Mutant RAS Selectively Promotes Sensitivity of Myeloid Leukemia Cells to Apoptosis by a Protein Kinase C-dependent Process

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ABSTRACT

RAS mutations arise at high frequency in human malignancy and have been shown to play a role in the disruption of both normal differentiation and proliferation. In addition, RAS influences a number of intracellular signaling pathways, which impinge on proteins that regulate programmed cell death. In this study, we have examined whether this oncogene can influence the activation of the apoptotic process induced by a range of therapeutic agents used to treat leukemia, and we have identified the downstream targets of RAS mediating the observed changes in sensitivity. Using myeloid leukemia cells (P39) retrovirally transduced with mutant H-RAS, we found that the influence of this oncogene was highly dependent on the inducer used: whereas RAS had no significant effect on spontaneous apoptosis or on the response to the cytotoxic drugs (doxorubicin or 1β-arabinofuranosylcytosine), P39-RAS cells showed a strongly augmented response to all-trans-retinoic acid (ATRA) in both the induction of apoptosis and differentiation. Because, under some circumstances, RAS has been associated with promoting apoptosis, we examined whether the activation of this kinase by mutant RAS could be responsible for the augmented response to ATRA. However, constitutive activation of RAS did not alter the apoptotic sensitivity of these cells, making it unlikely that RAS promotes apoptosis by stimulating this kinase. Nor did we find that BCL-2 was differentially down-regulated in P39-RAS cells. Rather, we found that the activation of protein kinase C (PKC) by low-dose phorbol ester could almost entirely recapitulate transformation by RAS, in terms of promoting both apoptosis and differentiation after treatment with ATRA. Moreover, the RAS-induced phenotype could be completely abolished by a specific inhibition of PKC under conditions that had no effect on the response of control cells. In conclusion, we have shown that mutant RAS promotes differentiation-associated cell death in P39 cells by stimulating the activity of PKC, which is itself an important regulator of myeloid differentiation. PKC activation, in turn, powerfully synergizes with the PKC-independent action of ATRA. This work identifies a possible explanation for the ability of this oncogene to promote myeloid differentiation of hematopoietic cells. Clinically, it raises the possibility that although leukemias expressing mutant RAS may not show an altered response to cytotoxic agents, they may show enhanced sensitivity to differentiation therapy with ATRA.

INTRODUCTION

Changes in the expression of genes directly involved in the control of the apoptotic process, such as the BCL-2 family (1), have been shown to diminish the sensitivity to induction of apoptosis and so contribute to malignant growth; however, other oncogenes associated with malignancy can also indirectly influence this process. RAS is one of the most commonly mutated oncogenes in cancer, occurring at high frequency in lung and colon cancer as well as in myeloid leukemia (2). Its precise role in malignancy is generally unclear, although there is evidence to suggest that this oncogene influences both the proliferative and differentiation of normal cells (3, 4). In addition, there is evidence that this oncogene may influence the process of apoptosis. RAS has already been implicated in cell signaling in apoptosis induced by various stimuli, such as tumor necrosis factor α (5), FAS (6), and viral infection (7) as well as mechanical stress (8). Constitutive activation of RAS has been reported either to inhibit apoptosis (9, 10) or to promote apoptosis (5, 7), depending on both the inducer and the cell type. For example, in fibroblasts, activated RAS has been reported to protect against DNA-damaging drugs (11, 12), whereas it potentiates apoptosis induced by tumor necrosis factor α (13, 14). In hematopoietic cells, mutant RAS promotes spontaneous apoptosis during erythroid development (3), which may partly explain the increased frequency of apoptosis in preleukemic marrow (15). In immortal hematopoietic cells, however, this oncogene can alleviate growth factor dependence and thereby reduce the frequency of apoptosis on factor withdrawal (10, 16). These divergent consequences of RAS activation probably reflect the fact that this molecule impinges on a variety of pathways that regulate apoptosis via its complex network of downstream effectors (17). Recent research has already identified that two of these, RAF and phosphatidylinositol 3'-kinase, can influence apoptosis induced by constitutive c-MYC expression. In this context, the activation of phosphatidylinositol 3'-kinase induced a protective effect, whereas RAS-dependent activation of RAF promoted apoptosis (18). Another target of RAS, mitogen-activated protein kinase kinase kinase 1 (19), is also involved in regulating apoptosis via stimulation of the SAPKs9 (20). In addition to these, indirect downstream targets of RAS, such as PKC, can also influence apoptosis (21).

Because much of the therapeutic strategy for treating leukemia involves overcoming the block in apoptosis, we set out to examine whether the activation of RAS affects the response of AML blasts to a range of agents, both cytotoxic drugs and differentiation inducers, and to identify the downstream targets of RAS mediating the observed changes in sensitivity. To study this, we have made use of a recently described AML cell line, P39, which is sensitive to the induction of apoptosis by a variety of inducers (22). We show here, for the first time, that constitutively activated RAS strongly potentiates the response to ATRA in terms of the induction of differentiation and apoptosis, without affecting the sensitivity to doxorubicin or ara-C. We also show that this increased sensitivity to ATRA is mediated through a PKC-dependent mechanism that is not affected by the activation of RAF.

MATERIALS AND METHODS

Retroviral Vectors and Producer Lines. The retroviral vector-expressing mutant H-RAS has been described previously (23). In this vector (based on DOL), the expression of human mutant H-RAS (valine at codon 12) was driven by the Moloney murine long terminal repeat, whereas the expression of the neomycin gene from transposon TnlO (also present in the vector) was transcribed from the SV40 early region promoter. Vector containing the neomycin gene alone served as a control. Retrovirus was generated by expressing this

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The abbreviations used are: SAPK, stress-activated protein kinase; PKC, protein kinase C; AML, acute myeloid leukemia; ATRA, all-trans-retinoic acid; ara-C, 1β-arabinofuranosylcytosine; TPA, 12-O-tetradecanoylphorbol-13-acetate; MFI, mean fluorescence intensity; RAR, retinoic acid receptor; RXR, retinoid X receptor.
constructs in the amphotropic packaging line ψ-CRIP. The retroviral producer lines were a gift from David Wynford-Thomas (University of Wales College of Medicine). The cDNA of activated RAF was generated by PCR of the catalytic domain (24) from the complete c-Raf sequence (ATCC 4105D) using the oligonucleotide primers 5'-CCCCGATCATCAGGACAGAGATCTCAAGCTATTAGG-3' (sense) and 5'-GGGGAATTTTCTCAGAAGACAGGGCAGGCTCGGGGAC-3' (antisense). The sense primer incorporates a BamHI restriction site, an in-frame ATG, and a complementary sequence to codons 333–341. The antisense primer incorporates a complementary sequence to codons 672–685 and an EcoRI restriction site. The PCR product was directionally cloned into the gag/pol cloning site of the PM5neo vector (a gift from Carol Stocking, Heinrich Pette Institute, Hamburg, Germany) to create PM5ΔRAF-neo. In this vector, human activated RAF and neo expression was driven by the myeloproliferative sarcoma virus long terminal repeat (RFL from the full-length genomic transcript and neo from the subgenomic spliced RNA). The integrity of this construct was confirmed by sequencing. Retrovirus was generated from PM5ΔRAF-neo by expressing this construct in the ecotropic packaging line GP+E86 (a gift from Dina Markowitz, Columbia University, New York, NY). Virus from these cells was subsequently used to infect the corresponding amphotropic producer line GP+envAM12.

Cell Culture and Infection. The myelomonocytoid cell line P39 (Ref. 22; a gift from Yatara Yoshida, Kyoto University, Kyoto, Japan) was derived from an AML (M2) and cultured in RPMI 1640 containing 10% FCS at 37°C in a humidified atmosphere of 5% CO2. For retroviral infection, these cells were seeded at 1 × 105 cells/ml onto pre-established monolayers of producer cells (see the description above). Cocultivation was carried out for 48 h in the presence of 4 μg/ml polybrene (Sigma, Poole, United Kingdom) with an equal volume of fresh medium (as described above) added after 24 h. Nonadherent cells were then harvested and cultured in fresh medium for an additional 24 h. Infected cells were selected by culture in the presence of the eukaryotic antibiotic G418 (Life Technologies, Inc., Paisley, United Kingdom) at a concentration of 800 μg/ml. To confirm the transforming potential of the PM5ΔRAF-neo retrovirus, NIH3T3 cells were infected by exposure to a 0.4-μm-filtered retroviral supernatant. Infected cells were selected in G418 as described above. NIH3T3 cells infected with PM5ΔRAF-neo, but not PM5-neo, were morphologically transformed and gave rise to tumors in nude mice. All retrovirally transduced cultures were negative for replicative virus, as judged by the absence of transmissible virus from G418-resistant cultures (which were extensively passaged after infection) and by the absence of amphotropic env sequences, as demonstrated by PCR of the DNA from these cell lines using the env-specific primers described previously (25).

The Induction and Inhibition of Apoptosis. Exponentially growing cells were used in all experiments. For the induction of differentiation with ATRA or vitamin D3 (Sigma), cells at a density of 1 × 105 cells/ml were cultured in the presence of 1 μM inducer for up to 4 days. Cells were treated with doxorubicin, ara-C, or TPA (all from Sigma) at a density of 2 × 105 cells/ml at the indicated concentrations for the indicated time period. Transient (10-min) exposure to TPA was carried out at 37°C. Cells were then washed twice in PBS before being returned to culture. In some experiments, the PKC-specific inhibitor, GF109203X (Ref. 26; Calbiochem, Nottingham, United Kingdom), was added at the same time as the inducer at a concentration of 2 μM. This inhibitor is structurally similar to staurosporine but is highly selective for the inhibition of PKC. Where treatment with the inducer caused cells to become adherent, the proportion of adherent cells was counted. The nonadherent portion of the culture was first removed by aspirating and gently rinsing the culture vessel; viable cells were then counted on the basis of trypan blue exclusion. The adherent fraction was then dislodged and counted by tapping the side of the flask in the presence of a minimal volume of PBS. Both adherent and nonadherent fractions were pooled for subsequent analysis.

Analysis of Markers of Differentiation, BCL-2 Expression, and the Frequency of Apoptosis. Cells stained for cell surface phenotype were suspended in PBS containing 1% bovine serum albumin (Sigma) to 4 × 106 cells/ml. Cell suspension (50 μl) was then incubated with the recommended concentration of antibody for 30 min on ice. Antibodies conjugated to FITC were CD11b (Serotec, Oxford, United Kingdom) and CD54 (Cymbus Bioscience, Southampton, United Kingdom); antibodies conjugated to phycoerythrin were CD14 and HLA-DR (Becton Dickinson, Oxford, United Kingdom). After staining, the cells were resuspended in ice-cold medium containing 1 μg/ml 7-amino actinomycin D (Molecular Probes, Eugene, OR). For the immunostaining of BCL-2, cells were first fixed in 2% paraformaldehyde for 15 min on ice and then washed in PBS and incubated with FITC-conjugated anti-BCL2 monoclonal antibody, as recommended by the supplier (DAKO, High Wycombe, United Kingdom), in medium containing PBS with 0.25% saponin, 0.1% Tween 20, and 5% goat serum. All stains were controlled with an appropriate isotype-matched control reagent. Cells were analyzed on a FACSCalibur cytometer (Becton Dickinson) equipped with an argon ion laser using the CellQuest acquisition program.

For the analysis of DNA content, cells were washed in PBS and fixed in 70% ethanol for 30 min on ice and then subsequently overnight at -20°C. Fixed cells were washed free of alcohol, resuspended in PBS containing 40 μg/ml propidium iodide (Molecular Probes) and 100 μg/ml RNase Type I-AS (Sigma), and incubated at 37°C for 30 min and analyzed on the above-described cytometer within 20 min. For a morphological assessment of apoptosis, cytospin preparations were made that were stained with a modified Wright-Giemsa stain (Bayer, Newbury, United Kingdom). P39 cells undergoing apoptosis exhibit a morphology typically associated with programmed cell death: condensed chromatin, apoptotic bodies, decreased cell size, and an intact plasma membrane. The percentage of apoptotic cells was calculated by counting 100–200 cells and recording the number of cells with clearly distinguishable apoptotic morphology.

Data Analysis. The acquired flow cytometric data were analyzed using WinMDI (Joe Trotter, Scripps Institute, La Jolla, CA). Cell cycle analysis was carried out using the pragmatic approach of Watson et al. (27). Copies of this program can be obtained from CytomterUK (http://www.cf.ac.uk/uuwmc/hg/hoy). Values of the MFI of cell surface antigen expression were calculated by subtraction of the background fluorescence of identically treated cells stained with an isotype-matched control reagent. Cells stained brightly with 7-amino actinomycin D were excluded from the analysis. In the analysis of DNA content, doublets were excluded on the basis of pulse width, as were particles with a DNA content of less than 10% of 2n (28). Cells with a DNA content of less than 2 SDs from the G0 peak (defined on identically stained untreated cells) were designated as apoptotic. The coefficient of variation of the G0 peak on untreated cells was <5% in all cases. The significance of difference was tested using the paired t test.

Northern Analysis. To demonstrate the expression of mutant H-RAS and activated RAF in P39 cells, total RNA was isolated using RNAzol (AMS Biotecnology, Witney, United Kingdom) according to the manufacturer’s instructions. In the case of P39-RAS cells and the corresponding control cells, a polyadenylen enrichment of total RNA was carried out using a mRNA purification kit (Qiagen, Crawley, United Kingdom). RNA (20 μg) was then loaded onto a 1% agarose formaldehyde gel. Northern blotting was carried out as described previously (29). The probe for H-RAS mRNA was generated by the PCR of exon 2 from the full-length genomic sequence in clone pbc-N1 (American Type Culture Collection, Rockville, MD) using primers 5’ CATTGATGGGAGACGTCTGGT7’ (sense) and 5’TGTACTGGTGGATGTCCTCAAAAG3’ (antisense). The full-length insert (see the description above) was used to probe for activated RAF expression. In each case, the same membrane was subsequently rehybridized using a probe for β-actin RNA (Oncor, Gaithersburg, MD). Fig. 1 demonstrates that low and moderate levels of transgene expression were detected in P39-RAS and P39-Raf cells, respectively.

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MUTANT RAS SELECTIVELY PROMOTES APOPTOSIS

The Effect of Other Low Molecular Weight Inducers on the Induction of Apoptosis in P39-RAS Cells. We next determined whether transformation by RAS increased the susceptibility of these cells to apoptosis after exposure to other low molecular weight inducers of differentiation. As previously reported (22), vitamin D₃ however, these cells did not express detectable levels of the monocytic antigens CD14 and HLA-DR (data not shown).

Because it has previously been reported that mutant RAS can potentiate apoptosis via stimulation of RAF (18), we determined whether constitutively active RAF could recapitulate the effect of mutant RAS. The results in Figs. 2 and 3 demonstrate that this was not the case, and that in this context, these cells behaved identically to control cells (expressing neo alone). This result makes it unlikely that RAS potentiates the effect of retinoic acid through the stimulation of RAF. We also examined the possibility that RAS may differentially modulate BCL-2 expression. Consistent with previous reports (30, 31), ATRA induction significantly reduced the expression of BCL-2 in P39 cells (P < 0.05); however, there was no difference in the extent of BCL-2 down-regulation between control and P39-RAS cells or P39-RAF cells (Fig. 4).

RESULTS

Mutant RAS but not Activated RAF Promotes Differentiation and Apoptosis Induced by ATRA. We first investigated whether the expression of mutant RAS altered the response of P39 cells to low molecular weight inducers of differentiation. We examined the response to ATRA using two types of end points: (a) induction of apoptosis (as assessed by the measurement of DNA content and by morphology); and (b) induction of differentiation (as determined by the expression of the cell surface antigens CD54 and CD11b).

ATRA efficiently induced both CD54 and CD11b expression on P39-neo cells over a 4-day period (Fig. 2, top panels). An analysis of DNA content and morphology (Fig. 3) showed that this was accompanied by an increased frequency of apoptotic death. Similar results were obtained whether this was assessed on the basis of the proportion of cells with a subdiploid DNA content or by morphological scoring of cytospin preparations (Fig. 3, B and C). Transformation by mutant RAS, itself, had no effect on the surface phenotype or the rate of proliferation, as indicated by the surface marker expression and the percentage of cells in S phase on day 0 (Figs. 2 and 3). However, the expression of this oncogene had a marked effect on the response of these cells to ATRA: both the rate of differentiation and the induction of apoptosis were greatly accelerated. The expression of differentiation markers was augmented by ~10-fold after 2 and 4 days of induction (Fig. 2). In addition, the frequency of apoptotic death was also significantly increased at both time points by between 2- and 4-fold (Fig. 3, A and B). By a comparison of the frequency of apoptosis after 4 days across a range of ATRA concentrations, we found that P39-RAS cells were 300 times more sensitive to ATRA with regard to apoptosis induction than were control cells (data not shown). We further observed that, in contrast to control cells, P39-RAS cells became adherent after 2 days of induction (35 ± 15%). The adherence of these cells suggested monocytic differentiation; however, these cells did not express detectable levels of the monocytic antigens CD14 and HLA-DR (data not shown).

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induces monocytic differentiation of P39 cells, with low levels of associated apoptotic death. The expression of mutant RAS did not affect the apoptotic sensitivity of these cells in the presence of vitamin D₃ (data not shown). On the other hand, treatment of P39-neo cells with the PKC agonist TPA rapidly induced apoptotic death, expression of CD11b and CD54 (but not CD14 or HLA-DR), and adhesion to plastic in a manner similar to the effect of ATRA on P39-RAS cells (Fig. 5). P39-RAS cells responded in a similar fashion; however, these cells showed an approximate 10-fold increase in sensitivity to this agent in terms of the induction of apoptosis (Fig. 5A). The induction of differentiation markers and adhesion was also potentiated (Fig. 5B).

This suggests that the stimulation of PKC activity in these cells promotes apoptosis, and that transformation by RAS sensitizes these cells to PKC-dependent programmed cell death. To confirm that the induction of apoptosis was due to the activation of PKC and not to subsequent down-regulation by prolonged exposure to TPA, we carried out the induction with TPA for 10 min only, after which the cells were washed free of inducer and returned to culture. Transient exposure to TPA also resulted in extensive cell death at levels not significantly different from those produced by a 48-h exposure: at 100 nM TPA, the frequency of apoptosis was 33 ± 4.5% for P39-neo and 50 ± 3.7% for P39-RAS cells. This indicates that the apoptotic program is initiated soon after exposure to TPA as a result of PKC activation.

**RAS Potentiates ATRA-induced Apoptosis and Differentiation through a PKC-dependent Process.** The increased sensitivity of P39-RAS cells to phorbol ester raised the possibility that constitutive activation of RAS may promote differentiation and cell death by the stimulation of PKC. To substantiate the role of PKC in mediating the effects of RAS, in this context, we examined the effect of ATRA in the presence of the highly selective PKC inhibitor GF109203X (26) on control and P39-RAS cells. At concentrations of GF109203X that did not affect the proliferative capacity of any of the cell lines (2 μM), there was no significant effect on control cells (P39-neo) in terms of the induction of apoptosis (Fig. 6A) or cell surface antigen expression (Fig. 6, B and C), with the exception of a slight reduction in CD54 expression on day 4 (P < 0.05). This indicates that the effect of ATRA on control cells was practically PKC independent. On the other hand, PKC inhibition had a dramatic effect on the response of P39-RAS cells. The level of apoptotic death was reduced by 2-3-fold on day 2 and day 4 of ATRA induction; the extent of this reduction meant that there was no significant difference between the response of P39-RAS cells in the presence of the inhibitor and P39-neo cells without the inhibitor (Fig. 6A). Likewise, the expression of the differentiation antigens CD54 and CD11b was also markedly inhibited (Fig. 6, B and C). These data demonstrate that the effect of this oncogene, in this context, could be blocked by PKC inhibition, and that P39-RAS cells seem to exhibit two separate responses to ATRA, leading to apoptosis and differentiation. The first involves a PKC-independent process (as in control cells), and the second is a PKC-dependent process only exhibited by P39-RAS cells.

If the enhanced response of P39-RAS cells to ATRA was due entirely to the activation of PKC, we reasoned that we should be able to mimic the effect of transformation by this oncogene merely by coinducing control cells with TPA. We used a concentration of TPA (0.6 nM) that alone had little effect on the induction of apoptosis (Fig. 7A) or on differentiation antigen expression (Fig. 7, C and D). However, when P39-neo cells were coinduced with ATRA, their response was strongly potentiated, to the extent that in the presence of TPA, P39-neo cells responded in an almost identical fashion to P39-RAS cells in the presence of ATRA alone (see dashed lines connecting
ever, we observed no change in the sensitivity to either compound as cells. Both of these compounds efficiently induced apoptosis; howwe therefore assessed the ability of ara-C and the topoisomerase tumor cells to deoxycytidine analogues and topoisomerase inhibitors has been reported that mutant RAS may enhance the sensitivity of activity of these cells to other types of therapeutic agents. Recently, it out to determine whether mutant RAS increased the apoptotic sensi-

despite expression induced (Fig. 1C). As might be expected, the response of these bars in Fig. 7). Under coinduction conditions, P39-neo cells even became adherent to an extent similar to that of P39-RAS cells without TPA (Fig. 7B). The only feature of P39-RAS that was not fully recapitulated in TPA-treated P39-neo cells was the level of CD54 expression induced (Fig. 7C). As might be expected, the response of P39-RAS cells was also augmented in the presence of TPA, but to a much lesser extent than that of P39-neo cells. Taken together with the inhibition data, these results demonstrate that the effect of mutant RAS expression in promoting the response to ATRA could be almost entirely accounted for by the stimulation of PKC.

The Expression of Mutant RAS Does Not Affect the Susceptibility to DNA Damage Induced by Cytotoxic Agents. We next set out to determine whether mutant RAS increased the apoptotic sensitivity of these cells to other types of therapeutic agents. Recently, it has been reported that mutant RAS may enhance the sensitivity of tumor cells to deoxycytidine analogues and topoisomerase inhibitors (32); we therefore assessed the ability of ara-C and the topoisomerase inhibitor doxorubicin to induce apoptosis in P39-neo and P39-RAS cells. Both of these compounds efficiently induced apoptosis; however, we observed no change in the sensitivity to either compound as a result of mutant RAS expression (Fig. 8). Therefore, these results indicate that P39-RAS cells are not generally more sensitive to inducers of apoptosis but are specifically sensitized to inducers that activate or synergize with PKC.

DISCUSSION

We have studied the influence of oncogenic RAS on the susceptibility to apoptosis of the human leukemia cell line P39 in response to a variety of inducers. A general conclusion of this work is that the influence of this oncprotein differs according to the inducer. In the case of the cytotoxic drugs doxorubicin and ara-C, mutant RAS had no significant effect, whereas it strongly potentiated the effects of ATRA and TPA. These distinct responses probably reflect the fact that each of these inducers acts by different mechanisms. RAS may or may not influence their effects, depending on whether it impinges on the signaling pathways used by these inducers. Doxorubicin and ara-C both induce DNA damage: doxorubicin produces protein-associated DNA cleavage in mammalian cells and inhibits the catalytic action of topoisomerase II (33); and in the case of ara-C, the active metabolite (ara-C triphosphate) acts as a competitive inhibitor of DNA polymerase and again induces strand breaks. However, the mechanism by which these compounds induce apoptosis is not well understood. A potential mechanism of action is via their ability to trigger ceramide generation. This second messenger is thought to be important in stimulating the SAPK cascade, which in turn induces apoptotic death (34). Both doxorubicin (35) and ara-C (36) may initiate apoptosis through this mechanism. RAS may impinge on this pathway through its interaction with mitogen-activated protein kinase kinase kinase 1 (19), which acts upstream of SAPKs; however, there was no evidence in this work to suggest that RAS could radically affect the ability of these cytotoxic drugs to induce apoptosis. Support for this comes from a recent study that demonstrated that the activation of SAPKs, in response to alkylating reagents, was independent of RAS (37). The influence of mutant RAS on the response to deoxycytidine analogues and topoisomerase inhibitors has also been examined from a clinical perspective. Here again, there seems to be no clear correlation between patients with detectable RAS mutations and their response to treatment. Of the larger studies, some have shown that RAS mutations are associated with improved survival, whereas others have demonstrated no such correlation (see Refs. 38 and 39 and the references therein).

Conversely, this study indicates that mutant RAS can strongly
influence the response to retinoic acid. Retinoic acid acts via the steroid/RAR family of nuclear transcription factors. To date, two families of RARs have been described: (a) the RAR-α/β/γ family; and the RXR-α/β/γ family. Heterodimers of RAR/RXR (and homodimers of RXR) bind to specific sequences known as retinoic acid response elements (reviewed in Ref. 40). However, the actual trigger mechanism by which retinoids are able to induce apoptosis is not clear. Retinoic acid has been reported to reduce the expression of BCL-2 in myeloid cells (30, 31), and we also observed this with P39 cells. However, there was no difference in the extent of the reduction of expression in P39-RAS cells, indicating that this was not the only factor controlling viability. Although we did not identify the mechanism by which apoptosis was triggered in these cells in this study, we did determine the downstream target of RAS signaling that potentiated the response to ATRA.

One of the best established downstream targets of RAS, which has been implicated in regulating survival and differentiation, is RAF (41–43). We therefore examined the possibility that the phenotype elicited by mutant RAS was recapitulated by constitutively active RAF. We found that this oncogene had no demonstrable influence on survival or differentiation in either the presence or the absence of the inducer. This suggests that alternative downstream target(s) are involved. Here, we turned our attention to the PKC family of enzymes, because these are a candidate group of signaling molecules that have been reported to be influenced by both RAS and retinoic acid. In the former case, RAS has been reported to stimulate PKC activity in a variety of contexts (44–46), possibly by promoting diacylglycerol formation through the activation of phospholipase D (47). PKC activation also seems to result from treatment with ATRA in hematopoietic cells (48). Two lines of evidence indicated that the potentiation of apoptosis and differentiation induced by mutant RAS in the presence of ATRA was mediated through PKC activity: (a) TPA, which stimulates the conventional and novel isoforms of PKC (49), was able to mimic the effect of transformation by RAS in control cells; and (b) the treatment of P39-RAS cells with an inhibitor to these PKC isoforms completely abolished the effects of mutant RAS expression. These results argue that the augmentation of differentiation and apoptosis consequential to RAS activation in these cells could be almost entirely accounted for by the stimulation of PKC activity. Interestingly, PKC inhibition had no effect on control cells; therefore, in these cells, there is no indication that the response to ATRA was dependent on PKC. P39-RAS cells therefore exhibit two distinguishable pathways leading to differentiation and apoptosis: (a) a PKC-independent pathway mediated by ATRA; and (b) a PKC-dependent pathway promoted by the activation of RAS. Because mutant RAS expression alone had no effect on these cells, it follows that the induction of PKC activity by itself was insufficient to trigger a response. Indeed, we were able to precisely mimic this situation by applying a low concentration of TPA to the control cells. This had little or no effect on differentiation or apoptosis, but in combination with ATRA, a strong synergistic effect was observed of the same magnitude as that observed for P39-RAS cells responding to ATRA alone. An increased basal PKC activity in P39-RAS cells was also indicated by their augmented sensitivity to TPA induction.

One possible explanation for the potentiated response to ATRA is that PKC-dependent signals from mutant RAS cooperate at a transcriptional level with retinoid receptors to promote differentiation and apoptosis. The fact that both ATRA and high-dose TPA promoted a similar pattern of response in terms of the induction of apoptosis and cell surface antigen expression supports the notion that these distinct pathways have overlapping sets of transcriptional targets. The only qualitative difference between the responses to ATRA and TPA was that the latter also promoted cell adhesion. The fact that P39-RAS cells became adherent in response to ATRA alone provided further evidence that this TPA-activable signal was operating in these cells. An alternative explanation is that ATRA may cooperate in the induction of the PKC-mediated response, as has been previously reported (50).

The mechanism by which PKC triggers apoptosis is not known, and the effects of PKC activation on cell survival are highly divergent, depending on the context and the nature of the inducer (21). What is more clearly established is a role for PKC in the induction of differentiation of both myeloid cells (51, 52) as well as other cell types (49). Therefore, in the context of this study, the activation of PKC may have no direct influence on apoptosis but may merely promote differentiation; cell death may therefore be triggered by the induction of a defective development program. Support for this comes from the observation that if these cells are protected from apoptosis by an overexpression of BCL-2, they nevertheless fail to undergo further differentiation (either morphologically or immunophenotypically), even after extensive culture. Therefore, RAS may potentiate apoptosis as a consequence of stimulating PKC-mediated differentiation, and indeed, mutant RAS can itself promote myeloid differentiation, not only of P39 cells, but also of U937 and FDC-P1 cells (53, 54), suggesting a link between activated RAS, PKC activity, and the induction of differentiation, as seems to be the case for keratinocytes (46). Furthermore, RAS mutations are most closely associated with the more-differentiated myeloid leukemias (M4; Ref. 55). A question that arises from this study is whether the tendency of this oncogene to promote differentiation could be exploited by using differentiation therapy. ATRA alone has not proved therapeutically effective for myeloid malignancy, other than for acute promyelocytic leukemia. However, the activation of RAS is unlikely to be the basis for the positive response of this type of leukemia (56). On the other hand, preliminary clinical data suggest that ATRA may be a useful adjunct to low-dose ara-C (57, 58). Although the basis for its efficacy has yet to be examined, it suggests that there is a potential to exploit a latent prodifferentiation signal within leukemia cells expressing mutant RAS.

In conclusion, these results demonstrate that the expression of mutant RAS can have a powerful and selective effect on the sensitivity to therapeutic agents, depending on their mechanism of action. Specifically, we have shown that in P39 cells, oncogenic RAS can potentiate PKC activity, and that this in turn can synergize with therapeutic inducers of differentiation such as ATRA.

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