A Lymphoma Growth Inhibitor Blocks Some but Not All Prolactin-stimulated Signaling Pathways*

Hiroko Yamashita‡, Jun Xu‡, Rebecca A. Erwin§, Andrew C. Larner¶, and Hallgeir Ruii

From the ‡Department of Pathology, Uniformed Services University of the Health Sciences School of Medicine, Bethesda, Maryland 20814, ¶Department of Integrative Biology, Pharmacology and Physiology, University of Texas, Houston, Texas 77030, and §§Department of Immunology, Lerner Research Institute, The Cleveland Clinic Foundation, Cleveland, Ohio 44195

Cytokines and hormones activate a network of intracellular signaling pathways to regulate cell division, survival and differentiation. In parallel, a series of growth inhibitory mechanisms critically restrict cell population sizes. For example, mitogens can be opposed in crowded cell cultures through contact-inhibition or by autocrine release of antiproliferative substances. Here, we characterize a small, heat-stable growth inhibitor secreted by a rat T lymphoma line when cultured at high cell density. Short term incubation (<60 min) of prolactin-responsive Nb2 lymphoma cells at high density selectively blocked prolactin stimulation of p42/p44 mitogen-activated protein kinases and transcription factors Stat1 and Stat3 but not prolactin activation of Stat5 or the tyrosine kinase Jak2. The selective effects of cell density on prolactin signaling were reversible. Furthermore, exposure of cells at low density to conditioned media from cells incubated at high density had the same inhibitory effects on prolactin signaling. This selective inhibition of discrete prolactin signals was mimicked by short term preincubation of cells at low density with stauorosporine or genistein but not with bis-indoyl maleimide, cyclic nucleotide analogs, calcium ionophore A23187, or phorbol 12-myristate 13-acetate. A heat-stable, proteinase K-resistant, low molecular weight factor with these characteristics was recovered from high density culture medium. The partially purified inhibitor suppressed Nb2 cell growth with a sigmoidal concentration response consistent with a saturable, receptor-mediated process.

Cell proliferation, differentiation, and death are controlled in a coordinated manner by soluble factors, extracellular matrix proteins, and direct cell-cell contact (1, 2). These extracellular regulators influence a series of positive and negative intracellular signaling pathways that translate into a net cellular response. Several mechanisms for growth suppression coexist in normal cells. Direct cell-cell contact inhibition and growth suppression through local secretion of inhibitors work to limit proliferation (3). Although some of these physiological mechanisms may become dysfunctional and thus contribute to malignant transformation and tumor progression, many remain operative in transformed cells. For example, loss of sensitivity to transforming growth factor-β, an autocrine growth inhibitor, is frequently observed in malignant cells (4), whereas contact-inhibition or cell density-induced growth inhibition may remain intact (5, 6). During studies of prolactin-stimulated pathogenesis in rat Nb2 lymphoma cells, we observed that cell crowding selectively inhibited some but not all prolactin-inducible signals, suggesting the existence of a cell density-dependent mechanism for modulation of discrete prolactin signals.

Nb2 lymphoma cells are of early T cell lineage and originated in the lymph node of an estrogenized male rat (7). These cells represent a widely used model for PRL-induced signal transduction and gene regulation (8–10). Despite expressing a mutant PRL receptor that lacks an internal portion of the cytoplasmic domain (11), Nb2 cells respond to PRL by activating multiple signaling pathways. Stimulation of the receptor-associated tyrosine kinase Jak2 (12, 13) is followed by activation of several signaling proteins, including latent cytoplasmic transcription factors Stat1, Stat3, Stat5a, and Stat5b (14–17), and several signaling proteins, including latent cytoplasmic transcription factors Stat1, Stat3, Stat5a, and Stat5b (14–17), and the GH-induced pathway (18–20). In addition, the Sre-tyrosine kinase Fyn (21), the phosphatidylinositol-3 kinase pathway (22), and protein kinase C (23) have also been reported to be activated by PRL in Nb2 cells.

We now demonstrate that short term incubation of Nb2 cells at high cell density selectively blocked PRL stimulation of p42/p44 MAPK and transcription factors Stat1 and Stat3 but did not affect PRL activation of Stat5 or Jak2 tyrosine kinase. This response to cell crowding was reversible and was mimicked at low cell density by short term exposure to conditioned high density medium, stauorosporine or genistein. A low molecular weight, heat-stable, and proteinase K-resistant factor with these characteristics was recovered from conditioned high density culture medium. Of particular importance, the partially purified factor also inhibited cell growth in a saturable dose-dependent fashion, suggesting operation through a receptor-mediated mechanism.

EXPERIMENTAL PROCEDURES

Materials—Ovine PRL (NIDDK-oPRL-19, AFP-9221A) was supplied by the National Hormone and Pituitary Program, NIDDK, the NICHD, and the U.S. Department of Agriculture. Polyclonal rabbit antisera specific to peptides corresponding to the unique COOH termini of Stat1a, Stat3, Stat5a, and Stat5b were generated as described previously (17, 24). In addition, a polyclonal rabbit antisemur that recognized both the 92–94-kDa α-forms and the COOH-terminally truncated 82–84-kDa β-forms of Stat5ab was raised against a peptide derived from the originally published sequence of sheep Stat5a as described.

* This work was supported by National Institutes of Health Grant RO1 DK52013-01A1 and Uniformed Services University of the Health Sciences Grant R074HF. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

† To whom correspondence should be addressed: Dept. of Pathology, Uniformed Services University of the Health Sciences School of Medicine, Bethesda, MD 20814. Tel.: 301-295-3801; Fax: 301-295-1640; E-mail hru@usuhs.mil.

‡ The abbreviations used are: PRL, prolactin; MAPK, mitogen-activated protein kinase; HDM, high density medium; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; PKC, protein kinase C.
Cell Density-associated Growth Inhibitor

previously (17). Each antiserum was used for detection of rat Stat proteins by immunoprecipitation and immunoblotting. Monoclonal anti-phosphotyrosine antibody 4G10 and polyclonal rabbit antisera to Jak2 were purchased from Upstate Biotechnology (catalog numbers 05-231 and 06-255, respectively). Affinity-purified rabbit antibodies to active MAPK were purchased from Promega (catalog number V667A).

Cell Culture and Treatment—The Nb2 cell line (7) was originally developed by Dr. Peter Gout (Vancouver, Canada), the Nb2-SP clone used in this study was generously provided by Dr. Henry Friesen (Manitoba, Canada). Cells were grown in RPMI 1640 medium (Mediatech, catalog number 15-040-LM) containing 10% fetal calf serum (In- tergen, catalog number 1020-90), 2 mM l-glutamine, 5 mM HEPES, pH 7.3, 0.1 mM non-essential amino acids, 1 mM sodium pyrophosphate, 50 mM sodium fluoride, 1 mM sodium orthovanadate, 1% Triton X-100, 1 mM phenylmethylsulfonyl fluoride, 5 μM aprotinin, 1 μg/ml pepstatin A, and 2 μg/ml leupeptin. Cell lysates were rotated end over end at 4 °C for 60 min, and insoluble material was pelleted at 12,000 × g for 30 min at 4 °C. Depending on the experiment, clarified lysates were incubated rotating end over end for 3 h at 4 °C with polyclonal rabbit antisera (3 μl/ml) to individual Jak2 and Stat proteins as specified. Antibodies were captured by incubation for 60 min with protein A-Sepharose beads (Amersham Pharmacia Biotech, catalog number 17-0780-01), and proteins were analyzed by SDS-polyacrylamide gel electrophoresis and immunoblotting as described previously (17), using polyclonally di-fluoride membranes (Millipore, catalog number IPVH 00010) and horseradish-peroxidase-conjugated secondary antibodies in conjunction with enhanced chemiluminescence substrate mixture (Amersham Pharmacia Biotech, catalog number RPN2106) and x-ray film.

Preparation of Conditioned High Density Medium (HDM)—Nb2 cells that had been cultured in PRL-free starvation medium for 20–24 h at a density of 1–5×10⁵ cells/ml were brought to a high cell density of 4.0 × 10⁶ cells/ml and incubated for 4 h in the same culture medium at 37 °C. The cells were then incubated for 45 min at 4 °C, and the conditioned medium was passed through a 0.22-μm filter and used fresh unless indicated otherwise. For some experiments, conditioned HDM was boiled for 10 min, followed by incubation on ice for 30 min with subsequent clarification by centrifugation at 12,000 × g for 30 min at 4 °C to remove precipitated proteins. For dialysis experiments, dialysis membranes with a molecular weight cutoff of 1000 Da were used, and the dialysis medium was either 100 volumes of starvation medium for removal of low molecular weight material or four volumes of water for recovery of the low molecular weight fraction.

Cell Proliferation Assays—Nb2 cells were dispensed into 96-well microtiter plates at 1×10⁵ cells/100 μl and exposed for 48 h to either control medium or conditioned HDM in some experiments or in dose-response experiments to various dilutions of the reconstituted low molecular weight material derived from boiled and clarified HDM. A number of viable cells were measured metabolically by the Promega cell proliferation assay using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) as described previously (25). Colorimetric analysis was performed using a semiautomatic plate reader from Dynatech Laboratories (MR 600) at absorbance 590 nm.

RESULTS

Selectivity in High Cell Density of PRL-inducible Phosphorylation of Stat1, Stat3, and ERK1/2 but not Jak2 and Stat5—To examine the effect of cell crowding on PRL-induced signal transduction, Nb2 cells were incubated at increasing concentrations (range 0.25–4.0×10⁶ cells/ml) for 45 min, with or without prolactin (100 nM) added during the final 15 min. Cells were maintained in suspension by manual agitation every 5 min during the entire incubation period. PRL-inducible tyrosine phosphorylation of Jak2, Stat1, Stat3, and Stat5 was subsequently analyzed by immunoblotting of immunoprecipitated proteins from detergent cell lysates. Parallel samples of whole cell lysates were also immunoblotted with anti-phospho-MAPK antibodies to detect activated ERK1 and ERK2. In addition, reblotting of the same or parallel samples for protein levels were performed as indicated.

tyrosine phosphorylation of Jak2, Stat1, Stat3, and Stat5 was subsequently analyzed by immunoblotting of immunoprecipitated proteins from detergent cell lysates. Parallel samples of whole cell lysates were also immunoblotted with anti-phospho-MAPK antibodies to detect activated ERK1 and ERK2. Immunoblotting with anti-phosphotyrosine antibodies or anti-phospho-specific MAPK antibodies showed that PRL-inducible activation of Stat1, Stat3, and ERK1/2 was significantly inhibited by increasing cell density, particularly at 2–4 × 10⁸ cells/ml (Fig. 1, lanes g–j, panels 3, 5, and 7 as numbered from the top). In contrast, PRL-inducible tyrosine phosphorylation of Jak2 and Stat5 remained unaffected by cell density (Fig. 1, lanes a and b versus i and j, panels 1 and 9). In parallel, basal levels of ERK1/2 activation were also significantly suppressed under high cell density conditions (Fig. 1). Reblotting of the same or duplicate samples for protein levels did not reveal any systematic changes in protein levels during the 45-min experiment period that could explain the marked reductions in signals by either Stat1, Stat3, or MAPK.

Previously, we had established by electrophoretic mobility shift assays that PRL-activated Stat5 and Stat1 from Nb2 cells form distinguishable complexes with an oligonucleotide probe derived from the rat β-casein promoter, whereas PRL-activated Stat3 does not bind to this DNA sequence (17, 26, 27). Parallel electrophoretic mobility shift analyses of Stat1 and Stat5 binding to this β-casein promoter probe showed a corresponding selective inhibition by cell crowding of PRL-inducible Stat1 activation.

The Selective Inhibition at High Cell Density of PRL Activation of Stat1, Stat3, and ERK1/2 Is Rapid and Reversible—Having demonstrated that a selective uncoupling of certain PRL-induced signals occurred at high cell density, we examined how rapidly this inhibition of PRL-inducible Stat1 and

2 H. Yamashita, J. Xu, and H. Rui, unpublished data.
stimulation with (3 activation of Stat1, Stat3, and ERK1/2 is rapid and reversible. a parallel samples of Nb2 cells were incubated at low density (0.5 \times 10^8 cells/ml) and/or high density (4 \times 10^9 cells/ml) for a combined period of 75 min at 37 °C with (+) or without (−) 100 nm ovine PRL added during the final 15 min. At variable times before the end of the 75-min incubation period, cells were switched from low density (0.5 \times 10^9 cells/ml) to high density (4 \times 10^9 cells/ml) by centrifugation and volume reduction, so that the individual cell samples were crowded for periods ranging from 0 to 75 min as indicated. Clarified cell lysates were immunoprecipitated (IP) with antibodies to Jak2, Stat1a, or Stat3, then blotted with anti-phosphotyrosine antibodies (α-PY). Nb2 cells were incubated at 37 °C for 30 min at low cell density (0.5 \times 10^6 cells/ml); lanes a and b) or high cell density (4 \times 10^9 cells/ml; lanes c−f) before stimulation with (+) or without (−) 100 nm of ovine PRL for 15 min at low cell density (lanes a, b, e, and f) or high cell density (lanes c and d). Clarified cell lysates were immunoprecipitated with antibodies to Jak2, Stat1α, Stat3, or Stat5, then blotted with anti-phosphotyrosine antibodies (α-PY). Parallel samples of whole cell lysates were also immunoblotted with α-pMAPK antibodies.

Stat3 tyrosine phosphorylation occurred, and whether the inhibitory effect could be reversed by dilution of the cells. Incubation of cells at high density (4.0 \times 10^8 cells/ml) for varying times up to 75 min, with or without PRL added during the final 15 min of each period, showed that whereas PRL-inducible Jak2 tyrosine phosphorylation was unaffected by cell crowding over the entire period, PRL-inducible tyrosine phosphorylation of both Stat1 and Stat3 was significantly inhibited after 30 min and completely after 75 min (Fig. 2A).

We also investigated whether this inhibition of discrete PRL signals was reversible upon dilution of cells. Nb2 cells were preincubated at high density (4.0 \times 10^8 cells/ml) for 30 min, followed by either continued incubation at high cell density or dilution to low cell density (0.5 \times 10^8 cells/ml) for 15 min in the presence or absence of PRL. Parallel control cells were also incubated at low cell density for the entire 45-min period with or without PRL during the final 15 min (Fig. 2B, lanes a and b). PRL-inducible tyrosine phosphorylation of Stat1 and Stat3 and ERK1/2 activation, but not Jak2 and Stat5 tyrosine phosphorylation, was significantly inhibited as observed previously when incubated at high density for 45 min (Fig. 2B, panels 1–5, lanes a–d). However, this selective inhibition of PRL-inducible Stat1, Stat3, and ERK1/2 phosphorylation was completely reversed by dilution for 15 min to low density after preincubation for 30 min at high cell density (Fig. 2B, panels 2–4, lanes e and f).

Replotting of samples for protein expression levels were consistent with the previous data shown in Fig. 1, demonstrating that density-induced inhibition of Stat1, Stat3, and ERK1/2 signals could not be explained by systematic reductions in protein levels (data not shown). Based on these experiments, we conclude that the inhibitory effect of cell crowding on discrete PRL signals was rapid and reversible. Because of the rapid reversal of signal suppression upon dilution to low cell density, and the short time at high cell density needed for establishing the inhibitory effect, we favored a direct mechanism that exclusively involved cell-cell contact. However, it remained possible that the effect of cell crowding also included the release of a soluble suppressor substance.

A Low Molecular Weight Factor in Conditioned High Density Medium Selectively Modulates PRL Signals—To directly test whether a soluble mediator could account for the observed effects, we analyzed the effect of conditioned HDM on PRL signals in cells incubated at low density (0.5 \times 10^8 cells/ml). HDM was generated by incubating parallel cultures of Nb2 cells at high density (4.0 \times 10^9 cells/ml) for 60 min at 37 °C followed by removal of cells by centrifugation. Contrary to our expectation, short term incubation of cells at low cell density in freshly generated HDM also led to selective suppression of PRL-inducible tyrosine phosphorylation of Stat1 and not of PRL-inducible Jak2 activation (Fig. 3A, lanes a–f). These observations suggested that a soluble factor was indeed present in freshly generated HDM that could mediate the suppression caused by cell crowding and showed that increased cell-cell contact per se was not required to achieve the inhibitory effect. Furthermore, these experiments showed that the soluble inhibitory activity was stable, because the activity persisted in HDM that had been stored for 24 h at 4 °C (Fig. 3A, lanes g and h) and in HDM that had been boiled for 10 min followed by centrifugation at 12,000 × g for 30 min (Fig. 3A, lanes i and j).

On the other hand, the soluble inhibitory activity was lost upon dialysis of boiled and clarified HDM against 100 volumes of starvation medium at 4 °C for 12 h using a molecular weight cutoff of 10 kDa (Fig. 3A, lanes k and l). Collectively, these results indicated that a heat-stable low molecular weight factor present in HDM could mediate the inhibition of discrete PRL signals induced by cell crowding.

We then tested whether this small heat-stable factor could be recovered from the dialysis medium. Conditioned HDM was boiled, clarified, and dialyzed against 4 volumes of water overnight at 4 °C using a molecular weight cutoff of 10 kDa. The dialysate was discarded, and the resulting dialysis fluid containing low molecular weight material was lyophilized and restored with water to a stock concentration corresponding to 10 × HM. This concentrated low molecular weight fraction of HDM was diluted 1:10 in cell culture medium and specifically tested for its ability to affect PRL activation of Jak2, Stat1, and ERK1/2. In control samples, incubation at high cell density was associated with inhibition of PRL-inducible Stat1 and ERK1/2 but not Jak2 signals (Fig. 3B, lanes a–d). Likewise, cells incubated at low cell density in medium containing the reconstituted low molecular weight fraction of HDM also showed selective inhibition of PRL-inducible phosphorylation of Stat1 and ERK1/2 without affecting Jak2 phosphorylation (Fig. 3B, lanes e and f). In addition, this reconstituted dialysis medium inhibited basal activity of ERK1/2 (Fig. 3B, panel 3, lane e), thus also mimicking this particular effect of cell crowding. On the other hand, no inhibition was seen in cells incubated in control medium containing the low molecular weight fraction from low density starvation medium, which had also been boiled, clarified, dialyzed, and reconstituted correspondingly (Fig. 3B, lanes g and h). We conclude that a small heat-stable activity that specifically mimicked the effect of cell crowding could be
Low MW correspoding low molecular weight fraction from control medium (A). The molecular weight fraction from boiled conditioned high density medium that had been either freshly generated (Fresh) or reclarified by centrifugation at 12,000 g/ml) at 37 °C for 1 h, reboiled for 5 min, and dialyzed using a molecular weight cutoff of 10 kDa against 100 mM ovine PRL during the final 15 min at either low cell density (lanes a–d) or high cell density (lanes e–h). Cell lysates were immunoprecipitated (IP) with antibodies to Jak2 or Stat1 and Stat3, albeit not as markedly as staurosporine (Fig. 3C). Moreover, the mimicry of cell crowding by inhibiting PRL-induced tyrosine phosphorylation of Stat1 and Stat3, while having little or no effect on Jak2 activation (Fig. 4B). In contrast, the more specific protein kinase C (PKC) inhibitor, bis-indolylmaleimide (50 μM), did not affect PRL signals, suggesting that the effect of staurosporine is not mediated by inhibition of PKC. On the other hand, genistein (200 μM), a tyrosine kinase inhibitor, also inhibited PRL-induced Stat1 and Stat3, albeit not as markedly as staurosporine (Fig. 4B). No effect was observed after pretreatment with any of the other agents, which included the calcium ionophore A23187 (1 μM), 8-Br-cAMP (1 mM), 8-Br-cGMP (1 mM), the cyclic nucleotide phosphodiesterase inhibitor isobutylmethylxanthine (100 μM), and the protein kinase C activator phorbol 12-myristate 13-acetate (1 μM). Furthermore, the mimery of cell crowding by staurosporine and genistein also included resistance of PRL-inducible Jak2, Stat1, and Stat3 signals in Nb2 cells. Cells were maintained at low density and were preincubated with pharmacologically active concentrations of each agent for 30 min before PRL was added for another 15 min. The results of these studies are presented in Fig. 4, A and B, and showed that the broad spectrum protein kinase inhibitor, staurosporine (500 nM), specifically mimicked the effect of cell crowding by inhibiting PRL-induced tyrosine phosphorylation of Stat1 and Stat3, while having little or no effect on Jak2 activation (Fig. 4B). In contrast, the more specific protein kinase C (PKC) inhibitor, bis-indolylmaleimide (50 μM), did not affect PRL signals, suggesting that the effect of staurosporine is not mediated by inhibition of PKC. On the other hand, genistein (200 μM), a tyrosine kinase inhibitor, also inhibited PRL-induced Stat1 and Stat3, albeit not as markedly as staurosporine (Fig. 4B). No effect was observed after pretreatment with any of the other agents, which included the calcium ionophore A23187 (1 μM), 8-Br-cAMP (1 mM), 8-Br-cGMP (1 mM), the cyclic nucleotide phosphodiesterase inhibitor isobutylmethylxanthine (100 μM), and the protein kinase C activator phorbol 12-myristate 13-acetate (1 μM). Furthermore, the mimery of cell crowding by staurosporine and genistein also included resistance of PRL-inducible tyrosine phosphorylation of Stat5a and Stat5b (Fig. 4C, as well as sensitivity of basal and PRL-inducible ERK1/2 activation as recently reported by us and others (29, 30). Thus, staurosporine and to some extent genistein mimicked cell crowding modulation of PRL signals, whereas a series of other

recovered by dialysis of HDM that had been boiled and clarified.

To address whether the inhibitory factor present in boiled and clarified HDM was a peptide, we tested its sensitivity to proteinase K, a general endoprotease that cleaves nonspecifically after any hydrophobic, aliphatic, or aromatic amino acid (28). For these experiments, boiled and clarified HDM or corresponding control medium (pH 7.3) were incubated with proteinase K (50 μg/ml) for 1 h at 37 °C, followed by reboiling for 10 min to inactivate the enzyme. Consistent with the previous data, preincubation of cells with boiled and clarified HDM suppressed the PRL-inducible Stat1 signal (Fig. 3C, lanes e and f) when compared with pretreatment with control medium from low density cultures that had been boiled only, or boiled and treated with proteinase K (Fig. 3C, lanes a–d). However, treatment of boiled and clarified HDM with proteinase K did not neutralize the endogenous inhibitor (Fig. 3C, lanes g and h). As a positive control to verify that the proteinase K used was enzymatically active in this biochemical setting, conditioned low density medium was spiked with prolactin (10 nM) and incubated for 30 min at 37 °C in the presence or absence of proteinase K. As shown in Fig. 3D, PRL-induced tyrosine phosphorylation of Jak2 was abolished after proteinase K treatment (lane c versus lane d). Because proteinase K cleaves peptide bonds after more than half of the natural amino acids (28), these observations suggested that the low molecular weight factor may not be a peptide. However, further physicochemical analysis and purification is needed to determine the structure of this factor.
agents had no effect. It is therefore possible that the soluble inhibitor acts by a mechanism shared by staurosporine and genistein to regulate PRL signals. Because staurosporine inhibited growth at low molecular weight factor derived from boiled clarified conditioned high density medium. Nb2 cells were cultured for 48 h in the presence of increasing concentrations of the partially purified factor derived from the low molecular weight fraction after dialysis of boiled and clarified conditioned HDM. The concentration of the partially purified inhibitory factor is presented as units relative to its concentration in conditioned HDM, i.e. the unit 1.00 represents 1×HDM. Cell numbers were assayed by the MTT method, and the data are presented as percent growth inhibition relative to cells treated with corresponding fractions derived from dialysis of boiled and clarified control culture medium. Error bars indicate the mean ± S.E. (n = 3).

**DISCUSSION**

This study describes a small heat-stable growth inhibitor secreted by a rat T lymphoma line when cultured at high cell density. The data also suggested that this inhibitor selectively and reversibly suppressed some, but not all, signaling pathways downstream of PRL receptors. Specifically, short term incubation of Nb2 lymphoma cells at high density blocked PRL stimulation of mitogen-activated protein kinases ERK1/2 and transcription factors Stat1 and Stat3 but did not affect PRL activation of Stat5 or the tyrosine kinase Jak2. These effects of
cell crowding were reversible and could be imitated by incubation of cells in conditioned high density culture medium at low cell density. A heat-stable and proteinase K-resistant low molecular weight factor (<10 kDa) with these characteristics was recovered from the conditioned high density medium. This factor suppressed basal and PRL-induced MAPK activation and inhibited cell growth with a sigmoidal concentration response consistent with a saturable mode of action.

Common Mechanism of Discrete Signal Modulation by Cell Crowding, Staurosporine and Genistein?—The discriminating suppression of PRL signals by the growth inhibitor present in conditioned high density medium could under low density culture conditions be specifically mimicked by the broad spectrum protein kinase inhibitor, staurosporine. Short term (30 min) pretreatment of cells with staurosporine was associated with selective inhibition of PRL-inducible Stat1, Stat3, and ERK1/2 signals, with little or no effect on PRL-inducible Stat5 and Jak2 signals. A lesser but similar selective inhibition of PRL signals was induced by the more specific tyrosine kinase inhibitor, genistein. In contrast, PRL signals were not modulated by pretreatment of cells with the PKC inhibitor bis-indoylmaleimide, the PKC activator phorbol 12-myristate 13-acetate, the calcium ionophore A23187, or several cyclic nucleotide mimetics. Collectively, these pharmacological data raise the possibility that cell crowding, staurosporine, and possibly genistein modulate PRL signals through a common and PKC-independent mechanism. One simple model would involve inhibition by staurosporine, genistein, and cell crowding of a tyrosine kinase that acts downstream of Jak2 and is required for PRL-induced activation of Stat1, Stat3, and MAPK. Such a role may be played by one or more Src tyrosine kinases. Both staurosporine and genistein inhibit tyrosine kinases of the Src family (30, 31), and Fyn and Lck is activated by PRL in Nb2 cells (21). In parallel studies of Shc, an adaptor protein that we have shown to couple PRL-receptors to the Grb2-Sos-Ras-MAPK pathway in Nb2 cells (19), supported this notion. PRL-inducible tyrosine phosphorylation of Shc was also inhibited after pretreatment with high cell density, staurosporine, or genistein.3 We are currently testing the possibility that Fyn or other Src tyrosine kinases represent common targets for cell crowding, genistein, and staurosporine in Nb2 cells.

A tyrosine kinase acting downstream of Jak2 might critically mediate PRL-induced activation of Stat1/3 and the Shc-Ras-MAPK pathway by two alternative mechanisms. On the one hand, Stat1, Stat3, and Shc could be direct substrates for a density-regulated tyrosine kinase. Alternatively, this putative kinase could phosphorylate tyrosyl docking sites within the receptor complex that are critical for bringing Stat1/3 and/or Shc within reach of the catalytic domain of the Jak2 kinase. Future examination of the sensitivity of specific phosphorylation sites within the prolactin receptor to inhibition by high cell density, staurosporine, and genistein may therefore be highly informative.

Another question that we have addressed is whether density-associated inhibition of MAPK in Nb2 cells may selectively suppress tyrosine phosphorylation of Stat1 and Stat3 but not Stat5. We recently demonstrated that the MEK-1 inhibitor, PD98059, blocked prolactin-induced MAPK activation in Nb2 lymphocytes without affecting inducible Stat5a and Stat5b tyrosine phosphorylation (29). However, further investigation showed that PD98059 did not inhibit prolactin-induced tyrosine phosphorylation of either Stat1, Stat3, or Stat5, despite effectively inhibiting MAPK activation.3 These experiments therefore did not suggest any causal link between MAPK inhibition and the selective inhibition of Stat1/3 tyrosine phosphorylation at high cell density but rather indicated that the two inhibitory events occur in parallel.

A final mechanistic matter relates to the observed lack of sensitivity of PRL-induced Jak2 tyrosine phosphorylation to kinase inhibitors. The specific resistance of Jak2 tyrosine phosphorylation to both staurosporine and genistein in Nb2 lymphocytes may suggest that Jak2 per se is not sensitive to these inhibitors. To our knowledge, the sensitivity of Jak2 to staurosporine or genistein has not been analyzed under stringent conditions in cell-free Jak2 kinase assays. The observation that staurosporine may inhibit cytokine-induced Jak2 tyrosine phosphorylation in some cellular models, e.g. growth hormone-induced Jak2 phosphorylation in fibroblasts (32), could reflect inhibition of one or more secondary tyrosine kinases in that certain cell lines contribute significantly to Jak2 tyrosine phosphorylation on multiple sites.

Context-sensitive Modulation of Discrete Signaling Pathways Downstream of PRL Receptors—The extracellular matrix is an important external modulator of cell fate (1) and is known to influence PRL signaling. Specifically, for PRL to activate Stat5 and induce lactogenic differentiation of mammary epithelial cells, a laminin-containing extracellular matrix was required that suppressed a prolactin receptor-associated protein tyrosine phosphatase (33, 34). The current observations on Nb2 lymphoma cells extends this phenomenon by describing a discriminative pattern of external regulation of individual signal transduction pathways downstream of PRL receptors. Provided that the observed changes in signaling repertoire have biological consequences for Nb2 cells, the modulated pathways may be viewed as sensors of the degree of cell crowding. The fact that significant quantitative changes occurred in some, but not all, PRL signals at different cell densities may also be directly linked to the associated growth inhibition. Although such a causal relationship remains to be proven, the observed suppression of MAPK activities may represent the most critical growth inhibitory component. Furthermore, the rapid uncoupling or heterologous desensitization of discrete PRL signals by an autocrine factor during cell crowding may reflect only one of many commonly operating mechanisms of external modulation of particular transduction pathways. Increased awareness of context-sensitive signaling pathways should also caution against simplistic interpretations of signaling data.

The observed resistance of Jak2 and Stat5 to inhibition at high cell density solidifies the Jak2-Stat5 axis as a distinct pathway as proposed by Groner and colleagues (35, 36). We have also recently shown that selective stimulation of Jak2 in Nb2 cells by the anti-apoptotic drug, aurantricarboxylic acid (ATA), exclusively led to activation of Stat5a and Stat5b, but not of Stat1 and Stat3 (26). Likewise, in this study, staurosporine treatment affected PRL-inducible activation of Stat1 and Stat3 but not Jak2 and Stat5. Therefore, accumulating evidence suggests that PRL activation of Jak2-Stat5 can be physiologically and pharmacologically dissociated from PRL activation of Stat1 and Stat3 in Nb2 cells. However, a clearer understanding of the biological roles of each of these pathways is needed to shed light on their involvement in disease progression and to establish a more specific pharmacological basis for their manipulation.

Characterization of the High Density Medium-associated Inhibitor—Thus far, the majority of growth inhibitory factors isolated from cultured cells have been thermolabile polypeptides, including transforming growth factor-β, interferons, glial maturation factor-β, and mammastatin (for review, see Ref. 37). We have described the partial purification of a heat-stable low molecular weight inhibitory activity from conditioned high

---

3 H. Yamashita and H. Rai, unpublished data.
cell density culture medium. The activity was also resistant to extended treatment with the nonselective endoprotease, protease K. It is therefore possible that the factor is not a peptide. Proteinase K is active over a wide pH range, is insensitive to metal ions, and cleaves peptide bonds nonselectively after nonpolar or aromatic amino acids (28), which account for more than half of the natural amino acids. It will now be important to determine whether the endogenous inhibitor is indeed of nonpeptidyl nature, or whether it is a peptide without or leukotrienes represent one candidate class of molecules, because eicosanoids can inhibit cell growth (38), are quickly released, and activate specific receptors (39). Further chemical analysis and purification of this factor is being pursued.

Relevance of the Endogenous Growth Inhibitor to Cancer Therapy—Could identification of this soluble inhibitor from Nb2 lymphoma cells cultured at high density lead to new drugs against cancer? The success of antiproliferative interferons as adjuvant treatment for several types of malignancies has demonstrated the usefulness of natural growth inhibitors in cancer therapy (40). The observations that the endogenous inhibitor suppressed MAPK activities and may operate through a mechanism shared by staurosporine are encouraging. MAPKs are hyperactive in a large number of tumors (41), and at least two staurosporine analogues, 7-hydroxystaurosporine and N-benzoylstaurosporine, are currently in clinical trials for cancer treatment (42, 43). Because staurosporine inhibits a variety of protein kinases and induces apoptosis in many cell types (44, 45), more selective analogues may have fewer adverse effects and be less toxic. It will therefore also be important to establish to what extent the endogenous inhibitory activity described here will mimic all of the effects of staurosporine. A possible antiproliferative effect on cells of lymphoid origin would also be of direct relevance for pharmacologic immunosuppression.

REFERENCES

1. Lin, C. Q., and Bissell, M. J. (1993) FASEB J. 7, 737–743
2. Juliano, R. (1996) Bioessays 18, 911–917
3. Fagotto, F., and Gumbiner, B. M. (1996) Dev. Biol. 180, 445–454
4. Fynan, T. M., and Reiss, M. (1993) Crit. Rev. Oncog. 4, 493–540
5. Harel, L., Chatelan, G., and Golde, A. (1984) J. Cell. Physiol. 119, 101–106
6. Coldwell, W. T., Wong, Y. V., Dore-Duffy, P., Freedman, M. S., and Antel, J. P. (1992) J. Neurol. Sci. 110, 178–185
7. Gout, P. W., Beer, C. T., and Noble, R. L. (1980) Cancer Res. 40, 2433–2436
8. Too, C. K., Murphy, P. R., and Friesen, H. G. (1989) Endocrinology 124, 2185–2192
9. Vu-Lee, L. Y. (1997) Proc. Soc. Exp. Biol. Med. 215, 35–52
10. Buckley, A. R., Rao, Y. P., Buckley, D. J., and Gout, P. W. (1994) Biochem. Biophys. Res. Commun. 204, 1158–1164
11. Ali, S., Pellegrini, I., and Kelly, P. A. (1991) J. Biol. Chem. 266, 20110–20117
12. Rui, H., Kirken, R. A., and Farrar, W. L. (1994) J. Biol. Chem. 269, 5364–5368
13. Rui, H., Lebrun, J. J., Kirken, R. A., Kelly, P. A., and Farrar, W. L. (1994) Endocrinology 134, 1289–1291
14. Sidis, Y., and Horwitz, N. D. (1994) Endocrinology 134, 1979–1985
15. David, M., Petrinic, E. F., Ill, Igarashi, K., Feldman, G. M., Finchlomb, D. S., and Larner, A. C. (1994) Proc. Natl. Acad. Sci. U. S. A. 91, 7174–7178
16. DaSilva, L., Rui, H., Erwin, R. A., Howard, O. M. Z., Kirken, R. A., Malabarra, M. G., Hackett, R. H., Larner, A. C., and Farrar, W. L. (1996) Mol. Cell. Endocrinol. 117, 131–140
17. Kirken, R. A., Malabarra, M. G., Xu, J., Liu, X., Farrar, W. L., Hennighausen, L., Larner, A. C., Grimley, P. M., and Rui, H. (1997) J. Biol. Chem. 272, 14098–14103
18. Clevegner, C. V., Torigoe, T., and Reed, J. C. (1994) J. Biol. Chem. 269, 5559–5565
19. Erwin, R. A., Kirken, R. A., Malabarra, M. G., Farrar, W. L., and Rui, H. (1995) Endocrinology 136, 3512–3518
20. Rao, Y.-P., Buckley, D. J., and Buckley, A. R. (1995) Cell Growth Diff. 6, 1235–1244
21. Clevegner, C. V., and Medaglia M. V. (1994) Mol. Endocrinol. 8, 674–681
22. Berlanga, J. J., Guilililo, O., Buteau, H., Applanat, M., Kelly, P. A., and Edery, M. (1997) J. Biol. Chem. 272, 20550–20552
23. Rui, H., Xu, J., Mehta, S., Fang, H., Williams, J., Deng, F., and Grimley, P. M. (1998) J. Biol. Chem. 273, 28–32
24. Liu, X., Robinson, G. W., and Hennighausen, L. (1996) Mol. Endocrinol. 10, 1496–1506
25. DaSilva, L., Howard, O. M. Z., Rui, H., Kirken, R. A., and Farrar, W. L. (1994) J. Biol. Chem. 269, 18267–18270
26. Rui, H., Xu, J., Mehta, S., Fang, H., Williams, J., Deng, F., and Grimley, P. M. (1998) J. Biol. Chem. 273, 28–32
27. Schaber J. S., Fang, H., Xu, J., Grimley, P. M., and Rui, H. (1998) Cancer Res. 58, 1914–1919
28. Forschung, B., and Darmstadt, E. M. (1974) Eur. J. Biochem. 47, 91–97
29. Yamashita, H., Xu, J., Erwin, R. A., Farrar, W. L., Kirken, R. A., and Rui, H. (1998) J. Biol. Chem. 273, 30218–30224
30. Carey, G. B., Liberti, J. P. (1995) Arch. Biochem. Biophys. 316, 179–189
31. Tamaoka, T. (1991) Methods. Enzymol. 201, 340–347
32. Gong, T. W., Meyer, D. J., Liao, J., Hodge, C. L., Campbell, G. S., Wang, X., Billestrup, N., Carter-Su, C., Schwartz, J. (1998) Endocrinology 139, 1863–1871
33. Streuli, C. H., Edwards, G. M., Del communiste, M., Whitelaw, C. B., Burdon, T. G., Schindler, C., and Watson, C. J. (1995) J. Biol. Chem. 270, 21639–21644
34. Edwards, G. M., Wilford, F. H., Liu, X., Hennighausen, L., Djiane, J., and Streuli, C. H. (1998) J. Biol. Chem. 273, 3483–3500
35. Gouilleux, F., Wakao, H., Mundt, M., and Groner, B. (1994) EMBO J. 13, 4361–4369
36. Barachmand-Pour, F., Meinke, A., Groner, B., and Decker, T. (1998) J. Biol. Chem. 273, 12567–12575
37. Johnson, T. C. (1994) Pharmacol. Ther. 62, 247–265
38. Marnett, L. J. (1992) Cancer Res. 52, 5575–5589
39. Smith, W. (1997) Adv. Exp. Med. Biol. 400B, 989–1011
40. Einhorn, S., and Strander, H. (1993) Med. Oncol. Tumor Pharmacother. 10, 25–29
41. Svarmawan, V. S., Wang, H.-Y., Nuovo, G. J., and Malbon, C. C. (1997) J. Clin. Invest. 99, 1478–1483
42. Shao, R. G., Cao, C. X., Shimizu, T., O’Connor, P. M., Kohn, K. W., and Pommier, Y. (1997) Cancer Res. 57, 4029–4035
43. van Ginj, R., van Tellingen, O., de Clippeleir, J. J., Hillebrand, M. J., Boven, E., Vermorken, J. B., ten Brok Huinink, W. W., Schwertz, S., Graf, P., and Beijnen, J. H. (1995) J. Chromatogr. B. Biomed. Appl. 667, 269–276
44. Ruegg, U. T., and Burgess, G. M. (1989) Trends. Pharmacol. Sci. 10, 218–220
45. Bertrand, R., Solary, E., O’Connor, P., Kohn, K. W., and Pommier, Y. (1994) Exp. Cell. Res. 211, 314–321