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Retinoic acid induces nuclear accumulation of Raf1 during differentiation of HL-60 cells

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Abstract

All trans-retinoic acid (RA) is a standard therapeutic agent used in differentiation induction therapy treatment of acute promyelocytic leukemia (APL). RA and its metabolites use a diverse set of signal transduction pathways during the differentiation program. In addition to the direct transcriptional targets of the nuclear RAR and RXR receptors, signals derived from membrane receptors and the Raf-MEK-ERK pathway are required. Raf1 phosphorylation and the prolonged activation of Raf1 persisting during the entire differentiation process are required for RA-dependent differentiation of HL-60 cells. Here we identify a nuclear redistribution of Raf1 during the RA-induced differentiation of HL-60 cells. In addition, the nuclear accumulation of Raf1 correlates with an increase in Raf1 phosphorylated at serine 621. The serine 621 phosphorylated Raf1 is predominantly localized in the nucleus. The RA-dependent nuclear accumulation of Raf1 suggests a novel nuclear role for Raf1 during the differentiation process.

Introduction

Retinoic acid (RA) regulates differentiation and cell cycle progression in a variety of cellular contexts including the acute myelomonocytic leukemia (M2) model cell line HL-60. RA treatment of HL-60 cells induces a series of phenotypic markers consistent with induced differentiation. Early expression of CD38 can be detected within 24 hours of treatment, followed subsequently by expression of CD11b at 48 hours post RA treatment and entry into G₀ cell cycle arrest and the ability of mature myeloid cells to perform a respiratory burst at approximately 72 hours of treatment. In acute promyelocytic leukemia patients, RA treatment results in complete remission in 92 – 95% of patients [1]. RA and its metabolites induce the systematic integration of a series of signal transduction pathways, in addition to the direct transcriptional targets of the RAR and RXR nuclear receptors. Those pathways include the involvement of membrane receptors such as the heterotrimeric G-protein coupled receptor BRL1, and also of the Raf/MEK/ERK kinase module [2].

Early responses to RA include the MEK dependent activation of ERK2 within 4 hours of RA treatment. Significantly, the MAPK signal is not the transient signal characteristic of mitogenic MAPK signaling, but is a durable protracted signal which must be sustained to elicit

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differentiation. Indeed, ERK activation persists during the entire 72 hour differentiation process [2–6]. The persistent activation of ERK is consistent with a positive feedback loop with upstream components of the pathway including Raf1 (v-raf-1 murine leukemia viral oncogene homolog 1). Raf1 expression, kinase activity and phosphorylation continue to increase during the 72 hour differentiation process. Overexpression of activated Raf1 synergistically cooperates with RA to induce differentiation of HL-60 cells [2,7]. Inhibition of the Raf/MEK/ERK kinase cascade with the small molecule inhibitor, PD98059, at the level of MEK blocks the persistent upstream activation of Raf1 and the RA-induced phenotype. The RA dependent activation of the Raf/MEK/ERK kinase cascade results in an atypical maintenance of the Raf1 kinase phosphorylation and activity. This atypical behavior of Raf1 motivated us to examine Raf1 localization related to phosphorylation level.

Raf is a serine threonine kinase that functions as a regulatory link between the cell membrane associated RAS GTPases and the downstream effector MAPK kinase cascade. This critical regulatory link functions as a switch determining cell fate decisions including proliferation, differentiation, apoptosis, survival and oncogenic transformation and itself is regulated by a complex series of coordinated events including subcellular localization, protein phosphorylation, as well as inter- and intra- protein interactions. The inactive form of Raf is found within the cytosol as a complex with 14-3-3 proteins and autoinhibitory interactions are maintained between the N-terminal regulatory region and the C-terminal catalytic domains. Relief of the autoinhibition of Raf by phosphorylation at sites within the catalytic region results in increased kinase activity [8]. Raf is phosphorylated by a diverse set of protein kinases including protein kinase A and C, Src related kinases, as well as the MAP kinases in both positive and negative feedback loops. Raf phosphorylation within the kinase domain at sites 340 and 341 have been shown to induce Raf kinase activity [8], while phosphorylation of additional sites have been shown to regulate Raf in both a positive and negative way dependent on the system utilized. Phosphorylation to other sites, such as Ser-338 has been shown to not be required for Raf1 activation [9].

Raf interacts with a diverse set of proteins including upstream regulatory proteins, downstream effectors and multiple scaffolding proteins (reviewed in [10,11]). Upstream Raf regulatory proteins include RAS which initiates relocalization of Raf from the cytoplasm to the cell membrane where a complex series of phosphorylation events occur. Downstream effectors include the substrates MEK, and the nuclear localized Retinoblastoma protein (Rb). In addition Raf1 interacts with a set of regulatory scaffolding proteins including 14-3-3, KSR1 and Raf1 kinase inhibitory protein (RKIP). These regulatory scaffolds are phosphorylation dependent and regulate Raf1 sub-cellular localization. To better understand the regulatory role of Raf, we looked for correlations between phosphorylation and subcellular localization of Raf and here we report three fold evidence for Raf1 and p621Raf1 nuclear accumulation during the RA-induced differentiation of HL-60 cells. The most established conventional paradigms of Raf activation place Raf in the cytosolic compartment and at the plasma membrane. The Raf accumulation in the nucleus reported here is thus novel and motivates expansion of the current concepts of Raf action and capabilities.

Materials and Methods

Cells and Culture Conditions

Human myeloblastic leukemia HL-60 cells were maintained in RPMI 1640 supplemented with 5% heat inactivated fetal bovine serum (both: Invitrogen, Carlsbad, CA) and 1x antibiotic/antimycotic (Sigma, St. Louis, MO) in a 5% CO₂ humidified atmosphere at 37°C. Cultures were treated with 2μM retinoic acid (Sigma, St. Louis, MO) while at a density of 0.2×10^6 cells/ml. The samples were collected 48h after RA treatment. Normal bone marrow cells were

harvested from femurs of C57BL6 mice. Bone marrow was flushed with a 21ga needle into RPMI 1640 supplemented with 5% FBS.

Multispectral imaging of Raf localization

HL-60 cells were harvested at indicated times fixed in 4% paraformaldehyde for 15 minutes and washed with PBS. Cells were then centrifuged to a pellet and resuspended in staining buffer as described in [12]. Cells were stained with FITC-anti-Raf1 or FITC-anti-p621Raf1 (Santa Cruz Biotechnology, Santa Cruz, CA) and co-stained with DRAQ5 (red nuclear dye). 5,000 cell images were collected on an ImageStream multispectral imaging flow cytometer (Amnis-Seattle, WA) at a rate of 50–100 cells per second. All samples were collected with 100 mW of laser power, bright field in channel 5, with a minimum object area threshold of 100 pixels to eliminate collection of only the smallest debris. Cells with saturating levels of FITC (less than 5% of the sample) were also eliminated from collection. Single color controls were used to create a compensation matrix that was applied to all experimental files to correct for spectral crosstalk. Image-based quantification of Raf1 or pRaf1 nuclear localization of Raf1 was performed using the IDEAS analysis software (Amnis-Seattle, WA) as previously described [13,14]. The Similarity score is derived from the log-transformed Pearson's correlation coefficient of the pixel values of the DRAQ5 and Raf images. If Raf is nuclear, the two images will be similar and the Raf/DRAQ similarity index will be a positive value. Cells with predominantly cytoplasmic Raf would have anti-similar Raf1 and DRAQ5 images and thus have lower Similarity values [15].

Flow Cytometry

Whole cell flow cytometry assays were performed as previously described using a formaldehyde-triton X-100 Methanol method [12]. Cells were analyzed on a BD Biosciences LSRII and data was analyzed using BD Bioscience's FACSDiva software and Treestar's FlowJo. Nuclei for flow cytometry were isolated from HL-60 cells resuspended in hypotonic lysis buffer as previously described [2,3] for 10 minutes on ice. Cells were then stained for 2 hours at 4°C with the indicated antibody, microcentrifuged to a pellet and resuspended in hypotonic lysis buffer. Cells were then analyzed on a BD Biosciences LSRII using the FITC channel, where the cells are excited at 488 nm with emission collected through a 525/10 nm band pass filter.

Western blot analysis

After centrifuging the cells at 600g, 4°C, 5 minutes, the cell pellet was washed twice with PBS and suspended either in M-PER Mammalian Protein Extraction Reagent (Thermo Scientific) – for the total lysate samples, or in sodium citrate–Triton X100 hypotonic solution – for the nuclei isolation as previously described [2,3]. The samples in hypotonic solution were centrifuged after 30 minutes to pellet the nuclei. The supernatant was kept as the cytosolic fraction and the nuclear fraction was lysed in RIPA buffer (Sigma). All lysis buffers had protease and phosphatase inhibitors (Sigma). Cell lysates (15ug) were resolved on 12% polyacrylamide gel and then transferred onto polyvinylidene difluoride membrane, subsequently probed with p621Raf1 (Invitrogen), β actin (Cell Signaling) and histone 3 (Cell Signaling) at 4°C overnight, followed by washing and probing with the appropriate horseradish protease-conjugated secondary antibody (Cell signaling).

Immunofluorescence Microscopy

The HL-60 cells as well as the mouse bone marrow cells were laid on a slide using a Shandon Cytospin3 (Thermo Scientific), at a speed of 700 rpm for 1 min and immediately fixed in 4% paraformaldehyde for 30 minutes at room temperature. Slides were washed three times in PBS, and cells were permeabilized in cold methanol for 15 minutes. Slides were washed three times

with PBS and the indicated primary antibody was applied over-night at 4°C. The secondary antibody used was FITC conjugated. The slides were counterstained with DAPI (4',6-diamidino-2-phenylindole), and mounted with prolong anti-fade mounting media (Molecular probes). Images were captured on a Zeiss LSM-510 Meta Confocal Microscope System, at 40X oil magnification, and numerical aperture of objective lenses of 1.3, at room temperature. The scan zoom was 3.8. PMT and Zeiss LSM510, V4.0 software were used for data acquisition.

Results

RA induces cellular redistribution of Raf1

In many systems Raf1 is a regulatory link between the membrane associated growth factor receptors and the MAP kinase cascade. In order to gain insight into the regulation of Raf1 during the differentiation process, we looked for correlations between sub-cellular localization of total Raf1 and its phosphorylated forms. An image based flow cytometry method, Image Stream, was utilized to identify RA-induced changes in Raf1 cellular localization. Changes in the cellular distribution of Raf1 were determined in HL-60 cells by comparing the similarity of Raf1 distribution with the nuclear compartment. Raf1 was visualized with a FITC labeled antibody, the nuclear compartment identified with Draq5, and the membrane associated region by a bright field image. Raf1 is frequently localized at the plasma membrane where it associates with activated Ras, and we were able to detect changes in the redistribution of Raf1 from the cytoplasm and to a more nuclear Raf1 profile. Images of Raf1 distribution in HL-60 cells are shown and quantified in Figure 1. Figure 1A shows representative brightfield, FITC, DRAQ5 and FITC/DRAQ5 composite images of cells collected with the ImageStream. Nuclear localization of Raf1 was measured using the Similarity score, which quantifies the correlation between the Raf1 and nuclear DNA images for each cell (Figure 1B). Cells with predominantly cytoplasmic Raf distributions are gated with R5, cells with predominantly nuclear distribution are gated with R4. Fisher's Discriminate Ratio (Rd) was used to represent the degree to which measured distributions can be distinguished from one another, and is given by the following formula:

$$Rd = (\text{MedianExp} - \text{MedianNeg}) / (\text{SDExp} + \text{SDNeg})$$

Compared to untreated HL-60 cells, 48 hour RA treatment induces a redistribution of Raf1 into the nuclear compartment (figure 1). This nuclear redistribution is consistent with Raf1 activation and persists for 72 hours after treatment (data not shown). The number of cells with low Raf/DRAQ5 similarity indexes is the dominant type found in untreated cultures of HL-60 cells. Thus, differentiating cells in RA induces a quantifiable increase in Raf1 nuclear localization (Rd 0.45). By contrast TPA, which is a known activator of Raf, fails to cause nuclear localization (Rd -0.01), suggesting a novel regulatory mechanism for Raf1 during RA-induced differentiation. Figure 1B illustrates the histograms for a sample of each treatment group. Figure 1C summarizes the statistical analysis for the data set presented in figure 1B, and shows results of the similarity score statistical analysis for the percent of cells with predominantly nuclear or cytoplasmic Raf defined by R4 or R5 respectively. Integrating the Raf signal over the entire cell showed no significant change in total Raf induced by RA after 48h (data not shown).

RA induces Raf 621 phosphorylation and p621Raf is in the nucleus

In order to identify Raf1 phosphorylation sites that correlated with the nuclear translocation during RA-induced differentiation, the phosphorylation status of S621, S259, S338 and S340/341 were assessed by flow cytometry. Previous data has shown a RA-dependent increase in the kinase activity of Raf and an increase in the phosphorylation status of Raf1 [2]. Compared

to a no primary antibody control baseline, most untreated HL-60 cells show above baseline staining levels of both p621 and p259 Raf1 staining, suggesting significant amounts of p621 and p259 phosphorylation (figure 2). Although, Raf1 phosphorylation at S259 was detected in HL-60 cells, there was no apparent change during RA-induced differentiation, suggesting a change in phosphorylation at S259 is not required for the nuclear translocation of Raf1. Interestingly, we were unable to detect a significant amount of S338 or S340/341 phosphorylation in HL-60 cells (data not shown). Consistent with the nuclear accumulation of Raf1, the RA treated cells showed increased phosphorylation at S621 when the entire cell was assessed by flow cytometry (figure 2). It should be noted that the baseline percentage of cells with high levels of p621 Raf and nuclear localized Raf were variable. However, RA consistently increased both nuclear localization of Raf1 and p621 phosphorylation in experiments that were completed more than three times.

As previously reported, Raf1 kinase activity is required [16] for RA induced differentiation of HL-60 cells, and RA induces the phosphorylation of Raf1 at serine-621 [2]. A correlation between the nuclear localization of Raf and the regulatory C-terminal phosphorylation site at serine-621 was identified. Representative images of untreated or RA-treated cells probed for p621 Raf and collected on the ImageStream are shown in Figure 3A. 95% of both untreated and RA treated HL-60 cells contained nuclear p621Raf (Figure 3B). The distribution of S621 phosphorylated Raf1 was predominantly nuclear, again suggesting an alternative role for Raf1 during RA induced differentiation. The Raf1 and p621Raf1 nuclear localization pattern is the same for cells in all phases of the cell cycle (data not shown), so the relocalization observed after RA treatment did not just reflect enrichment for G1 where Raf may have been more nuclear.

The nuclear enrichment in p621 Raf1 during RA treatment was confirmed by two cell fractionation methods. Nuclei were isolated from HL-60 cells and the nuclear component of p621 Raf1 was quantified by flow cytometry (figure 4). The p621 signal in RA-treated nuclei shows a shift corresponding to an increase in the amount of p621 Raf1 per nucleus. Consistent with previous data the amount of p621 Raf1 associated within the nucleus accumulated in RA-treated HL-60 cells. Western blot analysis of whole cell extract confirms the RA-dependent increase of p621 phosphorylation (figure 5, lanes 1 and 2). The Western blot confirms the existence of p621Raf1 in isolated nuclei (p621 Raf1 quantified by flow cytometry). β -actin and histone 3 were used as loading control.

Confocal microscopy was used to further confirm the presence of p621Raf1 within the nucleus. Untreated HL-60 cells were stained with p621Raf1- FITC and counter stained with DAPI to identify the nuclei for co-localization studies (figure 6). These images clearly indicate localization of Raf1 within both the cytoplasmic and nuclear components of HL-60 cells. As expected, a proportion of cytoplasmic p621 Raf1 was associated with the plasma membrane, consistent with the targeting of Raf1 to membrane bound Ras. In addition to the plasma membrane associated fraction of Raf1, there was a significant fraction of p621 Raf1 that co-localized with the DAPI stained chromatin (figure 6). Analysis of 1.2 micron Z-stacked confocal image sections confirms the presence of p621 Raf1 within the DAPI labeled portion of the nuclei (data not shown). In order to determine if the nuclear localization of Raf1 was restricted to HL-60 cells, we examined fresh mouse bone marrow cells stained with p621Raf1 which were also counter stained with DAPI. The incidence of nuclear Raf1 was not restricted to HL-60 cells and was clearly observed in a population of normal cells found in the fresh mouse bone marrow prep (figure 6, lower panel). The punctuate nuclear staining of Raf1 in the population of primary mouse bone marrow cells shown in figure 6 suggests a functional role for nuclear p621Raf1 and a potential role during the differentiation process.

Discussion

Here we provide ImageStream, flow cytometry, Western blot, and confocal microscopy evidence for a nuclear localization of both total Raf1 and p621Raf1. The localization correlates with retinoic acid – induced protracted Raf and MAPK activation and differentiation of HL-60 human myeloblastic leukemia cells. In these cells, RA is known to regulate the expression of several membrane receptors. These include CD38 [17], BLR1/CXCR5 [2,18], FcγRII [19], and c-FMS [20,21]. In each case increased expression of the receptor caused enhanced MAPK signaling and enhanced cellular differentiation in response to RA, as well as 1,25-dihydroxyvitamin D3. This suggests that these receptors cooperate to create the durable MAPK signal needed to elicit differentiation [2–6]. RA has also been shown to regulate the expression of certain adaptor molecules known to regulate MAPK signaling, in particular Dok1 and 2 [22], SLP-76 [23], and c-CBL [24]. Our laboratory has been shown that SLP-76 complexes with c-FMS and that c-CBL complexes with CD38 to cooperatively drive ERK activation and enhance RA-induced differentiation. Sprouty4 has been shown to directly interact with Raf1 [25] and with c-CBL [26]. In the classical model of Raf activation, Raf translocates to the membrane where it binds RAS and subsequently phosphorylates MEK1/2 starting thus the cellular response to the extracellular ligands. Raf kinase trapping to Golgi (RKTG) downregulates ERK signaling by trapping Raf1 [27] as well as B-Raf [28]. We have found that HL-60 cells have significant basal levels of p621 and p259 (figure 2); with RA treatment, the amount of p621 Raf1 increases with more going in the nucleus, p259 is unaffected by RA, and total Raf1 preferentially locates into nuclei with RA (figures 1–4). We have previously reported that the increase in total p621Raf1 is apparent as early as 6h post RA treatment and continuously increases during the 96h of observation [2]. The predominantly nuclear localization of p621Raf1 in both RA- treated and untreated cells reported here indicates an increase in nuclear p621 Raf1 that occurs early during differentiation and persists. The fact that p259 levels are not affected by RA treatment suggests that the role of Raf1 here is not dependent on enhancing plasma membrane association, since Ser-259 dephosphorylation was reported to induce Raf-1 membrane accumulation [29]. Both in HL-60 and PC12 cells, differentiation is accompanied by prolonged ERK activation. In PC12 cells, Raf1 is associated with transient ERK activation, leading to cell proliferation, whereas B-Raf activation was reported to correlate with prolonged ERK activation and differentiation [30]. We report here that Raf1 prolonged activation that characterizes RA-induced differentiation occurs with the unanticipated nuclear localization both of total Raf1 and of p621Raf1, suggesting a novel differentiation related nuclear function for Raf1.

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Anti-Raf1

Untreated

RA treated - 48hrs

Untreated

R4 - 7.02%

R5 - 57.3%

Mean - 0.17

SD - 0.65

RA

R4 - 27.8%

R5 - 21.8%

Mean - 0.81

SD - 0.76

Rd = 0.45

TPA

R4 - 7.21%

R5 - 57.1%

Mean - 0.16

SD - 0.65

Rd = -0.01

Figure 1. Whole cell image stream analysis for total Raf1 and DRAQ5 localization: Retinoic acid induces Raf1 nuclear redistribution in HL-60 cells

To assess cytoplasmic versus nuclear distribution in RA or TPA treated and untreated cells, ImageStream analysis of FITC anti-Raf1 and DRAQ5-labeled cells was performed. Representative brightfield, FITC, DRAQ5, and FITC/DRAQ5 composite images are shown in (A). The correlation (as measured by the Similarity score) between the nuclear DNA/Raf1 image pair was quantified on a per cell basis and plotted for all single cells in (B). Cells with a high degree of nuclear Raf1 are identified with the R4 gate; and R5 is the cytoplasmic Raf1. The percentage of cells that contained significant amounts of cytoplasmic and nuclear staining is plotted in (C) which is a graphical representation of the results of the similarity score statistical analysis for the three different samples (untreated, RA or TPA treated samples presented in (B)). The percentage of cells with predominantly nuclear Raf1, defined as a similarity score in R4, and predominantly cytoplasmic Raf1, defined as a similarity score in R5, is shown.

Figure 2. Flow cytometric analysis of HL-60 total (nuclear and cytoplasmic) Raf1 p-259 and p-621 phosphorylation status
48h RA-treatment induces p621Raf1 phosphorylation, but not p259Raf1 phosphorylation.

Figure 3. Whole cell ImageStream analysis for pS621Raf1 localization

Untreated or RA-treated (48 hr) cells were stained with FITC- anti p621Raf1 and DRAQ5 and analyzed for p621 nuclear localization using ImageStream cytometry. – Representative brightfield, FITC, DRAQ5 and merged FITC/DRAQ5 images are shown in (A). As determined by the p621Raf1/DRAQ5 Similarity score, p621 Raf1 is localized to the nucleus in the majority of untreated or RA-treated cells (B).

Nuclear phospo S-621 Raf1

Figure 4. RA induced nuclear enrichment of phosphorylated S-621 Raf

Cytometric analysis of phosphorylated 621Raf within isolated HL-60 nuclei shows a significant fluorescence intensity shift with RA treatment, an expression of RA-induced pRaf1 enrichment in the nucleus.

Figure 5.

Western blot analysis shows that phosphorylated 621Raf is more abundant in nuclear fraction, and the total amount is increased by RA treatment. Western blot analysis of total cell and nuclear extracts from RA untreated or treated cells probed for p621Raf1 and reprobed for β actin and histone 3. pRaf(S621) expression normalized to actin (pRaf(S621)/ β actin, middle panel) and to histone 3 (pRaf(S621)/histone 3, lower panel) is shown.

Figure 6. Nuclear localization of phosphorylated S621 Raf. Confocal images of RA treated HL-60 cells and normal mouse bone marrow co-stained with DAPI

In both HL-60 and mouse bone marrow cells, pS621Raf1 co-localizes with DNA. DAPI staining for nuclear DNA (left), FITC staining for p621Raf1 (middle) and merged confocal images (right).