRLR) were resuspended in McCoy’s 5 A medium supplemented with or without growth factors as indicated. The number of viable cells was determined at the different time points by counting the number of cells that excluded trypan blue.

Preparation of Membrane Fractions—Cells (20 × 10^6) were washed twice with 5 ml of ice-cold phosphate-buffered saline. Cells were resuspended in 1 ml of cold membrane lysis buffer (20 mM Tris-HCl, pH 8.0, 2 mM MgCl2, 1 mM EDTA, 1 mM β-mercaptoethanol) supplemented with 1 mM sodium orthovanadate and 100 units/ml aprotinin. Cells were then broken by passage through a 31-gauge needle and then centrifuged for 5 min at 1,000 rpm at 4 °C to remove nuclei. The supernatant fraction was then centrifuged for 30 min at 15,000 rpm, 4 °C to obtain a membrane fraction. The supernatant was carefully removed, and the pellet of membrane fraction was resuspended once again through a 31-gauge needle. Membrane proteins (100 μg) were resolved on 8% polyacrylamide gel, transferred to Immobilon membranes, and immunoblotted using anti-FLAG M2 monoclonal antibody (Sigma) or rabbit anti-FLAG probe polyclonal IgG (Santa Cruz Biotechnology).

Analysis of DNA Fragmentation Due to Apoptosis—Cells were pelleted by centrifugation and washed once with phosphate-buffered saline. Cells (10^6) were lysed in 400 μl of DNA lysis buffer (10 mM Tris, pH 7.5, 10 mM EDTA, 0.5% SDS, 100 μg/ml RNase A, 4 μg/ml proteinase K) and incubated at 37 °C for 6 h. DNAs were extracted from cell lysates with 400 μl of phenol/chloroform/i sovereignty alcohol (25:24:1) mix. The salt concentration was raised to 0.25 mM sodium acetate, and the DNA was precipitated by the addition of 1 ml of 100% EtOH. Following incubation at −20 °C, the DNA pellets were pelleted and then resuspended with 50 μl of TE (10 mM Tris, pH 7.4, 1 mM EDTA) with 20 μg/ml RNase A. DNAs were quantitated spectrophotometrically, and DNA fragmentation was examined by running 10 μg of DNA on a 1.5% agarose gel.

Constitutive Activation of the PRLR
RESULTS

Construction of the Δ178 Deletion Mutant—Mutation or deletion of the ligand binding domain of numerous cytokine receptor family members has been shown to result in their constitutive activation (7). To determine whether this would also hold true for the PRLR, a region of the extracellular domain of the hPRLR was deleted, and the ability of this deletion mutant to induce growth factor-independence was assessed. A cDNA clone of the hPRLR was digested with BglII and NcoI, resulting in a loss of 178 amino acids in the extracellular domain of the receptor. These restriction sites were chosen because they were unique restriction sites, they allowed removal of the majority of the extracellular ligand-binding domain, they retained the WSXWS motif, and they maintained the proper reading frame when the overhangs were filled and religated. This deleted version of the hPRLR will be referred to as hPRLRΔ178 or Δ178. The region of the hPRLR deleted is indicated in Fig. 1. The cDNAs of both the wild type hPRLR and the hPRLRΔ178 mutant were inserted into the eukaryotic expression vector pcDNA3. Both DNA molecules were introduced into the growth factor-dependent murine myeloid cell line 32Dc13, and G418-resistant clones were isolated and expanded for use in the described studies. The 32Dc13 cell line is absolutely dependent upon the presence of an exogenous source of mIL-3 for proliferation and viability (28). When cultured in the absence of IL-3 for 12–16 h, one can detect DNA fragmentation typical of cells undergoing apoptosis, and by 48–60 h no viable cells remain. Cells transfected with the hPRLR construct (32D/hPRLR cells) were capable of proliferating in mIL-3, hPRL, or oPRL; however, the parental 32Dc13 cells only proliferated in mIL-3. In contrast, cells transfected with the hPRLRΔ178 mutant (32DΔ178) were capable of proliferating in the absence of an exogenous growth factor. Single cell-derived clones of both 32D/hPRLR and 32DΔ178 cells were obtained by plating the cells in semisolid media containing the appropriate growth factors and picking individual colonies for expansion in liquid media. A second set of constructs was made in which the FLAG epitope tag was added to the C-terminal end of the hPRLR and the Δ178 deletion mutant. All studies reported in this paper used these single cell-derived clones expressing either the untagged or FLAG-tagged molecules. Identical results were obtained with both the untagged and FLAG-tagged molecules; however, the majority of the data shown below was generated using cells expressing the untagged molecules.

The hPRLR Δ178 Deletion Mutant Induces Growth Factor-independent Proliferation—The proliferation of 32Dc13, 32D/hPRLR, and 32DΔ178 cells were examined in media supplemented with 7.5% fetal calf serum, media supplemented with 7.5% fetal calf serum and 10% WEHI-3 conditioned media (a source of IL-3), or media supplemented with 7.5% fetal calf serum and 200 ng/ml oPRL (Fig. 2). All three cell lines proliferated in media supplemented with WEHI-3 conditioned media; by 72 h, the cell number of each cell line rose from 4 × 10^6 cells to approximately 25–35 × 10^6 cells. There was no significant difference in the proliferation rate of cells transfected with either hPRLR or the Δ178 mutant of the hPRLR (Fig. 2A). When cultured in media containing 7.5% fetal calf serum but lacking mIL-3, only the 32DΔ178 cells increased in number; the number of viable 32Dc13 cells decreased, while the number of viable 32D/hPRLR cells remained approximately the same (Fig. 2B). We suspect that the serum used in this study must have contained low levels of PRL, since the 32D/hPRLR cells remained viable, while the 32Dc13 cells died during the first 3 days in culture. The 32D/hPRLR cells die after 5–6 days in culture in the absence of exogenously provided PRL (data not shown). We suspect that low levels of PRL present in fetal calf serum might support the viability of 32D/hPRLR cells for longer than that of the 32Dc13 cells, which require IL-3 for proliferation and viability. Examination of the growth of the same cells in media supplemented with 7.5% fetal calf serum plus...
200 ng/ml oPRL revealed that the parental 32Dcl3 cells decreased in number, while both the 32DΔ178 and the 32D/hPRLR cells increased in number (Fig. 2C). The results shown are an average of three different studies. Similar data were obtained with five different independent clones of each cell type.

hPRLR and hPRLR Δ178 Proteins Are Expressed in Transfected 32Dcl3 Cells—Expression of the hPRLR or the Δ178 deletion mutant in the transfected 32Dcl3 cells was demonstrated by Northern blot analysis (Fig. 3, A and B). Twenty micrograms of total cellular RNA was resolved by agarose gel electrophoresis, the separated RNAs were transferred to a nylon membrane, and the blot was probed with either a probe for the human PRLR cDNA or a GAPDH cDNA probe to demonstrate equal loading of the gel. Although there was no RNA species detected in the parental 32Dcl3 cells, a single species of RNA was detected in the cells transfected with either the hPRLR or the Δ178 expression vector (Fig. 3A). Five clones of cells that were transfected with the FLAG-tagged hPRLR molecule had equivalent amounts RNA detected with the hPRLR probe (Fig. 3A, lanes 1–5). The five clones of 32Dcl3 cells transfected with the FLAG-tagged Δ178 deletion mutant had similar amounts of RNA detected with the PRLR probe; however, it appeared that there was less RNA than in the cells expressing the hPRLR molecule (Fig. 3A, lanes 7–11). The differences in the sizes of the RNAs observed in the 32D/hPRLR versus the 32DΔ178 cells were consistent with the size of the deletion that had been introduced into the hPRLR cDNA molecule. Reprobing the blot in Fig. 3A with the GAPDH probe revealed that roughly equal amounts of RNA were loaded in all but the 32Dcl3 cell sample (Fig. 3B). There clearly was more GAPDH RNA present in lane 6, indicating that more 32Dcl3 cell RNA was loaded in the gel shown in Fig. 3.

To demonstrate the presence of the hPRLR and Δ178 proteins in the transfected cells analyzed by Northern blot analysis, we used immunoblot analysis with an anti-FLAG epitope tag monoclonal antibody. In Fig. 3C, the expression of the hPRLR protein in the transfected cells was demonstrated. A membrane fraction was isolated from these 32Dcl3 and 32D/hPRLR cells, 100 μg of membrane protein was resolved by SDS gel electrophoresis, and the blot was subjected to immunoblotting with anti-FLAG antibody. Clones 6, 9, and 10 of the 32D/hPRLR cells appeared to have nearly equivalent levels of the hPRLR protein, while clones 7 and 8 appeared to have lower levels of the hPRLR protein (Fig. 3C, lanes 13–17).

We were also able to detect expression of the Δ178 protein in transfected cells by immunoblotting (Fig. 3D). Whole cell lysates of 32DΔ178 cells were prepared and were precleared with an irrelevant antibody (a monoclonal antibody directed against the β subunit of the IL-3 receptor (27). The lysates were then immunoprecipitated with agarose-conjugated anti-FLAG M2 antibody, and the immunoprecipitated proteins were resolved on an SDS gel. Immunoblotting was with an unconjugated anti-FLAG monoclonal antibody. Approximately equal amounts of the Δ178 protein were present in all four clones analyzed, although clone 12 might have slightly less protein (Fig. 3D, lanes 19–22). The sizes of the proteins detected in the different cell lines were consistent with that expected. Since different approaches were used to detect the presence of the FLAG-tagged protein molecules in the two different cell types, we cannot directly compare the amount of Δ178 protein with that of the hPRLR. For reasons that we do not understand at this time, we were not able to detect the hPRLR by immunoprecipitating whole cell lysates, nor were we able to detect the Δ178 protein in membrane fractions prepared from 32DΔ178 cells (data not shown).
anti-phosphotyrosine immunoblot analysis. The pattern of tyrosine-phosphorylated proteins was examined following stimulation of 32Dc13, 32D/hPRLR, and 32DΔ178 cells with either mIL-3 or oPRL for 0, 5, or 15 min. Stimulation of either 32Dc13 or 32D/hPRLR cells with mIL-3 resulted in the rapid appearance of proteins with molecular masses corresponding to 120–150, 95, 52, 46, and 42 kDa (Fig. 4, lanes 1–3 and 7–9). The 120–150-kDa region includes many different proteins including the β-subunit of the IL-3 receptor (30–32), JAK2 (33), CBL (26), SHIP (34, 35), and CAS. The 52- and 46-kDa proteins probably represent two of the isoforms of SHC (36), and the 42-kDa protein probably represents ERK2 (37). The observed 95-kDa protein does not represent STAT5 but instead represents an unidentified molecule whose phosphorylation is detected in a variety of cells stimulated with many different growth factors (data not shown). We do not routinely observe phosphorylated STAT molecules using the approach described in Fig. 4 (data not shown). In contrast to both 32Dc13 and 32D/hPRLR cells, numerous tyrosine-phosphorylated proteins were observed in unstimulated 32DΔ178 cells (Fig. 4, lane 4). Stimulation of 32DΔ178 cells with mIL-3 appeared to increase the extent of phosphorylation of both SHC and ERK2 (Fig. 4, lanes 4–6).

As would be expected, since these cells lack the PRLR, stimulation of 32Dc13 cells with 100 nM oPRL did not result in the appearance of tyrosine-phosphorylated proteins (Fig. 4, lanes 10–12). Lanes 10–18 of Fig. 4 were exposed longer than lanes 1–9, to allow for the detection of PRL-induced phosphorylation of proteins in lanes 16–18; however, this resulted in a higher background. This is most evident in lanes 10–15 of Fig. 4. Despite this higher background, it is clear that PRL did not stimulate an increase in the phosphotyrosine-containing proteins in 32Dc13 cells (Fig. 4, lanes 10–12). Stimulation of the 32D/hPRLR cells with oPRL resulted in a significant increase in the level of phosphorylated proteins (Fig. 4, lanes 16–18); most notable were proteins with molecular masses expected to correspond to SHC and JAK2. We do not know why there are more tyrosine-phosphorylated proteins present in unstimulated 32D/hPRLR cells than in unstimulated 32Dc13 cells. Consistent with the results described above, we observed the constitutive phosphorylation of proteins with sizes that correlate with known signaling molecules in unstimulated 32DΔ178 cells. We were surprised to observe an increase in the phosphorylation of proteins in the 120–150-kDa range following stimulation of these cells with oPRL (Fig. 4, lanes 13–15). Based upon the size of this protein, we would expect that this protein would correspond to JAK2 (see below). We believe that the increase in the phosphorylation of this band is due to the presence of another growth factor in the preparations of oPRL used in these studies, since rhPRL did not stimulate an increase in JAK2 phosphorylation (see below); however, in studies not shown here, oPRL did increase the phosphorylation of JAK2 (data not shown). These data demonstrate that the expression of the Δ178 mutant of the hPRLR in 32Dc13 cells results in the constitutive phosphorylation of numerous proteins, and consistent with data in Fig. 2, stimulation with mIL-3 resulted in the increased phosphorylation of several proteins. Consistent results were obtained in four different studies, and similar results were also obtained with the original pool of uncloned 32DΔ178 and 32D/hPRLR cells studied prior to isolation of single cell-derived clones.

Is the JAK/STAT Pathway Constitutively Activated in 32DΔ178 Cells?—The binding of either IL-3 or PRL to its respective receptor has been shown to result in the rapid activation of JAK2 tyrosine kinase (33, 38–40). Activation of Janus family tyrosine kinases, such as JAK2, results in the phosphorylation and activation of a family of proteins known as STATs. STAT molecules are cytosolic proteins that become tyrosine-phosphorylated following activation of Janus family kinases, dimerize, and translocate to the nucleus, whereupon they bind to specific DNA elements and activate transcription (1). To determine whether JAK2 was constitutively activated in 32DΔ178 cells, anti-JAK2 immunoprecipitates were subjected to anti-phosphotyrosine immunoblotting (Fig. 5). The tyrosine phosphorylation of JAK2 has been associated with the catalytic activation of the enzyme. Stimulation of 32Dc13 cells with mIL-3 resulted in the phosphorylation of JAK2 (Fig. 5, lanes 1–3). In a similar fashion, stimulation of 32D/hPRLR cells with mIL-3 resulted in the phosphorylation of JAK2, although the signal was not as robust as that seen with 32Dc13 cells (Fig. 5, lanes 7–9). In the absence of mIL-3 stimulation, tyrosine-phosphorylated JAK2 was readily detectable in 32DΔ178 cells, and mIL-3 stimulation increased the phosphorylation of JAK2 approximately 4–5-fold, as indicated by densitometric measure-

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2. S. M. Anderson, unpublished data.
Fig. 5. JAK2 is tyrosine-phosphorylated in the absence of growth factor stimulation in 32DΔ178 cells. A, JAK2 was immunoprecipitated from 32Dcl3 cells (lanes 1–3 and 10–12), 32DΔ178 clone 2 cells (lanes 4–6 and 13–15), and 32D/hPRLR clone 1 cells (lanes 7–9 and 16–18) stimulated with either 100 units/ml mIL-3 (lanes 1–9) or 1 μg/ml rhPRL (lanes 10–18). Cells were stimulated for 0, 5, or 15 min prior to lysis, and the time of stimulation is indicated at the top of each lane. Cell lysates were immunoprecipitated with a polyclonal antibody specific for JAK2, and the precipitated proteins were resolved on an 8% polyacrylamide gel. Immunoblotting was with anti-phosphotyrosine monoclonal antibody 4G10. The position of JAK2 is indicated by the arrow on the right. B, equal levels of JAK2 protein were demonstrated by running 100 μg of whole cell lysate on an 8% polyacrylamide gel and immunoblotting the proteins with anti-JAK2 antibody. Lane numbers are indicated at the bottom of each lane.

Fig. 6. STAT5 is tyrosine-phosphorylated in the absence of growth factor stimulation in 32DΔ178 cells. A, STAT5 was immunoprecipitated from 32Dcl3 cells (lanes 1–3 and 10–12), 32DΔ178 clone 2 cells (lanes 4–6 and 13–15), and 32D/hPRLR clone 1 cells (lanes 7–9 and 16–18) stimulated with either 100 units/ml mIL-3 (lanes 1–9) or 1 μg/ml rhPRL (lanes 10–18). Cells were stimulated for 0, 5, or 15 min prior to lysis, and the time of stimulation is indicated at the top of each lane. Cell lysates were immunoprecipitated with a polyclonal antibody specific for STAT5, and the precipitated proteins were resolved on an 8% polyacrylamide gel. Immunoblotting was with anti-phosphotyrosine monoclonal antibody 4G10. The position of STAT5 is indicated by the arrow on the right. B, the blot shown in A was reprobed with anti-STAT5 antibody to demonstrate the amount of STAT5 protein in each sample. Lane numbers are indicated at the bottom of each lane.

The constitutive activation of JAK2 in 32DΔ178 cells suggested to us that the constitutive activation of STAT molecules might also be observed in these cells. STAT5 was immunoprecipitated with a polyclonal antibody specific for STAT5, and its phosphorylation was examined by anti-phosphotyrosine immunoblotting. Consistent with the results shown in Fig. 5, tyrosine-phosphorylated STAT5 was present in unstimulated 32DΔ178 cells (Fig. 6A, lane 4). Little or no phosphorylated STAT5 was detected in unstimulated 32Dcl3 or 32D/hPRLR cells (Fig. 6A, lanes 1 and 7). Although stimulation of 32Dcl3 and 32D/hPRLR cells with IL-3 resulted in an increase in the amount of phosphorylated STAT5, we did not observe a corresponding increase in STAT5 phosphorylation in the 32DΔ178 cells (Fig. 6A, lanes 4–6). Stimulation of 32D/hPRLR cells with rhPRL resulted in a robust increase in STAT5 phosphorylation, consistent with that observed following stimulation of the same cells with mIL-3 (Fig. 6A, lanes 16–18). No tyrosine-phosphorylated STAT5 was detected in unstimulated or rhPRL-stimulated 32Dcl3 cells, consistent with the absence of the PRLR in these cells. Tyrosine-phosphorylated STAT5 was detected in unstimulated 32DΔ178 cells, and stimulation with rhPRL resulted in a decrease in the amount of phosphorylated STAT5 (Fig. 6A, compare lanes 13–15). Although this result is consistent with decreased phosphorylation of JAK2 in these cells described in Fig. 5, the mechanism behind the decreased phosphorylation of both JAK2 and STAT5 in 32DΔ178 cells stimulated with rhPRL is not clear. There is no reason to suspect that rhPRL would be down-regulating the levels of the Δ178 protein; however, that remains a possibility. It is clear that rhPRL does not induce a decrease in the amount of either JAK2 or STAT5 protein. The levels of STAT5 protein present in unstimulated and IL-3-stimulated 32Dcl3, 32DΔ178, and 32D/hPRLR cells were not significantly different (Fig. 6, bottom panel).

Activation of Mitogen-activated Protein Kinase in 32DΔ178
Cells—Cytokine stimulation has been reported to activate both ERK1 and ERK2, members of the MAP kinase family; this is true for both PRL (41, 42) and IL-3 (37). Catalytic activation of both ERK1 and ERK2 requires the phosphorylation of two amino acids, Thr\(^{183}\) and Tyr\(^{185}\) (43), and the activation of ERKs can be examined through the use of an antibody that recognizes these dually phosphorylated forms of these enzymes. The phosphorylation of MAP kinase was examined in stimulated and unstimulated 32Dcl3, 32DhPRLR, and 32D\(\Delta 178\) cells by immunoblotting with the anti-ACTIVE MAP kinase antibody, and the levels of ERK1 and ERK2 were examined by immunoblotting with a polyclonal antibody directed against both ERK1 and ERK2 (Fig. 7). Stimulation of 32Dcl3 and 32DhPRLR cells with mIL-3 resulted in the activation of both ERK1 and ERK2 as indicated by its reactivity with anti-ACTIVE MAP kinase (lanes 4–6 and 13–15), and 32DhPRLR clone 1 cells (lanes 7–9 and 16–18) were stimulated with either 100 units/ml mIL-3 (lanes 1–9) or 1.0 \(\mu\)g/ml rhPRL (lanes 10–18). Cells were stimulated for 0, 5, or 15 min prior to lysis, and the time of stimulation is indicated at the top of each lane. Fifty micrograms of total cellular protein were run on an 8% polyacrylamide gel. The proteins were transferred to an Immobilon membrane, and the filter was immunoblotted with either anti-ACTIVE antibody, which specifically recognizes the activated form of ERK2 and ERK1 (top), or with a polyclonal anti-MAP kinase antibody that recognizes both ERK1 and ERK2. The positions of ERK1 and ERK2 are indicated by the arrows on the right in each panel. Lane numbers are indicated at the bottom of each lane.

Numerous proteins have been reported to become phosphorylated on tyrosine residues following stimulation of cells with PRL. These include an adapter protein known as SHC (44). We have examined the phosphorylation of SHC in 32D\(\Delta 178\) cells to determine whether the presence of the activated PRLR resulted in its constitutive phosphorylation. SHC was immunoprecipitated from all three cell lines and immunoblotted with an anti-phosphotyrosine monoclonal antibody (Fig. 8). Although three forms of SHC are known to exist, only two forms were observed to become phosphorylated following IL-3 stimulation of either 32Dcl3 or 32DhPRLR cells (Fig. 8, lanes 1–3 and 7–9). We suspect that these may represent the 52- and 46-kDa forms of SHC, although they do not migrate at their reported molecular weights. Both of these forms of SHC were also phosphorylated in unstimulated 32D\(\Delta 178\) cells, and stimulation with mIL-3 increased their extent of phosphorylation (Fig. 8, lanes 4–6). Stimulation of 32Dcl3 cells with rhPRL did not result in the appearance of tyrosine-phosphorylated SHC (data not shown). Stimulation of 32DhPRLR cells with rhPRL resulted in the increased phosphorylation of both forms of SHC (Fig. 8, lanes 14 and 15); however, compared with the studies with mIL-3, rhPRL does not induce as robust a phosphorylation of SHC. A longer time of exposure was required to detect the phosphorylation of SHC induced by rhPRL. This longer exposure time can be appreciated by comparing the apparent amount of phosphorylated SHC present in the 32D\(\Delta 178\) cells in both panels of Fig. 8 (compare lanes 4 and 10). The constitutive phosphorylation of two forms of SHC was observed in unstimulated 32D\(\Delta 178\) cells, and stimulation with rhPRL did not result in a dramatic increase in the amount of phosphorylated SHC. A doublet of tyrosine-phosphorylated proteins in the 140–150-kDa range was observed to co-immunoprecipitate with SHC in cells stimulated with mIL-3 (Fig. 8, lanes 2, 3, 5, 6, 8, and 9). Although this protein is not detected in lanes 10–15 of Fig. 8, longer exposures revealed the presence of this phosphoprotein.

We suspect that this protein represents SHIP, the SH2-containing phosphatidylinositol 5-phosphatase that co-immunoprecipitates with SHC (34, 35, 45).

The \(\Delta 178\) Deletion Mutant Can Also Induce Growth Factor-independent Proliferation of the Nb2 Cell Line—To demonstrate that the effects of the \(\Delta 178\) deletion mutant were not specific to the 32Dcl3 cell line, similar studies were also con-

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**Constitutive Activation of the PRLR**

**Fig. 7. Constitutive activation of MAP kinase in unstimulated 32D\(\Delta 178\) cells.** 32Dcl3 cells (lanes 1–3 and 10–12), 32D\(\Delta 178\) clone 2 cells (lanes 4–6 and 13–15), and 32DhPRLR clone 1 cells (lanes 7–9 and 16–18) were stimulated with either 100 units/ml mIL-3 (lanes 1–9) or 1.0 \(\mu\)g/ml rhPRL (lanes 10–18). Cells were stimulated for 0, 5, or 15 min prior to lysis, and the time of stimulation is indicated at the top of each lane. Fifty micrograms of total cellular protein were run on an 8% polyacrylamide gel. The proteins were transferred to an Immobilon membrane, and the filter was immunoblotted with either anti-ACTIVE antibody, which specifically recognizes the activated form of ERK2 and ERK1 (top), or with a polyclonal anti-MAP kinase antibody that recognizes both ERK1 and ERK2. The positions of ERK1 and ERK2 are indicated by the arrows on the right in each panel. Lane numbers are indicated at the bottom of each lane.
Constitutive activation of the PRLR

Constitutive phosphorylation of SHC in unstimulated 32DΔ178 cells. 32Dcl3 cells (lanes 1–3), 32DΔ178 clone 2 cells (lanes 4–6 and 10–12), and 32D/hPRLR clone 1 cells (lanes 7–9 and 13–15) were stimulated with either 100 units/ml IL-3 (lanes 1–9) or 0.5 μg/ml rhPRL (lanes 10–15). Cells were stimulated for 0, 5, or 15 min prior to lysis, and the time of stimulation is indicated at the top of each lane. The lysates were immunoprecipitated with a polyclonal antibody to SHC, and the immunoprecipitated proteins were resolved on an 8% polyacrylamide gel. The resolved proteins were transferred to an Immobilon membrane and immunoblotted with antiphosphotyrosine monoclonal antibody 4G10. The positions of molecular mass markers are indicated on the left, and the positions of SHC are indicated on the right. The immunoblot was reprobed with a monoclonal antibody to SHC, revealing equal amounts of SHC in each of the different cell lines under each of the conditions employed in this experiment (data not shown). Lane numbers are indicated at the bottom of each lane.

Constitutive phosphorylation of SHC in unstimulated Nb2 cells, Nb2 (46). The Nb2 cell line was transfected with expression vectors encoding either the full-length hPRLR or the Δ178 deletion mutant, transfected cells obtained by selection for resistance to G418, single cell-derived clones obtained as described above. Consistent with the results described above, transfection of the Nb2 cell line with the Δ178 expression vector resulted in the appearance of a population of cells that grew in the absence of PRL. As shown in Fig. 9, the proliferation of Nb2 cells was stimulated by the addition of oPRL, although some proliferation of Nb2 cells was observed when cells were cultured in the medium containing horse serum alone. This apparently reflects the presence of low amounts of lactogenic hormones in the preparation of horse serum used in these studies. The Nb2Δ178 cells proliferated in both the absence and presence of exogenous oPRL (Fig. 9). Nb2Δ178 cells did not proliferate as fast in the absence of additional oPRL as did the cells cultured in the presence of oPRL (Fig. 9). This is not surprising, since, unlike the 32Dcl3 cells, Nb2 cells do have an endogenous PRLR.

The activation of two different signaling molecules was also examined in Nb2 cells expressing the Δ178 deletion mutant, JAK2 and ERKs. Nb2/hPRLR clone 7 and Nb2ΔΔ178 clone 4 cells were stimulated with rat PRL for 0–15 min, cell lysates were prepared, and the phosphorylation of JAK2 was examined as described in Fig. 5 above. A small but readily detectable amount of tyrosine-phosphorylated JAK2 was detected in the unstimulated Nb2Δ178 clone 4 cells, and the amount of tyrosine-phosphorylated JAK2 increased following stimulation with rat PRL (Fig. 10A, lanes 1–9). In contrast, very little, if any, tyrosine-phosphorylated JAK2 was detected in these cells following stimulation with rhPRL (Fig. 10A, lanes 10–15). Consistent with the data shown in Fig. 5, as well as all previous studies on the activation of JAK2 by PRL (33, 38–40), there was no difference in the amount of JAK2 in either of the cell lines at any of the time points examined (data not shown).

The amount of activated ERK1 and ERK2 was also examined in both Nb2Δ178 clone 4 and Nb2/hPRLR clone 7 cells using the same approach utilized in Fig. 7. Both cell lines were stimulated with 100 nM oPRL for 0–15 min, whole cell lysates were prepared, and immunoblots were analyzed with either anti-ACTIVE MAP kinase antibody or anti-MAP kinase antibody. As shown in Fig. 10B, a low amount of activated ERK1 and ERK2 was present in unstimulated Nb2Δ178 clone 4 cells, and the amount of activated ERK did not increase following stimulation with oPRL (Fig. 10B, lanes 10–12). In contrast, no activated ERK1 or ERK2 was detected in unstimulated wild type Nb2 cells or Nb2/hPRLR clone 7 cells; however, there was a dramatic increase in the amount of activated ERK following PRL stimulation, as indicated by immunoblotting. There was no change in the amount of either ERK1 or ERK2 in any of the cell lines at any of the time points examined (Fig. 10C). These data are consistent with the results obtained with the 32DΔ178
cells examined in Fig. 7. In both studies there was a low level of activated ERK present in cells expressing the Δ178 deletion mutant, and stimulation with either oPRL or rhPRL did not stimulate an increase in the amount of activated ERK.

Expression of the Δ178 Mutant of the PRLR Suppresses Apoptosis of 32Dcl3 Cells following Cytokine Withdrawal—As noted above, the proliferation and viability of 32Dcl3 cells is critically dependent upon stimulation with exogenous IL-3. Cultivation of these cells in the absence of growth factors results in their death within 48–60 h. Numerous oncogenes are able to render 32Dcl3 cells growth factor-independent with a concomitant suppression of apoptosis (47–52). We were interested in determining whether expression of the Δ178 deletion mutant of the PRLR suppressed apoptosis. Both 32Dcl3 and 32DΔ178 cells were cultured in the presence or absence of 10% WEHI-3 conditioned media for 0–72 h. DNA was isolated from these cells at various time points, and the induction of DNA fragmentation was assessed by agarose gel electrophoresis. DNA fragmentation was detected in 32Dcl3 cells cultured in the absence of mIL-3 by 16 h, and the 200-base pair ladder was evident at 36 h (Fig. 11, lanes 1–7). Cultivation of 32Dcl3 cells in mIL-3 did not result in DNA fragmentation (Fig. 11, lanes 8–11). No DNA fragmentation was observed in the 32DΔ178 clone 2 cells, regardless of whether they were cultured in the presence or absence of mIL-3 (Fig. 11, lanes 12–22). These results indicate that in addition to being growth factor-independent, cells expressing the Δ178 deletion mutant of the hPRLR do not undergo apoptosis following growth factor withdrawal.

DISCUSSION

The binding of PRL to its receptor activates a specific series of signaling events within the cell. The critical initial event appears to be the ligand-induced dimerization of the receptor leading to the activation of specific tyrosine kinases (53, 54). These kinases include both the JAK2 tyrosine kinase (33, 38–40) and the src-related tyrosine kinase Fyn (55). Activation of JAK2 leads to the phosphorylation and activation of the tran-
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transcription factor STAT5 (56, 57). STAT5, also known as mammary gland factor, is required for the induction of specific transcription products such as β-casein (56–58). Other signaling pathways that are activated include Ras, Raf, and MAP kinase (42, 44, 59) as well as phosphatidylinositol 3-kinase (60). The means by which these other signaling pathways are regulated has not been demonstrated; however, it is possible that they may lie downstream of src-like tyrosine kinases. Numerous other proteins become tyrosine-phosphorylated following activation of the PRLR, including the receptor itself (33, 38–40), the SH2-containing adapter protein SHC (44), and the insulin receptor substrate-1 (61). With the exception of insulin receptor substrate-1, these proteins and enzymes appear to be critical in regulating the response of all cells to growth factors and cytokines.

In this report, we have demonstrated that deletion of the extracellular ligand binding domain of the human PRLR results in the constitutive activation of the receptor. Evidence supporting this conclusion includes the following: 1) expression of this truncated receptor leads to growth factor-independent proliferation of a growth factor-dependent myeloid cell line; 2) an elevated level of tyrosine-phosphorylated proteins was observed in unstimulated 32DΔ178 cells; 3) constitutive activation of both JAK2 and STAT5 was observed in unstimulated 32DΔ178 cells; 4) ERKs were constitutively activated in the absence of IL-3 or PRL stimulation; 5) the SH2-containing adapter protein SHC was phosphorylated in unstimulated 32DΔ178 cells; and 6) in contrast to 32DcI3 cells, which undergo apoptosis upon IL-3 withdrawal, 32DΔ178 cells did not undergo apoptosis under the same conditions. Similar data were obtained when the Δ178 deletion mutant was expressed in the Nb2 cell line, indicating that these results are not cell type-specific. These data clearly support the conclusion that deletion of the majority of the extracellular domain of the PRLR results in the constitutive activation of this receptor. The majority, if not all, of the proteins involved in signal transduction by the PRLR, also appear to be activated/phosphorylated by the Δ178 deletion mutant. To date we have not observed the phosphorylation of any novel proteins that have not been observed to be phosphorylated following the binding of PRL to its receptor. This suggests that the constitutively active mutant of the PRLR utilizes the same normal signaling pathways used by the PRLR. Although identical results were obtained in the analysis of five independent clones of 32D/hPRLR cells, five different clones of 32DcI3 cells expressing the FLAG-tagged hPRLR, five different clones of Δ178 cells, and five independent clones of cells expressing the FLAG-tagged Δ178 protein, we cannot rule out the possibility that additional genetic changes have occurred to these cells during the selection of these cells. Secondary genetic changes in the 32DcI3 cells expressing the hPRLR cannot be readily identified, and for them to be present in all of the independently derived clones would suggest that there must be a tremendous genetic pressure for them to occur. DNA sequence analysis would be required to determine whether additional mutations have occurred in the Δ178 protein; DNA sequence analysis is beyond the scope of the present investigation but will be examined in the future.

Other investigators have previously described the constitutive activation of the rabbit PRLR following deletion of amino acids 103–203. The biological assay utilized by these authors was the transcriptional activation of a reporter gene construct in which the promoter of the β-lactoglobulin gene was fused to the chloroamphenicol acetyltransferase reporter gene (22). This constitutively activated receptor has also been shown to induce expression of β-casein in HC11 in the absence of PRL stimulation (22). The ability of this mutant receptor to induce transcription of a gene known to be induced by PRL, in a growth factor-independent manner, demonstrates that this deletion mutant of the PRLR has acquired some of the properties of an activated growth factor receptor. These authors did not examine the ability of their activated receptor mutant to activate downstream signaling molecules such as JAK2, STAT5, ERKs, or any other signaling proteins known to lie downstream of the PRLR (22). Furthermore, they did not report the ability of their activated receptor to induce growth factor-independent proliferation of PRL-dependent cell lines (22).

Gourdou et al. (22) have reported that the deletion of amino acids 103–203 (domain S2) resulted in the constitutive activation of the PRLR; however, the deletion of amino acids 3–103 (domain S1) or 3–203 (S1,S2) did not result in the activation of the receptor. Based upon these data, Gourdou et al. have suggested that sequences in the S1 region can activate the PRLR and that sequences in the S2 region function to suppress the activity of S1 (22). We do not believe this hypothesis of Gourdou et al. to be correct, since the Δ178 we have generated is very similar to the S1,S2 deletion used in their studies (see below).

As noted above, our strategy in making the Δ178 deletion mutant was to delete as much of the extracellular ligand binding region as possible and to leave the WSXS motif intact. The major difference between our Δ178 deletion mutant and the inactive 3–203 deletion mutant made by Gourdou et al. is that our deletion mutant contains the WSXS sequence, while their deletion mutant lacks this conserved sequence (Fig. 1). The three-dimensional structure of the human PRLR bound to human growth hormone indicates that the extracellular domain of the receptor forms two β-sheet barrels (62). The WSXS sequence is thought to be critical in maintaining the structure of the β-sheet barrel adjacent to the plasma membrane (62). We hypothesize that this WSXS sequence is absolutely critical for the constitutive activation of our Δ178 mutant. Our model predicts that dimerization of receptors containing deletions of the extracellular ligand binding domains is driven by the hydrophobic interaction between the WSXS sequence on one receptor molecule with the same sequence on a second receptor molecule. Such interactions would remove four tryptophan side chains from water and provide sufficient energy to make this a stable interaction. Since the tryptophan residues of the WSXS sequence are normally buried, these side chains normally would not contribute to the dimerization of receptor monomers. This model would predict that these tryptophan residues could be mutated to other hydrophobic amino acids, such as phenylalanine. Replacement of the tryptophans with charged amino acids, however, should render these constitutively activated receptors nonfunctional. This hypothesis will be directly tested by mutation of the amino acids predicted to be critical in the ligand-independent receptor dimerization. We do not believe that the S2 region functions as a repressor of the S1 region as suggested by Goudou et al. (22), since our mutant essentially deletes both S1 and S2.

Although it is clear that the PRLR is able to induce proliferation of the Nb2 rat pre-T lymphoma cell line as well as induce proliferation of factor-dependent myeloid cell lines such as those utilized in this study, it is not clear whether the PRLR can stimulate proliferation of other cells, such as normal mammary epithelial cells. Although other investigators have demonstrated that PRL can stimulate the proliferation of a human breast cancer cell line (63, 64), it is not clear whether PRL alone can stimulate the proliferation of mammary epithelial cells in the mammary gland. Both PRL and its receptor play a critical role in the development of the mammary gland and the induction of lactation (65, 66). Due to the complexity of growth factors and steroid hormones that influence the development of
We also thank Dr. Paul Kelly for providing the human PRLR cDNA and Colorado Cancer Center DNA Sequencing Core Facility in support of proliferation of cells in this tissue. We believe that we have the question of whether PRL and its receptor can stimulate gland (65, 67); however, analysis of these mice does not answer PRLR or STAT5a clearly demonstrate that the PRLR signal fibroblasts or mammary epithelial cells. Future studies will draw. These properties are among those one would expect of cells expressing this constitutively activated version of the PRLR do not undergo apoptosis when growth factor is withdrawn. These properties are among those one would expect of an activated oncogene; however, we have not demonstrated that the Δ178 deletion mutant is able to transform either fibroblasts or mammary epithelial cells. Future studies will address the oncogenic potential of this constitutively active mutant of the PRLR.

Acknowledgments—We acknowledge the services of the University of Colorado Cancer Center DNA Sequencing Core Facility in support of this research. We thank Elizabeth Burton for discussions on this project and Dr. Arthur Gutierrez-Hartmann for comments on this manuscript. We also thank Dr. Paul Kelly for providing the human PRLR cDNA and Dr. Andrew Larner for providing the anti-STAT5 antibody.

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