The Protein Tyrosine Kinase p59^{fyn} Is Associated with Prolactin (PRL) Receptor and Is Activated by PRL Stimulation of T-Lymphocytes

Charles V. Clevenger and Maxine V. Medaglia

Department of Pathology and Laboratory Medicine
University of Pennsylvania Medical Center
Philadelphia, Pennsylvania 19104

The clonal expansion of antigen-stimulated T-lymphocytes during an immune response is mediated by several lymphokines. Strong evidence now exists that the neuroendocrine hormone PRL is necessary, but not sufficient, for T-cell proliferation. Little is known, however, of the signal transduction mechanisms of the PRL receptor (PRLR) within T-cells. We demonstrate here that PRL stimulation of the T-cell line Nb2 induced the concentration- and time-dependent activation of the protein tyrosine kinase p59^{fyn}, but not of four other *src* family protein tyrosine kinases. Activation of *fyn* was also observed in Concanavalin-A-primed peripheral blood lymphocytes stimulated with PRL and in Nb2 cells incubated with anti-PRLR antibodies. The activation of *fyn* by PRL stimulation correlated with Nb2 cell proliferation. Immunoblot analysis of anti-*fyn* and anti-PRLR immune complexes revealed an association between each PRLR isoform and p59^{fyn}. These studies demonstrate for the first time an association between the PRLR and a *src* family protein tyrosine kinase affiliated with signal transduction. (Molecular Endocrinology 8: 674–681, 1994)

INTRODUCTION

The PRL receptor (PRLR) is a member of the growth factor receptor superfamily to which the interleukin-2–7 (IL-2–7), granulocyte/macrophage-colony-stimulating factor (CSF), granulocyte-CSF, and erthryopoietin receptors belong. Like other members of this superfamily, the functions of the PRL-PRLR complex are pleiotropic. Although best known for its functions in the terminal differentiation of mammary tissue, recent data indicate that the PRLR can modulate immune response. Manipulation of serum PRL levels can profoundly influence the immune response (1–7) *in vitro* and *in vivo*. Increases in PRL have been observed during allograft rejection (8), and a role for PRL in systemic lupus erythematosus has been suggested (9, 10). At the molecular level, the PRLR is expressed on all cells within the human (11) and murine (12) immune system; in addition, human T-cells can synthesize PRL (11, 13). *In vitro*, T-lymphocytes appear to use PRL in an autocrine manner, and PRL is necessary for T-cell proliferation and passage into the S-phase (14).

Like other members of the growth factor receptor superfamily, the mechanisms of PRLR signal transduction have remained poorly characterized. Although three isoforms of the PRLR that differ in the length of their cytoplasmic domain have been cloned, none of these has a demonstrable enzymatic activity (15–17). Several reports have alternatively suggested that GTP-binding proteins, adenylate cyclase, protein kinase-C, or the sodium/hydrogen antiport may participate in this event (18–21). More recent data have suggested that protein phosphorylation of tyrosine residues is induced by PRL stimulation (22, 23). This would suggest that an unidentified protein tyrosine kinase (PTK) may be involved in PRLR signal transduction.

The data presented here demonstrate that the PRLR is associated with the *src* family PTK, p59^{fyn}, and that stimulation of the PRL-responsive T-cell line Nb2 and Concanavalin-A (Con-A)-primed peripheral blood lymphocytes (PBL) with this hormone induces the activation of this PTK. These data suggest that *fyn* may serve during PRL stimulation as a signaling intermediary necessary for PRL-induced tyrosine phosphorylation and proliferation in T-lymphocytes.

RESULTS

PRL stimulation of Nb2 cells has been demonstrated to induce the phosphorylation on tyrosine residues of multiple proteins (22, 23). Analysis of immunoblots probed with antiphosphotyrosine in our laboratory also revealed a cross-reactive, 55- to 60-kilodalton (kDa) phosphoprotein (24). The mol wt of this phosphoprotein was appropriate for a PTK of the *src* family, which have

0888-8809/94/0674-0681$03.00/0
Molecular Endocrinology
Copyright © 1994 by The Endocrine Society

Downloaded from https://academic.oup.com/mend/article/8/6/674/2714991 by guest on 20 August 2022

been found in association with other members of the growth factor receptor family, *i.e.* the IL-7 receptor and the β-chain of the IL-2 receptor (25, 26). To determine which of the *src* family PTKs commonly found in T-cells (27) were present in Nb2 cells, immunoblot analysis was performed on Nb2 lysates, which revealed the presence of the PTK's *lck*, *fyn*, *src*, *lyn*, and *yes* (data not shown). Subsequently, activation of the PTKs within these immunoprecipitates was assessed by autokinase assay on lysates from Nb2 cells maintained under optimal growth conditions [*i.e.* in medium containing 10% fetal calf serum (FCS)]. Under these conditions, activation of *lck* and *fyn* was observed, but no detectable autokinase activity of *src*, *lyn*, or *yes* was noted.

The data shown in Fig. 1 suggested that, of five *src* family PTKs found in T-cells, only *lck* or *fyn* might be activated during PRL-induced growth. To examine the activation of these kinases by PRL under defined conditions, Nb2 cells were starved for 48 h in a PRL-deficient, chemically defined medium before stimulation with PRL and harvest of cells at various time intervals. Immunoprecipitation of lysates obtained from these cells with either anti-*fyn* or anti-*lck* followed by *in vitro* kinase assay revealed a transiently inducible activation of *fyn* kinase, as shown in Fig. 2A. Increases in the autophosphorylation of *fyn* were seen within 5 min of stimulation and peaked at approximately 20 min, reaching a level approximately 12-fold greater than that seen in resting cells (Fig. 2B). In comparison, no increase in *lck* autokinase activity occurred during PRL stimulation; in fact, the low levels of *lck* autokinase activity observed in resting cells became undetectable after PRL stimulation. Immunoblot analysis of Nb2 lysates from cells stimulated under similar defined conditions, however, revealed that the levels of $p59^{fyn}$ kinase during PRL stimulation varied by less than 25% (Fig. 3). This would indicate that the increases in *fyn* kinase activity during PRL stimulation represented a true change in the specific activity of this enzyme.

The concentration dependence of the PRL-stimulated *fyn* autokinase and substrate kinase activities was examined and correlated with Nb2 proliferation. As shown in Fig. 4, PRL-stimulated increases in *fyn* autokinase

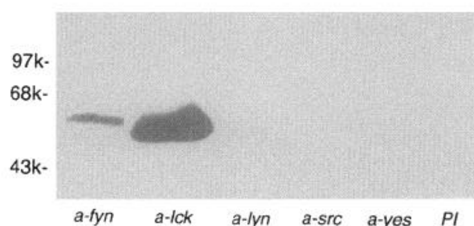

Fig. 1. *Src* Family PTK Phenotype of Nb2 Cells

Lysates from Nb2 cells maintained in 10% FCS were immunoprecipitated with antibodies directed against members of the *src* family of tyrosine kinases or preimmune (PI) serum. The autokinase activity of the immunoprecipitates was examined by incubation with [γ-^{32}P]ATP for 15 min, with subsequent analysis by SDS-PAGE. Mol wt, indicated on the *left*, are in kilodaltons.

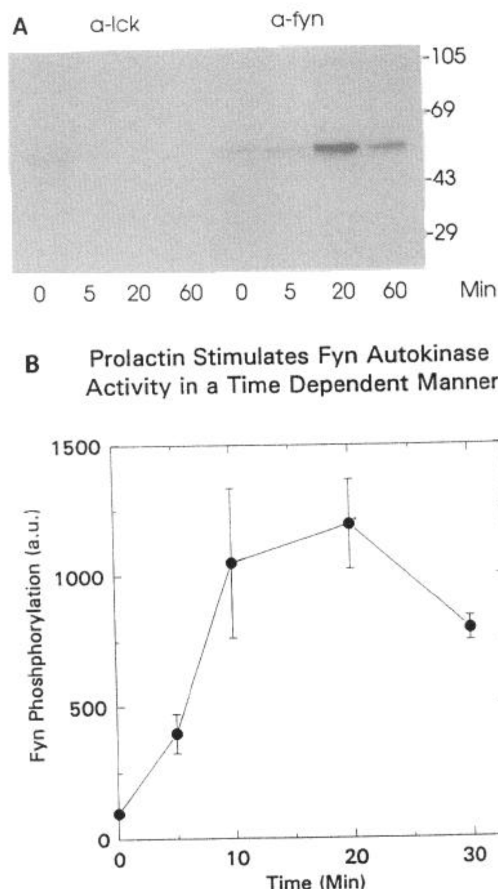

Fig. 2. PRL Stimulation of Nb2 Cells in Defined Conditions Induces a Time-Dependent Activation of *fyn*, but not *lck*, Autokinase Activity

A, Nb2 cells starved for 48 h in a defined PRL-deficient medium were stimulated with 1 ng PRL/ml for various time intervals. The lysates from 2×10^6 cells were immunoprecipitated with anti-*fyn* or anti-*lck* antiserum or preimmune serum before autokinase assay for 2 min with [γ-^{32}P]ATP and analysis by SDS-PAGE. Autokinase assay of preimmune immunoprecipitates revealed the absence of autokinase activity (data not shown). B, Time course of autokinase activation of *fyn*. Nb2 cells were stimulated with 1 ng PRL/ml and harvested at various times before immunoprecipitation and autokinase assay. Quantitation of the autoradiographs was accomplished by a Molecular Dynamics scanning laser densitometer, and the volumetric measurements are presented in arbitrary units. The data represent the mean of three experiments ± SEM.

and substrate kinase occurred in a concentration-dependent manner, with *fyn* autokinase activity peaking at approximately 0.1–0.3 ng PRL/ml, whereas *fyn* substrate kinase activity peaked at approximately 0.3–1.0 ng PRL/ml (Fig. 4C). Thus, the activation of *fyn* by PRL paralleled PRL-stimulated Nb2 cell proliferation (Fig. 4D), albeit at an ED_{50} approximately 10-fold less. These data would indicate that PRL regulates *fyn* activity, and that such activity may contribute to Nb2 cell proliferation.

To explore the possibility that *fyn* associates with the PRLR in Nb2 cells, anti-PRLR and anti-*fyn* immunopre-

Downloaded from https://academic.oup.com/mend/article/8/6/674/2714991 by guest on 20 August 2022

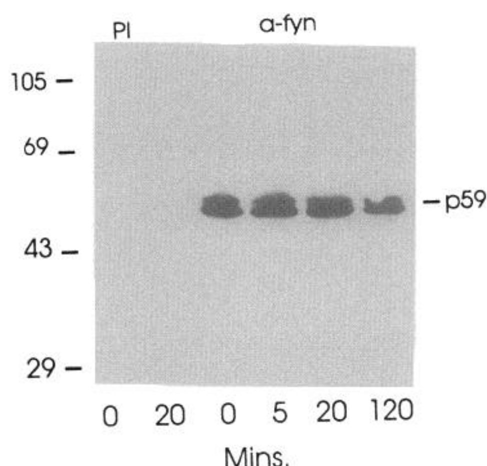

Fig. 3. Relative Levels of *fyn* Protein Are Unchanged by PRL Stimulation

Nb2 cells were starved for 48 h before stimulation with 1 ng PRL/ml for various time intervals. The lysates from approximately 5 × 10^5 cells were subjected to SDS-PAGE before immunoblot analysis with either anti-*fyn* or preimmune (PI) serum. Densitometric analysis revealed that each band was within ±25% of the aggregate mean; such minimal variation cannot account for the magnitude of activation of *fyn* autokinase activity seen in Fig. 2. As has been previously described (56), the *lower band* present in these immunoblots may represent a proteolytic product or isoform of *fyn* that is soluble in SDS only, hence its absence from lysates obtained with Nonidet P-40 (*i.e.* Figs. 1, 2A, 4A, 5, 6, and 7).

cipitates were obtained from Nb2 lysates and subjected to semiquantitative immunoblot analysis with anti-*fyn* or anti-PRLR antibodies, using enhanced chemiluminescence as an antigen-antibody detection method (Fig. 5). These data demonstrated that approximately 10% of *fyn* within the Nb2 cell is associated with the PRLR in anti-PRLR complexes (Fig. 5, *right side*) and revealed that each of the PRLR isoforms (short, intermediate, and long) is associated with the *fyn* (Fig. 5, *left side*). Furthermore, approximately equal levels of *fyn* were associated with the PRLR in resting and PRL-stimulated cells, indicating that *fyn*-PRLR complexes are preformed before PRL stimulation.

Although Nb2 cells provide an excellent system for examining PRLR signal transduction, these cells represent a transformed lymphocyte line, capable of proliferating in response to PRL alone (28). As PRL alone is incapable of driving the proliferation of most normal resting lymphocytes (12), the effect of PRL on *fyn* activation was examined during the mitogen stimulation of normal PBL in defined medium (Fig. 6). Anti-*fyn* immunoprecipitates were obtained from resting and Con-A-stimulated PBL lysates and subjected to autokinase assay. Both resting and continuously Con-A-stimulated PBL were refractory to the induction by PRL of *fyn* autokinase activity. The lysates from PBL, transiently primed with Con-A and rested 24 h before PRL stimulation, however, demonstrated a significant increase in *fyn* autokinase activity.

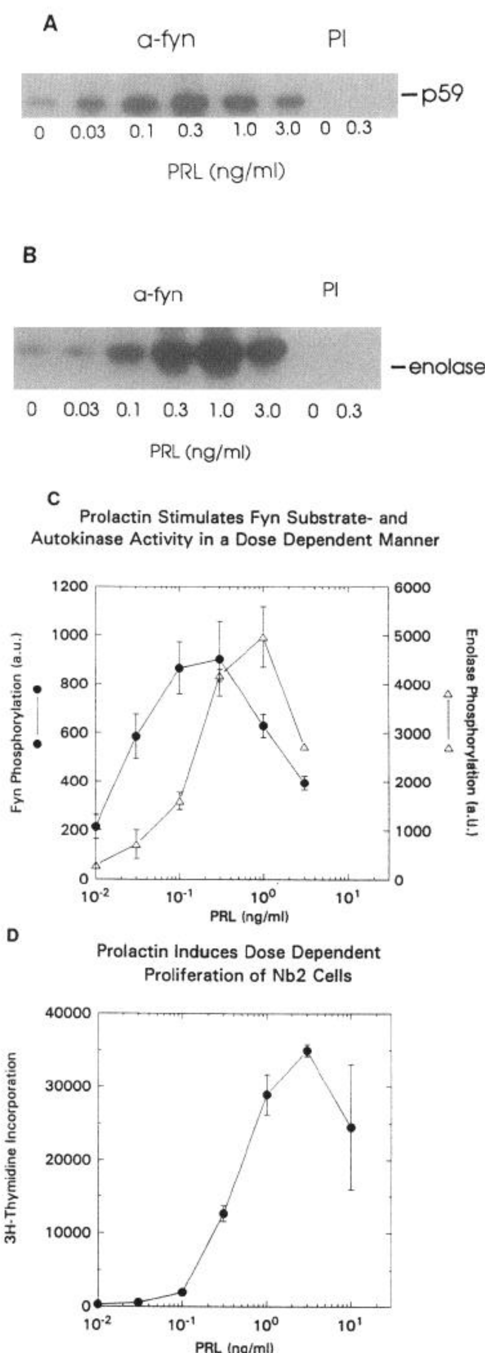

Fig. 4. Comparison of PRL Concentration Dependence of *fyn* Activation with Nb2 Cellular Proliferation

Nb2 cells were stimulated with varying concentrations of PRL for 20 min before immunoprecipitation with anti-*fyn* or preimmune (PI) serum or for 48 h before [^{3}H]thymidine incorporation studies. The immunoprecipitates were subjected to autokinase (A) or substrate kinase (B) assay. The substrate kinase assay used 5.0 μg acid-denatured enolase/assay. Quantitation of the data presented in part in A and B is shown in C, and the proliferation studies are presented in D. The data presented in both C and D represent the mean of three experiments ± SEM. Units for the y-axis in D are in incorporated counts per min.

Downloaded from https://academic.oup.com/mend/article/8/6/674/2714991 by guest on 20 August 2022

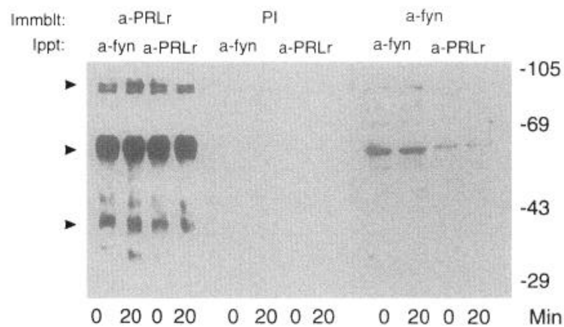

Fig. 5. Anti-PRLR Immune Complexes Contain *fyn*

Starved or PRL-stimulated cell lysates were immunoprecipitated with either anti-*fyn* or anti-PRLR antibodies. After clearing immunoglobulin from denatured immune complexes, the immunoprecipitated (Ippt) proteins were subjected to immunoblot (Immblt) analysis with anti-*fyn* or anti-PRLR antibodies or preimmune (PI) serum. Control experiments using isotype-matched control monoclonal antibody were comparable to those obtained using preimmune serum (data not shown). The *arrowheads on the left side* demonstrate each of the PRLR isoforms migrating at approximately 42, 62, and 82 kDa.

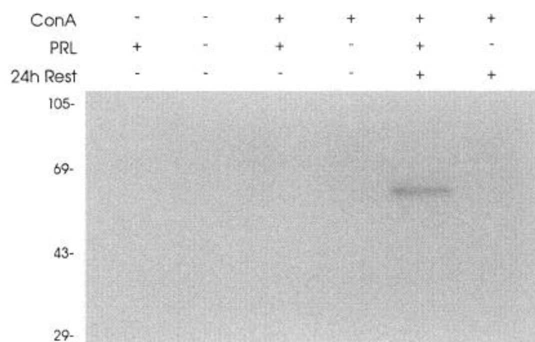

Fig. 6. Activation of *fyn* Kinase by PRL Occurs after Transient Con-A Stimulation

One × 10^6 PBL/ml defined medium were cultured in the presence or absence of 0.5 μg Con-A/ml for 24 h (ConA ±). At this point, one set of Con-A-stimulated PBL cultures was washed twice in defined medium, placed into medium lacking Con-A (24h Rest ±), and incubated with the other cultures for an additional 24 h. At the end of 48 h of incubation, half of the cultures received 10 ng human recombinant PRL/ml for 20 min (PRL ±). After this, all cultures were harvested, and cell lysates were subjected to autokinase assay after immunoprecipitation with anti-*fyn* antibody.

Previous data demonstrated that the proliferation of Nb2 cells may be induced by their incubation with anti-PRLR antibody in the presence of a secondary cross-linking antibody (29). These data have been interpreted to indicate that the antibody-induced dimerization of PRLR may induce cell surface signaling in a manner analogous to the hypothesized PRL-PRLR complex. To examine whether antibody-induced dimerization could induce the activation of *fyn* autokinase activity, Nb2 cells were incubated with 500 pM anti-PRLR antibody and a 1:1000 dilution of antimouse antibody, which has been previously documented to maximally induce Nb2 cell proliferation (29). As shown in Fig. 7, cross-linked anti-PRLR antibody effectively stimulated the autokinase activity of *fyn* immunoprecipitates, indicating that such cross-linking effectively stimulates PRLR signaling. In the absence of anti-PRLR antibody, the anti-mouse antibody did not induce appreciable autokinase activity (data not shown).

DISCUSSION

The results presented here demonstrate that of five *src* family PTKs examined, only $p59^{fyn}$ was activated by PRL in Nb2 cells. Nb2 lysates from cells maintained in FCS-containing medium, demonstrated significant *lck* autokinase activity; however, under defined conditions, activation of this kinase by PRL was negligible. The activation of *lck* in FCS-maintained Nb2 cells may be due to the presence of other growth factors in FCS, such as IL-2, which can activate *lck* (26). The autophosphorylation activity of *fyn*, unlike that of *lck*, was induced by PRL stimulation under defined conditions in a transient time-dependent manner. This stimulation was due to an increase in the specific activity of *fyn*, as immunoblot analysis of Nb2 lysates revealed a minimal change in the level of *fyn* protein during the time course examined here. Dose-dependent increases in both the autophosphorylation and substrate kinase activities were shown to roughly parallel the dose-dependent proliferative response of Nb2 cells to PRL. The kinetics demonstrated here are similar to the activation of other *src* family PTKs (*i.e. lyn* and *lck*) by other ligand-stimulated (IL-2 or IL-3) members of the growth factor receptor superfamily (30). The temporally transient and rapid activation of *fyn* by PRL indicates that this PTK may participate in PRLR signaling. PRL stimulation can induce the expression of immediate early gene expression, such as interferon regulatory factor-1 and *c-myc*

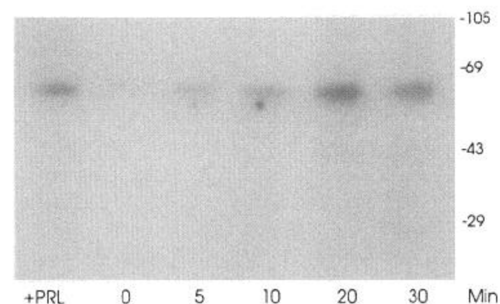

Fig. 7. Incubation of Nb2 Cells with Anti-PRLR Antibody under Cross-Linking Conditions Induces *fyn* Autokinase Activity

Nb2 cells were starved for 48 h before stimulation with 500 pM U6 anti-PRLR antibody in the presence of a 1:1000 dilution of goat antimouse antibody for various time intervals. Some cells were alternatively stimulated with 1 ng PRL/ml for 20 min (+PRL). Lysates from these cells were immunoprecipitated with anti-*fyn* antibody and subjected to autokinase assay.

Downloaded from https://academic.oup.com/mend/article/8/6/674/2714991 by guest on 20 August 2022

in Nb2 cells (31, 32), and is necessary for the cell cycle-dependent expression of histone-H3 and cyclin-B at the G1/S-phase transition in cloned T-lymphocytes (14). Besides its role in triggering PRLR signaling, the PRL ligand has been shown to translocate into the nucleus of IL-2-stimulated T-lymphocytes during G1-phase progression and appears to be necessary at this site for G1/S-phase traverse via a yet unidentified mechanism (12, 33). Hypothetically, the signaling afforded by the PRLR-associated *fyn* may contribute to the expression of immediate early genes, whereas nuclear PRL may participate in the expression of genes necessary for G1/S-phase traverse.

Immunoblot analysis of anti-PRLR immunoprecipitates revealed that *fyn* was complexed with each PRLR isoform. These complexes appeared to be preformed, *i.e.* they were present in resting cells. Preformed signaling complexes are not unique to the PRLR, but have also been found in other members of the growth factor receptor superfamily. One example is the β-chain of the IL-2 receptor, which has been found to preassociate with either $p56^{lck}$ or $p53/56^{lyn}$ (26, 34). The immunoprecipitation of *fyn* with each of the PRLR isoforms suggests that the structural determinants common to each of these isoforms may be responsible for this interaction. A growth factor receptor superfamily member that also associates with *fyn* is the IL-7 receptor (25, 35). A hydrophobic proline-rich region in the amino-terminal end of the cytoplasmic domain is present in each of the PRLR isoforms and the IL-7 receptor and may represent a potential *fyn*-binding site (25, 36). This region (the so-called box 1) is also present in other members of the growth factor receptor family, such as the IL-2β, IL-3, IL-4, GH, erythropoietin, and granulocyte-CSF receptors, and in gp-130 and, thus, could represent a site for PTK association (36). It is not clear which determinants within *fyn* are necessary for its interaction with the PRLR. Unlike the receptor for platelet-derived growth factor (37, 38), the PRLR lacks the enzymatic activity to autophosphorylate itself, and initial reports (22, 23) indicate that the PRLR itself is not significantly phosphorylated on tyrosine residues during PRL stimulation. Thus, it seems unlikely that *fyn*-PRLR interaction was mediated through the SH2 domain of *fyn*, as occurs with the *fyn*-platelet-derived growth factor complex. Recent data have indicated that the *fyn*-CD3ζ-chain interaction does not require the SH2 domain of *fyn*. Instead, a 10-amino acid sequence from the unique amino-terminal domain of *fyn* was found necessary for ζ-chain interaction (39). It is interesting to note that this unique region of *fyn* and the PRLR box 1 both lie in their respective amino-terminal ends adjacent to the plasma membrane. Thus, an interaction between these two loci is spatially conceivable. Alternatively, the interaction of *fyn* with the PRLR isoforms could be limited to one or two of the PRLR isoforms. *Fyn*'s "apparent" immunoprecipitation with each isoform, therefore, could be due to its interaction with a PRL-induced complex of heterodimeric isoforms, which cannot be entirely excluded on the basis of the data presented here.

The data presented in Fig. 5 also reveal that the long form of the PRL receptor is expressed in the Nb2 cell. These data have been additionally confirmed at the level of RNA expression by dideoxynucleotide sequencing of polymerase chain reaction products obtained from the reverse transcription of Nb2 total RNA (data not shown). This stands in contrast to a previous report (17) that indicated the presence in Nb2 of only the short and intermediate forms of the PRL receptor. The data presented here might suggest that only one of the two PRL receptor alleles is mutated in Nb2. Alternatively, in the absence of published data regarding the genomic organization of the PRL receptor locus, such data could argue that the intermediate form of the PRL receptor is not a mutant, as previously hypothesized (17); instead, the intermediate form may result from the splicing of a yet unidentified exon for the intermediate form or the expression of a separate PRL receptor locus.

The mechanism through which the PRLR activates *fyn* is uncertain. Recent data have indicated that certain anti-PRLR antibodies can induce the proliferation of Nb2 cells (29). From these data, it has been speculated that the dimerization/aggregation of PRLRs is necessary for signal transduction and proliferation, analogous to postligand-binding events seen in other related receptors (40). The data presented in Fig. 7 support the notion that dimerization, whether ligand or antibody induced, is capable of initiating PRLR signal transduction. It is possible that PRLR dimerization could bring *fyn* (or other kinases) into the close spatial proximity necessary for trans-phosphorylating activation. Alternatively, dimerization could bring *fyn* into close proximity to a protein-tyrosine phosphatase, similar to CD45 (41, 42), which, in turn, could activate *fyn* by the dephosphorylation of a regulatory tyrosine, such as Tyr^{531}.

Currently, *fyn* is known to associate with three receptor complexes on T-lymphocytes: the ζ-chain of the T-cell antigen receptor-CD3 (TCR) (43, 44), the IL-7 receptor (25), and the PRLR. The microenvironment surrounding *fyn* within these receptor complexes may well determine the substrates/secondary signals of the activated kinase. Thus, activation of the *fyn*-IL-7 complex has been shown to induce phosphatidylinositol 3-kinase (p85 and p110) activity (25), whereas stimulation of the ζ-chain-TCR complex induces phosphorylation of CD5 (p69) (45). Phosphorylation on tyrosine residues of multiple proteins, *i.e.* p40, p55–60, p97, and p120–130, have been observed by analysis of immunoblots of PRL-stimulated Nb2 lysates with antiphosphotyrosine antibodies (22, 23). The data presented here and elsewhere by our laboratory (24) indicate that the p55–60 phosphoprotein is $p59^{fyn}$, whereas preliminary data from our laboratory indicate that the p40 phosphoprotein represents mitogen-activated protein kinase. Recent data (46–48) now indicate that the 130-kDa protein-tyrosine kinase JAK2 is associated and activated by stimulation of the receptors for erythropoietin, IL-3, and GH and in a very recent report (57) with the PRLR. Whether JAK2 participates in the PRL stimulation of *fyn* is uncertain due to the lack of published data

Downloaded from https://academic.oup.com/mend/article/8/6/674/2714991 by guest on 20 August 2022

characterizing the activation of JAK within the 5 min of growth factor stimulation (*i.e.* the temporal window for early *fyn* activation). Although JAK2 is phosphorylated within 30 sec in response to GH stimulation (46), its activation has been demonstrated only after 60 min of GH stimulation. Similarly, the activation of JAK2 by IL-3 or erythropoietin has been shown only after 10 min of stimulation (47, 49). Thus, although it is possible that JAK2 kinase activity may stimulate *fyn* activity, temporal data supporting this theory are not currently present. Furthermore, data from our laboratory (not shown) have failed to demonstrate the phosphorylation of a p120–130 protein in either cell lysates or anti-JAK2 immunoprecipitates from Nb2, despite the use of four distinct antiphosphotyrosine antibodies, including monoclonal antibody 4G10. It is possible that the Nb2 line used in our laboratory differs from those Nb2 lines used to characterize PRL-induced tyrosine phosphorylation (22, 23). If so, this may indicate that JAK2 activity is sufficient, but not necessary, for early PRLR signaling, and that other tyrosine kinases, such as *fyn*, could subserve a similar role, as seen in other systems (30, 34).

The activation of *fyn* in normal PBL cultured in defined medium by PRL (Fig. 6) was demonstrated to occur after transient Con-A stimulation. The inability of PRL to stimulate *fyn* autokinase activity in resting G0-phase PBL is not unexpected, as previous data have indicated the inability of PRL to initiate proliferation in resting lymphocytes, perhaps due to the low level of PRLR present on resting lymphocytes (12). The lack of PRL-stimulated *fyn* activity in continuously Con-A-stimulated lymphocytes may be due to the previously demonstrated down-regulation of PTK activity after prolonged receptor cross-linking (50). Nevertheless, the data indicate that transiently Con-A-stimulated PBL respond to PRL by the biochemical activation of *fyn*. This is consistent with previous physiological data that have demonstrated the requirement for PRL during the lectin-stimulated mitogenesis of lymphocytes *in vitro* (3) and the G1-S-phase traverse of cloned T-cells (33). Thus, it is possible to conceive that during an immune response, competent lymphocytes (*i.e.* antigen-activated G1-phase) require signals from the PRL-PRLR complex, perhaps in part mediated by *fyn*, before G1-S-phase transition and clonal expansion can occur. Alternatively, the PRL-induced activation of *fyn* may initiate other cellular events, such as the synthesis and secretion of lymphokines.

The PRL-induced activation of the PRLR-associated serine/threonine kinase p72/74^{raf} has also been recently described by our laboratory (24). Significant tyrosine phosphorylation of *raf*-1 does not occur during PRL stimulation; thus, it seem unlikely that *raf*-1 serves as a direct substrate for *fyn* under such conditions. It is entirely possible, however, that either of these two kinases may modify the activity, via phosphorylation, of tyrosine or serine/threonine phosphatases, which, in turn, could modulate the activity of the other kinase. How these two kinases may modulate PRL-induced gene expression in T-cells (14, 31, 32) or the nuclear translocation of PRL during IL-2 stimulation (12, 33) is open to investigation and seems likely to yield insights into the molecular basis for the biological effects of the PRL/PRLR complex in T-lymphocytes.

MATERIALS AND METHODS

Cell Culture and Labeling

The rat T-cell lymphoma line Nb2/11c (referred to hereafter as Nb2) was maintained in Fisher's medium containing 10% bovine FCS (a rich source of PRL), 10% gelding serum, 10^{-4} M β-mercaptoethanol, and penicillin/streptomycin (28). The Nb2 line has served as an excellent model for studying PRL-stimulated proliferation, as its growth can be maintained in defined medium in the presence of either PRL or IL-2 (14, 33). Flow cytometric analysis of Nb2 cells for the presence of surface Fc receptors using phycoerythrin-conjugated anti-Fc receptor antibodies was negative (data not shown). For experimental purposes, the cells were rested for 48 h before PRL stimulation in chemically defined medium lacking PRL, consisting of Dulbecco's Modified Eagle's Medium with high glucose supplemented with glutamine, sodium selenite, linoleic acid, and transferrin (ITS+ supplement, Calbiochem, La Jolla, CA), as previously described (14). Unless otherwise specified, the PRL used in these studies was purified rat PRL obtained from the NIDDK. Human recombinant PRL was a gift from Genzyme (Cambridge, MA). [^{3}H]Thymidine labeling of stimulated Nb2 cells was accomplished by previously described methods (14). Briefly, after 44 h of culture, PRL-stimulated Nb2 cells (6×10^4 cells in 200 μl chemically defined medium) were labeled for 4 h with 0.5 μCi [^{3}H]thymidine. Normal donor human PBL were isolated using the Ficoll-Hypaque technique and were placed into culture at 1×10^6 cells/ml defined medium. For cross-linking studies, a 1:1000 dilution of goat antimouse antiserum (Tago) was used.

Immunoprecipitation and Immunoblotting

After PRL stimulation, 1×10^7 cells were promptly washed with ice-cold PBS and lysed in lysis buffer consisting of 10 mM Tris (pH 7.6), 50 mM NaCl, 30 mM sodium pyrophosphate, 50 mM NaF, 2.5 mM EDTA, 1 mM Na_3VO_4, 1 mM phenylmethylsulfonylfluoride, 0.23 U aprotonin/ml, 10 μg leupeptin/ml, 1 μm pepstatin, and 2% Nonidet P-40 at 4 C. After removal of debris by centrifugation, 500 μl lysate were precleared with 60 μl of a 1:1 mixture of protein-A-protein-G-conjugated Sepharose beads (Gibco, Grand Island, NY). Immunoprecipitations using the precleared lysates were then performed using anti-*fyn* (3 μl C12; Santa Cruz Biotechnology, Santa Cruz, CA); anti-*lck*, anti-*lyn*, or anti-*src* (3 μl; gifts from Dr. John Reed) (30); and anti-*yes* (5 μl; gift from Dr. Joe Bolen); all of which were rabbit antiserum that have been characterized extensively and effectively immunoprecipitated their respective antigen from Nb2 lysates (data not shown). Similar results in autokinase assays were obtained with a rabbit anti-*fyn* antiserum received as a gift from Dr. John Reed (30, 34). In addition, immunoprecipitations were performed with murine monoclonal antibodies directed against the PRLR (5 μl U6; gift from Dr. P. Kelly) (51) or 10 μl α-7a (gift from Dr. J. Porter; both antibodies yielded similar results in autokinase assays) (52). Antigen-antibody complexes were isolated by the addition of 50 μl protein-A/G beads. After three washes with lysis buffer, the beads were resuspended in 2-fold concentrated (2 ×) Laemmli buffer with mercaptoethanol, boiled, and analyzed on 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), as described previously (53).

Immunoblot analysis was performed as previously described (33). For analysis of cell lysates, 5×10^5 cells in 2 × Laemmli buffer with mercaptoethanol were loaded per lane.

Downloaded from https://academic.oup.com/mend/article/8/6/674/2714991 by guest on 20 August 2022

Antigen was labeled with a 1:2000 dilution of anti-*fyn* antiserum or a 1:1000 dilution of the anti-PRLR (U6). The primary antibody concentrations were known to be in excess, as multiple blots could be probed with the same antibody dilution with no effect on band intensity (data not shown). Antigen-antibody complexes were then detected using enhanced chemiluminescence (ECL kit, Amersham, Arlington Heights, IL).

In Vitro Autokinase and Substrate Kinase Assays

Autokinase activities were measured using a modification of a previously described method (54). Briefly, washed immunoprecipitates (as described above) were washed once with low salt buffer [10 mM Tris (pH 7.0), 100 mM NaCl, and 100 μm Na_3VO_4]. The protein-A/G-Sepharose beads were then suspended in 30 μl autokinase buffer [25 mM Tris (pH 7.0), 10 mM $MnCl_2$, and 1 μM unlabeled ATP] with 10 μCi [γ-^{32}P]ATP. After 2 min at room temperature, the reaction was stopped by the addition of 2 × Laemmli buffer with mercaptoethanol, and the reaction products were analyzed on 10% SDS-PAGE. The gels were treated for 1 h with 1 M KOH at 55 C to remove labile phosphate groups at residues other than tyrosine before autoradiography. Pilot experiments indicated that 2 min of autokinase reaction were within the linear range of this assay for the kinases examined; however, longer incubations (as in Fig. 1) were used at times for maximal sensitivity.

Substrate kinase activity was measured using a previously described enolase assay (55). Briefly, washed immunoprecipitates were resuspended in 30 μl substrate kinase buffer [10 mM Tris (pH 7.0), 100 mM NaCl, 10 mM $MnCl_2$, and 100 μM Na_3VO_4] with 10 μCi [γ-^{32}P]ATP and 5 μg enolase (Boehringer Mannheim, Indianapolis, IN; mol wt, 46,900 daltons), which had been denatured with 50 mM acetic acid at 37 C for 15 min. After 15 min at room temperature, the reactions were terminated with 2 × Laemmli buffer with mercaptoethanol, and the reaction products were analyzed on 10% SDS-PAGE. Gels were treated with KOH, as described above, before autoradiography. Quantitation of all kinase reactions was accomplished using a Molecular Dynamics scanning laser densitometer (Sunnyvale, CA).

Acknowledgments

The authors wish to thank Winnie Ngo for excellent technical assistance.

Received November 17, 1993. Revision received February 16, 1994. Accepted March 8, 1994.

Address requests for reprints to: Charles V. Clevenger, M.D., Ph.D., Department of Pathology and Laboratory Medicine, 6 Founders Pavilion, University of Pennsylvania Medical Center, 3400 Spruce Street, Philadelphia, Pennsylvania 19104

This work was supported by NIH Grant R29 AI-33510 (to C.V.C.).

REFERENCES

1. Bernton EW, Meltzer MS, Holaday JW 1988 Suppression of macrophage activation and T-lymphocyte function in hypoprolactinemic mice. Science 239:401–404
2. Glick B 1984 Interrelation of the avian immune and neuroendocrine systems. J Exp Zool 232:671–682
3. Hartmann DP, Holoday JW, Bernton EW 1989 Inhibition of lymphocyte proliferation by antibodies to prolactin. FASEB J 3:2194–2202
4. Nagy E, Berczi I 1978 Immunodeficiency in hypophysectomized rats. Acta Endocrinol (Copenh) 89:530–537
5. Nagy E, Berczi I, Wren GE, Asa SL, Kovacs K 1983 Immunomodulation by bromocryptine. Immunopharmacology 6:231–243
6. Berczi I, Nagy E, Kovacs K, Horvath E 1981 Regulation of humoral immunity in rats by pituitary hormones. Acta Endocrinol (Copenh) 98:506–513
7. Nagy E, Berczi I 1991 Hypophysectomized rats depend on residual prolactin for survival. Endocrinology 128: 2776–2784
8. Carrier M, Emery RW, Wild-Mobley J, Perotta NJ, Russell DH, Copeland JG 1987 Prolactin as a marker of rejection in human heart transplantation. Transplant Proc 29:3442–3443
9. McMurray R, Keisler D, Kanuckel K, Izui S, Walker SE 1992 Prolactin influences autoimmune disease activity in the female b/w mouse. J Immunol 147:3780–3787
10. Cross RJ, Bryson JS, Roszman TL 1992 Immunologic disparity in the hypopituitary dwarf mouse. J Immunol 148:1347–1352
11. Pelligrini I, Lebrun J-J, Ali S, Kelly PA 1992 Expression of prolactin and its receptor in human lymphoid cells. Mol Endocrinol 6:1023–1031
12. Clevenger CV, Russell DH, Appasamy PM, Prystowsky MB 1990 Regulation of IL2-driven T-lymphocyte proliferation by prolactin. Proc Natl Acad Sci USA 87:6460–6464
13. Neal KD, Montgomery DW, Truong TM, Yu-Lee L-Y 1992 Prolactin gene expression in human thymocytes. Mol Cell Endocrinol 87:R19–R23
14. Clevenger CV, Sillman AL, Hanley-Hyde J, Prystowsky MB 1992 Requirement for prolactin during cell cycle regulated gene expression in cloned T-lymphocytes. Endocrinology 130:3216–3222
15. Boutin JM, Jolicoeur C, Okamura H, Gagnon J, Edery M, Shirota M, Banville D, Dusanter-Fourt I, Djiane J, Kelly PA 1988 Cloning and expression of the rat prolactin receptor, a member of the growth hormone/prolactin receptor gene family. Cell 53:69–77
16. Boutin JM, Edery M, Shirota M, Jolicoeur C, Lesueur L, Ali S, Gould D, Djiane J, Kelly P 1989 Identification of a cDNA encoding a long form of prolactin receptor in human hepatoma and breast cancer cells. Mol Endocrinol 3:1455–1461
17. Ali S, Pelligrini I, Kelly PA 1991 A prolactin-dependent immune cell line (Nb2) expresses a mutant form of prolactin receptor. J Biol Chem 266:20110–20117
18. Too CK, Murphy PR, Friesen HG 1989 G-proteins modulate prolactin- and interleukin-2-stimulated mitogenesis in rat Nb2 lymphoma cells. Endocrinology 124:2185–2192
19. Rillema JA, Tarrant TM, Linebaugh BE 1989 Studies on the mechanism by which prolactin regulates protein, RNA, and DNA synthesis in NB2 node lymphoma cells. Biochim Biophys Acta 1014:78–82
20. Rayhel EJ, Hughes JP, Svihla DA, Prentice DA 1990 Growth and protein phosphorylation in the Nb2 lymphoma: effect of prolactin cAMP and agents that activate adenylate cyclase. J Cell Biochem 43:327–337
21. Banarjee R, Vonderhaar BK 1992 Prolactin-induced protein kinase C activity in a mouse mammary cell line (NOG-8). Mol Cell Endocrinol 90:61–67
22. Rillema JA, Campbell GS, Lawson DM, Carter-Su C 1992 Evidence for a rapid stimulation of tyrosine kinase activity by prolactin in Nb2 rat lymphoma cells. Endocrinology 131:973–975
23. Rui H, Djeu J, Evans GA, Kelly PA, Farrar WL 1992 Prolactin receptor triggering: evidence for rapid tyrosine kinase activation. J Biol Chem 267:24076–24081
24. Clevenger CV, Torigoe T, Reed JC 1994 Prolactin induces rapid phosphorylation and activation of prolactin receptor associated Raf-1 kinase in a T-cell line. J Biol Chem 269:5559–5565
25. Venkitaraman AR, Cowling RJ 1992 Interleukin 7 receptor functions by recruiting tyrosine kinase p59fyn through a

Downloaded from https://academic.oup.com/mend/article/8/6/674/2714991 by guest on 20 August 2022

segment of its cytoplasmic tail. Proc Natl Acad Sci USA 89:12083–12087
26. Hatakeyama M, Kono T, Kobayashi N, Kawahara A, Levin S, Perlmutter RM, Taniguchi T 1991 Interaction of the IL-2 receptor with src-family kinase p56lck: identification of novel intermolecular association. Science 252:1523–1528
27. Bolen JB, Rowley RB, Spana C, Tsygankov AY 1992 The *src* family of tyrosine kinases in hemopoietic signal transduction. FASEB J 6:3403–3409
28. Gout PW, Beer CT, Noble RL 1980 Prolactin-stimulated growth of cell cultures established from malignant Nb rat lymphomas. Cancer Res 40:2433–2436
29. Elberg G, Kelly PA, Djiane J, Binder L, Gertler A 1990 Mitogenic and binding properties of monoclonal antibodies to the prolactin receptor in Nb2 rat lymphoma cells. J Biol Chem 265:14770–14776
30. Torigoe T, O'Conner R, Fagard R, Fischer S, Santoli D, Reed JC 1992 Regulation of SRC-family protein tyrosine kinases by interleukins, IL-2, and IL-3. Leukemia 6:94S–97S
31. Yu-Lee L-Y 1990 Prolactin stimulates transcription of growth-related genes in Nb2 T lymphoma cells. Mol Cell Endocrinol 68:21–28
32. Yu-Lee L-Y, Hrachovy JA, Stevens AM, Schwarz LA 1990 Interferon-regulatory factor 1 is an immediate-early gene under transcriptional regulation by prolactin in Nb2 T cells. Mol Cell Endocrinol 10:3087–3094
33. Clevenger CV, Altmann SW, Prystowsky MB 1991 Requirement of nuclear prolactin for interleukin-2-stimulated proliferation of T lymphocytes. Science 253:77–79
34. Torigoe T, Saragovi HU, Reed JC 1992 Interleukin 2 regulates the activity of the lyn protein-tyrosine kinase in a B-cell line. Proc Natl Acad Sci USA 89:2674–2678
35. Goodwin RG, Friend D, Ziegler SF, Jerzy R, Falk BA, Gimpel S, Cosman D, Dower SK, March CJ, Namen AE 1990 Cloning of the human and murine interleukin-7 receptors: demonstration of a soluble form and homology to a new receptor superfamily. Cell 60:941–951
36. Murakami M, Narazaki M, Hibi M, Yawata H, Yasukawa K, Hamaguchi M, Taga T, Kishimoto T 1991 Critical cytoplasmic region of the interleukin 6 signal transducer gp130 is conserved in the cytokine family. Proc Natl Acad Sci USA 88:11349–11353
37. Twamley GM, Kypta RM, Hall B, Courtneidge SA 1992 Association of *fyn* with the activated platelet-derived growth factor receptor: requirements for binding and phosphorylation. Oncogene 7:1893–1901
38. Kypta RM, Goldberg Y, Ulug ET, Courtneidge SA 1990 Association between the PDGF receptor and members of the *src* family of tyrosine kinases. Cell 62:481–492
39. Gauen LKT, Kong ANT, Samelson LE, Shaw A 1992 p59fyn tyrosine kinase associates with multiple T-cell receptor subunits through its unique amino-terminal domain. Mol Cell Biol 12:5438–5446
40. Cunningham BC, Ultsch M, DeVoss AM, Mulkerrin MG, Clauser KR, Wells JA 1991 Dimerization of the extracellular domain of the human growth hormone receptor by a single hormone molecule. Science 254:821–825
41. Mustelia T, Pessa-Morikawa T, Autero M, Gassman M, Andersson LC, Gahmberg CG, Burn P 1992 Regulation of the p59fyn protein tyrosine kinase by the CD45 phosphotyrosine phosphatase. Eur J Immunol 22:1173–1178
42. Shiroo M, Goff L, Biffen M, Shivnan E, Alexander D 1992 CD45 tyrosine phosphatase-activated p59fyn couples the T cell antigen receptor to pathways of diacylglycerol production, protein kinase C activation and calcium influx. EMBO J 11:4887–4897
43. Da Silva AJ, Yamamoto M, Zalvan CH, Rudd CE 1992 Engagement of the Tcr/CD3 complex stimulates p59fyn activity: detection of associated proteins at 72 and 120–130 KD. Mol Immunol 29:1417–1425
44. Samelson LE, Phillips AF, Luong ET, Klausner RD 1990 Association of the *fyn* protein-tyrosine kinase with the T-cell antigen receptor. Proc Natl Acad Sci USA 87:4358–4362
45. Burgess KE, Yamamot M, Prasad KVS, Rudd CE 1992 CD5 acts as a tyrosine kinase substrate with a receptor complex comprising T-cell receptor zeta chain/CD3 and protein-tyrosine kinases p56lck and p59fyn. Proc Natl Acad Sci USA 89:9311–9315
46. Witthuhn BA, Quelle FW, Silvennoinen O, Yi T, Tang B, Miura O, Ihle JN 1993 JAK2 associates with the erythropoietin receptor and is tyrosine phosphorylated and activated following stimulation with erythropoietin. Cell 74:227–236
47. Argetsinger LS, Campbell GS, Yang X, Witthuhn BA, Silvennoinen O, Ihle JN, Carter-Su C 1993 Identification of JAK2 as a growth hormone receptor-associated tyrosine kinase. Cell 74:237–244
48. Silvennoinen O, Witthuhn BA, Quelle FW, Cleveland JL, Yi T, Ihle JN 1993 Structure of the murine Jak2 protein tyrosine-kinase and its role in interluekin 3 signal transduction. Proc Natl Acad Sci USA 90:8429–8433
49. Prigent SA, Stanley KK, Siddle K 1990 Identification of epitopes on the human insulin receptor reacting with rabbit polyclonal antisera and mouse monoclonal antibodies. J Biol Chem 265:9970–9977
50. Veillette A, Bookman MA, Horak EM, Bolen JB 1988 The CD4 and CD8 T cell surface antigens are associated with the internal membrane tyrosine protein kinase $p56^{lck}$. Cell 55:301–308
51. Katoh M, Raguet S, Zachwieja J, Djiane J, Kelly PA 1987 Hepatic prolactin receptors in the rat: characterization using monoclonal antireceptor antibodies. Endocrinology 120:739–749
52. Sissom JM, Eigenbrodt ML, Porter JC 1988 Anti-growth action on mouse mammary and prostate glands of monoclonal antibody to prolactin receptor. Am J Pathol 133:589–595
53. Clevenger CV, Epstein AL, Bauer KD 1987 Modulation of the nuclear antigen p105 in lymphocytes as a function of cell cycle progression. J Cell Physiol 130:336–343
54. Williams NG, Roberts TM, Li P 1992 Both p21*ras* and pp60v-*src* are required, but neither alone is sufficient, to activate the *raf*-1 kinase. Proc Natl Acad Sci USA 89:2922–2926
55. Reed JC, Yum S, Cuddy MP, Turner BC, Rapp UR 1992 Differential regulation of the p72–74 *raf*-1 kinase in 3T3 fibroblasts expressing ras or src oncogenes. Cell Growth Differ 2:235–243
56. Quill H, Riley MP, Cho EA, Casnellie JE, Reed JC, Torigoe T 1992 Anergic Th1 cells express altered levels of the protein tyrosine kinases p56lck and p59fyn. J Immunol 149:2887–2893
57. Rui H, Kirken RA, Farrar WL 1994 Activation of receptor-associated tyrosine kinase JAK2 by prolactin. J Biol Chem 269:5364–5368

Downloaded from https://academic.oup.com/mend/article/8/6/674/2714991 by guest on 20 August 2022