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Amplification of B cell receptor–Erk signaling by Rasgrf-1 overexpression in chronic lymphocytic leukemia

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Abstract
Rasgrf-1 is a guanine exchange factor (GEF) that catalyzes the exchange of GDP for GTP. In a RNA microarray analysis of chronic lymphocytic leukemia (CLL) specimens (n = 5), this gene was found to be overexpressed in CLL as compared to normal peripheral blood mononuclear cell (PBMC) CD19 + B cells (n = 3). CLL specimens (n = 29) expressed Rasgrf-1 RNA at levels 5–300-fold higher as compared to normal B cells. CLL specimens expressed a 75 kDa isoform that was smaller than the expected full-length protein (140 kDa) and the truncated variant had higher GEF activity. Knockdown of Rasgrf-1 in CLL specimens inhibited active GTP-bound Ras and the Ras/Erk/mitogen-activated protein kinase (MAPK) pathway. Rasgrf-1 was phosphorylated and activated by B cell receptor (BCR) signaling that increased its GEF function, and this phosphorylation was blocked by Src and Bruton’s tyrosine kinase (BTK) inhibitors. Rasgrf-1 is a novel GEF protein that has a role in BCR signaling and its overexpression further activates the Ras/Erk/MAPK pathway in CLL specimens.

Keywords: CLL, B cell receptor, guanine exchange factor, Rasgrf-1, Erk pathway

Introduction
The B cell receptor (BCR) is a crucial signaling molecule in chronic lymphocytic leukemia (CLL) and also determines the clinical behavior of the disease [1,2]. Signaling via the BCR inhibits apoptosis of CLL cells and stimulates the growth and survival of leukemic cells. Activation of BCR signaling is dependent upon the immunoglobulin V_H status of CLL specimens, and patients with CLL with high Zap-70 expression and non-mutated immunoglobulin V_H status are associated with progressive disease [3,4]. BCR signaling is initiated by the formation of a signalosome or a complex of kinases and adaptor molecules on the cytoplasmic tail of BCR associated immunoglobulin chains [1,2,5]. This results in phosphorylation of immunoreceptor tyrosine based activation motifs that subsequently activate phosphatidyl inositol 3-kinase (PI3K)/Akt, Lyn, Syk, Lck, Bruton’s tyrosine kinase (BTK), protein kinase C (PKC), extracellular signal-regulated kinase (Erk) and other signaling pathways [2,5–13].

The Erk pathway is one such pathway that is activated by BCR signaling [5,8], and chemokines [9,10] with leukemic cells frequently display constitutive activation of this pathway [5,14]. BCR signaling switches Ras to its active conformation with further downstream activation of Raf protein kinase, mitogen-activated protein kinase (MAPK) and Erk [15,16]. This requires activation of Ras guanine nucleotide-binding proteins that exist in an inactive GDP-bound form in unstimulated cells to switch to the active conformation of GTP-bound Ras [17,18]. The activity of Ras proteins is regulated by GTPase-activating proteins (GAPs) that increase GTPase activity and by GEFs (guanine nucleotide exchange factors) that increase the active form of Ras [18]. GEF proteins also function in T-cell receptor (TCR) signaling, and Rasgrp-1 is an example of a RasGEF protein that links TCR signaling to Ras [19,20] and Erk in T cells. Rasgrp-1 and Rasgrp-3 are examples of GEFs that regulate B cell receptor–Ras signaling in B cells [21]. BCR mediated Erk pathway activation suppresses apoptosis in CLL specimens via up-regulation of X-linked inhibitor of apoptosis (XIAP) [22,23] and post-translational modification of pro-apoptotic protein Bim [24]. A number of Erk pathway inhibitors also induce apoptosis, implying that Erk activation is required for survival of these leukemic cells [25].

In this article we report the expression and function of a novel gene, Rasgrf-1 [26], that is overexpressed in CLL specimens. This gene was identified from microarray analysis data of CLL specimens and is known to function as a GEF by removing the inactive GDP from Ras that in turn allows the active GTP to bind Ras. The study outlined here shows that overexpression of this GEF up-regulates the Ras/Erk pathway.

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in CLL specimens, thereby amplifying BCR mediated signaling. Rasgrf-1 is also phosphorylated and activated by BCR signaling, and this is inhibited by blocking two proximal BCR kinases, BTK and Src.

Methods

Cell culture and reagents
CLL specimens were obtained from patients at the West Los Angeles VA hospital hematology clinic after informed consent and Institutional Review Board approval. CLL specimens for this study were obtained from patients who had not received any prior treatment and had more than 90% CLL cells in the peripheral blood mononuclear cell (PBMC) isolate. CLL cells were isolated by a Ficoll gradient and stored in liquid nitrogen. Normal peripheral blood B cell control samples were isolated with a negative isolation kit (Dynal; Invitrogen, Carlsbad, CA) from PBMCs obtained from normal donors. All cell lines were obtained from the American Type Culture Collection (ATCC) and grown in the recommended culture media. Activation of the BCR was performed by crosslinking with a goat F(ab′)2 anti-human immunoglobulin M (IgM) antibody (SouthernBiotech, Birmingham, AL) at a concentration of 10 μg/mL for 10 min.

Microarray analysis
Microarray analysis was performed on CLL specimens from five treatment-naive patients and peripheral blood B cells from two normal donors. CLL specimens were obtained by Ficoll gradient of peripheral blood (more than 95% purity by flow cytometry). Normal B cells were obtained by a negative magnetic bead separation method from PBMCs from normal donors (more than 80% purity). Briefly, total RNA was isolated and purified. cDNA was obtained as per the manufacturer’s protocols and hybridized to the Affymetrix chip 133A. Hybridization was performed at the University of California Los Angeles (UCLA) microarray core facility and data analyzed using the Microsoft Excel software program. The microarray analysis data for two normal human B cell preparations was averaged and then employed to calculate the fold change in the signal intensity in CLL specimens.

Real-time polymerase chain reaction analysis
Taqman probes for Rasgrf-1 and actin were obtained from Applied Biosystems (Foster City, CA). Real-time polymerase chain reaction (PCR) was performed on CLL and normal B cell cDNA in a 30 μL reaction volume with Taqman master mix. To determine expression in other B cell malignancies, a lymphoma TissueScan PCR array was purchased from Origene (Rockville, MD) and real-time PCR performed with Rasgrf-1 and actin probes. Expression data were calculated using the method of Pfaff [27].

Western blot analysis
Cells were washed with cold phosphate buffered saline (PBS) and disrupted in lysis buffer (Cell Signaling, Beverly, MA) supplemented with protease inhibitor cocktail. Cells were lysed on ice and then sonicated for 10 s. Insoluble material was removed by centrifugation (10 000g, 10 min) and protein concentrations determined by BioRad DC protein assay. Samples were mixed with sodium dodecyl sulfate (SDS) sample buffer and 20–30 μg aliquots resolved on SDS/polyacrylamide gel electrophoresis (PAGE) gels. Rasgrf-1 antibody (immunogen is 350 C-terminus amino acids, total residues are 1273) was purchased from Proteintech (Chicago, IL), phospho-Rasgrf1 antibody (serine 929) from Santa Cruz Biotechnology (Dallas, TX), and total Erk, phospho-Erk, caspase 3, poly ADP ribose polymerase (PARP) and actin from Cell Signaling. Detection was performed with horseradish peroxidase (HRP)-conjugated secondary antibodies and chemiluminescence (ECL plus; GE Healthcare and LAS Mini imager; Fuji). Densitometry was performed with GelQuant software.

Rasgrf-1 siRNA knockdown
siRNA against Rasgrf-1 (ON-TARGET plus) and a scrambled control siRNA mix were obtained from Dharmacon (Fisher Scientific, Pittsburgh, PA), and used at a final concentration of 50 nM with Amaxa nucleofector (Nucleofector kit V, program U07). Control and Rasgrf-1 siRNA transfected specimens were treated identically, and after nucleofection were co-cultured with a stromal cell line HS-5 [28] to improve cell viability for 24 h. Cells were then removed from HS-5 cells and cultured for another 24 h and then analyzed by Western blot analyses.

Ras-GTP enzyme-linked immunosorbent assay
Active Ras was measured colorimetrically using the Ras GTPase Chemi enzyme-linked immunosorbent assay (ELISA) kit (Active Motif, Carlsbad, CA) according to the manufacturer’s instructions. Raf-RBD protein fused to glutathione S-transferase (GST) was coated on to the plates and CLL whole cell lysates were added. This was followed by the addition of Ras antibody and a HRP-linked secondary antibody. All experimental groups were run in triplicate. Chemiluminescence was read using a charge coupled device (CCD) camera system (ImageQuant LAS 4000; GE Healthcare Life Science, Pittsburgh, PA) within 15 min to minimize changes in signal intensity. Scanned gel was then analyzed with GelQuant software to obtain the relative density of the bands and reported as Ras activation units.

Inhibition of Src and BTK kinases
Src inhibitor dasatinib and BTK inhibitor ibrutinib were purchased from Selleckchem (Houston, TX). CLL specimens were treated with dasatinib (100 nM concentration) or ibrutinib (1 μM concentration) for 2 h for optimal inhibition and then treated with anti-IgM antibody for crosslinking BCR for 10 min. Cells were washed with cold PBS and lysates prepared for Western blot analysis. Anti-phospho BTK antibody (Tyr 223) and anti-phospho Src antibody (Tyr 416) were purchased from Cell Signaling.

Results

Microarray analysis of CLL specimens
To identify differentially expressed genes in CLL specimens as compared to normal B cells, five CLL specimens and
two normal B cell specimens were analyzed by microarray analysis. These patients all had monoclonal leukemic CD19-positive B cells that co-expressed CD5 and CD23 markers. The percentage of lymphocytes that were leukemic in these five cases ranged from 80 to 95%. The control normal B cells for the array were CD19 positive peripheral blood B cells purified by magnetic bead separation. Microarray analysis was performed on the Affymetrix chip 133A, as per the manufacturer’s protocol, and data analyzed for differentially expressed genes in CLL. The top 20 overexpressed genes are shown in Supplementary Table I to be found online at http://informahealthcare.com/doi/abs/10.3109/10428194.2014.898759, and the entire microarray data are deposited at GEO accession no. GSE 18026. Rasgrf-1 gene was found to be overexpressed in CLL specimens (position #20) as compared to normal B cells. This was selected for further analysis, as this GEF protein could potentially activate the Ras/Erk pathway in CLL.

**Expression of Rasgrf-1 RNA in lymphoid malignancies**

To confirm the expression of Rasgrf-1 in CLL specimens a quantitative real-time PCR analysis was performed on additional CLL specimens. Figure 1(A) shows Rasgrf-1 expression in CLL specimens of different clinical stages (Rai classification) relative to normal peripheral blood B cells. Total PBMCs and Epstein–Barr virus (EBV) transformed B cell line Raji had a low expression of Rasgrf-1 as compared to CLL specimens that had 11–270-fold higher expression than normal B cells. Rasgrf-1 expression was not related to the clinical stage of the disease as high expression was noted in all clinical stages. CLL specimens that were analyzed with microarray analysis are marked with an asterisk in Figure 1(A). A cDNA TissueScan PCR array with a number of different types of leukemias and lymphomas was next analyzed to determine the expression in other leukemias and lymphomas. In Figure 1(B) the Rasgrf-1 expression data are shown for various lymphomas and leukemias relative to the expression in normal lymph nodes (log scale, arbitrary value of 1). For comparison purposes the average fold expression in CLL specimens \( n = 29 \) from Figure 1(A) is also shown. CLL and the closely related small lymphocytic lymphoma (SLL) specimens had high Rasgrf-1 expression as compared to mantle and marginal zone lymphomas, with low to negligible expression in other lymphomas. The findings indicate that overexpression of Rasgrf-1 is characteristically associated with CLL/SLL and its overexpression is observed in all clinical stages of the disease process.

![Figure 1. Rasgrf-1 RNA expression in CLL and B cell malignancies. (A) Rasgrf-1 expression in primary CLL specimens from patients of different clinical stages (Rai classification). The data are relative to expression in normal peripheral blood B cells \( n = 3 \), arbitrary value of 1, adjusted to actin. Expression also shown for normal PBMCs \( n = 3 \) and Raji B cell line. Asterisks indicate CLL specimens that were analyzed by microarray analysis. (B) Rasgrf-1 RNA expression in a panel of B-cell malignancies and CLL. TissueScan lymphoma array was used to detect Rasgrf-1 RNA expression and the expression data normalized to expression in normal lymph node (L node). The bar diagram shows mean expression of Rasgrf-1 for each histology (log scale) with the number of specimens for each lymphoma shown in brackets. For comparison, expression in CLL specimens is shown as an average of 29 CLL specimens that were analyzed in (A).](image-url)
Expression of Rasgrf-1 by Western blot analysis

To determine Rasgrf-1 protein expression, Western blot analysis was performed on lysates from CLL specimens and cell lines. The Rasgrf-1 antibody recognizes the C-terminal region of the protein that also has the GEF activity domain. In Figure 2 (upper and lower panels), Western blot analysis confirmed the expression of Rasgrf-1 in CLL specimens (CLL specimens shown with number sign underlined) as compared to low or undetectable expression in B cell lines (Raji, Daudi), Jurkat, mantle cell lines (JeKo-1, Z138, Maver) and two normal B cell (CD19+−) isolates. The full-length Rasgrf-1 protein is 140 kDa; however, cells are known to express other additional isoforms as well, including 55 kDa, 75 kDa, 95 kDa and 120 kDa [26,29,30]. The Western blots in Figure 2 show that cells expressed more than one Rasgrf-1 isoform, and in the CLL specimens the antibody recognized a band at 100 and 75 kDa with a faint band at 140 kDa. Interestingly, the N-terminal domain of Rasgrf-1 harbors regulatory domains that are lost in the truncated smaller isoforms of Rasgrf-1, and these isoforms that retain the C-terminal region of the protein are known to function as more effective GEFs [31–33].

Rasgrf-1 knockdown inhibits active Ras in CLL specimens

BCR crosslinking in CLL results in activation of a number of pathways, including the Ras/ERK/MAPK pathway [5,7]. To ascertain whether Rasgrf-1 overexpression in primary CLL specimens activates the Ras/ERK/MAPK pathway via its function of removing inactive GDP from Ras, siRNA mediated Rasgrf-1 RNA knockdown was performed. Lysates from these knockdown CLL specimens were analyzed for active Ras by an ELISA method that quantifies active Ras. Four primary CLL specimens were transiently transfected with Rasgrf-1 siRNA or control siRNA as described, and lysates were analyzed for Rasgrf-1 expression. Figures 3(A)–3(D) show a significant decrease in the Rasgrf-1 signal on Western blot analysis, confirming its knockdown. In specimen CLL#23 [Figure 3(A)], Rasgrf-1 knockdown was observed in the 75 kDa isoform, which was the dominant isoform in this CLL specimen, while in CLL#6 both isoforms were expressed and knockdown was observed only in the 75 kDa isoform. This lack of siRNA mediated knockdown of the 100 kDa band indicates that in CLL specimens the 75 kDa band is the specific Rasgrf-1 band, and this band was analyzed in the follow-up experiments. Transfected cells were also cross-linked with anti-IgM antibody to activate BCR signaling, and lysates were then analyzed for active Ras by ELISA as described above [lower panels, Figures 3(A)–3(D)]. The siRNA inactivation of Rasgrf-1 resulted in a significant decrease (mean and standard deviation [SD], Figure 3, p-values 0.06, 0.007, 0.8 and 0.04) in active Ras in the CLL specimens that were not stimulated with BCR crosslinking as compared to the control siRNA transfected cells. Activation of BCR increased active Ras in CLL specimens, indicating activation of this pathway, and CLL specimens with Rasgrf-1 knockdown were also found to have decreased active Ras levels (p-values 0.02, 0.05, 0.01 and 0.016). This decrease in active Ras demonstrates that Rasgrf-1 expression is required for Ras to be in the GTP bound active conformation in CLL specimens.

Inhibition of Ras/Erk/MAPK pathway with Rasgrf-1 knockdown

siRNA mediated Rasgrf-1 knockdown CLL specimens were further analyzed to investigate the function of Rasgrf-1. For CLL#23, #6 and #27, identical lysates that showed a decrease in active Ras by ELISA were used. Western blot analysis of BCR crosslinked Rasgrf-1 knockdown (75 kDa band shown) CLL specimens (#23, #6, #27 and #26 and additional specimens #9, #39) revealed a reduction in phospho-Erk signal as compared to the control siRNA transfected cells [Figure 4(A)]. As inhibition of the Ras/Erk pathway in CLL specimens also results in growth...
inhibition and apoptosis, this was analyzed in knocked down cells. As there was low viability after Amaxa electroporation, a direct assessment of apoptosis in Rasgrf-1 siRNA transfected cells with flow cytometry could not be performed. Instead all the transfected cells were lysed and Western blot analysis was performed to detect apoptosis. Lysates were analyzed for apoptosis markers, cleaved caspase 3 and PARP in non-crosslinked Rasgrf-1 knockdown CLL specimens. The numbers at the bottom of the gels in Figures 4 represent the relative density of the band as normalized to actin. An increase in cleaved caspase 3 and PARP cleavage [increase in intensity of the 75 kDa band] are indicative of apoptosis, and an increase in PARP cleavage [increase in intensity of the 100 kDa band upon knockdown only the 75 kDa band is shown in (C) and (D)]. The numbers below the gel figures are the relative densitometry numbers depicting Rasgrf-1 knockdown. Lower panels [bar diagram] show data from active Ras ELISA performed on the lysates of siRNA knockdown cells. Experiment was performed twice with similar results (mean and SD, p = p-values).

**BCR activation phosphorylates Rasgrf-1**

The GEF proteins play an important role in the Ras pathway and are themselves subject to regulation as well [18]. The phosphorylation of Rasgrf-1 is described in other model systems and is known to activate its GEF function [34,35]. To determine whether there was a similar activation mechanism in CLL specimens, we analyzed the phosphorylation of serine 929 residue (C-terminus region) with a phospho-specific Rasgrf-1 antibody. To stimulate the Ras/Erk pathway, BCR crosslinking was performed and cells analyzed for Rasgrf-1 phosphorylation. Figures 5(A) and 5(B) show data from CLL specimens (mean and SD, p = p-values).

**Figure 3. siRNA knockdown of Rasgrf-1 reduces active Ras in CLL specimens.** Four primary CLL specimens were transfected with control siRNA (Ctrl Si) or Rasgrf-1 siRNA by Amaxa protocol (A-D). B-cell receptors of transfected cells were cross-linked by adding anti-IgM antibody (+ IgM) and cells analyzed by Western blot analysis (upper panel) for Rasgrf-1 signal. Decrease in intensity was observed in the 75 kDa band. The band at 100 kDa is marked by an arrow in (A) and (B). As there is no decrease in intensity of the 100 kDa band upon knockdown only the 75k Da band is shown in (C) and (D). The numbers below the gel figures are the relative densitometry numbers depicting Rasgrf-1 knockdown. Lower panels [bar diagram] show data from active Ras ELISA performed on the lysates of siRNA knockdown cells. Experiment was performed twice with similar results (mean and SD, p = p-values).
the 75 kDa band and was not observed in the smaller Rasgrf-1 isoforms, as the antibody is specific for the serine 929 residue.

Activation of Src and BTK is an early step that initiates BCR signal transduction [1,2,6]. These two kinases were analyzed as potential upstream effectors of Rasgrf-1 phosphorylation at serine residue 929. Src inhibitor dasatinib (100 nM concentration [36,37]) and BTK inhibitor ibrutinib (1 μM concentration [38]) were added to CLL specimens in culture at optimal concentrations for 2 h. CLL specimens were then treated with anti-IgM antibody to crosslink the BCR and cells analyzed by Western blots. This pretreatment of cells inhibited the phosphorylation of Src and BTK as expected and shown earlier [36 – 38] [Figures 5(A) and 5(B)]. Both inhibitors blocked the phosphorylation of Src and BTK to varying degrees [Figure 5(B)] and also blocked Rasgrf-1 phosphorylation. These proximal Src kinases could potentially directly or indirectly phosphorylate Rasgrf-1 upon BCR activation and thereby further increase its GEF activity.

Discussion

We report on the overexpression of a GEF protein Rasgrf-1 in CLL specimens. High Rasgrf-1 expression is consistently observed across clinical stages of CLL, including early stage CLL specimens, implying an important role of this gene in CLL biology. This expression is also specific to CLL and to the related SLL, as other B cell malignancies such as diffuse large B-cell lymphoma and follicular lymphoma show minimal expression by real-time PCR analysis. This GEF protein enhances BCR mediated Erk activation in CLL specimens as it increases the dissociation of inactive GDP from Ras, thus allowing the formation of active GTP-Ras. This occurs by a number of mechanisms: first the gene is overexpressed in CLL specimens, and second the majority of Rasgrf-1 isoforms expressed in CLL specimens lack the N-terminus regulatory regions of the protein that increase its GEF activity [31,32]. Third, our results show that BCR signaling phosphorylates Rasgrf-1 at serine residue 929 that is also known to further increase its GEF function [33,35].

Microarray analysis of CLL specimens has been reported by a number of groups to identify novel differentially expressed genes and signaling pathways [39–43]. Fibromodulin and Rasgrf-1 up-regulation in CLL specimens was also reported by a number of groups [40–43]. Fibromodulin is a member of a family of small interstitial proteoglycans and a component of the extracellular matrix that may also

Figure 4. siRNA mediated Rasgrf-1 knockdown alters BCR signaling in CLL specimens. (A) Primary CLL specimens #29, #30, #33, #27 and #26 were transfected with control (Ctrl Si) or Rasgrf-1 siRNA by Amaxa nucleofection and analyzed by Western blot analysis 48 h after transfection. Figure shows Western blot analysis of Rasgrf-1, phospho-Erk (P-ERK), total Erk in CLL specimens with and without IgM crosslinking. The numbers below phospho-Erk bands represent relative densitometric values of the phosphor-Erk bands (Ctrl siRNA vs. Rasgrf-1 siRNA). (B) Western blots for cleaved caspase 3, PARP and actin in non-cross-linked Rasgrf-1 knockdown and control siRNA transfected CLL specimens. Numbers below the caspase 3 blot show relative intensities of caspase 3 bands while numbers below the PARP Western blot are ratios of the upper (full length) and lower (cleaved) bands.
Rasgrf-1 overexpression in CLL

Figure 5. Rasgrf-1 phosphorylation with BCR crosslinking. (A, B) Western blot analysis of seven primary CLL specimens that were treated with dasatinib (Src inhibitor, 100 nM) and ibrutinib (1 μM BTK inhibitor) for 2 h followed by BCR crosslinking. P-Rasgrf-1 signal (75 kDa band) is observed with BCR crosslinking that also increases P-Erk signal in CLL specimens. Pretreatment of cells with dasatinib and ibrutinib inhibits P-Rasgrf-1 and P-Erk signals. (B) Western blots for P-Src (Phospho-Src) and P-BTK (phosphor-Bruton tyrosine kinase) confirm activity of the inhibitors as there is an inhibition of P-Src and P-BTK with inhibitor treatment, respectively. Phospho specific Rasgrf-1 antibody only identifies the 75 kDa Rasgrf-1 band.

Regulate transforming growth factor-β (TGF-β) activities by sequestering TGF-β into the extracellular matrix [44]. siRNA knockdown of this gene results in apoptosis of CLL, indicating its role in CLL survival [45]. Cyclin D1 is also highly up-regulated in CLL specimens and is up-regulated by the Wnt pathway that is active in CLL specimens [46]. Rasgrf-1 amplification was further analyzed, as it activates Ras and the Erk/MAPK pathway.

The function of Rasgrf-1 has been studied in other tumor models; in rat cells this gene cooperates with Ras to increase colony formation [47], and in the rhabdomyosarcoma model, inactivation of Rasgrf-1 results in inhibition of growth and metastatic potential of tumor cells [48]. In rhabdomyosarcoma cell lines, inactivation of Rasgrf-1 inhibits MAPK activity and the chemokine SDF-1 (CXCL12) is no longer able to increase the metastatic potential of cells. There is also evidence of its activation in response to G-protein coupled receptor signaling, and by signaling via receptor and non-receptor tyrosine kinases [26]. Interestingly the gene is imprinted in mice and humans, and this overexpression in CLL specimens could indicate a loss of imprinting process in leukemic cells [49]. Expression of a number of Rasgrf-1 transcripts and proteins has been described, including a 100 kDa, 75 kDa, 64 kDa and 55 kDa [26,32,50], and the proteins retain the active C-terminus region that includes the CDC25 domain that is sufficient and necessary for Ras nucleotide exchange activity. The N-terminus domains of Rasgrf-1 are considered regulatory domains, as there is evidence that they control the catalytic activity of the C-terminus CDC25 domain [31,51].

Previous reports have identified a number of Ras regulatory proteins that play a role in lymphoid cell signaling [19–21]. Rasgrp-1 and Rasgrp-3 are GEFs that are expressed in T and B cells and have a role in T cell receptor and BCR signaling. These GEFs also have a CDC25 catalytic domain as well as the Ras exchange motif (REM) that is also observed in the Rasgrf-1 protein. Overexpression of Rasgrp-1 in Jurkat cells increases their sensitivity to TCR stimulation and results in increased interleukin-2 (IL-2) production [19,20]. Rasgrp-1 and Rasgrp-3 double knockout mice were found to be defective in BCR mediated Ras/Erk signaling, indicating their role in BCR signaling [21]. Signaling via the GEFs in B cells can also be pro-apoptotic, as was shown for Rasgrp and SOS, two GEFs involved in production of RasGTP [52]. In our study, Rasgrf-1 mediated Erk activation that has an anti-apoptotic effect in CLL specimens.

BCR signaling activates the ERK pathway, which is a critical survival signal in CLL as leukemic cells undergo apoptosis with this pathway inhibition. Inhibition of this pathway activation by flavopiridol and vitamin D analogs results in down-regulation of anti-apoptotic genes such as Bcl-2 and Mcl-1 [25,53] and apoptosis. Besides BCR mediated Erk activation, this pathway is also activated by chemokines such as CXCL12 and by co-culturing cells with stromal cells [11,14,54]. Inhibition of the ERK pathway with sorafenib, a MEK inhibitor, down-modulates the antiapoptotic gene Mcl-1 [14] and results in apoptosis. Our experiments showed that knockdown of Rasgrf-1 resulted in PARP and cleaved caspase expression in a number of primary CLL specimens, and indicates that blocking this pathway alone results in
apoptosis. The signaling pathways in CLL specimens requiring Ras activation would require the GEF family of proteins [55] to be active, and Rasgrf-1 is an example of such a GEF protein that maintains Ras in the active conformation, thereby activating the Ras/Erk pathway.

Activation of the Rasgrf-1 protein is known to be associated with its phosphorylation of serine/threonine residues [34,35]. Previous studies have shown that cyclic adenosine monophosphate (cAMP)-dependent protein kinase is involved in this phosphorylation of residues that cluster around the C-terminus REM [35]. To investigate whether BCR signaling could itself phosphorylate Rasgrf-1 and further activate its GEF function its phosphorylation status was assessed. The study shows that BCR cross-linking results in phosphorylation of Rasgrf-1 at serine 929 at the C-terminus region of the protein. A similar example of phosphorylation and activation of a GEF is activation of the proto-oncogene product Vav [56]. Vav is phosphorylated by Lck that in turn catalyzes GDP/GTP exchange on Rac-1. This phosphorylation/activation of Rasgrf-1 by BCR signaling further enhances its GEF activity and increases downstream signaling via the Erk pathway.

BCR signaling is triggered by phosphorylation of the immunoreceptor tyrosine-based activation motifs (ITAMs) that results in recruitment of Src, Lyn, Syk and Lck and their association with adaptor molecules (BLNK) and other kinases such as BTK [1,2] (Figure 6). This complex of proteins is also called a signalosome, and activates a number of downstream signaling pathways in B cells and CLL specimens. As Src and BTK are early events in BCR signaling, they were investigated as potential effectors of Rasgrf-1 phosphorylation/activation. Also, the non-receptor tyrosine kinase Src is known to phosphorylate Rasgrf-1 in other model systems [57]. Inhibition of these kinases by dasatinib (Src inhibitor) and ibrutinib (BTK inhibitor) blocked Rasgrf-1 phosphorylation, suggesting that these kinases function upstream of Rasgrf-1 and directly or indirectly phosphorylate and activate Rasgrf-1, which further increases its GEF activity. In our study BTK and Src inhibitors were more effective in inhibiting ERK signaling as compared to Rasgrf-1 knockdown. This could be due to a number of reasons, including incomplete knockdown of Rasgrf-1 in all the cells and the presence of other GEF proteins in CLL cells with a similar function. The Src and BTK inhibitors have cytotoxic activity in this leukemia, and inhibit a number of BCR activated pathways including Erk activation [36,38], and are currently in clinical trials as well.

The schematic in Figure 6 outlines the major signaling pathways (PI3K, PKC and nuclear factor kB [NFkB]) that are activated by formation of the CLL signalosome. The novel GEF protein Rasgrf-1 plays a role in activation of the Erk pathway by increasing the formation of active Ras. Inactivation of this GEF with subsequent apoptosis in CLL specimens points toward a vital role of Rasgrf-1 in CLL biology. Interestingly, Ras mutations have not been reported in CLL specimens [58]; however, increased activity of this GEF protein can result in a similar downstream effect. Rasgrf-1 function can be inhibited by Src and BTK inhibitors in CLL specimens as they inhibit the functioning of the signalosome upstream of Rasgrf-1. Ras–Rasgrf-1 interaction is a potential therapeutic target, and inhibitors of this interaction can be developed to inhibit the active Erk pathway in CLL and in other tumor models.

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Potential conflict of interest: Disclosure forms provided by the authors are available with the full text of this article at www.informahealthcare.com/lal.

References


**Supplementary material available online**

Supplementary Table showing top 20 over expressed genes