A MAPK-positive Feedback Mechanism for BLR1 Signaling Propels Retinoic Acid-triggered Differentiation and Cell Cycle Arrest

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Jianrong Wang and Andrew Yen

From the Department of Biomedical Sciences, Cornell University, Ithaca, New York 14853

MAPK signaling is required for retinoic acid (RA)-triggered G0 cell cycle arrest and cell differentiation, but the mechanism is not well defined. In this study, RA is found to cause MAPK activation with sustained association of RAF to MEK or ERK, leading to a MAPK-dependent accumulation of p21Waf1/Cip1 and binding to CDK2 blocking G1/S transition. BLR1, a chemokine receptor, was found to function as a critical component of RA-triggered MAPK signaling. Unlike wild-type parental cells, RA-treated cells failed to show RAF and consequential MEK and ERK phosphorylation, failed to accumulate CDK inhibitors that control G1/S transition, and failed to differentiate and arrest in response to RA, whereas ectopically overexpressing BLR1 enhanced MAPK signaling and caused accelerated RA-induced differentiation and arrest. Ectopic overexpression of RAF enhanced BLR1 expression in response to RA, whereas inhibition of RAF or MEK by inhibitors or knockdown of RAF by short interfering RNA diminished RA-induced BLR1 expression and attenuated differentiation and growth arrest. Ectopic expression of the RAF CR3, the catalytically active domain, in the BLR1 knock-out restored RA-induced MAPK activation and the ability to differentiate and arrest, indicating that RAF effects MAPK signaling by BLR1 to propel differentiation/arrest. Taken together, RA induces cell differentiation and growth arrest through activation of a novel MAPK pathway with BLR1 as a critical component in a positive feedback mechanism that may contribute to the prolonged MAPK signaling propelling RA-induced cell cycle arrest and differentiation.

MAPK2 signaling was historically found as the effector of peptide growth factor–induced mitogenesis through the classical RAF/MEK/ERK axis. MAPK signaling has now been implicated in a wide variety of processes. Enigmatically it may also cause cell growth arrest and differentiation. How the signal is evoked to cause arrest and differentiation in contrast to mitogenesis is not well defined. Retinoic acid is a form of vitamin A that has known roles governing cell growth and differentiation in embryogenesis, nutrition, and chemotherapy of cancer. It can cause leukemic cell growth arrest and differentiation in a process that depends on sustained MAPK signaling (1–3), but the origin and regulation of that signaling are not well understood. It has thus been an elusive goal to find a paradigm that might rationalize how retinoic acid gives rise to the sustained MAPK signal that is needed to propel arrest and differentiation or a potential receptor that is seminal for that signal.

BLR1, also known as CXCR5, is a putative serpentine heterotrimeric G protein–coupled CXC chemokine receptor. BLR1 was first found in a differential screen comparing malignant Burkitt lymphoma cells against Epstein-Barr virus-transformed normal B lymphocytes (4). It was thus a potential determinant of the malignant phenotype of Burkitt lymphoma. The sequence is highly related to the interleukin-8 receptor. In humans BLR1 expression was originally found restricted to mature resting B cells and a subset of T-helper memory cells (5, 6). The pathology of BLR1 knock-out mice suggested a role for BLR1 in lymphocyte migration and activation (7). However, its function is still unclear, and a clear demonstration of its ability to regulate cellular proliferation or differentiation remained elusive. Although originally implicated as a determinant of B cell malignancy, its cellular functions have not been elucidated. Expression of the BLR1 is induced by retinoic acid, and there is evidence such receptors can utilize MAPK signaling (8, 9). BLR1 may thus be of relevance to the mechanism of action of RA.

As a known regulator of cell differentiation and proliferation, RA regulates BLR1 transcription through a novel retinoic acid–response element (RARE) that differs from the canonical AGGTCA half-sites. The RARE is a 17-bp 5′-element containing two GT boxes located ~1 kb upstream of the transcriptional start (10). Ligand-dependent binding of RET and retinoid X receptor to this RARE precipitates assembly of a complex containing Ret1, NFATc3, and CREB2 bound to their cognate sites 3′ of the GT box element. After recruitment of these tethered downstream transcription factors into the complex, CREB2 dissociates, leaving a transcriptionally active complex. The RARE in the BLR1 promoter suggests that it may have a role in mediating the effects of retinoic acid on cell differentiation and the cell cycle.

Treating HL-60 human myeloblastic leukemia cells with RA causes early expression of BLR1, enhanced ERK activation in...
the MAPK signaling pathway, and subsequently terminal cell differentiation along the myeloid lineage and G0 cell cycle arrest (8, 9). In contrast to classical MAPK signaling in response to peptide growth factors where a transient MAPK signal leads to mitogenesis, MAPK signaling here is prolonged and leads to differentiation and G0 arrest (1–3, 11). Inhibiting MAPK signaling blocks RA-induced differentiation indicating that it is necessary for RA to induce differentiation and arrest. This is consistent with the paradigm that although a short MAPK signal is mitogenic, a prolonged MAPK signal leads to differentiation and/or growth arrest as proposed earlier by Cohen and co-workers (12) in studies on the action of nerve growth factor. This paradigm has been validated in RA-treated HL-60 cells. Significantly, the question of how the MAPK signal originates or is sustained in response to RA is still outstanding. The relatively slower onset of MAPK activation in response to RA suggests a dependence on gene expression. Ectopic expression of BLR1 in stable transfectants resulted in enhanced ERK activation compared with parental HL-60, suggesting BLR1 may be a candidate RA-targeted gene important to RA-induced MAPK activation (8, 9). The BLR1 stable transfectants underwent accelerated terminal myeloid differentiation and G0 arrest in response to RA, further suggesting a functional consequence of RA-induced BLR1 expression consistent with the known role of MAPK in propelling RA-induced cell differentiation and G0 arrest.

BLR1 thus emerges as an attractive candidate for mediating the cell differentiation and cell cycle arrest attributed to RA. To test this, a BLR1 knock-out of HL-60 cells was created in the presently reported studies. Treating these cells with RA would thus elicit all effects except those dependent on BLR1 expression. Because WT HL-60 cells do not express BLR1 until treated with RA, the untreated knock-out would suffer no deleterious or potentially unanticipated effects because of loss of BLR1. RA failed to induce RAF, MEK, or ERK activation in the knock-out cells. Unlike WT cells, RA also failed to increase expression of p21^{Waf1/Cip1} and its binding to CDK2, and the cells failed to cell cycle arrest or terminally differentiate. Ectopic expression of the RAF CR3 domain, which is the catalytically active kinase domain devoid of the N-terminal negative regulatory regions, in the BLR1 knock-out cells restored ERK activation and rescued their ability to undergo differentiation and G0 arrest in response to retinoic acid. Overexpression of RAF in WT cells enhanced RA-induced BLR1 expression, whereas down-regulation of RAF attenuated BLR1 expression consistent with MAPK-positive feedback regulation of BLR1 expression. Inhibiting RAF or MEK inhibited RA-induced ERK activation and BLR1 expression, indicating that transcriptional activation of BLR1 depended initially on basal levels of MAPK signaling. Because MAPK signaling was enhanced by RA-induced BLR1 expression, a positive feedback loop could thus propel BLR1 expression and amplify MAPK signaling, offering a potential mechanism of prolonged BLR1 signaling that contributes to the prolonged MAPK signal needed to elicit cell differentiation and arrest in response to RA. Consistent with such a loop, inhibiting MEK was found to inhibit RAF activation. The data thus demonstrate a function for BLR1 and its attendant RAF signal in propulsion of RA-induced cell differentiation and G0 cell cycle arrest through a positive feedback loop using the RAF/MEK/ERK MAPK signaling pathway driven by BLR1. This feedback loop amplifies basal levels of MAPK signaling to promote BLR1 expression and propel differentiation and arrest. This establishes a new function for BLR1 and a clear demonstration of a role in regulating cell differentiation and the cell cycle.

**EXPERIMENTAL PROCEDURES**

**Reagents**—Antibodies against ERK1/2 and phospho-ERK1/2, MEK1/2 and phospho-MEK1/2, RAF1 and phospho-RAF1(Ser-259), CDK2, p21^{Waf1/Cip1}, and β-actin were purchased from Cell Signaling (Beverly, MA); the antibody against phospho-RAF1(Ser-621) was from BIOSOURCE/Invitrogen; allophyocyanin-conjugated anti-CD11b was from BD Biosciences; retinoic acid, Ras inhibitor farnesyltransferase inhibitor 277 (FTI-277), RAF inhibitor GW5074, MEK inhibitor PD98059, 12-O-tetradecanoylphorbol-13-acetate (TPA) were from Sigma; DCB reagent 5-(and-6)-chloromethyl-2′,7′- diarylchlorodihydrofluorescein diacetate, acetyl ester, and G418 were from Invitrogen; radioactive materials were from PerkinElmer Life Sciences; PCR primers were from Operon (Huntsville, AL); mutagenesis kit was from Stratagene (La Jolla, CA); PCR kit was from Clontech. ProFound co-immunoprecipitation kits were purchased from Pierce. siRNAs was ordered from Qiagen (Valencia, CA).

**Cell Culture**—HL-60 human myeloblastic leukemia cells and their derivatives, including wild-type, BLR1 knock-outs, BLR1 stable transfectants, RAF1 stable transfectants, and RAF1/CR3 stable transfectants, were cultured in RPMI 1640 medium supplemented with 5% heat-inactivated fetal bovine serum (Invitrogen) in a 5% CO2 humidified atmosphere at 37 °C and maintained in continuous exponential growth. Experimental cultures were initiated by reculturing exponentially growing cells at a density of 0.2 × 10^6 cells/ml and sampled at the indicated times for different analyses as described.

**RT-PCR and Northern Analysis**—Total RNA was extracted from HL-60 cells by using a RNA-Beet kit (Tel-test, Friendswood, TX) according to the manufacturer’s protocol. RT-PCR for cloning of BLR1 ORF or RAF1 ORF or RAF1 CR3 coding sequences was performed with TITANIUM One-step RT-PCR kit with a pair of primers at 5′ or 3′ of the BLR1 cDNA sequence 5′CTGGGAGCTGGGACAGATTGGACAAC3′ and 5′GAACGTGGTGGGAAGGTTGCC3′. The RT-PCR amplified coding sequences for BLR1 exon 2 was used as a probe for Northern detection. The protocols for RT-PCR and Northern analysis were described previously (10).

**Generation of BLR1 Knock-out Cell Line**—To generate a BLR1 knock-out cell line, a human genomic BLR1 fragment was cloned from HL-60 cells by PCR. The BLR1 targeting vector includes a total of 5571 bp of genomic sequence, EGFP gene, neomycin resistance gene (Neo), and cytomegalovirus promoter-driven herpes simplex virus thymidine kinase gene (CMV-TK). The BLR1 gene consists of two exons encoding a 372-amino acid protein. The first exon encodes only 17 amino acids, and separated by a 10-kb intron, the second exon encodes the majority of the protein, including most known functional domains and all three putative phosphorylation sites. Homologous recombination between the targeting vector and the wild-type BLR1 allele results in deletion of a 148-bp region of the
BLR1 gene in exon 2 and replacement of this sequence with CMV-EGFP gene followed by neomycin (thymidine kinase promoter directed). After RA treatment, the disrupted BLR1 gene is expected to yield a 1.9-kb BLR1-EGFP fusion mRNA molecule that contains only 189 bp representing the N-terminal 63 amino acids of BLR1 and then EGFP. In the disrupted BLR1 gene reading frame, the thymidine kinase promoter-directed Neo open reading frame has a stop codon followed by a poly(A) signal, so sequences downstream of Neo will no longer be transcribed. As a result, in BLR1 knock-out cells (BLR1−) the full-length BLR1 protein of 372 amino acids is lost, and expression of only 63 amino acids from exon 1 (17 amino acids) and 5′ exon 2 (46 amino acids) occurs under RA induction. Sall-linearized targeting vector was electroporated into HL-60 cells, and clones were selected in 1 mg/ml G418 and 2 μM gancyclovir for 3 weeks. Serial dilution under the same selection pressure was used to derive clones for screening. The clones were examined by PCR using a forward primer at Neo and the reverse primer at BLR1 exon 2. Eight positive clones contained integration of the homologously recombined targeting vector. To get a biallelic knock-out, the positive clones underwent selection with a higher concentration of G418 (2 mg/ml) while continuing 2 μM gancyclovir for another month. Southern analysis of these positive clones using a 1250-bp probe spanning BLR1 exon 2 and its flanking sequence of genomic DNA after a BamHI and HindIII site was described. As a result, in Neo open reading frame has a stop codon followed by a poly(A) gene reading frame, the thymidine kinase promoter-directed Neo gene expression is expected to yield a 1.9-kb BLR1-Neo-ORF-EGFP fusion mRNA (data not shown). 2) Raf1 stable transfectants were generated using the pEIE vector. The Raf1 open reading frame was amplified by RT-PCR from HL-60 cells with a pair of primers, forward primer 5′ GGATCCATGGAGCACAGCCGGC3′ and reverse primer 5′ GGATCCCTAGAAGACAGGAGGCCAGGCGG3′. The Raf1 ORF was then cloned into the BamHI site of the pEIE vector, and the proper orientation of the insert was confirmed by restriction analysis and sequencing. After transfection by DEAE-dextran added electroporation, Raf1 transfectant cells were selected with G418 selection for 3 weeks and further sorted by cytometry. The enhanced Raf1 expression was then confirmed by Western analysis. 3) Raf1 CR3 stable transfectants were generated using a pIRESpuro3 vector (Clontech). The cDNA for the kinase domain (CR3) of Raf1 with an added ATG translation start codon was isolated by RT-PCR from total RNA extracted from HL-60 cells with a pair of primers, forward primer 5′ GGGAATATGTCAAGCGGAAAACCCCC3′ and reverse primer 5′ GGATCCCTAGAAGACAGGAGGCCAGGCGG3′. The amplified fragment was then cloned into EcoRI and BamHI sites of the pIRESpuro3 vector, which contains a puromycin marker after the internal ribosome entry site. This allowed selection of CR3-transfected BLR1− cells, which already expressed Neo and EGFP. The Raf1 CR3 DNA construct was sequenced and transfected into BLR1 knock-out cells by DEAE-dextran-aided electroporation and was subject to selection with puromycin (10 ng/μl) for 4 weeks. Western analysis of the selected cells confirmed expression of the CR3 domain protein. Similarly, ectopic expression of BLR1 in BLR1 knock-outs was achieved by constructing plasmid pIRESpuro3-BLR1 (BamHI is the site where BLR1 ORF was cloned in pIRESpuro3) and stably transfecting pIRESpuro3-BLR1 into BLR1 knock-outs, followed by the above same selection with puromycin.

**siRNA Interference—**Human siRNAs to specifically interfere with Raf1 expression were designed based on siRNA design software ordered from Qiagen (Valencia, CA), siRNA sequence 1, 5′ AAGGCCACCAGGCCACCTTTT3′ (sense strand), and siRNA sequence 2, 5′ AAGGCCACGCCTTGTGAGAT3′ (sense strand). The two siRNAs both targeting human RAf gene were pooled at equal molarity with a final concentration for each siRNA of 20 nM. For each treatment, 5 million HL-60 cells were transfected with a total of 40 nM of siRNAs using Nucleofector solution (Amaxa, Gaithersburg, MD) using a BioRad Gene Pulser. RA was added immediately after transfection for the designated times for Western or cytometric analysis.  

**Co-immunoprecipitation—**50 μg of antibody against each bait protein was immobilized in coupling gel, and each 50 μg of cell lysates prepared from different cell lines with or without 1 μM of RA treatment was incubated with the antibody-immobilized coupling gel using a ProFound co-immunoprecipitation
and incubated at 37 °C, 5% CO2 for 20 min. Samples were analyzed using an LSRII flow cytometer (BD Biosciences). The threshold to exceed for positive staining was set so that 95% of control untreated cells were negative.

**Cell Growth Arrest Assay**—The distribution of cells in the cell cycle was measured by flow cytometric analysis of hypotonic propidium iodide-stained nuclei to determine the percentage of cells with G1/0 DNA content as described previously (1, 16).

**RESULTS AND DISCUSSION**

RA Induces BLR1 Expression, Prolonged RAF/MEK/ERK Activation, and Increased p21Waf1/Cip1 Expression and Its Binding to CDK2—To assess how loss of BLR1 affects RA-induced MAPK signaling and subsequent changes in the expression and interactions of cyclin-dependent kinases (CDKs) and their inhibitors, the RA-induced responses were first characterized in WT HL-60. As reported previously (8, 9), RA induces G0 cell cycle arrest and functional differentiation, evidenced by inducible oxidative metabolism characteristic of terminally mature myeloid cells. Onset is apparent at about 48 h, and most cells are differentiated and arrested after 72 h of RA treatment. As shown in Fig. 1A, RA induced BLR1 expression with low levels detectable after ~9 h, progressively increasing thereafter, peaking at ~48 h, and still persisting at 72 h before declining. Western blots (Fig. 1E) showed that although requiring hours to become detectable, RA significantly enhanced phosphorylation of RAF (Ser-621) and induced phosphorylation of MEK (Ser-217/221) and ERK (Thr-202/Tyr-204). Cells not treated with RA had no detectable changes in phosphorylation of these three kinases (data not shown; see also Fig. 6 for ERK activation in untreated control versus RA). Interestingly, RA also induced an increase in total MEK expression that roughly coincided with when MEK was phosphorylated, suggesting RA could regulate MEK phosphorylation and/or expression levels in HL-60 cells (Fig. 1B).

The RA-induced phosphorylated MEK and phosphorylated ERK co-immunoprecipitated with phosphorylated RAF(Ser-621) and induced phosphorylation of MEK (Ser-217/221) and ERK (Thr-202/Tyr-204). Cells not treated with RA had no detectable changes in phosphorylation of these three kinases (data not shown; see also Fig. 6 for ERK activation in untreated control versus RA). Interestingly, RA also induced an increase in total MEK expression that roughly coincided with when MEK was phosphorylated, suggesting RA could regulate MEK phosphorylation and/or expression levels in HL-60 cells (Fig. 1B).

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**Differentiation Assays**—1) For DCF assay, one-half million cells were collected by centrifugation and resuspended in 500 μl of phosphate-buffered saline. 1 μl of TPA and 1 μl of DCF, 5-(and-6)-chloromethyl-2',7'-dichlorodihydrofluorescein diacetate, acetyl ester (Invitrogen), were added to each sample and incubated at 37 °C, 5% CO2 for 20 min. Samples were analyzed by flow cytometry as described previously (15). 2) For NBT assay, the percentage of cells capable of inducible oxidative metabolism, a functional marker for mature myelomonocytic cells, was measured as the percentage of cells capable of reducing NBT to intracellular formazan because of superoxide as described previously (16, 17). Cells were harvested and scored by microscopy for formazan. 3) For CD11b staining, the percentage of cells expressing the myelomonocytic cell surface differentiation-specific marker CD11b was measured by immunofluorescence using allophycocyanin-conjugated CD11b antibody and flow cytometry. One-half million cells were collected by centrifugation and resuspended in 200 μl of phosphate-buffered saline. 5 μl of allophycocyanin-conjugated CD11b antibody was added, and the sample was incubated at 37 °C, 5% CO2 for 1 h. Samples were analyzed using an LSRII flow cytometer (BD Biosciences). The threshold to exceed for positive staining was set so that 95% of control untreated cells were negative.
RA induced transcriptional activation of p21Waf1/Cip1 (23). Nei-
by a Southern blot of HindIII-digested genomic DNA using a probe against the region shown by the
under the mutant allele. Positive clones produced an expected PCR-amplified band of 757 bp.
undergoing differentiation and growth arrest. WT HL-60,
transcriptional start running to the stop and poly(A) of the EGFP.
confirmed by Northern analysis. The 1.9-kb transcript detected in successful recombinants with an exon 2 probe represents the sequence from the
wild-type allele. The successful homologous recombinant clones show two expected bands of 3561 and 668 bp; the wild-type clones show one band of 3877
bp, and the heterozygous clones show three expected bands of 3877, 3561, and 668 bp. D, Northern analysis. Successful homologous recombination was
confirmed by Northern analysis. The 1.9-kb transcript detected in successful recombinants with an exon 2 probe represents the sequence from the
BLR1 locus indicated by the thin black line. B, PCR screening. Integration of the targeting vector was identified by PCR with the primers shown by arrows (Fig. 2A)
under the mutant allele. Positive clones produced an expected PCR-amplified band of 757 bp. C, Southern analysis. Homologous recombination was identified
by a Southern blot of HindIII-digested genomic DNA using a probe against the region shown by the thin black line across exon 2 of the
BLR1 gene in the wild-type allele. The successful homologous recombinant clones show two expected bands of 3561 and 668 bp; the wild-type clones show one band of 3877
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confirmed by Northern analysis. The 1.9-kb transcript detected in successful recombinants with an exon 2 probe represents the sequence from the
BLR1 transcriptional start running to the stop and poly(A) of the EGFP. E, ectopic expression of BLR1 in BLR1 knock-outs restores response to RA and capability of
undergoing differentiation and growth arrest. WT HL-60, BLR1−, and BLR1/BLR1+ cells were treated with or without RA for the designated time. Ectopic
expression of BLR1 in the BLR1 knock-outs restores CD11b expression, production of TPA-inducible reactive oxygen species, and G0 growth arrest in response
to RA. Data represent the means ± S.D. for three replicate experiments (*, p < 0.05 versus control for the same designated time; **, p < 0.01 versus control).

FIGURE 2. Generation of BLR1 knock-out HL-60 cells. A, schematic representation of gene targeting in the BLR1 locus by homologous recombination. HL-60
cells were transfected with a vector containing a green fluorescent protein cassette and a neomycin resistance cassette for positive selection. Negative
selection of cells harboring randomly integrated vector was done by gancyclovir sensitivity conferred by a thymidine kinase gene. Black boxes indicate coding
DNA; H indicates a HindIII site; RARE denotes retinoic acid-response element. Probes for Southern and Northern analyses are directed at regions of the wild-type
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Figure 2A shows a schematic diagram of gene targeting in the BLR1 locus by homologous recombination. HL-60 cells were transfected with a vector containing a green fluorescent protein cassette and a neomycin resistance cassette for positive selection. Negative selection of cells harboring randomly integrated vector was done by gancyclovir sensitivity conferred by a thymidine kinase gene. Black boxes indicate coding DNA; H indicates a HindIII site; RARE denotes retinoic acid-response element. Probes for Southern and Northern analyses are directed at regions of the wild-type locus indicated by the thin black line. B, PCR screening. Integration of the targeting vector was identified by PCR with the primers shown by arrows (Fig. 2A) under the mutant allele. Positive clones produced an expected PCR-amplified band of 757 bp. C, Southern analysis. Homologous recombination was identified by a Southern blot of HindIII-digested genomic DNA using a probe against the region shown by the thin black line across exon 2 of the BLR1 gene in the wild-type allele. The successful homologous recombinant clones show two expected bands of 3561 and 668 bp; the wild-type clones show one band of 3877 bp, and the heterozygous clones show three expected bands of 3877, 3561, and 668 bp. D, Northern analysis. Successful homologous recombination was confirmed by Northern analysis. The 1.9-kb transcript detected in successful recombinants with an exon 2 probe represents the sequence from the BLR1 transcriptional start running to the stop and poly(A) of the EGFP. E, ectopic expression of BLR1 in the BLR1 knock-outs restores response to RA and capability of undergoing differentiation and growth arrest. WT HL-60, BLR1−, and BLR1/BLR1+ cells were treated with or without RA for the designated time. Ectopic expression of BLR1 in the BLR1 knock-outs restores CD11b expression, production of TPA-inducible reactive oxygen species, and G0 growth arrest in response to RA. Data represent the means ± S.D. for three replicate experiments (*, p < 0.05 versus control for the same designated time; **, p < 0.01 versus control).
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**FIGURE 3. Disruption of BLR1 causes loss of RA-induced MAPK activation, p21\(^{Waf1/Cip1}\) expression, and its subsequent binding to CDK2.**

A. Western blot of phospho-RAF (Ser-621) in BLR1− cells treated with 1 μM of RA for the indicated times. β-Actin was used as a lane loading control. B. Western blot of phospho-RAF (Ser-621) in WT HL-60, BLR1−, and BLR1+ cells were treated with 1 μM of RA for 12 h. Western analysis of the cell lysates was performed with antibodies against phospho-RAF, MEK1/2, MEK2/1, and ERK1/2 in response to RA. WT HL-60, BLR1−, and BLR1+ cells were treated with 1 μM of RA for 12 h. Western analysis of the cell lysates was performed with antibodies against phospho-RAF, MEK1/2, MEK2/1, and ERK1/2. C. Knock-out of BLR1 causes loss of RA-induced MAPK activation, p21\(^{Waf1/Cip1}\) expression, and its subsequent binding to CDK2, as reported previously (8, 9). D. Knock-out of BLR1 blocks RA-induced p21\(^{Waf1/Cip1}\) expression. Western blots (top to bottom) of CDK2, p21\(^{Waf1/Cip1}\), and β-actin loading control in untreated control or RA-treated WT HL-60 and BLR1− cells. E. Knock-out of BLR1 obviates p21\(^{Waf1/Cip1}\) binding to CDK2 seen by immunoprecipitation using CDK2 as bait. Western blot of p21\(^{Waf1/Cip1}\) for untreated control and RA-treated WT HL-60 and BLR1− cells. F. Growth of WT HL-60, BLR1−, and BLR1+ cells treated for 0, 24, 48, and 72 h with 1 μM RA.

homologous recombination (Fig. 2A); the stably transfected clones isolated by serial dilution screened by PCR using primers detecting insertion by homologous recombination (Fig. 2B); the bi-allelic recombinants selected by intensified G418 selection (2 mg/ml) in the presence of gancyclovir identified by Southern blotting (Fig. 2C); as well as loss of the BLR1 transcript and expression of the disrupted BLR1 transcript in homozygous recombination clones verified by Northern analysis (Fig. 2D). Flow cytometry was used to confirm EGFP expression in all of the cells thereby verifying BLR1 disruption. The bi-allelic knock-out cells, hence called BLR1−, had no discernible differences in growth rate or cell cycle distribution compared with wild type cells, as expected because BLR1 is not expressed in untreated HL-60 cells (data not shown).

To test if the BLR1 knock-outs are functional for RA response and to further confirm the role of BLR1 in RA-induced cell growth arrest and differentiation, ectopic expression of BLR1 in the BLR1 knock-outs was achieved by stable transfection. The results show that ectopic expression of BLR1 in the BLR1 knock-outs restored the expression of the differentiation marker CD11b (Fig. 2E, left panel), TPA-inducible production of reactive oxygen species (Fig. 2E, middle panel), and Go growth arrest (Fig. 2E, right panel) in response to RA.

To determine how loss of BLR1 affected RA-induced MAPK signaling and subsequent p21\(^{Waf1/Cip1}\) binding to CDK2, BLR1− cells were treated with RA and the consequential MAPK signaling and p21\(^{Waf1/Cip1}\) binding to CDK2 were analyzed by Western blotting and co-immunoprecipitation. RA induced a transient phosphorylation of RAF1 at Ser-621 in BLR1 knock-outs but not the strong persistent response in WT HL-60. Western blotting using a longer development to bring out a weaker signal showed a transient phosphorylation of RAF1 at Ser-621 that peaked at ~6 h and decayed away afterward (Fig. 3A). The same cells without RA showed no change in their low level basal RA (Ser-621) phosphorylation. The Ser-259, Ser-296, Ser-338, Ser-340, Ser-494, Tyr-341, and Thr-491 phosphorylation sites of RAF1 in the knock-out did not show any detectable change in phosphorylation with or without RA during this time course as was the case for WT HL-60 cells (data not shown). Measur
loss of RA-induced enhanced p21\textsuperscript{Waf1/Cip1} expression, as shown by Western blotting (Fig. 3D). RA-induced p21\textsuperscript{Waf1/Cip1} binding to CDK2 detectable in immunoprecipitates from WT HL-60 was also lost in BLR1- cells (Fig. 3E). Consistent with this, RA-treated BLR1- cells failed to retard growth compared with BLR1+ positive cells (Fig. 3F). Thus knocking out BLR1 to preclude its RA-induced expression resulted in loss of a durable RAF/MEK/ERK signal and subsequent failure to induce expression of p21\textsuperscript{Waf1/Cip1} and its complex with CDK2.

To determine the effect of knocking out BLR1 and its attendant protracted MAPK signal on RA-induced differentiation, BLR1- cells, WT HL-60, and BLR1+ cells were treated with RA and analyzed for induced differentiation. Differentiation was assayed by expression of CD11b, a cell surface marker, and also by inducible oxidative metabolism, a functional marker for terminal myeloid differentiation, using flow cytometry. RA induced expression of CD11b in WT HL-60, but not in BLR1- cells (Fig. 4B). Fig. 4A shows typical flow cytometric dual

FIGURE 4. Disruption of BLR1 causes failure of RA-induced differentiation and growth arrest. A, representative flow cytometric scatter plots of CD11b expression (vertical) versus EGFP (horizontal) verifying EGFP expression in transfectants for untreated control (Ctrl) or RA-treated cells. WT HL-60, BLR1- and BLR1+ cells were treated with or without 1 \(\mu\)M RA for 72 h, and expression of CD11b was determined by flow cytometry. B, CD11b expression in cells cultured for 72 h with (RA) or without (control) 1 \(\mu\)M RA. Expression of CD11b was impaired in BLR1- cells and enhanced in BLR1+ cells. The means and error bars from three repeats are shown. C, representative DCF fluorescence histograms for WT HL-60, BLR1- and BLR1+ cells treated (RA) or not (control) with 1 \(\mu\)M RA for 72 h. HL-60 and BLR1+ cells show a pronounced increase because of RA characterizing induced differentiation, but BLR1- cells do not. D, functional differentiation assayed by DCF staining for HL-60, BLR1-, and BLR1+ cells treated for 0, 24, 48 and 72 h with 1 \(\mu\)M RA. The means and error bars for three repeats are shown. Loss of BLR1 cripples induced differentiation (BLR1-), whereas overexpression enhances it (BLR1+). E, representative DNA histograms of WT HL-60, BLR1- and BLR1+ cells that were untreated control or RA treated for 72 h. BLR1- cells did not show an enrichment in the percentage of cells with G\textsubscript{1} DNA (G\textsubscript{1}%) by RA compared with untreated control cells, whereas ectopic expression of BLR1 enhanced the increased G\textsubscript{1}% by RA: WT HL-60 (73%) and BLR1+ cells (84%). F, percentage of WT HL-60, BLR1-, and BLR1+ cells in G\textsubscript{1} treated with 1 \(\mu\)M RA for 0, 24, 48, and 72 h. Loss of BLR1 caused failure to undergo G\textsubscript{0} arrest, whereas ectopic BLR1 expression enhanced G\textsubscript{0} arrest by RA (revealed by enrichment in the percentage of G\textsubscript{1} DNA cells). Data represent the means ± S.D. for three replicate experiments (*, \(p < 0.05\) versus control for the same designated time; **, \(p < 0.01\) versus control).
Novel MAPK Signaling Pathway through BLR1

Inhibiting RAF or MEK-inhibited MAPK activation and BLR1 expression and cell differentiation. A, inhibiting RAF1 or MEK diminishes ERK activation in HL-60 and BLR1 + cells. WT HL-60, BLR1 −, and BLR1 + cells were pretreated with RAF inhibitor FTI-277 (FTI, 3 μM), RAF1 inhibitor GW5074 (GW, 5 μM), or MEK inhibitor PD98059 (PD, 2 μM) for 16 h and re-treated with the same inhibitor at the same concentration with 1 μM RA treatment. C, control. None of the inhibitors as used inhibited cell growth or compromised cell viability using this previously described treatment protocol (1). The cells were harvested after 12 h of RA treatment for Western analysis of phospho-ERK1/2 or total ERK1/2. 10 μg of total protein was loaded per lane. GW5074 and PD98059, but not FTI-277, inhibited ERK activation after RA treatment. Total ERK1/2 was unaffected. RA-functional differentiation of HL-60 cells treated with designated inhibitors (untreated control, FTI-277, GW5074, and PD98059 measured after 0, 24, 48, 72, and 96 h of RA treatment by reduction of NBT because of inducible superoxide production. GW5074 and PD98059, but not FTI-277, inhibited differentiation in response to RA. The means and error bars from three repeats are shown. C, Western analysis of phospho-RAF1 (Ser-621) (top panel) and RAF1 in WT HL-60 (2nd panel), BLR1 − and BLR1 + cells that were treated (RA) or not (C) with RA for 24 h. Loss of BLR1 crippled RA-induced phosphorylation of RAF1 at Ser-621, whereas ectopic expression of BLR1 enhanced RAF1 phosphorylation compared with WT HL-60 cells. Total RAF1 was unaffected. β-Actin was a lane loading control. D, Northern analysis of RA-induced BLR1 expression in HL-60 cells treated with RA as in panel C. RA inhibited BLR1 expression in WT HL-60 cells. E, Western analysis of activated phospho-RAF1 for WT HL-60, BLR1 −, and BLR1 + cells treated with RA for 12 h in the absence (−) or presence (+) of the MEK inhibitor, PD98059 (PD). In WT HL-60 and BLR1 + cells, the MEK inhibitor blocked RA-induced phosphorylation of RAF1 at Ser-621. β-Actin was the lane loading control. RA induces BLR1 expression which causes enhanced RA phosphorylation at serine 621 with MEK and ERK activation, resulting in the prolonged MAPK signaling leading to accumulation of p21\textsuperscript{Waf1/Cip1} and its binding to CDK2. This suggests that BLR1 and its attendant RAF signaling through the MAPK pathway are critical to propulsion of cell differentiation and arrest in response to RA.

RAF Regulates BLR1 Expression, and Ectopic Expression of RAF Activity in BLR1 Knock-outs Rescues RA-induced MAPK Pathway Activation and Cell Differentiation and Arrest—To test the functional need for RAF in eliciting the differentiation in response to RA, the effect of inhibiting RAF on RA-induced cell differentiation was determined. WT HL-60 cells were treated with the RAF-specific inhibitor, GW5074, to inhibit RAF and then RA to try to induce differentiation. The GW5074 inhibited RA-induced ERK activation, indicating that it was effective as administered (Fig. 5A). Unlike control cells without histograms of DCF fluorescence). Loss of BLR1 thus crippled RA-induced differentiation in BLR1 − cells compared with HL-60 cells that do harbor BLR1.

To determine whether RA-induced cell cycle arrest also depended on BLR1, the distribution of WT HL-60, BLR1 −, and BLR1 + cells in the G1/S, S, and G2/M phases after RA treatment was measured by flow cytometry (Fig. 4, F and E shows typical DNA histograms for untreated control and RA-treated WT HL-60, BLR1 −, and BLR1 + cells). Consistent with the results for differentiation, whereas WT HL-60 cells arrested and accumulated in G1 after treatment with RA, the BLR1 − cells failed to undergo G0 arrest. Arrest was enhanced for BLR1 + cells, consistent with previous studies showing ectopic BLR1 expression enhanced induced differentiation and arrest (8, 9). The cell cycle data corroborate the earlier data (Fig. 3F) showing that the RA-treated BLR1 − cells continued to grow in contrast to the growth retardation of the WT HL-60 and BLR1 + cells in response to RA. As for RA-induced differentiation, loss of BLR1 prevented RA-induced cell cycle arrest and growth inhibition. BLR1 expression induced by RA was thus necessary for RA to cause RAF/MEK/ERK MAPK signaling and then cell differentiation and G0 cell cycle arrest.

These data suggest a paradigm where RA induces BLR1 expression which causes enhanced RA phosphorylation through the MAPK pathway, critical to propulsion of cell differentiation and arrest in response to RA.

FIGURE 5. Inhibiting RAF or MEK-inhibited MAPK activation and BLR1 expression and cell differentiation. A, inhibiting RAF1 or MEK diminishes ERK activation in HL-60 and BLR1 + cells. WT HL-60, BLR1 −, and BLR1 + cells were pretreated with RA inhibitor FTI-277 (FTI, 3 μM), RAF1 inhibitor GW5074 (GW, 5 μM), or MEK inhibitor PD98059 (PD, 2 μM) for 16 h and re-treated with the same inhibitor at the same concentration with 1 μM RA treatment. C, control. None of the inhibitors as used inhibited cell growth or compromised cell viability using this previously described treatment protocol (1). The cells were harvested after 12 h of RA treatment for Western analysis of phospho-ERK1/2 or total ERK1/2. 10 μg of total protein was loaded per lane. GW5074 and PD98059, but not FTI-277, inhibited ERK activation after RA treatment. Total ERK1/2 was unaffected. B, functional differentiation of HL-60 cells treated with designated inhibitors (untreated control, FTI-277, GW5074, and PD98059 measured after 0, 24, 48, 72, and 96 h of RA treatment by reduction of NBT because of inducible superoxide production. GW5074 and PD98059, but not FTI-277, inhibited differentiation in response to RA. The means and error bars from three repeats are shown. C, Western analysis of phospho-RAF1 (Ser-621) (top panel) and RAF1 in WT HL-60 (2nd panel), BLR1 − and BLR1 + cells that were treated (RA) or not (C) with RA for 24 h. Loss of BLR1 crippled RA-induced phosphorylation of RAF1 at Ser-621, whereas ectopic expression of BLR1 enhanced RAF1 phosphorylation compared with WT HL-60 cells. Total RAF1 was unaffected. β-Actin was a lane loading control. D, Northern analysis of RA-induced BLR1 expression in HL-60 cells treated with RA as in panel C. RA inhibited BLR1 expression in WT HL-60 cells. E, Western analysis of activated phospho-RAF1 for WT HL-60, BLR1 −, and BLR1 + cells treated with RA for 12 h in the absence (−) or presence (+) of the MEK inhibitor, PD98059 (PD). In WT HL-60 and BLR1 + cells, the MEK inhibitor blocked RA-induced phosphorylation of RAF1 at Ser-621. β-Actin was the lane loading control.

parameter plots of CD11b expression versus EGFP expression, which characterizes the transfected BLR1 − or BLR1 + cells, for untreated control and RA-treated cells. RA-induced expression was augmented in BLR1 + cells. The BLR1 − cells thus failed to express CD11b like the WT HL-60 in response to RA, whereas expression was enhanced in BLR1 + cells, indicating that BLR1 expression induced by RA drives expression of the CD11b cell surface differentiation marker. Differentiation was also assayed by a functional differentiation marker, inducible oxidative metabolism. Inducible reactive oxygen, superoxide, was measured by DCF (5-(and-6)-chloromethyl-2',7'-dichlorofluorescin) fluorescence using flow cytometry as described previously (15). Corroborating the results for CD11b, RA caused differentiation into cells capable of inducible oxidative metabolism in WT HL-60 but not BLR1 − cells (Fig. 4, D and C shows typical and ERK activation, resulting in the prolonged MAPK signaling leading to accumulation of p21\textsuperscript{Waf1/Cip1} and its binding to CDK2. This suggests that BLR1 and its attendant RAF signaling through the MAPK pathway are critical to propulsion of cell differentiation and arrest in response to RA.
inhibitor, which differentiated as expected, the GW5074-treated cells failed to terminally differentiate appropriately in response to RA (Fig. 5B). Differentiation was measured by inducible oxidative metabolism detected by a cytological assay using NBT reduction (1, 3). Inhibiting RAF also inhibited the otherwise enhanced ERK activation in BLR1+ cells, confirming that BLR1-driven ERK activation acts through RAF (Fig. 5A). RAF activity and its consequential ERK activation thus are needed for RA-induced differentiation. The loss of RAF signaling causing loss of ability to differentiate is consistent with the loss of RA-induced RAF phosphorylation in BLR1− cells and its enhancement in BLR1+ cells (Fig. 5C).

Allowing that MEK is a downstream activation target of RAF, then an obvious anticipation of this result is that inhibiting MEK would recapitulate similar results. Cells were ergo treated with the MEK-specific inhibitor, PD98059, in lieu of GW5074, and the same determinations were made. Inhibiting MEK inhibited RA-induced ERK activation, confirming that the PD98059 was effective as administered (Fig. 5A). The MEK-inhibited cells also failed to differentiate in response to RA as determined by the occurrence of inducible oxidative metabolism (Fig. 5B). Also, as for RAF inhibition, inhibiting MEK inhibited the BLR1-driven ERK activation in BLR1+ cells confirming the dependence of BLR1-driven ERK activation on RAF and MEK (Fig. 5A).

Although previous studies have indicated that RA does not induce RAS activation in HL-60 cells, the dependence of RA-induced differentiation on RAS was tested to confirm the anticipation that it would not affect RA-induced differentiation (24). The RAS inhibitor FTI-277 was used. It had no effect on RA-induced ERK activation in WT HL-60 or the BLR1+ cells where ERK activation is enhanced because of the ectopic BLR1 expression (Fig. 5A). It also as anticipated had no effect on RA-induced differentiation (Fig. 5B). Taken with previously reported results, the activation of MAPK signaling and propulsion of RA-induced differentiation attributed to BLR1 have no apparent RAS dependence. The apparent lack of RAS dependence for MAPK activation is consistent with other reports in different systems to that effect. For example calcium-induced activation of the RAF/MEK/ERK pathway leading to keratinocyte differentiation is independent of RAS activation (25). In other studies RAF has also been found to have activity other than through MEK and ERK, and in particular it can antagonize apoptosis signal-regulating kinase-1 through a MEK/ERK-independent mechanism, indicating the need to demonstrate the RAF/MEK/ERK signaling proposition explicitly for different cell contexts because it can vary (26). As used in the present assays, none of the inhibitors by themselves compromised cell growth or viability as described previously for this treatment protocol (1).

Interestingly RAF or MEK inhibition, also blocked RA-induced BLR1 expression (Fig. 5D). RA-induced BLR1 expression itself thus was propelled by MAPK signaling. Furthermore, inhibiting MEK inhibited RA-induced phosphorylation of RAF in WT HL-60 and partially in BLR1+ cells where there is ectopic BLR1 expression (Fig. 5E). The data may betray a positive feedback loop where BLR1 causes RAF-driven MEK and ERK activation which further drives (endogenous) BLR1 expression to sustain a feedback loop resulting in prolonged MAPK signaling. In this case inhibiting MEK, as was done in the present studies, would break the loop and cause loss of RAF phosphorylation at serine 621 as was observed.

To determine whether the pharmacological inhibitions would be corroborated with diminished RAF expression, siRNA knockdown of RAF1 was done. Western blotting (Fig. 6A) showed that RAF1 expression was effectively reduced by siRNA specific to RAF1. RA-induced BLR1 expression was diminished by siRNA knockdown of RAF, but not by control scrambled siRNA transfection, compared with WT HL-60 cells (Fig. 6A). Also corroborating the pharmacological results, the RAF1 knockdown cells both differentiated (Fig. 6B) and G0-arrested (Fig. 6C) slower than the WT or scrambled control siRNA transfectants. The knockdown results thus support the
pharmacological results and motivate the anticipation that overexpression of RAF would enhance RA-induced BLR1 expression. To test this, RAF1 stable transfectants were made. Western blotting (Fig. 7A) showed that the stable transfectants overexpressed RAF compared with the WT and vector control. The RAF1 transfectants had more phosphorylated RAF1 (Ser-621) also as a result of overexpression. RA-induced BLR1 expression was higher in the RAF1 stable transfectants than the WT or vector control cells, corroborating the anticipation. As expected, overexpression of RAF1 enhanced differentiation and growth arrest (data not shown). Hence knocking down RAF1 diminished RA-induced BLR1 expression, whereas overexpressing RAF1 enhanced RA-induced BLR1 expression. Because BLR1 signals through a MAPK cascade, there is an apparent positive feedback loop.

The indicated dependence of RA-induced differentiation on BLR1 and its RAF1 signaling motivates a test of whether ectopic expression of RAF1 activity in BLR1− cells can restore ERK activation and cell differentiation in response to RA. BLR1− cells were stably transfected with the RAF1 CR3 domain, which is the catalytically active kinase domain devoid of the N-terminal negative regulatory domains (27–30). Expression of the CR3 domain in BLR1− stable transfectants, hence called CR3/BLR1−, was verified by Western blotting (Fig. 8A). ERK activation in response to RA was restored in the CR3/BLR1− cells compared with parental BLR1− cells (Fig. 8B), indicating ERK signaling is functional in parent BLR1− cells. The Western blot shows that the resulting ERK activation was approximately comparable with WT HL-60 cells (Fig. 8B). The ectopic CR3 thus had the anticipated activity. With restoration of RA-induced MAPK signaling, RA-induced p21Waf1/Cip1 expression was also restored in CR3/BLR1− cells compared with parental BLR1− cells (Fig. 8B). Differentiation in response to RA was restored in CR3/BLR1− cells in contrast to parental BLR1− cells that fail to differentiate (Fig. 8C). Differentiation was measured by inducible oxidative metabolism using DCF fluorescence measured by flow cytometry. Ectopic expression of CR3 in BLR1− cells restored their ability to differentiate in response to RA. The means and error bars from three repeats are shown. D, percentage of cells with G0 DNA for WT HL-60, BLR1−, and CR3/BLR1− cells. WT HL-60, BLR1−, and CR3/BLR1− cells were treated with RA for 24, 48, and 72 h as detected by inducible oxidative metabolism measured by DCF fluorescence using flow cytometry. Stable expression of CR3 in BLR1− cells restored their ability to undergo G1 arrest. The means and error bars from three repeats are shown. D, percentage of cells with G0 DNA for WT HL-60, BLR1−, and CR3/BLR1− cells after 24, 48, and 72 h of RA treatment measured by flow cytometry. Ectopic expression of CR3 in BLR1− cells rescued the capability of the BLR1 knock-out cells to G0 arrest. The means and error bars from three repeats are shown.

The RAF1 transfectants had more phosphorylated RAF1 (Ser-621) also as a result of overexpression. RA-induced BLR1 expression detected by Northern blotting (Fig. 8D). Western blotting (Fig. 7) showed that the stable transfectants overexpressed RAF compared with the WT and vector control. The RAF1 transfectants had more phosphorylated RAF1 (Ser-621) also as a result of overexpression. RA-induced BLR1 expression was approximately comparable with WT HL-60 cells (Fig. 8D). The ectopic CR3 thus had the anticipated activity. With restoration of RA-induced MAPK signaling, RA-induced p21Waf1/Cip1 expression was also restored in CR3/BLR1− cells compared with parental BLR1− cells (Fig. 8B). Differentiation in response to RA was restored in CR3/BLR1− cells in contrast to parental BLR1− cells that fail to differentiate (Fig. 8C). Differentiation was measured by inducible oxidative metabolism using DCF fluorescence measured by flow cytometry. The kinetics and extent of differentiation closely mimicked those of WT HL-60 cells. Consistent with restoring RA-induced p21Waf1/Cip1 and the ability to differentiate, the CR3/BLR1− cells also regained the ability to undergo G1 arrest in response to RA (Fig. 8D). Ectopic expression of RAF activity in BLR1− cells thus restored ERK activation and rescued cell differentiation and G0 arrest in response to RA.
Interestingly, the MAPK signaling attributed to RA-induced BLR1 expression is associated with phosphorylation of RAF1 at Ser-621. Early reports showed that phosphorylation of Ser-621 negatively regulated the kinase activity of the isolated RAF1 kinase domain (31), regulated binding of 14-3-3 to RAF1, but that its phosphorylation did not change in response to certain stimuli (32). Other studies have shown that RAF1 can be activated in a 14-3-3- and Ras-independent manner, indicating that 14-3-3 binding is not necessarily a prerequisite for RAF1 activation (33). Later studies showed that in full-length RAF1 Ser-621 phosphorylation was a positive regulator of activation (34). It is thus evident that the function of Ser-621 is potentially complex and dependent on cell context. In different contexts it may function other than the now classical RAS/RAF/MEK/ERK cascade, and the complexity may depend on the nature of the signaling complex formed for a particular biological function. The availability of adaptor molecules specific to the context may thus be an important determinant of how RAF1 functions and the role of specific phosphorylation sites. The present results underscore this. For the case of RA-induced BLR1 expression-driven MAPK signaling, RAS is not necessary, and Ser-621 phosphorylation is associated with RAF1 binding to MEK and ERK and their activation. Furthermore, we observed that this was dependent on RA. Without RA, there was also no enhancement of MAPK signaling in BLR1 — cells expressing the RAF1 CR3 domain (data not shown), indicating the need for other RA-induced factors to create an active signaling complex and restore the cellular ability to growth arrest and differentiate. In this regard, we note that whereas we show that BLR1 signaling is a critical determinant of whether cells arrest and differentiate in response to RA, it is not the sole determinant. Blr1-transfected cells, although they have enhanced MAPK signaling, do not differentiate until treated with RA, indicating that although BLR1 and its RAF/MEK/ERK signaling are a critical component of the machinery induced by RA, it is not the only component. Other supporting pieces of the machinery relevant to BLR1 signaling and other cellular processes are clearly needed. It is, however, apparent that because overexpression of BLR1 accelerates RA-induced arrest and differentiation that availability of the receptor is rate-limiting. In contrast, the availability of agonistic ligand, which the serum-supplemented medium apparently provides in abundance, is not a limiting factor here. Interestingly, phosphorylation at other known RAF1 regulatory sites, in particular Ser-259, Ser-296, Ser-338, Ser-340, Ser-494, Tyr-341, and Thr-491, was only weakly detectable at best by Western blots and unaffected by RA (per 48 or 72 h of treatment) or BLR1 ectopic expression by stable transfection (data not shown), pointing to the potential significance of Ser-621 in forming the signaling complex important in the present cellular context. Supporting this, we found that administration of GW5076 blocked RA-induced RAF1 Ser-621 phosphorylation, ERK activation, and arrest and differentiation. The apparent importance of Ser-621 here is consistent with the earlier report (32) of its activating function. These findings motivate interest in the character of the signaling complex, and we are pursuing it, seeking in particular the identity of relevant adapters.

In summary, BLR1-stimulated RAF1, MEK, and ERK activation acts in a feedback loop to drive BLR1 expression that further stimulates MAPK signaling. Basal levels of MAPK signaling could thus be amplified with subsequent interaction between enhanced CDK inhibitors and CDK, leading to growth arrest and cell differentiation. The amplification occurs relatively slowly because of its dependence on transcription, and signaling is relatively protracted. BLR1 expression and its attendant RAF signaling thus perform a critical function in RA-induced cell differentiation and G0 arrest, providing a negative regulatory function on proliferation contrary to the premise of its discovery.

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REFERENCES

1. Yen, A., Roberson, M. S., Varvayanis, S., and Lee, A. T. (1998) Cancer Res. 58, 3163–3172
2. Yen, A., Sturgill, R., and Varvayanis, S. (2000) In Vitro Cell. Dev. Biol. 36, 249–255
3. Yen, A., Williams, M., Platko, J. D., Der, C., and Hisaka, M. (1994) Eur. J. Cell Biol. 65, 103–113
4. Dobner, T., Wolf, I., Emrich, T., and Lipp, M. (1992) Eur. J. Immunol. 22, 2795–2799
5. Forster, R., Emrich, T., Kremmer, E., and Lipp, M. (1995) Blood 84, 830–840
6. Kaiser, E., Forster, R., Wolf, I., Ebensperger, C., Kuehl, W. M., and Lipp, M. (1993) Eur. J. Immunol. 23, 2532–2539
7. Forster, R., Mattis, A. E., Kremmer, E., Wolf, I., Brem, G., and Lipp, M. (1996) Cell 87, 1037–1047
8. Battle, T. E., Levine, R. A., and Yen, A. (2000) Exp. Cell Res. 254, 287–298
9. Battle, T. E., Roberson, M. S., Zhang, T., Varvayanis, S., and Yen, A. (2001) Eur. J. Cell Biol. 80, 59–67
10. Wang, J., and Yen, A. (2004) Mol. Cell. Biol. 24, 2423–2443
11. Hong, H. Y., Varvayanis, S., and Yen, A. (2001) Differentiation 68, 55–66
12. Traverse, S., Gomez, N., Paterson, H., Marshall, C., and Cohen P. (1992) Biochem. J. 288, 351–355
13. Lamkin, T. J., Chin, V., Varvayanis, S., Smith, J. L., Sramkoski, R. M., Jacobberger, J. W., and Yen, A. (2006) J. Cell. Biol. 97, 1238–1338
14. Lamkin, T. J., Chin, V., and Yen, A. (2006) Annu. J. Hematol. 81, 603–615
15. Reiterer, G., and Yen, A. (2007) Cancer Res. 67, 7765–7772
16. Yen, A., Reece, S. L., and Albright, K. L. (1984) J. Cell. Physiol. 118, 277–286
17. Yen, A., Powers, V., and Fishbaugh, J. (1986) Leuk. Res. 10, 619–629
18. Aplin, A. E., Stewart, S. A., Assoian, R. K., and Juliano, R. L. (2001) J. Cell Biol. 153, 273–282
19. Chen, R. H., Sarnecki, C., and Blenis, J. (1992) Mol. Cell. Biol. 12, 915–927
20. Gille, H., Starrocks, A. D., and Shaw, P. E. (1992) Nature 358, 414–417
21. Lenormand, P., Sardet, C., Pages, G., L’Allemain, G., Brunet, A., and Pouyssegur, J. (1993) J. Cell Biol. 122, 1079–1088
22. Colch, W. (2000) Biochem. J. 351, 289–305
23. Tanaka, T., Suh, K. S., Lo, A. M., and De Luca, L. M. (2007) J. Biol. Chem. 282, 29987–29997
24. Katagiri, K., Hattori, S., Nakamura, S., Yamamoto, T., Yoshida, T., and Katagiri, T. (1994) Blood 84, 1780–1789
25. Schmidt, M., Goebeler, M., Posern, G., Feller, S. M., Seitz, C. S., Brocker, E. B., Rapp, U. R., and Ludwig, S. (2000) J. Biol. Chem. 275, 41011–41017
26. Chen, J., Fuji, K., Zhang, L., Roberts, T., and Fu, H. (2001) Proc. Natl. Acad. Sci. U. S. A. 98, 7783–7788
27. Cutler, R. E., Jr., Stephens, R. M., Saracino, M. R., and Morrison, D. K. (1998) Proc. Natl. Acad. Sci. U. S. A. 95, 9214–9219
28. Daum, G., Eisenmann-Tappe, I., Fries, H. W., Tropfsmair, J., and Rapp, U. R. (1994) Trends Biol. Sci. 19, 474–479
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