Retinoic Acid-induced Growth Arrest and Differentiation: Retinoic Acid Up-Regulates CD32 (FcγRII) Expression, the Ectopic Expression of Which Retards the Cell Cycle[1]

Jenifer Wightman, Mark S. Roberson, Thomas J. Lamkin, Susi Varvayanis, and Andrew Yen[2]

Department of Biomedical Sciences, College of Veterinary Medicine, Cornell University, Ithaca, New York 14853

Abstract

Retinoic acid is known to cause the cell cycle arrest and myeloid differentiation of HL-60 myeloblastic leukemia cells. Evidence suggesting the possible involvement of the FcγRII immunoglobulin receptor in mediating retinoic acid-induced growth arrest and differentiation of HL-60 cells is presented. HL-60 cells stably transfected with the Δ205 mutant polyoma middle T antigen, a largely debilitated polyoma middle T antigen, are known to undergo accelerated retinoic acid-induced growth arrest and differentiation compared with parental HL-60 cells. Δ205 transfected cells were compared with parental HL-60 cells by differential display to identify differentially expressed genes, which are regulated downstream of Δ205 and might facilitate cellular response to retinoic acid. Differential display revealed that the FcγRII immunoglobulin receptor was differentially expressed. HL-60 cells express FcγRIIA but not FcγRIIB. In parental HL-60 cells, retinoic acid up-regulated FcγRII expression, and FcγRII membrane protein expression increased concomitantly with retinoic acid-induced cell cycle arrest and differentiation. Ectopic expression of FcγRIIa1 in HL-60 cells retarded cellular progression through all phases of the cell cycle. For HL-60 cells stably transfected with FcγRIIa1, onset of retinoic acid-induced growth arrest and differentiation occurred in fewer cell cycles than for parental HL-60 cells. Similar results occurred with 1,25-dihydroxy vitamin D_3. Retinoic acid-induced tyrosine phosphorylation of various PAGE-detected protein bands in HL-60 cells was enhanced by cross-linking ectopically expressed FcγRIIa1 receptor. The known retinoic acid-induced sustained activation of various mitogen-activated protein kinase signaling molecules, including extracellular signal-regulated kinase 2, src-like kinases, and adapter molecules, may in part reflect induced expression of FcγRIIA, which is known to activate a similar ensemble of signaling molecules through its ITAM domain. The data suggest that retinoic acid induces increased FcγRIIA expression, which is of functional consequence in eliciting growth arrest and differentiation.

Introduction

Retinoic acid regulates cell division and differentiation in a variety of contexts (reviewed in Refs. 1–4). It is a well-known developmental morphogen that regulates embryonic *HOX* gene expression and determines spatial body axis orientation during embryogenesis. It is a necessary dietary factor, provided as a prohormone, for proper development in juveniles. It is also a cancer chemotherapeutic agent used in the differentiation induction therapy of acute promyelocytic leukemia, where it causes cell cycle arrest and myeloid differentiation (5). Retinoic acid and its retinoid metabolites are ligands for the RAR[3] and RXR classes of ligand-activated transcription factors, which are members of the steroid-thyroid hormone superfamily of nuclear receptors (Refs. 6 and 7, reviewed in Ref. 8). Retinoic acid can also regulate transcription by activating MAPK signaling (9–15). In particular it causes MEK-dependent activation of the ERK2 MAPK and subsequently RAF kinase activation in HL-60 leukemic cells. Although MAPK signaling is the prototypical mitogenic signal, it also propels retinoic acid-induced cell cycle arrest and differentiation (9, 10, 13, 14). However, retinoic acid-induced MAPK signaling is atypical compared with that commonly attributed to growth factors in both its prolonged duration and late RAF activation. Relevant to MAPK signaling activation, in HL-60 cells retinoic acid up-regulates the expression and activation of a variety of molecules known as potential positive regulators of MAPK signaling, in particular src-like kinases, including fgr, lyn (16, 17), and hck (18), and adapter molecules, including paxillin (19), CBL, Crkl (20), vav (21, 22), and SLP-76.[4] Retinoic acid can thus apparently cause the wholesale up-regulation of expression and activation of cellular machinery typically associated with mitogenic MAPK signaling. A prominent symptom of such signal activation is the tyrosine phosphorylation of a variety of cellular

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[2] To whom requests for reprints should be addressed, at Department of Biomedical Sciences, College of Veterinary Medicine, Cornell University, Ithaca, NY 14853.

[3] The abbreviations used are: RAR, retinoic acid receptor; RXR, retinoid X receptor; MAPK, mitogen-activated protein kinase; MEK, MAPK kinase; ERK, extracellular signal-regulated kinase; PDGF, platelet-derived growth factor; PI-3 kinase, phosphatidylinositol 3-kinase; RT-PCR, reverse transcription-PCR; ITIM, immunoreceptor tyrosine-based inhibition motif; ITAM, immunoreceptor tyrosine-based activation motif.
[4] A. Yen and S. Varvayanis, unpublished data.

proteins, such as these signaling molecules. It is, however, not clear how these various signaling molecules are activated by retinoic acid.

The molecular mechanism of action of retinoic acid has been studied in a variety of *in vitro* cell lines. The HL-60 human myeloblastic leukemia cell line is one of the archetype *in vitro* models used (Refs. 23 24, reviewed in Ref. 25). Derived from a patient with myeloblastic (French-American-British Classification, FAB M1) leukemia, HL-60 is an uncommitted hematopoietic precursor cell that grows avidly in culture. It can be induced to undergo G_0 cell cycle arrest and either myeloid or monocytic differentiation. Retinoic acid and DMSO, for example, induce G_0 arrest and myeloid differentiation, whereas 1,25-dihydroxy vitamin D_3 or sodium butyrate induce G_0 and monocytic differentiation. Treating a population of HL-60 cells with retinoic acid or 1,25-dihydroxy D_3 induces onset of G_0 arrest and mature myelomonocytic differentiation after approximately 48 h, a period corresponding to two division cycles in the subline studied. This period segregates into two sequential segments (26–29). The first 24 h, corresponding to the first division cycle in the presence of retinoic acid or 1,25-dihydroxy vitamin D_3, leads to a "precommitment" state, where cells are temporarily primed to differentiate without lineage specificity, even after removal of the retinoic acid or 1,25-dihydroxy vitamin D_3, although they continue to proliferate. During this time, retinoic acid causes activation of ERK2 and then RAF kinase (9, 14, 15). Consistent with this, 1,25-dihydroxy vitamin D_3 also causes ERK2 activation (15, 30). The second 24 h of treatment commits the cells to myeloid or monocytic differentiation, depending on whether retinoic acid or 1,25-dihydroxy vitamin D_3 is used. Activation of both RAR and RXR by receptor-selective retinoid ligands is needed to elicit G_0 arrest and mature myeloid differentiation, as well as ERK2 and RAF activation, with kinetics that are similar to activation by retinoic acid (14, 31, 32). In contrast, activation of just one class of retinoid receptors is much less effective with much slower and smaller effects. Retinoic acid-induced MAPK signaling also appears to be necessary to elicit differentiation and G_0 arrest because inhibition of MEK prevents retinoic acid-induced ERK2 and RAF activation and also blocks subsequent differentiation and G_0 arrest (9, 14). Consistent with this, retinoic acid-induced MAPK signaling is also needed for retinoic acid to induce hypophosphorylation of the RB tumor suppressor protein, a central cell cycle regulator (9, 15). In HL-60 cells, retinoic acid thus appears to induce prolonged MAPK signaling during the "precomittment" state to elicit differentiation and G_0 arrest.

Retinoic acid up-regulates the expression of various receptors associated with MAPK signaling in HL-60 cells. One of these is the c-FMS receptor for CSF-1, a cytokine that regulates myelomonopoiesis (33). c-FMS is a transmembrane tyrosine kinase receptor, which is a member of the PDGF subfamily (reviewed in Ref. 34). Ligand binding causes dimerization and autophosphorylation leading to RAS/RAF recruitment and activation of MAPK signaling. Early molecular regulators of this cascade correspond to the binding domains on the cytosolic domain of the receptor for src-like kinases, PI-3 kinase, and adapter molecules. Ectopic expression of c-FMS in HL-60 cells increases the amount of activated ERK2 (15). It also retards the cell cycle and accelerates G_0 arrest and differentiation in response to retinoic acid or 1,25-dihydroxy vitamin D_3 (35, 36). In addition, it enables cells to differentiate in response to less retinoic acid (37). c-FMS originated MAPK signaling thus appears able to propel retinoic acid-induced differentiation. Almost all of the MAPK signal enhancing capabilities of PDGF subfamily receptors, in particular activation of src-like kinases, PI-3 kinase, and phospholipase Cγ, as well as certain adapter molecules, are shared by the polyoma middle T antigen. Ectopic expression of polyoma middle T in HL-60 cells accelerates retinoic acid-induced differentiation as well as 1,25-dihydroxy vitamin D3-induced differentiation (38). Surprisingly, the Δ205 middle T mutant formed by deletion of histidine 205 to alanine 214 is crippled in its ability to activate src-like kinases, PI-3 kinase, and phospholipase Cγ but still enhances ERK2 activation when ectopically expressed in HL-60 cells (10). This is despite having its primary signal regulating capabilities abrogated. It also accelerates retinoic acid- and 1,25-dihydroxy vitamin D_3-induced differentiation. This motivates the question of what changes in gene expression this minimal viral antigen caused that facilitated cellular response to retinoic acid.

In addition to PDGF subfamily transmembrane tyrosine kinase receptors, retinoic acid can also up-regulate the expression of a heterotrimeric G-protein coupled receptor, BLR1 (12, 32), also known as CXCR5. In untreated HL-60 cells, BLR1 is not or is at most minimally expressed, but retinoic acid induces prominent expression within the first 24 h when "precommitment" priming occurs. BLR1 causes enhanced ERK2 activation when ectopically expressed in HL-60 cells. It also accelerates retinoic acid-induced and 1,25-dihydroxy vitamin D_3-induced cell differentiation. BLR1 and c-FMS thus have in common that they can cause MAPK signaling and accelerate both myeloid and monocytic differentiation when ectopically expressed, presumably through facilitating the "precommitment" priming of cells. An emerging rationalization is thus that retinoic acid may sustain its unusually prolonged MAPK signaling in part by inducing the expression of membrane receptors capable of MAPK signaling. This motivates interest in the identity of receptors that are retinoic acid-regulated and target the explicit src-like kinases and adapter molecules known to be regulated downstream of retinoic acid.

Another receptor that is now known to be associated with some of the same src-like kinases and adapter molecules believed to be downstream of retinoic acid is FcγRIIA. FcγRIIA is one of the FcγRII receptors, cluster designation CD32, which are members of the Fc receptor family (reviewed in Ref. 39). Signaling by FcγRIIA is of particular interest to the present considerations. Aggregation of FcγRIIA causes phosphorylation of src kinases, lyn and hck in monocytic THP-1 cells (40), as well as fgr in human neutrophils (41). It also causes phosphorylation of syk kinases in myelomonocytic HL-60 cells (42). FcγRIIA cross-linking also causes FcγRIIA phosphorylation and ERK2 activation within 0.5 or 2 min, respectively, in HL-60 cells (43). The adapter molecules, shc, SLP-76, vav (43), and cbl (43, 44), are also phosphorylated in HL-60 cells by receptor cross-linking.

Phosphorylations occur within minutes and are transient. The phosphorylated SLP-76 coprecipitates with vav (43), and the p56 src kinase and p72 tyrosine kinase coprecipitate with cbl (44). FcγRII cross-linking in monocytes also causes phosphorylation of the paxillin adapter (45). It is thus a striking coincidence that FcγRII signaling activates an ensemble of signaling molecules closely resembling the ensemble activated downstream of retinoic acid. In this regard, it is noteworthy that CD32 ligation can suppress the growth of B-lineage ALL cells (46).

The present communication reports that, using differential display to compare wild-type HL-60 *versus* HL-60 transfected with the Δ205 mutant polyoma middle T, expression of Δ205 regulated FcγRII expression. In HL-60 cells, which express FcγRIIA, retinoic acid was found to regulate FcγRII expression, increasing expression as cells underwent cell cycle arrest and differentiation. Stable transfectants of FcγRIIa1 were generated. Ectopic expression of FcγRIIa1 retarded the cell cycle, lengthening each phase of the cell cycle, G_1, S, and G_2-M, by ~50%. The transfected cells underwent more prominent growth arrest when treated with retinoic acid. In response to retinoic acid, the transfected cells also differentiated ~1 cell cycle faster than the parental cells when half-maximum population responses were compared. Cross-linking FcγRIIA increased the tyrosine phosphorylation of proteins caused by retinoic acid. It thus appears that retinoic acid may induce expression of the FcγRIIA receptor, which contributes to sustaining activation of src kinases and adapters implicated with regulating MAPK signaling, ultimately causing growth arrest and differentiation.

Materials and Methods

Cells and Culture Conditions. HL-60 human myeloblastic leukemia cells were continuously cultured in RPMI 1640 (Life Technologies, Inc., Grand Island, NY) supplemented with 5% FCS (Intergen, Purchase, NY) as described previously (9, 10, 12). These late-passage wild-type cells were originally a generous gift of Dr. Alan Sartorelli (Yale University, New Haven CT). They respond to both retinoic acid and 1,25-dihydroxyvitamin D_3 as described previously (9–13). Stock cells were maintained in 10-ml cultures that were initiated at a density of 0.2×10^6 cells/ml for 2 days, twice a week, and then 0.1×10^6 cells/ml for 3 days, once a week, to sustain constant exponential growth. FcγRIIa1 stable transfectants were maintained in cultures initiated at a density of 0.2×10^6 cells/ml for 3 days and then 0.1×10^6 cells/ml for 4 days. The stable transfectants were cultured under constant selective pressure with 1 mg/ml active G418 (Geneticin; Sigma Chemical Co., St. Louis, MO) added to the medium. Viability ascertained by exclusion of 0.2% trypan blue exceeded 90% in stock and experimental cultures.

Experimental 30-ml cultures were initiated at a cell density of 0.2×10^6 cells/ml with 10^{-6} M retinoic acid (Sigma) or 0.5×10^{-6} M 1,25-dihydroxy vitamin D_3 (Solvay Duphar B. V., Weesp, Netherlands) in serum-supplemented medium. Retinoic acid or 1,25-dihydroxy vitamin D_3 was added from a 10^{-3} M stock in ethanol stored at −20°C protected from light. G418 was not added to the medium of transfectants in experimental cultures, although stock cultures are maintained under continuous selective pressure. At the indicated times, cells were harvested to determine cell density, differentiation, cell cycle distribution, or Western analysis. Experiments shown are typical of two or more replicates, all using the same stable transfectant. In experimental cultures, untreated transfectants grew in the absence of G418 indistinguishably from stock cultures containing G418, indicating that the G418 *per se* had no effect on growth.

In the experiments where vinblastine was used to block cell cycle transit in G_2-M, HL-60 or FcγRIIa1 transfected cells were initiated in 30-ml cultures with 0.2×10^6 cells/ml. The cultures were incubated for at least 12 h to avoid any potential lag phase attributable to initiation of a new culture. Replicate 10-ml cultures derived from these were then treated with 10^{-7} M using a 10^{-3} M stock of vinblastine (Lymphomed, Deerfield, IL). Cells were harvested at 0 h (when vinblastine was added), 7, 8.5, 11, and 17 h for cell cycle analysis by flow cytometry as described below.

Assays of Growth and Differentiation. Assays of cell growth by measuring cell density and distribution in the cell cycle and assays of cell differentiation detected by inducible oxidative metabolism were performed as described previously (9, 10, 12). Cell density in experimental cultures was measured by repeated counts with a hemacytometer. The distribution of cells in the cell cycle was determined by flow cytometry using propidium iodide-stained nuclei. 0.5×10^6 cells were harvested at each indicated time and resuspended in 0.5 ml of hypotonic propidium iodide solution (0.05 mg/ml propidium iodide, 1 mg/liter sodium citrate, and 0.1% Triton X-100) and stored refrigerated and protected from light until analyzed. Flow cytometric analysis was done with a multiparameter dual laser fluorescence-activated cell sorter (EPICS; Coulter Electronics, Hialeah, FL) using 200 mW of 488 nm excitation from a tunable argon ion laser. Functional differentiation to a mature myelomonocytic phenotype capable of inducible oxidative metabolism was assayed by phorbol 12-myristate 13-acetate (Sigma) inducible oxidative metabolism, resulting in intracellular reduction of nitroblue tetrazolium to formazan by superoxide. 0.2×10^6 cells were harvested at the indicated times and resuspended in 0.2 ml of 2 mg/ml nitroblue tetrazolium in PBS containing 200 ng/ml phorbol 12-myristate 13-acetate in DMSO. The cell suspension was incubated for 20 min in a 37°C water bath and then scored using a hemacytometer for the percentage expressing intracellular purple formazan precipitated by superoxide. Only clear, morphologically intact cells were scored; discolored, yellow, or crenelated cells were discounted. Over 200 cells were counted per sample, and variation in replicates was routinely within 10%.

RNA Isolation. Total RNA was isolated using the RNeasy Midi kit (Qiagen, Valencia, CA) according the manufacturer's instructions. 60×10^6 cells were typically used per column. Isolated RNA was stored in autoclaved water at −80°C. It was quantitated in water by 260/280 absorbances. RNA integrity was verified by agarose gel electrophoresis and ethidium bromide staining to visualize the 18S and 28S ribosomal RNA bands.

Differential Display. Differential display-PCR was performed using isolated RNA and the RNA image kit following

Table 1 PCR primer sequences for detecting FcγRIIA, B, or C[a]

A	Forward: ATC CCA GAA ATT CTC CCG TTT G Reverse: TTC TGA TGG CAA TCA TTT GAC G Size: 328 bp
B	Forward: CAA GAA ATT TTC CCG TTC GGA Reverse: GAC AGC AGG TGC AGT CGG TTA Size: 627 bp (b1), 570 bp (b2), 627 bp (b3)
C	Forward: CAA GAA ATT TTC CCG TTC GGA Reverse: TTC TGA TGG CAA TCA TTT GAC G Size: 316 bp (c1), 330 bp (c2), 278 bp (c3), 363 bp (c4).

[a] PCR was done using 40 cycles and an annealing temperature of 63°C.

the manufacturer's instructions (GenHunter, Nashville, TN). Exponentially growing HL-60 cells and HL-60 cells transfected with the Δ205 mutant polyoma middle T antigen were compared by differential display. The stable transfectants were reported previously (10). All lanes were run in duplicate, and only differences that reproduced in replicates were considered positive. The primer combinations used were: $HT_{11}A$ as the 5′ end primer (antisense) in conjunction with HAP 1–8, 17–25, 27–37, and 39–50 as 3′ primers (sense). Forty of the 240 available primer combinations, corresponding to ~17%, from the kit were used. The differential display fragment was gel purified, PCR amplified with 40 cycles, cloned as a *Hin*dIII fragment into a pGEM-T vector, and sequenced at the Cornell DNA sequencing facility.

From the 40 primer combinations tested, 10 bands were selected for further analysis. The bands were excised from the dried gels and reamplified by PCR using the primer combinations that detected them. The product from each band was radiolabeled using random priming to probe Northern blots of total RNA from Δ205 transfected and parental HL-60 cells. The Northern analysis verified that two differential display bands corresponded to differentially expressed transcripts. To verify that each of the identified differential display sequencing gel bands consisted of a unique species, each band was cloned into a pGEM-T vector that was used to transform bacteria and derive clones. The inserted fragment derived from the differential display band was remobilized from plasmid in these clones and used to probe Northern blots of Δ205 transfected and parental HL-60 cells. One of the differential display bands contained multiple species and was discounted from further consideration. The other band yielded clones that consistently identified a 2.6-kb transcript associated with a less abundant 1.6-kb transcript. The primer combination that identified this band was 5′-AAG CTT ACG GGG T-3′ (forward) and 5′-AAG CTT TTT TTT TTT A-3′ (reverse).

PCR. Reamplification of cDNA probes was performed according to the manufacturer's instructions (RNAimage kit; GenHunter). RT-PCR was performed using the primer pairs given in Table 1 by essentially the same procedure, except that Superscript II (Invitrogen Life Technologies, Inc., Carlsbad, CA) reverse transcriptase was used instead of the Moloney murine leukemia virus reverse transcriptase supplied in the kit. A 40-cycle amplification was used. The annealing temperature was 63°C.

Northern Analysis. Isolated total RNA was separated on a 6.0% formaldehyde/1% agarose gel. RNA was transferred by capillary blotting to nylon membrane (Hybond-N or Hybond-XL, Amersham, Arlington Heights, IL; or SureBlot Nylon, Oncor, Gaithersburg MD), which were then baked at 80°C for 2 h (Hybond membrane) or 30 min (SureBlot). Blots were prehybridized at 42°C for at least 30 min in Ziphybe or Ultrahybe (Ambion, Austin, TX). Probe was radiolabeled with [$^{32}\alpha$P]dATP (New England Nuclear) using the Random Primed Labeling kit (Boehringer Mannheim, Indianapolis, IN) and used at a concentration of 1.5×10^6 cpm/ml of prehybridization/hybridization solution. Hybridization was done at 42°C for 3 h to overnight. Blots were then successively washed twice for 20 min with 2× SSC/0.1% SDS, once for 10 min with 1× SSC/0.1% SDS, and once for 10 min with 0.25× SSC/0.1% SDS, all at 65°C. Blots were autoradiographed using Kodak BioMax MS film (Eastman Kodak Company, Rochester, NY) with an intensifying screen at −80 C for 16 h to 14 days. Blots using SureBlot membranes were stripped using boiling 0.1× SSC, 0.1% SDS in 10 mM Tris (pH 7.0).

Expression Vector. The FcγRIIa1 cDNA cloned into pcEXV-3 (47), a generous gift of Dr. Jeffrey Ravetch (Rockefeller University, New York, NY), was mobilized as a *Eco*RI fragment and recloned into pZIP-NeoSV(X)1 (48). This expression vector has been used to transfect HL-60 previously, and the vector control stably transfected cells proliferate and differentiate in response to retinoic acid and 1,25-dihydroxy vitamin D_3 indistinguishably from wild-type HL-60 (10, 49).

Transfection. Transfection was performed by electroporation as described previously (49). Plasmid was isolated with the Qiagen Maxiprep kit (Valencia, CA) according to the manufacturer's instructions. 2×10^{12} plasmid copies and 4×10^6 cells in 0.4 ml of RPMI 1640 without serum were electroporated (Gene Pulser; Bio-Rad Laboratories, Hercules, CA) using 300 V and 500 μF capacitance in a 0.4-cm electrode gap cuvette. The time constant was 11.7. After electroporation, the cells were cultured in serum-supplemented medium for 2 days to allow their recovery from electroporation. All cells were then harvested and resuspended in fresh serum-supplemented medium containing 1 mg/ml active G418. The total pool of cells derived from the electroporation was thus subject to selection. Pooled transfectants were used to obviate clonal variation. By 25 days, the transfected cells resumed growth, but at a slower rate compared with the wild-type HL-60. The derivation of the slower growing cells after transfection and selection is consistent with loss of faster growing nontransfected HL-60 cells during selection.

Detection of Membrane CD32. Membrane-bound CD32 was isolated and detected by Western analysis using a modification of the method of Lorenzo *et al.* (50). Cells (30×10^6) were harvested from the indicated cultures, washed twice in cold PBS, resuspended in 1 ml of ice-cold lysis buffer [20 mM Tris HCl (pH 7.4), 5 mM EGTA, 1 mM phenylmethylsulfonyl fluoride, and 20 μM leupeptin], and disrupted with 150 strokes of a tight fitting Dounce homogenizer on ice. After emptying the Dounce homogenizer, it was rinsed with 0.5 ml of lysis buffer, which was added to the disrupted lysate. The lysate was centrifuged at 100,000 × *g* (45,000 rpm) at 4°C for 1 h (TLA-100.4 fixed angle rotor in an Optima TL Ultracen-

trifuge; Beckman, Palo Alto, CA). The pellet was resuspended in 225 μl of lysis buffer with 1% Triton X-100 added. The samples were solubilized overnight at 4°C with gentle agitation and clarified by centrifugation at 4 C for 15 min (13,000 rpm in a refrigerated microcentrifuge). The supernatant was added to an equal volume of 2× SDS buffer [125 mM Tris HCl (pH 6.8), 20% glycerol, 6% SDS, 0.71 M β-mercaptoethanol, and 0.25% bromphenol blue], heated in a boiling water bath for 5 min, vortexed briefly, and either subjected to SDS-PAGE or stored at −80°C.

SDS-PAGE was done using a 5% stacking gel and a 12% resolving gel using 29:1 acrylamide:bis. Samples were electrophoresed for 1650 V-hr, typically 150 V for 20 min and then 80 V for 20 h, once the sample was in the running gel. Proteins were electrotransferred from the gel to nitrocellulose membrane at 0.8 A for 1 h. (Trans Blot Cell; Bio-Rad) for Western analysis as described previously (49). Uniformity of lane loading and electrotransfer was confirmed by Ponceau S staining of the membrane. CD32 was detected with a mouse monoclonal antibody (clone FLI8.26; PharMingen, San Diego, CA) used at 2 mg/ml in 2.5% BSA, 0.05% Tween 20 in PBS with an overnight incubation at 4°C. Detection was performed using a horseradish peroxidase-conjugated secondary antimouse antibody (NA931; Amersham) and enhanced chemiluminescence (ECL kit, Amersham).

CD32 Cross-Linking and Phosphotyrosine Western Blotting. Thirty-ml cultures of HL-60 cells or HL-60 cells stably transfected with FcγRIIa1 were cultured for 48 h in the absence or presence of 10^{-6} M retinoic acid as described above. 2×10^6 cells were harvested, washed in 1 ml of RPMI 1640, and resuspended in 500 μl of RPMI plus either 8.4 μl of anti-FcγRIIA antibody to make a final concentration of 14 μg/ml [IV.3 mouse monoclonal antibody (Ref. 43); the 8.4-mg/ml stock, a generous gift of Drs. Michael Fanger and Ruth Craig, Dartmouth College (Hanover, NH), was used diluted 1:10] or 10 μl of PBS for a negative control. After incubation for 30 min on ice, the cells were washed again by centrifugation, supernatant with unbound antibody was aspirated, the cells were resuspended in 500 μl of RPMI plus 100 μg/ml of rabbit antimouse IgG antibody, whole molecule (M-7023, Sigma), and incubated for 20 min on ice and then 2 min at 37°C. The cells were centrifuged to a pellet and resuspended in lysis buffer [125 mM Tris-HCl (pH 6.8), 10% glycerol, 4% SDS, 0.006% bromphenol blue, and 2% β-mercaptoethanol], heated in a boiling water bath for 5 min, briefly vortexed, stored at −80 C, and then subjected to SDS-PAGE using a 10% resolving gel with 37.5:1, acrylamide:bis. After SDS-PAGE, Western blotting was performed with an antiphosphotyrosine antibody (pY99 SC-7720; Santa Cruz Biotechnology, Santa Cruz, CA), which was used at 0.2 μg/ml in 5% BSA with an incubation for 1 h at room temperature. Detection was performed as above for CD32. Using only the bivalent anti-FcγRIIA IV.3 antibody without the antimouse IgG antibody to further enhance receptor aggregation gave qualitatively similar, but quantitatively less enhanced changes detectable by phosphotyrosine Western blotting. This was consistent with a lesser degree of aggregation and signaling without the second antibody.

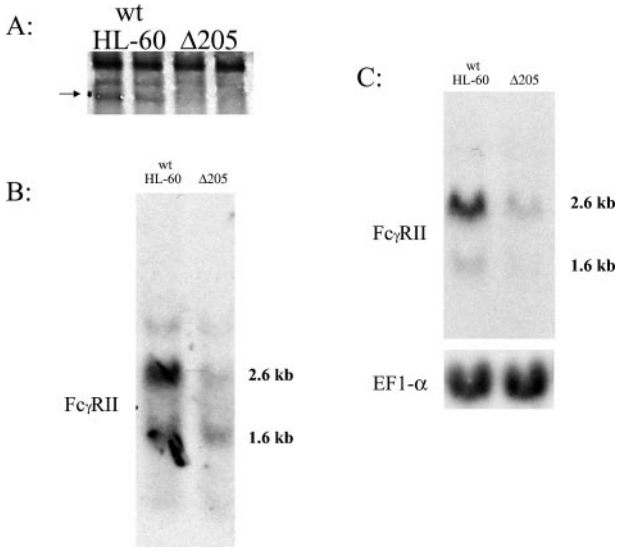

Fig. 1. A, differential display sequencing gel revealing a fragment that is differentially expressed between parental HL-60 cells and Δ205 mutant polyoma middle T antigen transfected HL-60 cells (*arrow*). The *four lanes* are replicate HL-60 and replicate Δ205 transfectant samples. *B,* Northern analysis using a PCR-amplified differential display fragment as a probe confirms that the fragment represents a differentially expressed ~ 2.6- and 1.6-kb mRNA. *C,* Northern analysis using a cloned differential display fragment as a probe confirms the same differentially expressed mRNA and verifies that the differential display fragment corresponds to a single species. *wt,* wild type.

Results

Expression of the Δ205 Mutant Polyoma Middle T Antigen Regulates Expression of the FcγRII Receptor. Because HL-60 cells stably transfected with the Δ205 mutant polyoma middle T antigen growth arrest and differentiate faster in response to retinoic acid, differential display was used to search for genes that are regulated by Δ205 expression and that facilitate retinoic acid-induced cellular growth arrest and differentiation. Total RNAs isolated from Δ205 transfected HL-60 and parental wild-type HL-60 were compared by differential display. From the 10 differentially expressed bands revealed by the 40 primer combinations tested, Northern analysis verified that two differential display bands corresponded to differentially expressed transcripts. Each band was cloned into a pGEM-T vector, and each of the cloned fragments was used to probe Northern blots of Δ205 transfected and wild-type HL-60. The cloned fragments from one band failed to identify any differentially expressed transcript. The cloned fragment from the other band identified a 2.6-kb transcript associated with a less abundant 1.6-kb transcript that was differentially expressed. Fig. 1*A* shows the differential display gel with replicate lanes for wild-type HL-60 and Δ205 transfected HL-60 cells. Only replicate bands that were consistently expressed or not were considered. Fig. 1*B* shows the Northern blot using the PCR amplified excised differential display sequencing gel band as a probe. Fig. 1*C* shows an analogous Northern blot probed with the cloned insert. Expression of the transcript was much higher in HL-60 cells than in Δ205 transfected HL-60. Equal loading of the gel lanes was verified by visualization of the

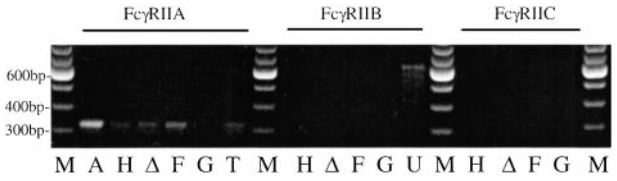

Fig. 2. Ethidium bromide-stained gel showing products from RT-PCR of total RNA from indicated sources using primers specific for FcγRIIA, FcγRIIB, or FcγRIIC. The primers and the size of their expected products are given in Table 1. *Lane M,* molecular weight markers (100-bp markers); *Lane A,* FcγRIIA-containing plasmid used as a positive control (A + control); *Lane H,* wild-type HL-60; *Lane Δ, Δ205 transfected HL-60;* Lane F, *FcγRIIa1-transfected HL-60;* Lane G, *αT3-1 gonadotroph cells used as a FcγRII-negative control (Fc − cell line);* Lane T, *THP-1 cells used as a FcγRIIA-positive control (A + cell line);* Lane U, *U937 cells used as a FcγRIIB-positive control (B + cell line). The PCR reaction underwent 40 cycles. Consistent with a previous report, HL-60 cells express FcγRIIA but no detectable FcγRIIB or FcγRIIC. Expected sizes of the three isoforms are: A, 328 bp; B, 600 bp; and C, 316 bp.*

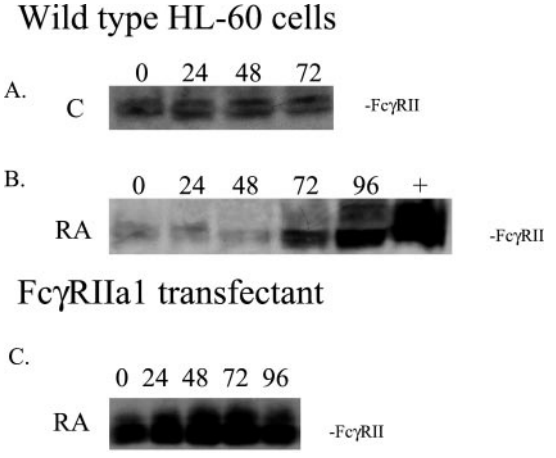

Fig. 3. Western blots of membrane-associated FcγRII in untreated (*A*) and retinoic acid-treated (*B*) HL-60 cells and in retinoic acid-treated FcγRIIa1 transfected HL-60 cells (*C*) at the indicated times (h). THP-1 cells in the last lane (+) of *A* were used as a positive control for detection of FcγRII. Retinoic acid caused increased expression of FcγRII by 72 h when significant conversion of the population to G_0 arrested differentiated cells occurred. The 0 h is untreated.

18S and 28S ribosomal RNA after ethidium bromide staining and by probing for the EF1α transcription factor, which is typically constitutively expressed in HL-60 cells at the same level, regardless of proliferation or differentiation state (12). The insert was sequenced, and comparison to GenBank sequences identified it as FcγRII, an immunoglobulin receptor also known by its cluster designation CD32.

FcγRII exists in three forms, FcγRIIA, B, and C, from three different genes. The FcγRIIA transcript can be either 2.6 or 1.6 kb, depending on which of the two transcriptional start sites is used (51, 52), whereas the FcγRIIB and C transcripts are 1.6 kb (47). Northern blots (Fig. 1) probed with a fragment common to FcγRII revealed a 2.6-kb transcript and a much less abundant 1.6-kb transcript, indicating that FcγRIIA was expressed. The lower abundance 1.6-kb transcript was either the alternative FcγRIIA transcript, the FcγRIIB transcript, or the FcγRIIC transcript. To ascertain whether the FcγRIIB or FcγRIIC were detectable, RT-PCR was done with total RNA from HL-60 cells using primers specific for each. Table 1 shows the primer sequences and the anticipated size of the PCR products. Fig. 2 shows the ethidium bromide-stained PCR products resolved on an agarose gel. Using A-specific primers, an appropriate size PCR product was detected for HL-60 cells and for the Δ205 stable transfectants, as well as for the FcγRIIa1 expression plasmid used for transfection (a positive control), the THP-1 and U937 cell lines which are known to have FcγRIIA (47), and also FcγRIIa1 transfected HL-60 (a stable transfectant to be described below). In contrast, the αT3-1 gonadotroph cell line, a negative control, showed no apparent product. Using B-specific primers, no product was detectable for HL-60 cells or the Δ205 transfectant, nor as expected for the FcγRIIa1 transfectants or the gonadotroph cell line. In contrast, U937 cells, which express FcγRIIB (47), showed an appropriate size PCR product. Using C-specific primers, no PCR product that demonstrated the C transcript was detectable for HL-60 cells or the Δ205 transfectants. None of the tested cells known to be negative for FcγRIIC expression yielded a detectable PCR product. Consistent with this, we have been unable to find any data in the literature that demonstrate that FcγRIIC is expressed in wild-type HL-60 cells. HL-60 cells thus express the FcγRIIA form of FcγRII. There was no detectable FcγRIIB or FcγRIIC. Although FcγRIIB contains an ITIM and is clearly functionally distinct from FcγRIIA, which contains an ITAM; FcγRIIC is also an ITAM-containing receptor for which no clear functional distinction from FcγRIIA has yet been established. The results show that the differentially expressed FcγRII is the A form. This is consistent with previous observations (43) that HL-60 cells express FcγRIIA.

Retinoic Acid Regulates FcγRII Protein and mRNA Expression. Because FcγRII is differentially expressed when comparing wild-type HL-60 and the Δ205 transfected HL-60, which arrest and differentiate faster, it is of interest to determine whether retinoic acid regulates FcγRII expression when causing HL-60 myeloid differentiation. This would contribute evidence to its potential involvement in retinoic acid-induced arrest and differentiation. HL-60 cells were cultured with 10^{-6} M retinoic acid and harvested at the indicated times for Western analysis of FcγRII expression in the membrane fraction. Fig. 3 shows Western blots of membrane-associated FcγRII. Fig. 3*A* shows that culture for 72 h by itself had no apparent effect on FcγRII expression. In contrast, Fig. 3*B* shows that FcγRII expression increased in retinoic acid-treated HL-60 cells. The increase was strongly evident by 72 h. This correlates with the occurrence of significant retinoic acid-induced cell cycle arrest and differentiation (see Figs. 9 and 10). It should be noted that Fig. 3, *A* and *B,* are different Western blots, and Fig. 3*B* was done with a shorter film exposure than Fig. 3*A* to avoid film saturation in the case of high induced expression. Consistent with HL-60 cells, expression of FcγRII also increased in retinoic acid-treated FcγRIIa1 transfected cells, although expression levels were consistently much higher. These cells are characterized below. Fig. 3*C* shows the Western blot for membrane associated FcγRII in FcγRIIa1-transfected cells. Because of the

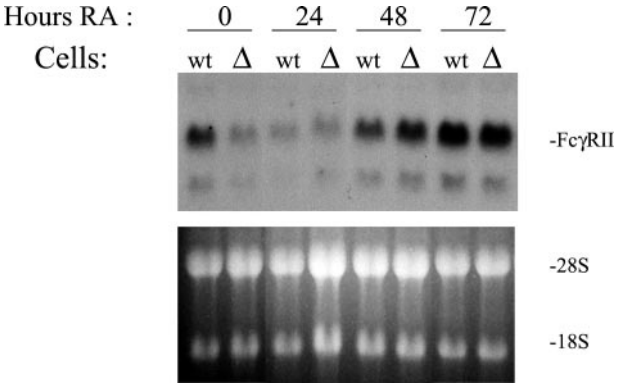

Fig. 4. Northern analysis of FcγRII expression in retinoic acid-treated HL-60 cells (*wt*) and Δ205 transfected HL-60 (Δ). HL-60 cells showed a transient decrease in expression to levels exhibited by Δ205 transfected cells at 24 h, but by 72 h there was increased expression for both cell lines, corresponding to increased protein. Ethidium bromide staining of the gel (*lower panel*) to reveal the 18S and 28S RNase bands was used to confirm uniform lane loading.

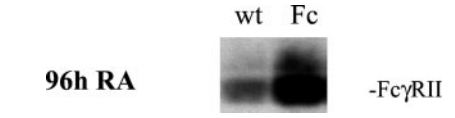

Fig. 5. Western analysis of FcγRII expression in HL-60 cells treated for 96 h with retinoic acid to increase FcγRII expression (*left lane*) and FcγRIIa1-transfected HL-60 (*right lane*). The transfected cells (*Fc*) expressed much greater levels of FcγRII than HL-60 cells (*wt*) where FcγRII expression had been increased by 96 h of retinoic acid treatment. Without retinoic acid treatment, the levels in HL-60 cells could not be visualized without grossly saturating the signal for the transfectant (data not shown). This verifies that the transfected cells ectopically expressed FcγRII protein (CD32).

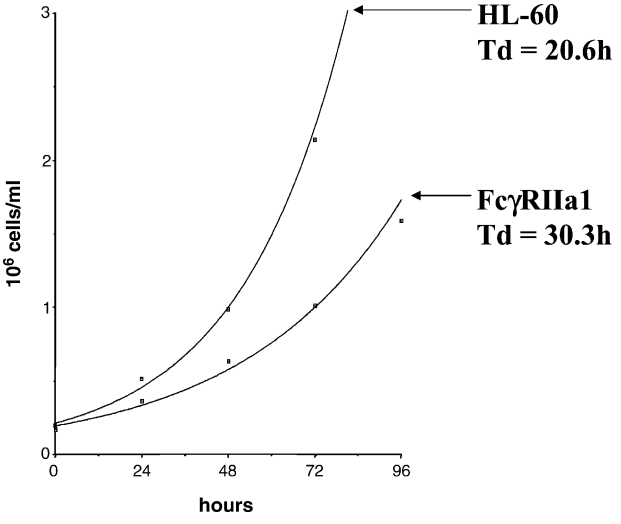

Fig. 6. Growth curves for HL-60 cells before and after stable transfection with FcγRIIa1. The *vertical axis* is million cells/ml. The *horizontal axis* is hours in culture. The FcγRIIa1 transfectants grew slower. A least squares fit to the exponential growth equation is shown in each case. The fit yielded a doubling time of 20.6 h for HL-60 cells and 30.3 h for the FcγRIIa1 transfectants with correlation coefficients of 0.992 and 0.987, respectively.

high expression levels in FcγRIIa1-transfected cells compared with HL-60 cells, the membrane-associated protein from only one-third as many FcγRIIa1-transfected cells was loaded per PAGE gel lane in Fig. 3*C* compared with HL-60 cells in Fig. 3, *A* and *B*. Interestingly the protein can appear as a doublet in HL-60 cells, suggesting the possibility that it may be subject to modification, *e.g.,* two reported glycosylations. Retinoic acid thus induced increased membrane-associated FcγRII in HL-60 cells as they underwent growth arrest and differentiation. As will be discussed below, the increase was unanticipated based on the original rationale of the differential display search and motivated pursuit of its potential functional significance.

To determine whether FcγRII protein expression correlated with increased FcγRII mRNA expression in retinoic acid-treated HL-60 cells, Northern analysis was performed. HL-60 and Δ205 transfected HL-60 were cultured with 10^{-6} M retinoic acid and harvested at the indicated times for Northern analysis of FcγRII expression. Fig. 4 shows the Northern blot probed with the cloned FcγRII-specific fragment described above. Expression of FcγRII in HL-60 cells transiently decreased at 24 h and then increased thereafter. By 72 h, elevated FcγRII mRNA expression correlated with increased FcγRII protein, as well as differentiation of approximately 75–80% of the population (see Fig. 10). Retinoic acid thus also increased FcγRIIA mRNA expression in the course of inducing cell cycle arrest and differentiation.

Ectopic Expression of FcγRIIa1 Retards the Cell Cycle. Because retinoic acid caused an increase in the expression of membrane bound FcγRII while inducing growth arrest and differentiation, the consequences of ectopic expression of FcγRIIa1 on proliferation were of interest. FcγRIIa1 is the transmembrane form of FcγRIIA, whereas FcγRIIa2 is a truncated soluble form. FcγRIIa1 was cloned into an expression vector that was used to make stable transfectants expressing FcγRIIa1. Fig. 5 shows that FcγRII membrane protein expression was clearly enhanced in the resulting stable transfectant. Fig. 6 shows that expression of FcγRIIa1 retards cell growth. HL-60 cells and FcγRIIa1 stable transfectants were initiated in culture, population density was determined every 24 h, and the growth curve was fitted by least squares to the exponential growth equation:

$$N(t) = N(0)\exp(kt)$$

where $N(t)$ is the cell density at time t, $N(0)$ is the starting cell density at time zero, and k is the growth constant given by $(\ln 2)/T_G$, where T_G is the doubling time. The calculated T_G for HL-60 cells is 20.6 h, consistent with previous recent determinations of its cell cycle duration/growth rate (10, 13). In contrast, the calculated T_G for FcγRIIa1-transfected HL-60 cells is 30.3 h. Ectopic expression of FcγRIIa1 thus apparently retarded the growth rate of HL-60 cells significantly.

During log linear growth, the percentage of HL-60 cells in G_1, S, and G_2-M was typically approximately 41.1, 40.4, and 18.5%, respectively, as determined by flow cytometry. The percentage of FcγRIIa1-transfected HL-60 in G_1, S, and G_2-M was typically approximately 40.7, 40.5, and 18.8%, respectively. (Fig. 7 shows typical DNA histograms.) The G_1,

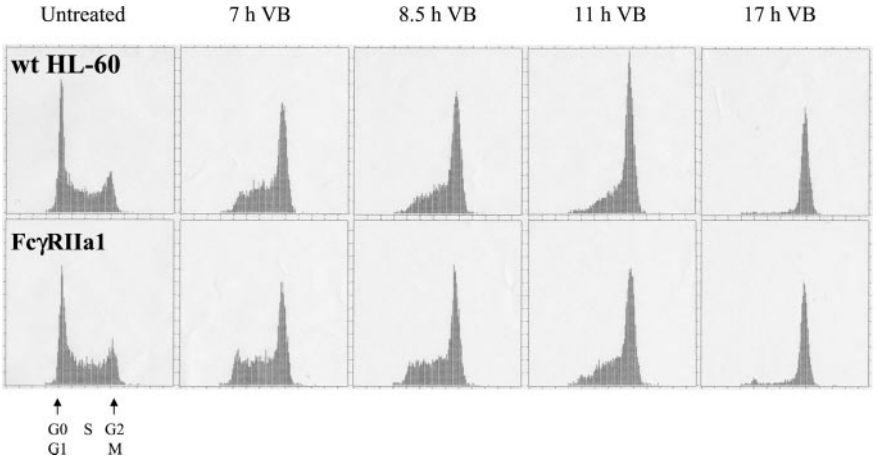

Fig. 7. DNA histograms showing relative number of cells (*vertical axis*) *versus* DNA content (*horizontal axis*) for HL-60 cells and FcγRIIa1 stable transfectants that were untreated or cultured with vinblastine (*VB*) for 7, 8.5, 11, and 17 h. The cells were exponentially proliferating when vinblastine was added at 0 h. Vinblastine blocks mitosis, and the histograms show that the FcγRIIa1 transfectants lost G_1 DNA cells and accumulated G_2 DNA cells slower than parental HL-60 consistent with their slower proliferation. The kinetic analysis is described in the text.

Table 2 Approximate cell cycle phase durations (h) for HL-60 cells and FcγRIIa1-transfected HL-60 cells

	G_1	S	G_2-M	T_G
HL-60	6.8	8.8	5.0	20.6
FcγRIIa1	10	12.8	7.5	30.3

S, and G_2 cell cycle phase durations computed in the Appendix from the flow cytometric data are 6.8, 8.8, and 5 h for HL-60 cells and 10, 12.8, and 7.5 h for FcγRIIa1-transfected HL-60 cells (Table 2). Each of the cell cycle phase durations in the stable transfectants was thus increased by ~50% compared with the parental HL-60.

To verify that the cell cycle was retarded by expression of FcγRIIa1, exponentially growing HL-60 cells and FcγRIIa1-transfected HL-60 cells were treated with vinblastine, which blocks cells in mitosis. The kinetics of accumulation of cells in G_2-M thus reflects the rate that they transit the cell cycle. If the above analysis is valid, then in the presence of vinblastine, the FcγRIIa1 transfectants should lose G_1 cells slower than HL-60 because they are transiting the cell cycle more slowly. Likewise HL-60 should accumulate G_2-M cells faster than FcγRIIa1 transfectants. The rate at which each cell line should accumulate in G_2-M can be predicted on the basis of their computed cell cycle phase durations as described in the Appendix. Concordance of the predicted and observed percentage of cells in G_2-M would confirm the cell cycle effects of FcγRIIa1 expression. Fig. 7 shows the DNA histograms of HL-60 cells and FcγRIIa1 stable transfectants at 0 h when they were exponentially proliferating and then after 7, 8.5, 11, and 17 h of culture with vinblastine. The FcγRIIa1-transfected cells had relatively more G_1 cells at all times. The observed *versus* calculated percentage of cells in G_2-M for HL-60 cells after 7, 11, and 17 h was 51% *versus* 50%, 70% *versus* 71%, and 89% *versus* 100%, respectively, corroborating that the cell cycle kinetics calculated above were a reasonable approximation. The observed *versus* calculated percentage of cells in G_2-M for FcγRIIa1-transfected cells after 7, 11, and 17 h was 44% *versus* 39%, 61% *versus* 53%, and 84% *versus* 75%, respectively, corroborating that the cell cycle kinetics calculated for FcγRIIa1 transfectants were a reasonable approximation. The observed accumulation of FcγRIIa1-transfected cells in G_2-M was thus slower than that observed for HL-60 cells, consistent with their calculated slower cell cycle kinetics. This analysis verifies that the slower population growth rate of the FcγRIIa1-transfected cells reflected retarded cell cycle progression and not a reduction in growth fraction of the population or the occurrence of significant cell death.

Ectopic Expression of FcγRIIa1 Enhances Retinoic Acid-induced Growth Arrest and Differentiation. Because retinoic acid induces FcγRII expression concomitant with growth arrest and differentiation and because ectopic expression of FcγRIIa1 retards the cell cycle, it is of interest to determine whether ectopic expression of FcγRIIa1 facilitates retinoic acid-induced growth arrest. HL-60 cells and FcγRIIa1 stable transfectants were initiated in culture with or without retinoic acid, and the population density of the cultures was determined at 24-h intervals. Fig. 8*A* shows the resulting growth curves. The FcγRIIa1 stable transfectants growth arrested much more abruptly in response to retinoic acid than the parental HL-60 cells. The initial population underwent one to two doublings after retinoic acid treatment in the case of the transfectants compared with approximately three doublings in the case of parental HL-60 cells. Consistent with this, 1,25-dihydroxy vitamin D_3 also caused a more prominent growth arrest for FcγRIIa1 transfectants (Fig. 8*B*).

The enhanced growth arrest in response to retinoic acid of the FcγRIIa1-transfected cells suggests that they might become more rapidly blocked in $G_{1/0}$. Cell cycle arrest evidenced by enrichment in the percentage of cells with $G_{1/0}$ DNA was determined by flow cytometry for the above cultures with and without retinoic acid. Because the cell cycle duration of the FcγRIIa1 transfectants is significantly longer than the cell cycle duration of parental HL-60 cells and because cells can only arrest in $G_{1/0}$ when they cycle around to it, it is necessary to normalize the time when $\%G_{1/0}$ is measured to the cell cycle duration (during exponential growth) specific for that cell line to determine when the cells begin to $G_{1/0}$ arrest relative to the number of cell cycles elapsed. Fig. 9*A* shows the results with the percentage of cells in $G_{1/0}$ as a function of time normalized to the cell cycle

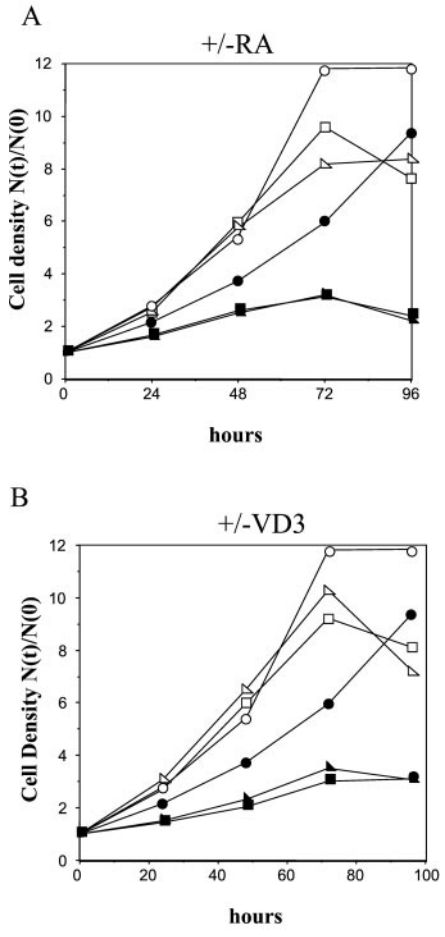

Fig. 8. Growth curves for HL-60 cells (*open symbols*) and FcγRIIa1-transfected HL-60 (*closed symbols*) without (*circle*) or with (*triangle* and *square* representing replicate experiments plotted together) retinoic acid (*+/−RA*; *A*) or 1,25-dihydroxy vitamin D_3 (*+/−VD3*; *B*) for the indicated hours in culture. The FcγRIIa1-transfected cells underwent a more prominent growth arrest compared with the parental HL-60 when treated with retinoic acid or with 1,25-dihydroxy vitamin D_3. The *vertical axis* is cell density represented as *N*(*t*)/*N*(0), the number of cells at time *t*, divided by the number of cells at time of initiation of culture, 0 h.

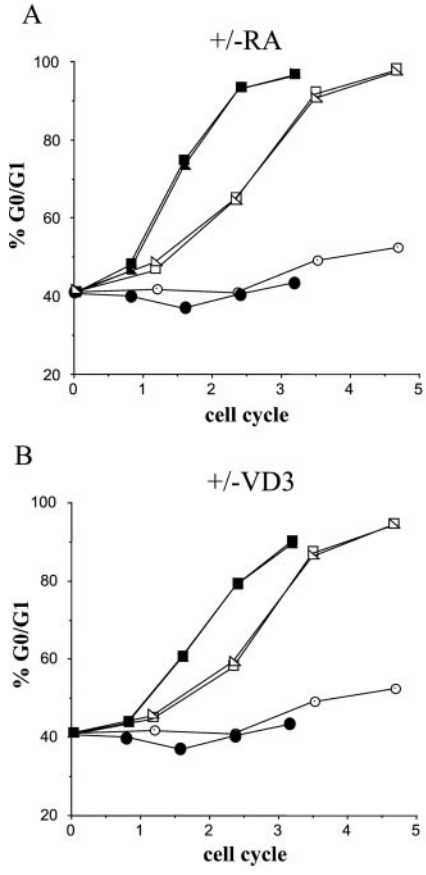

Fig. 9. Percentage of cells with G_{1-0} DNA for HL-60 cells (*open symbols*) and FcγRIIa1-transfected HL-60 (*closed symbols*) without (*circle*) or with (*triangle* and *square* representing replicate experiments plotted together) retinoic acid (*+/−RA*; *A*) or 1,25-dihydroxy vitamin D3 (*+/−VD3*; *B*) as a function of elapsed cell cycle durations. For each cell line, time in culture is thus normalized to the cell cycle duration specific for that cell line. The FcγRIIa1-transfected cells arrested in G_{1-0} approximately one cell cycle faster than the parental HL-60 cells.

duration specific for each cell line. It is apparent that the transfectants undergo $G_{1/0}$ cell cycle arrest faster than the parental HL-60 cells. Comparing when the half-maximum responses occur for each cell line shows that the transfectants arrested approximately one cell cycle sooner than the parental cells. Onset of $G_{1/0}$ cell cycle arrest was apparent for parental HL-60 cells after approximately two cell cycles, but onset was apparent for the transfectants after approximately one cell cycle. With ectopic expression of FcγRIIa1, retinoic acid thus began to arrest cells in $G_{1/0}$ after approximately one cell cycle instead of approximately two for parental HL-60 cells. Similar considerations apply to the case of 1,25-dihydroxy vitamin D_3-treated cells. Fig. 9*B* shows the corresponding results.

Because ectopic expression of FcγRIIa1 has an effect on retinoic acid-induced growth arrest and $G_{1/0}$ cell cycle arrest, it is of interest to determine whether it affects induced differentiation. Differentiation was assayed in the same cultures analyzed for growth and cell cycle kinetics above. Inducible oxidative metabolism, a functional marker for mature myelomonocytic cells, was used as a functional differentiation marker. If the induced differentiation is analyzed with duration of retinoic acid treatment renormalized to cell cycle durations (during exponential growth) specific for each cell line as shown in Fig. 10*A,* then it is apparent that the transfectants differentiate in fewer elapsed cell cycle durations than the parental HL-60 cells. Consistent with the case of induced $G_{1/0}$ cell cycle arrest, the half-maximum response for retinoic acid-induced functional differentiation occurred earlier by approximately one cell cycle duration because of ectopic expression of FcγRIIa1. It should be noted that had the data been plotted with the horizontal axis as hours in culture, then the retinoic acid-treated transfectants would then also show a consistently greater percentage of differentiated cells compared with wild type HL-60. A similar result occurred with 1,25-dihydroxy vitamin D_3 induced differentiation as shown in Fig. 10*B*.

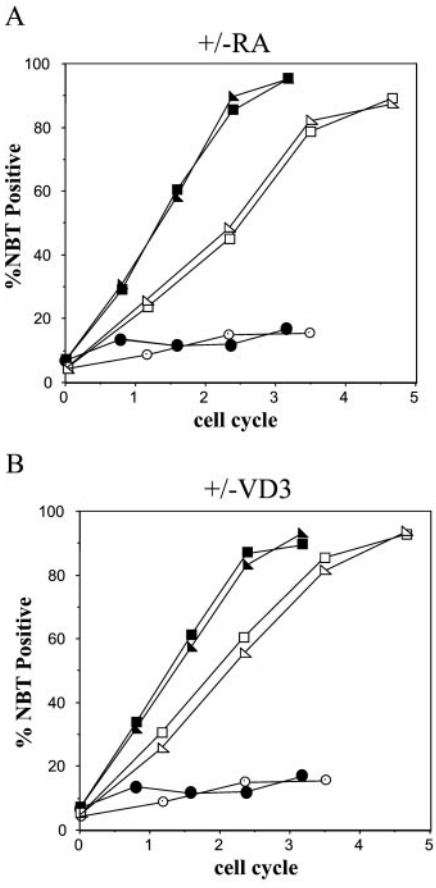

Fig. 10. Percentage of cells expressing the functional differentiation marker, inducible oxidative metabolism (% *NBT Positive*), for HL-60 cells (*open symbols*) and FcγRIIa1 transfected HL-60 (*closed symbols*) without (*circle*) or with (*triangle* and *square* representing replicate experiments plotted together) retinoic acid (+/−*RA*; *A*) or 1,25-dihydroxy vitamin D3 (+/−*VD3*; *B*) as a function of elapsed cell cycle durations. Time is thus normalized to the cell cycle duration specific for that cell line. Consistent with the induced growth arrest, the FcγRIIa1-transfected cells underwent differentiation in fewer cell cycles than the parental HL-60 cells when treated with either retinoic acid or 1,25-dihydroxy vitamin D3.

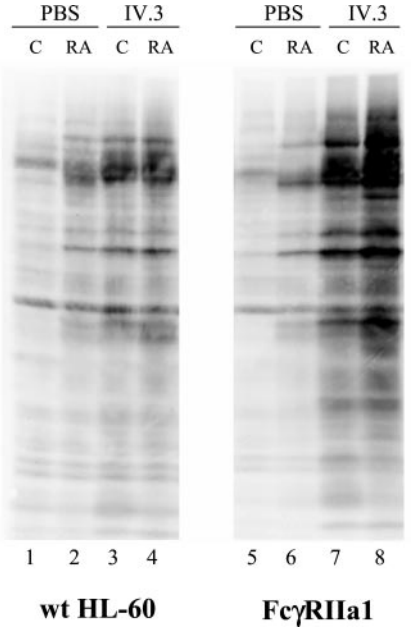

Fig. 11. Phosphotyrosine Western blot for wild-type (*wt*) HL-60 cells (*Lanes 1–4*) and FcγRIIa1 stable transfectants (*Lanes 5–8*). The cells were cultured in the absence (*C, Lanes 1, 3, 5,* and *7*) or presence (*RA, Lanes 2, 4, 6,* and *8*) of retinoic acid for 48 h and then treated with cross-linking antibody (*IV.3*; *Lanes 3, 4, 7,* and *8*) or PBS (*Lanes 1, 2, 5,* and *6*). Equal numbers of cells were loaded per lane. Uniformity of loading was confirmed by Ponceau S staining of the membrane.

Cross-Linking Ectopically Expressed FcγRIIa1 Enhanced the Tyrosine Phosphorylation of Proteins Caused by Retinoic Acid. FcγRIIA receptor signaling is known to cause rapid tyrosine phosphorylation of a variety of molecules. To confirm that the ectopically expressed FcγRIIa1 receptor was capable of signaling, the receptors were cross-linked to verify consequential protein tyrosine phosphorylation. HL-60 cells or FcγRIIa1 stable transfectants were cultured in the absence or presence of 10^{-6} M retinoic acid for 48 h. Cells were harvested in each of these four cases and then treated with FcγRIIA cross-linking antibodies or a PBS control. To aggregate the receptors and stimulate their signaling, cross-linking was done using the IV.3 mouse antibody (43) against FcγRIIA and a rabbit antimouse immunoglobulin antibody. The resulting eight cases were analyzed by phosphotyrosine Western blotting. Fig. 11 shows a representative blot. For HL-60 cells, retinoic acid caused the tyrosine phosphorylation of a variety of proteins [as seen by comparing *Lane 1* (control) and *Lane 2* (retinoic acid treated)]. Cross-linking of the receptor enhanced the phosphorylation of several of these [as seen by comparing *Lane 3* (control, cross-linked) and *Lane 4* (retinoic acid treated, cross-linked) with *Lane 2*]. For the FcγRIIa1 stable transfectants, the same was true. Stable transfection of FcγRIIa1 and cross-linking further increased the tyrosine phosphorylation of proteins [as seen by comparing *Lane 7* (control, cross-linked) and *Lane 8* (retinoic acid treated, cross-linked) with *Lanes 3* and *4,* which are the corresponding cases without ectopic receptor expression]. This confirms that the ectopically expressed receptor could signal with consequential protein tyrosine phosphorylation.

Interestingly, cross-linking appears to cause tyrosine phosphorylation of several of the same PAGE-detected protein bands, which also become tyrosine phosphorylated after retinoic acid treatment. This is seen comparing *Lane 2* (retinoic acid treated, not cross-linked) with *Lane 3* (untreated, cross-linked) for HL-60 cells; or *Lane 6* (retinoic acid treated, not cross-linked) with *Lane 7* (untreated, cross-linked) for the FcγRIIa1 transfectants. This is consistent with retinoic acid signaling resulting in the tyrosine phosphorylation of an ensemble of proteins, which are also phosphorylated by CD32 receptor signaling after receptor cross-linking. Experimentally enhancing the signaling from these receptors by cross-linking then increases the tyrosine phosphorylation of these proteins. Likewise, ectopic expression of the receptor to increase their numbers and then cross-linking results in further enhanced tyrosine phosphorylation of these proteins.

Discussion

Retinoic acid is known to regulate gene expression leading to growth arrest and differentiation of HL-60 myeloblastic leukemia cells. This process depends on MAPK signaling that can be regulated by retinoic acid-induced expression of receptors, including in particular c-FMS and BLR1, as well as ectopic expression of polyoma middle T antigens that activate MAPK signaling. Of the polyoma middle T antigens, the Δ205 mutant is largely debilitated in the principal signal activating capabilities of polyoma middle T but still causes ERK2 activation and accelerates retinoic acid-induced G_0 cell cycle arrest and differentiation. Δ205 is thus likely to cause the minimal changes needed by middle T to enhance cellular response to retinoic acid. Differential display was used to search for Δ205-induced changes in gene expression that would facilitate retinoic acid-induced growth arrest and cellular differentiation. Comparing wild-type HL-60 with Δ205 stably transfected HL-60 revealed that the FcγRII immunoglobulin receptor was differentially expressed. The Δ205-transfected cells expressed lower levels of FcγRII mRNA. Seeking evidence that changes in FcγRII were implicated in retinoic acid-induced growth arrest and differentiation, expression of membrane-associated FcγRII protein in retinoic acid-treated cells was characterized. Enhanced expression compared with untreated cells was prominent by 72 h when significant cell cycle arrest and differentiation were apparent in the population. To ascertain whether the retinoic acid-induced changes were of potential functional significance, FcγRIIa1 was ectopically expressed in HL-60 cells. The proliferation and response to retinoic acid of the stable transfectants was characterized. Expression of FcγRIIa1 retarded cell growth, slowing the progression of cells through all phases of the cell cycle. For the FcγRIIa1 expression levels achieved in the stable transfectants, the cell cycle was ~50% longer. Retinoic acid-induced growth arrest was enhanced in these transfectants. Differentiation was also enhanced. FcγRIIa1 expression caused incremental, but reproducible, increases in the percentage of differentiated cells as a function of duration of retinoic acid treatment. Normalizing the kinetics to the slower cell cycle of the transfected cells and comparing half-maximum population responses of the transfectants and wild-type HL-60 revealed that the transfectants differentiated approximately one cell cycle faster than wild-type HL-60, requiring one instead of two cell cycles for onset of differentiation. Consistent with this, induced G_0 arrest was likewise enhanced. As for retinoic acid-induced myeloid differentiation, essentially similar effects were observed for 1,25-dihydroxy vitamin D_3-induced monocytic differentiation.

In essence, the present results thus suggest that retinoic acid increases the expression of an immunoglobulin receptor, the increased expression of which retards cell growth. This receptor is known to cause tyrosine phosphorylation and activate an ensemble of MAPK signaling-associated molecules similar to that known to be activated by retinoic acid treatment. Interestingly, stimulation of FcγRIIA signaling by cross-linking caused enhanced tyrosine phosphorylation of an apparent small ensemble of proteins that are also tyrosine phosphorylated after retinoic acid treatment, suggesting that retinoic acid-induced FcγRIIA expression may contribute to the retinoic acid-induced activation of signaling molecules. Because retinoic acid-induced receptor expression is not prominent until 72 h, this contribution is most likely of relevance to propelling cellular events occurring after precommitment and late in the retinoic acid-induced cascade of events culminating in G_0 and differentiation. The results may thus provide a partial explanation for how retinoic acid causes sustained activation of these putative signaling molecules.

Analysis of signaling by Fc receptors is complicated by the potential interactions between these and other receptors, generating a variety of potential combinatorial signaling possibilities, depending on the composition of Fc and other receptors in the aggregate. Fc receptors exist for each class of antibodies, IgG (FcγR), IgA (FcαR), IgE (FcεR), IgM (FcμR), and IgD (FcδR) (reviewed in Ref. 39). Fc receptors that bind noncomplexed monomeric immunoglobulins are designated FcRI (high affinity receptors), and those that bind aggregated immunoglobulins or multivalent antigen-aggregated antibodies are designated FcRII (low affinity receptors). Fc receptors can be segregated by function into two groups; those that trigger cell activation and those that do not. Receptors that trigger activation consist of two types. Most are multichain receptors consisting of a ligand-binding FcRα subunit plus one or two common FcRγ subunits. FcRγ subunits contain a signal transduction domain, ITAM, consisting of two YxxL motifs flanking seven variable residues. A second type consists of a single chain containing an intracytoplasmic ITAM consisting of two YxxL motifs flanking 12 variable residues. These are FcγRIIA and FcγRIIC, which are unique to humans. FcγRIIA exists as a membrane receptor, FcγRIIa1, and a transmembrane deleted soluble form, FcγRIIa2. A third FcγRII subtype is FcγRIIB (existing as b1, b2, and b3 forms), which contains no ITAM and does not signal but can inhibit signaling by receptors that aggregate with it. Although less studied, FcγRIIC has no clearly demonstrable function that distinguishes it from FcγRIIa1. FcγRII receptors are collectively also given the cluster designation, CD32. The aggregation of ITAM-containing Fc receptors, such as by multivalent antigen-antibody complexes, results in tyrosine phosphorylation of their ITAMS as well as other signaling molecules, most notably src and syk family kinases, and activates MAPK signaling with its attendant consequential tyrosine phosphorylation of a variety of cellular proteins. Studies with single chain chimeras containing an ITAM suggest that aggregation of ITAMs themselves is sufficient to cause tyrosine phosphorylation of a variety of targets. It is not known what kinase(s) causes the phosphorylations after Fc receptor aggregation. It is noteworthy that Fc receptors can interact and regulate the signaling of other receptors, notably T-cell and B-cell immunoglobulin receptors; and also that Fc receptor signaling can be regulated by coaggregation with other receptors, notably CD45 which can inhibit FcγRIIA- and FcγRI-induced effects. The signaling by ITAM containing Fc receptors may also be balanced by ITIM containing Fc receptors, *i.e.,* FcγRIIB, which might also coaggregate with FcγRIIA. In HL-60 cells, however, we were unable to detect FcγRIIB expression. The combinatorial pos-

sibilities of FcγRIIA signaling are thus potentially complex. In addition, in the studies using ectopic expression of FcγRIIa1 that were presented here, a great increase in the cell surface density of FcγRIIa1 attributable to ectopic expression may itself cause receptor aggregation and spontaneously trigger signaling. We speculate that overexpression of FcγRIIa1 in some ways mimics the effects of retinoic acid-induced up-regulation of FcγRII expression and thus facilitates retinoic acid-induced growth arrest and differentiation. The data are consistent with the proposition that retinoic acid-induced up-regulation of FcγRII expression is of functional consequence in ultimately inducing growth arrest and differentiation.

Although FcγRII was found to be differentially expressed by Δ205 mutant polyoma middle T antigen transfected cells compared with parental wild-type HL-60 cells, it cannot be solely responsible for the ability of the Δ205 stable transfectants to growth arrest and differentiate faster than parental HL-60 in response to retinoic acid or 1,25-dihydroxy vitamin D_3. There are several reasons for believing this. The expression of FcγRIIA was lower in the Δ205 transfected cells, but in wild-type HL-60 cells, retinoic acid caused increased expression in the process of inducing growth arrest and differentiation. If altered FcγRII expression were the only relevant change induced by Δ205 expression, then one would anticipate that either: (*a*) retinoic acid induced increased FcγRII expression as observed and the Δ205 transfected cells had more FcγRII than the parental cells; or (*b*) Δ205 transfectants had less FcγRII expression as observed, and retinoic acid caused down-regulation of FcγRII. The effects of Δ205 and FcγRII on the growth of cells in the absence of inducer also differed. Ectopic expression of FcγRIIa1 in HL-60 cells slowed growth, but growth was essentially unaffected in Δ205 stable transfectants. Furthermore, although the Δ205 transfectants expressed less FcγRII than parental HL-60, they had more activated ERK2. The increased activated ERK2 and faster differentiation of Δ205 transfectants is anticipated from the increased activated ERK2 induced by retinoic acid in wild-type HL-60. However, increased ERK2 activation is not anticipated from the reduced FcγRII expression because FcγRIIA typically activates MAPK signaling when cross-linked. The differences between the Δ205 transfected cells and parental cells are thus not reconciled by the difference in FcγRII expression alone. The ectopic expression of Δ205 thus apparently must cause other changes in gene expression that affect the function of FcγRII. Nevertheless, the present differential display data implicate the *FcγRII* gene as a component of cellular response to retinoic acid and implicate it in particular as a regulator of the cell cycle.

Taken with previous data, the present results suggest a paradigm for retinoic acid-induced growth arrest and differentiation of HL-60 cells. In this case, retinoic acid causes activation of RARs and RXRs, as well as MEK with consequential ERK2 activation. These transcriptionally regulate the expression of a variety of genes. One subset of these genes are receptors that include BLR1, c-FMS, and FcγRIIA. These receptors all can activate MAPK signaling. Their sequential expression leads to sustained activation of MAPK signaling. The sustained MAPK signal activation attributed to retinoic acid is thus the sum of sequential waves of MAPK signal activation attributable to the sequence of retinoic acid-induced receptors that use MAPK signaling. The prolonged duration of the MAPK signal, acting in the context of other retinoic acid-induced gene changes, leads to down-regulation and hypophosphorylation of the RB protein and consequential G_0 arrest and differentiation. In this and other cell systems, notably PC12 cells, prolonging MAPK signal activation distinguishes it from the short activation duration that typically causes mitogenesis attributable to peptide growth factors. It will not escape attention that triggering these receptors is a means of augmenting the effects of retinoic acid.

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Appendix

Analysis of Cell Cycle Phases

Assuming that the cells are undergoing exponential growth with no cell death and no resting compartment, then the age-density function for the populations is given by:

$$n(a,t) = n(0,0)\exp(-k(a-t))$$

such that $0 \leq a \leq T_G$, where $n(a,t)$ is the age density function, a is age (hours) in the cell cycle, t is duration (hours) of culture, and k is the exponential growth constant as described previously (53–55). If G_1 is the duration of G_1, then the definite integral of $n(a,t)$ from 0 to G_1 divided by the definite integral from 0 to T_G gives the fraction of the population in G_1. Solution for G_1 as described previously (54, 55) gives:

$$G_1 = -k^{-1}\ln[1 - F_{G1}/2]$$

where F_{G_1} is the fraction of the population in G_1. Analogously:

$$S = -k^{-1}\ln[1 - (F_{G1} + F_S)/2] - G_1$$

Because the sum of the phase durations is T_G, then:

$$G_2\text{-M} = T_G - (G_1 + S)$$

Analysis of Mitotic Block

Assuming the cell cycle phase durations derived above, the kinetics of accumulation in G_2-M can be predicted for each cell line using the age density representation, $n(a,t)$ essentially as described previously (55). The calculation stems from the fact that if cells of cell cycle age 0 to ξ hours in the original population are not in G_2-M after a vinblastine block of X hours, then because cells of age 0 at the start of the block will be of age X after the block:

$$\xi = T_G - X - G_2\text{-M}$$

where G_2-M is the duration of G_2-M. As in calculating the duration of G_1 above, the definite integral of the age density function from 0 to ξ divided by the definite integral from 0 to T_G gives the fraction of cells not yet blocked in G_2-M. Hence:

$$1 - \{\int_{o}^{\xi} n(a',t)da'\}/\{\int_{o}^{T_G} n(a',t)da'\} = 1 - 2(1 - \exp(-k\xi))$$

gives the fraction of cells blocked in G_2-M after the X hour vinblastine block.

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