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Prolactin Receptor Signaling

Shared Components with the T-Cell Antigen Receptor in Nb2 Lymphoma Cells

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Previously, we reported that activation of the human prolactin receptor (PRLR) produced a protein phosphorylation pattern strikingly similar to that provoked by Concanavalin A (Con A), an activator of the T-cell antigen receptor (TCR). These results suggested that certain signaling components of the TCR may be shared by the activated PRLR. Additional studies here assessed the levels of TCR expression following PRLR stimulation and the effect of TCR activation on PRL-stimulated proliferation in lactogen-dependent pre-T Nb2-11 lymphoma cells. The results indicated that the TCR was expressed on the surface of approx 4% of exponentially proliferating and prolactin- (PRL) treated cells. In contrast, approx 45% of quiescent cells, cultured in the absence of PRL for 24 h, expressed the TCR at the cell surface, suggesting that lactogen withdrawal may up-regulate TCR cell-surface expression. Moreover, TCR activation with anti-CD3 antibodies attenuated PRL-stimulated Nb2-11 cell proliferation in a concentration-dependent manner. In other experiments, immunoprecipitation and immunoblotting of Nb2-11 lysates revealed that activation of the PRLR resulted in rapid tyrosyl phosphorylation of ZAP-70, a critical TCR-associated tyrosine kinase. In addition, ZAP-70 was found to associate transiently with the putative guanine nucleotide exchange factor and substrate, Vav, in PRL-treated cells. ZAP-70 was also found to associate constitutively with the PRLR; PRL stimulation provoked the transient recruitment of Vav to the complex. These observations suggest that PRL signaling reflects the transient formation of a PRLR-ZAP-70-Vav complex and its immunomodulatory actions involve diverse interactions that affect TCR expression and signaling mechanisms.

Key Words: Prolactin; signaling; T-lymphoma; T-cell antigen receptor; ZAP-70; prolactin receptor.

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Introduction

Prolactin (PRL), a peptide hormone primarily produced by the pituitary, has a wide variety of physiological functions, but is best known for its role in regulating the ontogeny and differentiated function of the mammary gland *(1)*. However, during the last decade, evidence has accumulated indicating that PRL also serves a salient role as a modulator of the immune response. Various laboratories have demonstrated that bioactive PRL can be produced by rodent, and human lymphocytes and the hormone, acting as a cytokine, can stimulate lymphoproliferation *(2–5)*. The PRL receptor (PRLR), which mediates hormone (cytokine) action, is ubiquitously expressed on hematopoietic cells and is recognized as a member of the hematopoietin/cytokine receptor superfamily on the basis of sequence homology *(6,7)*. Ligand-induced activation of members of this receptor family leads to mitogenesis in some systems, reflecting signaling through similar tyrosine and serine/threonine phosphorylation pathways *(6,7)*. Studies using PRL-dependent Nb2 lymphoma T-cell cultures *(8)* have implicated several proteins as mediators of the mitogenic response initiated by the hormone including: Janus kinase 2 (JAK2) tyrosine kinase *(9,10)*; mitogen-activated protein kinase (MAPK;*11*); $p59^{fyn}$ tyrosine kinase (Fyn;*12*); protein kinase C (PKC;*13*); phosphatidylinositol 3-kinase *(14)*; S6 kinase *(15)*; SHC signaling protein *(16)*; and the guanine-nucleotide proteins Ras *(17)* and $p95^{VAV}$ (Vav;*18*). Activation of one or more of these signaling intermediates by PRL is thought to form the basis of its comitogenic and antiapoptotic actions in hematopoietic cells *(19,20)*. Despite extensive investigation, the precise mechanisms

underlying the activation of the various signaling pathways by PRL, as well as their coupling to the actions of the hormone, have yet to be fully elucidated.

Although responsive to PRL, activation of T-lymphocytes is primarily initiated by recognition of processed antigen bound to a self major histocompatibility complex molecule via the cell-surface T-cell antigen receptor (TCR) presented by antigen-presenting cells. This process can be mimicked in vitro with the use of mitogens, such as the plant lectin, Concanavalin A (Con A), or stimulatory anti-TCR antibodies. Current evidence indicates that the TCR represents a protein heterodimer of either α and β or γ and Δ peptide chains, depending on their function and distribution. The TCR is associated with a number of additional proteins, including CD3 proteins (γ, Δ, and ε chains) and, in most cases, a homodimer of ζ chains. The functional interdigitation of these proteins comprises the TCR/CD3 complex *(21,22)*. Whereas recognition of antigen is mediated by the TCR α and β heterodimer, signals that initiate T-lymphocyte activation are transduced by other TCR-associated components. The latter involves phosphorylation catalyzed by multiple signaling enzymes, which recognize immunoreceptor tyrosine-based activation motifs (ITAMs) present within the intracellular domain of the TCR complex *(23,24)*. Following its activation, the complex is internalized by endocytosis and subsequently degraded, a process presumably reflecting PKC-mediated phosphorylation of its CD3 components *(25)*.

Phosphorylation of ITAMs within the TCR complex leads to recruitment and activation of a member of the Syk family of tyrosine kinases, the ζ chain-associated protein, ZAP-70 *(26)*. On activation, ZAP-70 binds to the TCR ζ chain via Src-like homology 2 (SH2) interactions *(27–29)*. The phosphorylation of ITAMs is crucial for ZAP-70 binding to the complex, since its inhibition markedly reduces binding and activation of the kinase *(30)*. Once bound, ZAP-70 associates with other signaling molecules also through SH2 interactions. These include members of the Src family of tyrosine kinases, Fyn and Lck, as well as the putative nucleotide exchange factor and oncoprotein, Vav *(31–33)*. As such, ZAP-70 is a central mediator of TCR function *(24,27)*.

Previously, we and others demonstrated that PRL stimulation of T-lymphocytes and Nb2 lymphoma cells results in phosphorylation of several kinases and other proteins that are similarly phosphorylated in response to TCR activation, including MAPK *(11,34)*, Fyn *(12,35)*, and Vav *(18,24)*. Recently, we reported that PRL stimulated phosphorylation of the human TCR complex and ZAP-70 as well as the tyrosine kinase in rat Nb2-11 lymphoma cells *(36)*. Taken together, these observations suggested that the mechanism by which PRL exerts its immunomodulatory effects involve, at least in part, components of the TCR/CD3 signal transduction pathway(s) *(36)*.

Lactogen-dependent rat pre-T Nb2 lymphoma cell lines *(8)* have become the experimental tool of choice for investigating PRL-provoked mitogenic signaling mechanisms *(37)*. Culturing the Nb2 cells in lactogen-deficient medium for 18-24 h arrests growth and leads to accumulation in the early G_1-phase of the cell cycle. The cells in such "quiescent" cultures respond to mitogenic stimulation by physiological concentrations of PRL in a partially synchronized manner *(8,38)*. This characteristic has facilitated the identification of several PRL signaling mediators *(9–18)* and immediate-early genes, such as pim-1, c-myc, and IRF-1, which are transcriptionally regulated by PRL *(39–41)*. Moreover, recent studies have indicated that the Nb2 lymphoma cells also express several TCR-component mRNAs *(42)*, and that PRL-stimulated Nb2 cell proliferation can be altered by treatment with plant lectins, such as Con A *(43)*. Therefore, Nb2 lymphoma cells provide an exemplary T-lymphocyte paradigm for investigating not only PRL-coupled mitogenic signaling mechanisms, but also interactions between PRL and the immune system *(37,43)*.

In the present study, we utilized Nb2-11 cells to investigate crosstalk between the signaling pathways of the PRLR and the TCR/CD3 complex. It was found that PRL withdrawal led to a marked increase in the expression of cell-surface CD3 proteins. Furthermore, treatment with TCR-activating, anti-CD3 antibodies markedly attenuated PRL-stimulated proliferation. Finally, evidence is presented to suggest that PRL signaling reflects the transient formation of a PRLR-ZAP-70-Vav complex.

Results

PRL Depletion Increases Nb2-11 Cell Surface TCR/CD3 Expression

Activation of the TCR complex is characterized by phosphorylation of its components and its internalization via endocytosis *(25)*. The latter process allows assessment of T-cell activation by determining the extent of reduction in cell-surface TCRs *(44)*. Since we had previously shown that treatment with PRL provoked phosphorylation of TCR complex components *(36)*, a possible role for the hormone in regulation of expression of this complex was investigated.

Expression of the TCR was assessed by flow cytometric analysis of Nb2-11 cells utilizing fluorescein isothiocyanate-conjugated (FITC) anti-CD3 antibodies, a method employed by others to evaluate downregulation of the activated TCR complex *(44)*. As shown in Fig. 1, approx 45% of quiescent Nb2-11 cells expressed the CD3 antigen at the cell surface. In contrast, only 4% of exponentially proliferating, lactogen-driven cells expressed CD3. Addition of growth-stimulating concentrations of PRL (20 ng/mL) to quiescent cultures followed by a 48-h incubation similarly led to low levels of CD3. These results indicate that PRL stimulation in Nb2-11 cells results in decreased expression of CD3, suggesting a functional interaction between the PRLR and CD3.

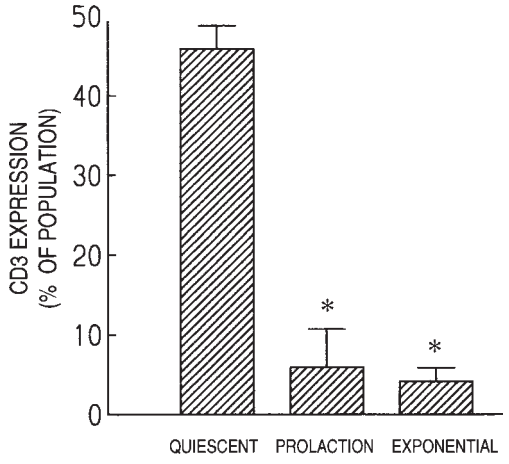

Fig. 1. Cell-surface CD3 expression in Nb2-11 cells. Quiescent, restimulated (PRL, 20 ng/mL for 48 h) or exponentially proliferating Nb2-11 cells (1×10^6 cells/mL) were evaluated for cell-surface TCR/CD3 expression by flow cytometric analysis using anti-rat CD3 FITC-conjugated antibodies as described in Materials and Methods. The data are expressed as percentages of cells expressing the CD3 epitope (mean + SE) and represent results obtained from three separate experiments.

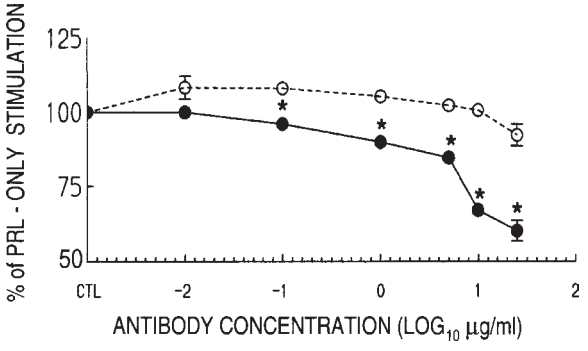

Fig. 2. Effect of incubation with anti-CD3 antibodies on PRL-stimulated proliferation quiescent Nb2-11 cells (1.2×10^5 cells/mL) were preincubated for 30 min with increasing concentrations of a preservative-free, anti-rat CD3 antibody (closed circles) or an IgG isotype control antibody (open circles). Cultures were then stimulated with PRL (10 ng/mL) and incubated for 48 h before cell density was determined as described in Materials and Methods. The results are expressed as the percentage of PRL-only treated cultures (no anti-CD3 antibody) obtained from triplicate samples and are representative of an experiment conducted at least three times. *$p < 0.001$ vs isotype control IgG.

Activation of the TCR Attenuates PRL-Stimulated Nb2-11 Cell Proliferation

To investigate whether activation of the TCR complex could affect PRL mitogenic signaling, the effect of receptor-activating anti-CD3 antibodies on PRL-stimulated Nb2-11 cell proliferation was evaluated. Quiescent cells were pretreated for 30 min with anti-CD3 antibodies prior to a 48 h of exposure to PRL (10 ng/mL). As shown in Fig. 2, treatment of the cells with the anti-CD3 antibody significantly reduced PRL-stimulated Nb2-11 cell proliferation

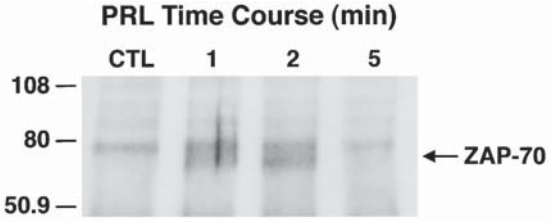

Fig. 3. PRL-induced tyrosyl phosphorylation of ZAP-70. Quiescent Nb2-11 cells (1.5×10^6 cells/mL) were incubated with PRL (20 ng/mL) and harvested at the times indicated. Tyrosyl phosphorylation of ZAP-70 protein was determined by immunoprecipitation of cell lysate proteins with an anti-ZAP-70 antibody, followed by SDS-PAGE fractionation, and immunoblotting using anti-phosphotyrosine as described in Materials and Methods. A representative autoradiograph of an experiment replicated five times is presented. CTL, control (no PRL).

in a concentration-dependent manner. Concentrations of anti-CD3 as low as 100 ng/mL significantly reduced PRL-stimulated proliferation, whereas 25 µg/mL attenuated the response to the greatest extent. Notably, addition of supraphysiological concentrations of PRL (up to 500 µg/mL) did not reduce the effect of anti-CD3, suggesting that the antibody most likely did not compete with the hormone for binding to the PRLR (data not shown). Moreover, treatment of the cells with an isotype control antibody did not interfere with PRL-stimulated mitogenesis, indicating that the inhibitory effects observed were specific for a TCR/CD3 interaction. Results from other experiments indicated that anti-CD3, when added to the cultures in the absence of PRL, neither affected viability of Nb2-11 cells nor induced their proliferation (data not shown). Thus, it appears that ligation of the TCR complex provides an inhibitory signal that impedes mitogenic signaling through the PRLR. These results, together with the observations presented in Fig. 1, provide evidence for a functional interaction between these two distinct receptors, which are both expressed on T-lymphocytes.

PRL Stimulates ZAP-70 Tyrosyl Phosphorylation in Nb2-11 Cells

We previously reported *(36)* that activation of the TCR and PRLR led to phosphorylation of ZAP-70, a tyrosine kinase required for TCR signaling *(27)*. To evaluate this effect of PRL further, quiescent Nb2-11 cells were incubated with PRL (20 ng/mL), and tyrosyl phosphorylation of ZAP-70 was assessed as an index of its activation (Fig. 3). Whereas in quiescent cultures, the levels of ZAP-70 tyrosyl phosphorylation were low, addition of PRL rapidly provoked increased phosphorylation of the enzyme. Within 1 min after PRL addition, increased tyrosyl phosphorylation of ZAP-70 was detected; maximal phosphorylation was observed from 1–2 min before returning toward control levels by 5 min. These results demonstrate that activation of the Nb2-11 cell PRLR is coupled to increased tyrosyl

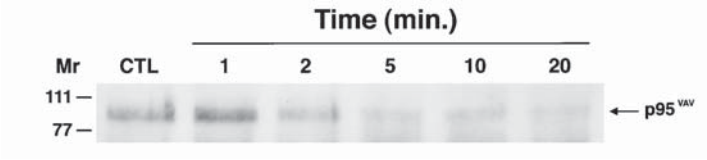

Fig. 4. ZAP/Vav association: Effect of PRL. Quiescent Nb2-11 cells (1×10^6 cells/mL) were incubated with PRL (20 ng/mL) and harvested at the times indicated. Cell lysate proteins were immunoprecipitated with anti-ZAP-70, SDS-PAGE fractionated and immunoblotted using an anti-Vav antibody as described in Materials and Methods. A representative autoradiograph of an experiment replicated at least three times is presented. CTL, control (no PRL).

phosphorylation of ZAP-70, a kinase that, when associated with the TCR complex in human and rodent T-cells, is essential for the signaling of that receptor.

ZAP-70 Transiently Associates with p95VAV in PRL-Stimulated Nb2-11 Cells

Since ZAP-70 appeared to play a role in both PRLR and TCR-coupled signal transduction, we sought to identify other proteins that could participate in signaling mediated by this tyrosine kinase. As reported by others, TCR activation involves an SH2-mediated interaction of the putative, nucleotide-regulatory substrate, Vav, with ZAP-70 *(45)*. Therefore, experiments were conducted to determine whether a complex composed of ZAP-70, and Vav could also be detected in PRL-stimulated cultures. Quiescent Nb2-11 cells were incubated with PRL (20 ng/mL) for time periods through 20 min. Cell lysates were immunoprecipitated with anti-ZAP-70, and then immunoblotted with an anti-Vav antibody (Fig. 4). The results indicate that in untreated, quiescent cells, Vav was associated with ZAP-70. Prolactin stimulation of the cultures further increased the association within 1 min. However, the increased interaction of ZAP-70 with Vav appeared to be transient, since it decreased from 2 to 20 min. Thus, PRL depletion of Nb2-11 cells apparently leads to an association of ZAP-70 and Vav. Restimulation of the cells with PRL rapidly enhances, then causes dissociation of Vav.

Association of ZAP-70 and Vav with the PRLR

The oncoprotein Vav has been previously linked to PRL signaling and has been demonstrated to associate with the PRLR in lactogen-treated Nb2 cells *(18)*. This observation, together with the demonstrated association of ZAP-70 with Vav (Fig. 4), suggested that this enzyme–effector complex may interact with the PRLR. Therefore, this potential interaction was assessed in PRL-stimulated Nb2-11 cells again over a 20-min time-course. Lysates obtained from PRL-stimulated cells were immunoprecipitated with anti-ZAP-70 and then immunoblotted utilizing anti-PRLR (U6) antibodies. As shown in Fig. 5A, ZAP-70 co-immunoprecipitated with the PRLR in quiescent Nb2-11 cells. Notably, other experiments in which immunoprecipitation was conducted with

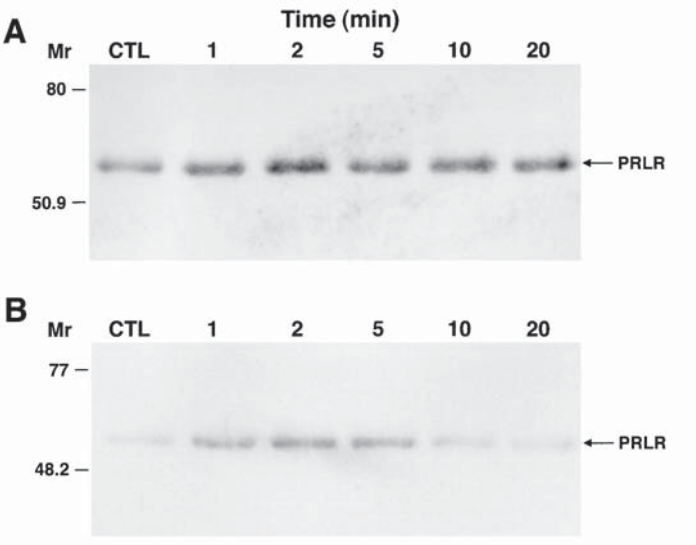

Fig. 5. Association of ZAP-70 and Vav with the PRLR. Quiescent Nb2-11 cells (1.5×10^6 cells/mL) were incubated with PRL (20 ng/ml) and harvested at the times indicated. Cell lysate proteins were immunoprecipitated with either anti-ZAP-70 **(A)** or anti-Vav antibodies **(B)**, fractionated by SDS-PAGE, and subjected to immunoblotting using the U6 anti-PRLR antibody as described in Materials and Methods. A representative autoradiograph of an experiment replicated at least three times is presented. CTL, control (no PRL).

anti-PRLR antibodies and fractionated proteins immunoblotted utilizing anti-ZAP-70 yielded similar results (not shown). Moreover, the results presented in Fig. 5B confirm that Vav associates with the PRLR in hormone-stimulated Nb2-11 cells as previously reported *(18)*. Together, these results suggest that the PRLR is associated with relatively low levels of ZAP-70 and Vav in quiescent Nb2-11 cells. Hormonal stimulation increased the interaction of the PRLR with these signaling intermediates, which are also key mediators of TCR-stimulated signal transduction.

Discussion

The results presented in this article demonstrate a functional interaction between the TCR and PRLR as well as a molecular association between the signaling intermediates, ZAP-70 and the Vav oncoprotein. Notably, these signaling molecules have been previously implicated in signal transduction mechanisms coupled to both receptors. PRL withdrawal increased expression of the cell surface TCR/CD3 complex in a substantial proportion of the Nb2-11 cells (Fig. 1). Moreover, activation of the TCR with anti-CD3 antibodies significantly attenuated PRL-stimulated Nb2-11 cell mitogenesis (Fig. 2), indicating that alterations in the activation status of either receptor results in functional consequences at the level of expression or cellular effects mediated by the other receptor. The lack of a mitogenic effect in response to anti-CD3 alone in Nb2-11 cells (not shown) is consistent with the inability of

T-cell selective lectins to stimulate proliferation in this model *(43)*.

Evidence from other T-cell systems indicates that lymphocyte activation by anti-CD3 antibodies causes downregulation of TCR complexes. In addition, ligation of a minimal proportion of TCR complexes is apparently required for cellular activation. The T-cell activation threshold can be more difficult to achieve in the presence of fewer TCRs available to sustain a triggering process *(44)*, such as during partial stimulation with a nonmitogenic anti-CD3 antibody. This observation may explain the results presented here in the Nb2-11 cells exposed to nonmitogenic anti-CD3 antibody. In this context, Smith et al. *(46)* demonstrated that an anergic state in pGL10 T-cells was induced by nonmitogenic anti-CD3 antibodies, which was sustained even after TCR levels rebounded toward levels observed in control cultures following their downregulation. Furthermore, although suppression of PRL-stimulated Nb2 cell proliferation by classical lymphocyte mitogens, such as phytohemagglutinin-P and pokeweed mitogen, has been reported, the inhibitory effect observed in that report was reversed by the addition of high PRL concentrations *(43)*. In contrast, high concentrations of PRL failed to overcome the growth inhibition provoked by anti-CD3 antibodies in the present study, indicating that this effect was most likely not owing to competition of the antibody with PRL for binding to the PRLR.

We have previously reported that PRL stimulates rapid tyrosyl phosphorylation of TCR/CD3 complex proteins in human T-cells *(36)*. However, the phosphorylation state of the TCR/CD3 constituents in Nb2-11 cells following PRL stimulation remains to be fully evaluated. The increase in TCR/CD3 complex expression following PRL depletion, coupled with the observation that TCR activation suppressed PRL-stimulated Nb2-11 cell proliferation, suggests that signaling components may be shared between these two seemingly disparate receptors. Thus, we previously demonstrated that either PRL or Con A similarly stimulated phosphorylation of TCR components and activated at least one signaling tyrosine kinase considered to be unique to the antigen receptor in T-cells (ZAP-70; *36*). This observation was extended in the present study to include the interaction of ZAP-70 with the PRLR and a putative nucleotide-regulatory oncoprotein, Vav. These observations lead us to propose that the PRLR and TCR complex may interact through a signaling "crosstalk" mechanism.

Stimulation of the PRLR has been shown to involve activation of multiple signaling cascades, including JAK2-STAT *(9)*, Ras-MAPK *(11,15–17)*, and PKC *(13)*. In addition, Clevenger and Medaglia *(12)* previously reported that Fyn, but not other members of the Src tyrosine kinase family, was rapidly activated in Nb2 cells following treatment with PRL. Comparison of the kinetics of activation of these signaling molecules suggests that MAPK activation closely follows stimulation of the receptor-associated tyrosine kinases, JAK2 and Fyn. Once activated, these signaling molecules initiate the sequence of events that lead to transcription of immediate-early genes, such as *pim-1 (39), bcl-2 (46)*, and *IRF-1 (41)*, thought to be required for a proliferative response.

Signaling by the TCR/CD3 complex also reflects protein phosphorylation, which leads to cellular activation. The TCR complex components responsible for transmitting these signals include the intracellular domains of the membrane-spanning CD3 together with the ζ chain dimers *(23,24)*. Activation of the Src kinases, Lck and Fyn, is thought to be the initial consequence of TCR stimulation; which Src kinase is activated depends on cell type. Activation of the Src kinase facilitates recruitment of ZAP-70 to the intracellular domain of the TCR complex, where it functions as an intermediate in signal processing. Recruitment of ZAP-70 appears to be selectively mediated by SH2 domain interactions *(47)*. The interplay among Syk family kinases, CD3, and ζ chains also involves SH2 binding domains that recognize ITAMs present within the intracellular portions of the TCR complex *(48)*. Specifically, TCR triggering has been shown to activate Fyn, which, in turn, catalyzes dual phosphorylation of ITAMs, subsequently leading to recruitment of ZAP-70 and its binding to both the ζ chain and Fyn through SH2 interactions *(26,31)*.

T-cell function, signaling, and responsiveness have been extensively studied in vitro with models exhibiting gain-of-function or lack of ZAP-70 *(30,49,50)*. Importantly, absent or mutated ZAP-70 in humans leads to severe immunodeficiency owing to impaired T-cell selection and lack of signaling *(51–53)*. As reported here, stimulation by PRL rapidly (1–2 min) increased the transient tyrosyl phosphorylation of ZAP-70 in Nb2-11 cells as well as augmented its association with the PRLR. These observations suggest that ZAP-70 may play a role as a mediator of early phosphorylation signals coupled to PRLR stimulation. Due to the abundance of evidence supporting interactions between Fyn and the TCR in numerous T-cell systems *(23,54-56)*, as well as between Fyn and the PRLR in Nb2 cells *(12)*, we conclude that activation of ZAP-70 is likely mediated by Fyn in the latter paradigm.

The stimulation by PRL of ZAP-70 tyrosyl phosphorylation led to further investigation of potential interactions of the enzyme with other TCR-associated effectors in Nb2-11 cells. Immunoprecipitation/immunoblot experiments revealed that ZAP-70 associated with Vav in quiescent cells; this affiliation was initially augmented, but then reduced within 2 min after PRL stimulation. In other T-cell models, TCR signaling reflects the association between ZAP-70 and Vav, which also has been demonstrated to occur through a SH2 interaction involving a single tyrosine residue in ZAP-70 *(33,57)*. Furthermore, Vav has been shown to be a direct substrate for ZAP-70 *(45)*. Importantly, mutation in ZAP-70 hinders its association with Vav and attenuates its tyrosyl phosphorylation *(57)*. Since Vav is

phosphorylated in response to PRL in Nb2 cells *(18)* and associates with ZAP-70 (Fig. 4), it may similarly represent a substrate for ZAP-70 in this pre-T cell line. Moreover, immunoprecipitate with anti-ZAP-70 antibodies revealed an additional, larger tyrosyl-phosphorylated band. Although speculative, it is possible that this protein may represent SLP-76, which binds Vav via SH2 interactions subsequent to its tyrosyl phosphorylation by ZAP-70 *(58)*.

Others have demonstrated that a complex of Vav and the PRLR is rapidly formed following PRL stimulation of Nb2 cells *(18)*. The observation that Vav and ZAP-70 associate in hormone-stimulated cells (Fig. 4) prompted us to evaluate whether the PRLR is also a component of the Vav-ZAP-70 complex. The results obtained indicated that ZAP-70 was constitutively associated with the PRLR. In addition, hormonal stimulation augmented this association. Although some studies have demonstrated that ZAP-70 affiliation with ζ chain ITAMs only occurs subsequent to TCR stimulation *(26)*, other results suggest that ZAP-70 constitutively associates with ζ chains presumably phosphorylated by Lck in unstim-ulated murine thymocytes and lymph node T-cells *(59,60)*. It is interesting to note that in Nb2 cells, Lck is constitutively autophosphorylated, likely reflecting its activation, but not affected by treatment with PRL *(12)*. It is clear that in T-cells, the association of ZAP-70 with intracellular domain ITAMs within the TCR complex is related to their phosphorylation state. The observations here suggest that specific tyrosine-containing motifs within the PRLR may be constitutively phosphorylated, perhaps by Lck, which would provide a potential docking site for ZAP-70. On PRLR stimulation, ZAP-70 may be phosphorylated by a Src kinase, most likely Fyn, which propagates signals to other downstream effectors. Further experiments are required to define fully the nature of the association between ZAP-70 and the PRLR in Nb2-11 cells.

In addition to an interaction between ZAP-70 and the PRLR, an association of the kinase with Vav was also observed. Moreover, as reported by Clevenger et al. *(18)*, PRL stimulated the transient affiliation of Vav with the PRLR. Therefore, it appears that PRLR signaling reflects activation of receptor-associated ZAP-70 and its potential phosphorylation of the putative nucleotide regulatory protein, Vav. Following its activation, Vav may transmit signals through other effectors or collaborate with other pathways known to be activated by PRL, such as JAK2-STAT or Ras-MAPK, to facilitate signaling to the nucleus.

Numerous investigations in T-cells, including Nb2 lymphoma lines, have indicated that PRLR signaling occurs primarily at the surface membrane (although a potentially important nuclear receptor exists), and that ZAP-70 is constitutively present at the inner leaflet of the plasma membrane where its activation occurs in association with the TCR/CD3/ζ complex *(61,62)*. Zap-70 is also recruited to the membrane from the cytoplasmic pool of this kinase *(63)*. Therefore, the inner leaflet of the membrane is likely to be the site of its interaction with the PRLR. Similarly, Vav is associated with the TCR complex at the cell membrane *(64,65)*, where its affiliation with the ZAP-70 substrate, SLP-76, mediates regulation of Il-2 induction *(58)* and activation of the Ras and calcium signaling pathways *(66)*. Taken together, these observations strongly suggest that the PRLR-ZAP-70-Vav complexes we report here exist at the inner leaflet of the plasma membrane. However, since ZAP-70 *(63)*, Vav *(18)*, and the PRLR *(67)* have been reported to be present within the cell nucleus, the possibility for their interaction at this site as well cannot be ruled out.

The precise mechanism by which the TCR, ZAP-70, and Vav interact to transmit signals leading to T-cell activation awaits clarification. However, it is clear that complex aggregates of receptors, signaling enzymes, as well as transcription factors collaborate to signal the intitiation of an immune response. In the present study, PRL-stimulated Nb2-11 cells were found to react similarly to cells activated by TCR stimulation. This observation strongly suggests a close communicative relationship in T-cells between the PRLR and components of the TCR complex.

Materials and Methods

Hormones, Antibodies and Reagents

Ovine PRL (NIDDK oPRL-20, AFP 10677C) was obtained from the National Hormone and Pituitary Program (Rockville, MD). FITC-conjugated anti-rat CD3 IgM monoclonal antibodies (MAbs) obtained from Caltag, Inc. (Burlingame, CA) were utilized for flow cytometric analysis of cell-surface CD3 expression. A no-azide/low-endotoxin anti-CD3 IgG antibody obtained from Pharmingen, Inc. (San Diego, CA) was used for experiments assessing effects of TCR complex activation on PRL-stimulated cell proliferation. IgG isotype control antibody was obtained from Sigma (St. Louis, MO). Anti-ZAP-70 and anti-$p95^{VAV}$ antibodies were obtained from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). A monoclonal anti-PRLR (U6) antibody was generously provided by Paul Kelly (INSERM, France). An anti-phosphotyrosine antibody (PY20) was obtained from Transduction Laboratories (Lexington, KY). Unless otherwise specified, all other reagents were of molecular biology-grade and obtained from Sigma.

Cell Culture

Cultures of cloned Nb2-11 lymphoma cells were maintained at 37°C in Fischer's medium containing 10% fetal bovine serum (Summit Biotechnology, Fort Collins, CO) as a source of lactogen, 10% horse serum (Summit Biotechnology) 2-mercaptoethanol (2-ME; 10^{-4} *M*), penicillin (50 U/mL), and streptomycin (50 μg/mL) as originally described by Gout et al. *(8)*. Exponentially proliferating cultures were rendered quiescent by incubating the cells

for 24 h in lactogen-free medium (Fischer's medium supplemented with 10% nonmitogenic gelding serum [ICN; Irvine, CA], 2-ME, and antibiotics). Restimulation of cultures was carried out by incubation with PRL (10–20 ng/mL; *38*).

Flow Cytometric Analysis of Nb2-11 Cell-Surface CD3 Antigens

Nb2-11 cells (1×10^6 cells/mL), which were exponentially proliferating, quiescent, or restimulated (48 h + PRL), were washed with phosphate-buffered saline (PBS) supplemented with 1% BSA. The cells were then incubated at 4°C in the dark for 30 min with anti-CD3-FITC. Cells were again washed, and then fixed in PBS containing 0.5% paraformaldehyde. Samples (10,000 cells) were analyzed for FITC-labeling using an Elite flow cytometer (Coulter Electronics Inc., Hialeah, FL).

TCR/CD3 Activation Studies

To determine the effect of TCR complex activation on PRL-stimulated proliferation, quiescent Nb2-11 cells (1.2×10^5 cells/mL) were preincubated for 30 min at 37°C with various anti-CD3 antibody concentrations. PRL (10 ng/mL) was then added and the cultures further incubated for 48 h. Cell density was determined by electronic cell counting (Coulter Electronics Inc.).

Immunoprecipitation and Immunoblotting

Subsequent to incubation of quiescent Nb2-11 cultures with PRL (20 ng/mL), cells (1.5×10^6 cells/mL) were washed in ice-cold PBS containing 1 m*M* sodium orthovanadate (Na_3VO_4). The cells were then lysed in a buffer containing 10 m*M* Tris (pH 7.6), 50 m*M* NaCl, 30 m*M* sodium pyrophosphate, 50 m*M* NaF, 2.5 m*M* EDTA, 1 m*M* Na_3VO_4, 1 m*M* phenylmethylsulfonyl-fluoride, 10 μg/mL each of leupeptin and aprotinin, 1 μ*M* pepstatin, and 2% Nonident P-40 (Pierce Chemical, Rockford, IL). Cellular debris was removed by centrifugation. The lysates were precleared by incubation with 25 μL of protein G agarose (Gibco-BRL, Grand Island, NY) for 1 h at 4°C. Total protein concentration of the lysates was determined utilizing the Bradford reagent (Bio-Rad, Hercules, CA). Equal concentrations of sample protein were immunoprecipitated with 25 μL protein G and anti-phosphotyrosine, anti-ZAP-70, or anti-Vav antibodies in a total volume of 500 μL. Immunoprecipitates were washed 3 times in lysis buffer, boiled for 5 min in Laemmli sample buffer (with 5% 2-ME), and fractionated on 10% SDS gels. Resolved proteins were transferred to Immun-Lite blotting membrane (Bio-Rad) and blocked overnight at 4°C with 5% nonfat dry milk. The membranes were incubated with anti-ZAP-70 (1/1000), anti-Vav (1/1000) or anti-PRLR (1/2000) antibodies for 2 h at 25°C. Antigen–antibody complexes were detected using an Immun-Lite chemiluminescence detection system (Bio-Rad).

Data Analysis

Levels of CD3 expression (Fig. 1) were assessed in individual cell cultures (1×10^6 cells/treatment) by flow cytometric analysis. This experiment was replicated three times and the results pooled ($n = 3$) prior to statistical analysis. Each proliferation experiment (Fig. 2) employed triplicate cultures with each antibody concentration and was also replicated three times. The mean values from each replicate experiment were pooled ($n = 3$) prior to conducting statistical analysis. All blotting experiments (Figs. 3–5) utilized individual cell cultures (2×10^7 cells/time-point) and were replicated at least three times. Statistical analysis was performed by ANOVA followed by the student Newman-Keuls posttest for multiple comparisons. Where applicable, the results are presented as means + SE.

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