Identification of an Interleukin-3-regulated Aldoketo Reductase Gene in Myeloid Cells Which May Function in Autocrine Regulation of Myelopoiesis*

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Yang Du†, Schickwann Tsai‡, Jonathan R. Keller¶, and Simon C. Williams†,**

From the †Department of Cell Biology and Biochemistry and the ‡Southwest Cancer Center at University Medical Center, Texas Tech University Health Sciences Center, Lubbock, Texas 79430, §Mount Sinai School of Medicine, New York, New York 10029, and the ¶Intramural Research Support Program, Scientific Applications International Corporation, NCI-Frederick Cancer Research and Development Center, Frederick, Maryland 21702

The EML hematopoietic progenitor cell line is a model system for studying molecular events regulating myeloid commitment and terminal differentiation. We used representational difference analysis to identify genes that are expressed differentially during myeloid differentiation of EML cells. One gene (named mAKRa) encoded a novel member of the aldoketo reductase (AKR) superfamily of cytosolic NAD(P)(H)-dependent oxidoreductases. mAKRa mRNA was detected in murine hematopoietic tissues including bone marrow, spleen, and thymus. In myeloid cell lines, mAKRa was expressed at highest levels in cells representative of promyelocytes. mAKRa mRNA levels increased rapidly in response to interleukin-3 over the first 24 h of EML cell differentiation when the cells undergo lineage commitment and extensive proliferation. mAKRa mRNA levels decreased later in the differentiation process particularly when the EML cells were cultured with granulocyte/macrophage colony-stimulating factor and retinoic acid to induce terminal granulocytic maturation. mAKRa mRNA levels decreased during retinoic acid-induced terminal granulocytic differentiation of the MPRO promyelocyte cell line. AKRs act as molecular switches by catalyzing the interconversion or inactivation of bioactive molecules including steroids and prostaglandins. We propose that mAKRa may catalyze the production or catabolism of autocrine factors that promote the proliferation and/or lineage commitment of early myeloid progenitors.

Starting from a population of pluripotent hematopoietic stem cells (PHSC),† the process of hematopoietic differentiation culminates in the production of multiple functionally and morphologically distinct blood cell types. The mature cells have relatively short lifespans and must be repopulated continuously from the pool of PHSC. Hematopoietic differentiation is a highly regulated process, and a growing number of regulatory molecules have been identified which are involved both in the normal differentiation process and also in the aberrant hematopoiesis seen in leukemia and other hematological disorders. For example, the hematopoietic growth factors recognize specific receptors on the surface of subsets of hematopoietic cells at different stages of development and stimulate their further differentiation, proliferation, and/or survival (1). In addition, many transcriptional regulators have been identified which are expressed in cell-specific patterns in hematopoietic cells and which are directly associated with hematopoietic differentiation or leukemia (2, 3).

Another class of factors which modulates hematopoiesis consists of small biologically active molecules such as retinoids, particularly retinoic acid (4), prostaglandins, and steroids (5, 6). With the notable exception of retinoic acid, which acts as a ligand for a family of nuclear hormone receptors, the mechanisms of action of such molecules are poorly understood. One model is that such molecules may act as autocrine or intracrine regulators of hematopoietic commitment, proliferation, and terminal maturation; thus, the identification of enzymes that catalyze the synthesis or catabolism of these molecules in hematopoietic cells is of great interest. Recently, for example, a member of a superfamily of intracellular oxidoreductases, the aldoketo reductases (AKRs), has been implicated in the synthesis of autocrine factors that may interfere with retinoic acid signaling during granulocytic differentiation (7).

Various techniques have been used to identify genes that encode regulators of hematopoietic differentiation. For example, genetic studies in mice revealed critical lineage-specific roles for transcriptional regulators such as C/EBPa (8), GATA-1 (9), PU.1 (10), and many others (3, 11). Elucidation of the genetic alterations underlying certain leukemias, including chromosomal translocations and more subtle mutations, has revealed the hematopoietic functions of proteins such as PLZF and AML-1 (3). In addition, new techniques have been developed to identify genes that are expressed differentially in two cell populations, including PCR-based methods such as differential display (12) and representational difference analysis.

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The nucleotide sequence(s) reported in this paper has been submitted to the GenBank™/EBI Data Bank with accession number(s) AF177941.

** To whom reprint requests should be addressed: Dept. of Cell Biology and Biochemistry, Texas Tech University Health Sciences Center, 3601 4th St., Lubbock, TX 79430. Tel.: 806-743-2524; Fax: 806-743-2990; E-mail: Simon.Williams@ttmehsc.edu.

† The abbreviations used are: PHSC(s), pluripotent hematopoietic stem cell(s); AKR, aldoketo reductase; C/EBP, CCAAT/enhancer-binding protein; PCR, polymerase chain reaction; RDA, representational difference analysis; RAR, retinoic acid receptor; SCF, stem cell factor; IL, interleukin; atRA, all-trans-retinoic acid; GM-CSF, granulocyte/macrophage colony-stimulating factor; BHK, baby hamster kidney; FBS, fetal bovine serum; CM, conditioned medium; GADD45, glyceraldehyde phosphate dehydrogenase; bp, base pair(s); DP, difference product; RACE, rapid amplification of cDNA ends; UTR, untranslated region; HSD, hydroxysteroid dehydrogenase; RAK, rat aldoketo reductase; NSAIDs, non-steroidal anti-inflammatory drugs.
Aldoketo Reductases and Myelopoiesis

The relative scarcity of PHSCs in bone marrow (0.01–0.005% of nucleated cells) and the difficulty in isolating pure populations of cells at specific stages of differentiation have hampered the molecular analysis of hematopoietic development (2). To address this problem, cell lines representative of different stages of hematopoietic differentiation have been established over the past few decades. In certain cases, including the human HL-60 (18) and murine 32DCl3.19 and M1 (20) cell lines, differentiation may be regulated in culture permitting the analysis of the role of putative regulatory genes. Shortcomings of these studies include the leukemic origin of many immortalized cell lines and their limited differentiative capacity. A pair of recently established murine cell lines, named EML and MPRO, permit the analysis of hematopoietic development over the whole spectrum of differentiation from the pluripotent progenitor cell to terminally differentiated granulocytes and macrophages (21, 22).

The EML cell line was established by forcing expression of a dominant negative retinoic acid receptor α (RARα) molecule in murine bone marrow cells and subsequently selecting cells that were dependent on stem cell factor (SCF) for survival (22). The resulting cell line expresses many markers of hematopoietic progenitor cells (Lin<sup>+</sup>, c-Kit<sup>+</sup>, Sca-1<sup>+</sup>) (23) and grows continuously in the presence of stem cell factor. Although a limited number of prepro-B cells and erythroid progenitors arise spontaneously in culture, myeloid differentiation is inhibited by the dominant negative RARα. This block can be overcome by culturing in the presence of the multifunctional cytokine interleukin-3 (IL-3) and pharmacological doses of all-trans-retinoic acid (atRA) (17, 22). Under these conditions, EML cells differentiate into promyelocytes over a period of 6–7 days, and differentiation-specific genes are expressed in patterns similar to those seen in bona fide hematopoietic progenitors (17, 24, 25). Terminal granulocytic differentiation can be achieved by removing IL-3 after 3 days and replacing it with granulocyte/macrophage colony-stimulating factor (GM-CSF) (22, 25). In this system, IL-3 promotes the survival and expansion of cells committed to the myeloid and other lineages (17, 22). atRA appears to act at two separate stages, first in combination with IL-3 to promote the appearance of early myeloid progenitors and second in combination with GM-CSF to promote the terminal maturation of committed granulocyte/monocyte progenitors (22, 26). The MPRO cell line was established using the same dominant negative RARα as EML but is arrested at the promyelocyte stage of granulocytic differentiation (21, 22). MPRO cells are GM-CSF-dependent, and terminal granulocytic differentiation is induced using high levels of atRA. Using these two cell lines, it should be possible to identify genes involved at various stages of granulocytic differentiation.

In this study we sought to identify genes that are activated during the initial stages of myeloid differentiation by using RDA to isolate genes whose expression is elevated 72 h after the addition of IL-3 and atRA to the EML culture. This time point was chosen because although many cells have become committed to the myeloid lineage at this stage, as measured by their responsiveness to GM-CSF (17), few cells have reached terminal maturation (25). We identified a novel member of the AKR superfamily and named it mouse aldoketo reductase α (mAKRa). Based on its expression pattern in EML and MPRO cells, and the ability of AKRs to metabolize molecules such as prostaglandins and steroids, we propose that it may function in the synthesis or catabolism of autocrine/intracrine factors that regulate myeloid differentiation.

**EXPERIMENTAL PROCEDURES**

**Cell Culture—** BHK-MKL, BHK-HM5, L (fibroblast), and NIH 3T3 (fibroblast) cell lines were maintained in Dulbecco’s modified Eagle’s medium (BioWhittaker, Walkersville, MD) supplemented with 10% fetal bovine serum (FBS, Atlanta Biological, Norcross, GA). P388 (pre-B lymphoblast), P388D1(IL-1) (macrophage), M1 (myeloid), WEHI-3 (melanoma/monocyte), and IC21 (macrophage) cell lines were cultured in RPMI 1640 (BioWhittaker) supplemented with 5% FBS. 32DCl3 (myeloblast), FDCP-1 (myeloid progenitor), and NFS-68 (promyelocyte) cells were cultured in RPMI 1640 plus 5% FBS supplemented with 20% WEHI-3-conditioned medium (CM) as a source of IL-3. M-NSF-68 (promyelocyte) cells were maintained in RPMI 1640 medium containing 10% FBS and 1% L cell CM as a source of macrophage colony-stimulating factor. GG2EE (macrophage) and WEHI 274.1 (monocyte) cell lines were maintained in RPMI 1640 containing 10% FBS and 10% BHK-HM5 CM as a source of GM-CSF. EML and EPRO cells were maintained in Iscove’s modified Dulbecco’s medium containing 10% FBS and 10% BHK-HM5 CM (as a source of SCF) or 10% BHK-HM5 CM, respectively.

Myeloid differentiation of EML cells was initiated by plating at 1 × 10<sup>6</sup> cells/ml in Iscove’s modified Dulbecco’s medium containing 20% horse serum, 10% WEHI-3 CM, 10% BHK-MKL CM, and 1 × 10<sup>-5</sup> M atRA (Sigma) for a 6-day period. Terminal granulocytic differentiation was induced by washing cells after 72 h in culture under the conditions described above and replating in Iscove’s modified Dulbecco’s medium containing 20% horse serum, 10% BHK-HM5 CM, and 1 × 10<sup>-5</sup> M atRA. Terminal granulocytic differentiation of MPRO cells was initiated by replating cells in fresh medium containing 1 × 10<sup>-7</sup> M all-trans retinoic acid. In all differentiation experiments cells were fed with fresh medium after 48 h and every 24 h thereafter. Cells were harvested at various times for RNA preparation.

**RNA Preparation and Northern Blotting—** Cellular RNA was prepared from mouse tissues and cell lines using a modified guanidine isothiocyanate/phenol extraction procedure and analyzed as described previously (27). Northern blots were analyzed on a Molecular Dynamics 4458i PhosphorImager for quantitation of relative mRNA levels. mAKRa mRNA levels were calculated after normalization to the level of glyceraldehyde phosphate dehydrogenase (GAPDH) mRNA. The mAKRa probe was a DpnII fragment that was isolated in the RDA procedure (see below), and the GAPDH probe was a 200-bp fragment amplified by PCR using the following oligonucleotide primers: GAPDH 5′-AAGTGGAGATCCACCGGATT and GAPDH 3′-TTGATGACAACTTCCCGT (24). The C/EBPβ probe was an 850-bp NeolHin III fragment containing the entire murine CDNA (24).

**RDA—** RDA (14) was carried out to identify genes expressed at higher levels in EML cells cultured in the presence of IL-3 and atRA for 72 h compared to unstimulated EML cells. Briefly, total RNA was isolated from EML cells cultured in the presence of SCF or in the presence of SCF, IL-3, and atRA, and poly(A)<sup>+</sup> RNA was isolated using the poly(A) spin mRNA isolation kit (New England Biolabs, Beverly, MA). Double-stranded cDNA was synthesized from 4 μg of poly(A)<sup>+</sup> RNA using a cDNA synthesis kit (Life Technologies, Inc.). Three rounds of RDA were performed using R-Bgl-12, R-Bgl-24, J-Bgl-12, J-Bgl-24, N-Bgl-12, and N-Bgl-24 adaptors and primers described previously (13, 14). The cDNAs described above were digested with DpnII and ligated to the R-Bgl adaptors. Amplifications were synthesized by 23 cycles of PCR amplification of the R-ligated cDNA fragments using the R-Bgl-24 oligonucleotide. DpnII digestion of the representation derived from EML cells cultured in the presence of SCF alone generated driver cDNA. Tester cDNA was prepared by DpnII digestion of the representation derived from EML cells cultured in the presence of SCF, IL-3, and atRA for 72 h followed by ligation of J-Bgl adaptors. The first subtractive hybridization was performed combining 40 μg of driver cDNA and 0.4 μg of tester cDNA (tester:driver = 1:100). An aliquot of the hybridization mix was amplified by PCR using the J-Bgl-24 primer. The PCR products were then digested with PvuI and cloned into the pBluescript vector (Stratagene, La Jolla, CA) and sequenced.

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rylated BamHI-digested vector. Eight unique clones were isolated, and six of these displayed the expected differential expression pattern in EML cells.2

Data-base searches with the sequence of one subclone (1603) revealed that it encoded a portion of a protein related to members of the AKR superfamily (28). The initial clone contained the coding sequence for 86 amino acids at the COOH terminus of the protein. Two rounds of nested 5'-rapid amplification of cDNA ends (RACE) reactions were carried out to isolate the 5' end of the cDNA. 5'-RACE was carried out on Marathon-Ready cDNA from mouse spleen using the Advantage cDNA PCR kit (both from Clontech) as described previously (24). The primers used for 5'-RACE reactions were: primer 1, CAG AAA TGA TGA TGT TTC CAT TCA CCA G; and nested primer, CAG GCA GAA GTC ATG ATT AGT TC. The resulting cDNA products were subcloned into pGEM-T easy vector (Promega, Madison, WI).

**Fig. 1.** Identification of differentially expressed genes in EML-C1 cells at 0 h and 72 h after the addition of IL-3 and atRA. Panel A, three rounds of RDA were carried out to identify cDNAs expressed at higher levels after 72 h of culture in IL-3 and atRA. An aliquot of starting cDNA and difference products from the three rounds of RDA were electrophoresed on a 1.2% agarose gel and stained with ethidium bromide. Distinct bands were evident after the third round of RDA, and each was isolated from the gel and subcloned. The sizes in base pairs of molecular weight markers are shown on the left of the ethidium bromide-stained gel. Panel B, the most intensely staining band (indicated with an **arrow**) was used to probe a Northern blot containing RNA from 0- and 72-h EML-C1 cells. The Northern blot confirmed that this cDNA represented a mRNA that was expressed at significantly higher levels in the 72-h sample (12–16-fold as determined by PhosphorImage analysis of three repetitions of this experiment).

**RESULTS**

**Identification of Genes That Are Expressed Differentially during Myeloid Differentiation of EML Cells by RDA—**RDA is a recently developed technique that combines the selective power of subtractive hybridization with the sensitivity of PCR to isolate cDNAs representing genes that are expressed differentially in two cell populations (14). Our experimental approach was designed to identify genes that encode potential regulators of the myeloid differentiation process, rather than genes expressed in terminally differentiated neutrophils and/or macrophages. Therefore, we used RDA to isolate cDNA clones that were expressed more robustly in EML cells 72 h after the addition of IL-3 and atRA versus unstimulated cells. At least eight distinct bands representing potential differentially expressed genes were evident after three rounds of RDA (Fig. 1). Six of these bands represented **bona fide** differentially expressed genes as assessed by Northern blotting (data not shown). Sequence analysis identified genes known to be expressed in mature myeloid cells (lysozyme), genes encoding enzymes involved in serotonin and histamine biosynthesis, and two genes whose identity is currently unknown.2 However, this report will focus on the gene represented by the most intense band in the DP3 lane (indicated with an **arrow** in Fig. 1A, lane 5). After subcloning, this band was used to probe an RNA blot containing treated and untreated EML RNA samples. A single mRNA species of approximately 1400 nucleotides was detected in both EML samples, and the level of this mRNA was 12–16-fold higher in EML at 72 h versus 0 h (Fig. 1B).

The cDNA clone was sequenced and found to contain an 86-amino acid open reading frame. Comparison with sequences in public databases revealed that the encoded protein had not been deposited previously and that it displayed significant sequence similarity to multiple members of the AKR superfamily. The AKRs are cytosolic enzymes that catalyze the NAD(P)(H)-dependent reduction of a variety of carbonyl compounds, including molecules implicated as regulators of hematopoietic differentiation such as steroids and prostaglandins (28, 30).

**Characterization of the mAKRa cDNA and Peptide—**To gain some insight into the identity and potential functions of mAKRa, we characterized the full-length cDNA and determined the relatedness of the mAKRa polypeptide to other AKR family members. The complete mAKRa sequence has been deposited in GenBank under accession number AF177041 and is depicted in Fig. 2. The 1108-nucleotide mAKRa cDNA includes 34 bases from the 5'-untranslated region (UTR) and 105 bases from the 3'-UTR. The longest open reading frame encodes a protein of 323 amino acids with a predicted molecular weight of 38,000 and a pl of 6.6.

A new nomenclature has been introduced recently for the AKR superfamily which assigns names to proteins based on their level of sequence similarity to other family members, and this information can be useful in predicting potential substrates (30). By carrying out pairwise comparisons between mAKRa and proteins representative of the seven families of AKRs, we determined that mAKRa is likely to be a member of the AKR1 family (data not shown). The AKR1 family is divided further into subfamilies (30), and Fig. 3 shows the alignment of mAKRa and representatives of four of the five subfamilies within the AKR1 subfamily, namely AKR1C9 (3α-HSD) (31), AKR1D2 (rat D4–3-ketosteroid 5β-reductase (32)), AKR1B4 (rat aldose reductase (33)), and AKR1A3 (rat aldehyde reductase (34)). In addition, this comparison includes three relatively uncharacterized AKR proteins (rat aldoketo reductase (RAKb, RAKd, and RAKf) that were originally identified based on their

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cross-reactivity to antisera raised against rat 3α-HSD (35).

This alignment highlights amino acid differences between mAKRa and each of the other proteins, and it can be seen that mAKRa is most similar to the three RAK proteins than to the more characterized family members. Previous studies have identified 19 amino acids that are either totally or highly conserved within the AKR superfamily (28). These positions are shaded in this alignment showing that 18 of the 19 residues are conserved in mAKRa. The only exception is residue 276, which is an arginine in most AKRs but is a glutamic acid in mAKRa.

Membership in an AKR subfamily is defined at the 60% sequence identity level (30). To determine whether mAKRa defines a new subfamily in the AKR1 family or is a novel member of an existing subfamily a dendrogram was constructed which displayed the degree of sequence identity among the eight proteins shown above (Fig. 4). As expected from the alignment in Fig. 3, mAKRa was most similar to the three RAK proteins, sharing between 87 and 92% identity. mAKRa (and each of the RAK proteins) shared between 64 and 67% sequence identity with AKR1C9 (3α-HSD) but less than 60% identity with each of the other three proteins (AKR1D2 (49–51%), AKR1B4 (48–49%), and AKR1A3 (42–43%). Similar levels of sequence identity were observed in comparing mAKRa with other members of each of these subfamilies (data not shown), indicating that mAKRa, along with the three RAK proteins, form a highly related group within the AKR1C subfamily.

mAKRa Expression Profiles in Tissues and Hematopoietic Cell Lines—Having shown that mAKRa was a novel member of the AKR superfamily, we carried out RNA blot analyses to identify tissues and cell types that express mAKRa mRNA. In a survey of mouse tissues prepared from fetal (14 days postcoitum) and adult animals, mAKRa mRNA was detected at high levels in fetal and adult liver and at low levels in adult kidney (Fig. 5A). Fetal liver is primarily a hematopoietic tissue at 14 days postcoitum, suggesting that mAKRa is expressed in normal hematopoietic cells. mAKRa-specific signals were also detected at lower levels in adult bone marrow, thymus, and spleen (Fig. 5B) but were undetectable in fetal lung and adult heart and brain.

Because the EML differentiation protocol used to isolate mAKRa causes differentiation along the myeloid lineage, we examined mAKRa expression in hematopoietic cell lines to test whether it is expressed in a lineage- or stage-specific manner. Total RNA was prepared from immortalized cell lines representative of various stages of myeloid differentiation and was analyzed by Northern blotting (Fig. 6). The strongest signals were detected in cells representative of the promyelocyte stage of granulocytic differentiation (EPRO, MPRO, and NFS-58); an intermediate signal was detected in the IL-3-dependent
FDCP-1 early myeloid progenitor line; and weak signals in M1 myelocyte, the WEHI-3 myelomonocyte cell lines, M-NFS-60, a macrophage CSF-dependent derivative of NFS-60 promyelocytes, and three macrophage cell lines (GG2EE, P388D1(IL-1) and IC-21). mAKRa mRNA was undetectable in WEHI 274.1 monocytes.

Having shown that mAKRa mRNA is present in normal hematopoietic tissues and is expressed at highest levels in cells at intermediate stages of the hematopoietic differentiation process, we returned to the EML cell line to investigate further the expression profile of mAKRa during experimentally induced differentiation. EML cells were cultured in the presence of IL-3 and atRA, and total RNA was prepared at various time points over a period of 144 h. Relative mAKRa mRNA levels were determined by Northern analysis (Fig. 7A). mAKRa mRNA levels were low in unstimulated cells (0 h) but increased rapidly to reach maximal levels after 24 h. In a series of experiments, mAKRa mRNA levels increased 12–16-fold (13.2-fold in this example) over the first 24 h, during which time commitment to the myeloid lineage and extensive proliferation occur. mAKRa mRNA levels remained relatively high over the subsequent 2 days and then decreased over the final 3 days of the differentiation time course to approximately 6.5-fold over the initial level (Fig. 7A).

Culturing EML cells in the presence of IL-3 and atRA promotes differentiation to the promyelocyte stage of the granulocytic lineage, but the addition of the more lineage-specific growth factor GM-CSF is required to achieve terminal granulocytic maturation (22, 25). Therefore, we carried out a second differentiation experiment in which IL-3 and SCF were removed after 72 h and replaced with GM-CSF. Relative mAKRa mRNA levels were again determined by Northern analysis (Fig. 7B). A similar pattern of expression was observed over the first 72 h; however, mAKRa levels decreased significantly after the addition of GM-CSF to below basal levels. To demonstrate that the cytokines were eliciting the appropriate response in these EML cells, both Northern blots were reanalyzed using a probe for C/EBP\(\epsilon\), a member of the CCAAT/enhancer-binding protein family of basic region/leucine zipper transcription factors (24). C/EBP\(\epsilon\) mRNA levels were undetectable in EML cells cultured in the presence of SCF alone and increased toward the later stages of the differentiation pathway.
way under both culture conditions (Fig. 7, A and B). C/EBP\(\epsilon\) mRNA levels increased dramatically during terminal granulocytic differentiation after IL-3 was removed from the culture and replaced by GM-CSF (Fig. 5 B). The data from the EML cell experiments indicated that mAKRa expression decreases during terminal granulocytic differentiation but did not distinguish between the relative contribution of atRA and GM-CSF to this effect. In addition, significant cell death is commonly observed in EML cell cultures after replacing IL-3 and SCF with GM-CSF (22). Therefore, it was not clear whether the decrease in mAKRa mRNA levels was caused by the death of mAKRa-expressing cells or by down-regulation of mAKRa expression in surviving GM-CSF-responsive cells. To address these questions we examined mAKRa expression levels during atRA-induced terminal granulocytic differentiation of the GM-CSF-dependent MPRO cell line. MPRO cells are dependent on GM-CSF for survival, and previous studies have shown that greater than 90% of the cells will mature into terminally differentiated granulocytes in 4–8 days after atRA stimulation (21, 25). MPRO cells were cultured in the presence of GM-CSF and atRA, and total RNA was harvested at 2-day intervals for Northern analysis (Fig. 8). High levels of mAKRa mRNA were detected in MPRO cells cultured in GM-CSF alone. However, mAKRa mRNA levels decreased during atRA-driven maturation to approximately 30% of the level in untreated cells after 2 days and to about 10% of maximal levels on subsequent days. Reanalysis of this blot with the C/EBP\(\epsilon\)-specific probe revealed the expected differentiation-related increase in C/EBP\(\epsilon\) mRNA levels after atRA addition and is consistent with the observation that expression of the cebp\(e\) gene is stimulated by atRA during terminal granulocyte maturation (38).

Factor-dependent Regulation of mAKRa Expression—Having shown that the decrease in mAKRa mRNA levels during terminal granulocytic maturation appears to be dependent on atRA, we examined lastly whether the initial increase in mAKRa mRNA levels during myeloid differentiation in EML cells was dependent on atRA or IL-3. For these studies, EML cells were grown in the presence of SCF and stimulated for various times up to 48 h with IL-3 alone, atRA alone, or the combination of IL-3 and atRA as a positive control. Total RNA was prepared at various times after factor addition and analyzed by Northern blotting (Fig. 9). mAKRa mRNA levels were unaffected by atRA alone but were significantly and rapidly elevated by IL-3 alone. The level of mAKRa mRNA after IL-3 treatment was similar to that seen with both IL-3 and atRA, suggesting that the rapid increase in mAKRa mRNA levels in the first 24 h of the differentiation process is primarily the result of IL-3-dependent increases in mAKRa transcription.

DISCUSSION

The commitment, proliferation, and maturation of the multiple blood cell lineages from the PHSC are controlled by a growing number of proteins including the hematopoietic growth factors (1), cytokines and chemokines (39), intracellular
protein kinases (40, 41), and nuclear transcription factors (2). In addition, a number of non-protein factors, including prostaglandins (5, 42), steroids, and retinoids (4) have been implicated as regulators of hematopoiesis. In a search for genes whose expression is activated during commitment and proliferation of myeloid progenitors, we isolated the gene encoding a novel member of the AKR superfamily of oxidoreductases, named mAKRa. Based on the expression pattern and potential function of mAKRa, we propose that it may play a role in the metabolism of small biologically active molecules, such as steroids or prostaglandins, which may function in the autocrine or intracrine regulation of myelopoiesis.

mAKRa Is a New Member of the AKR Superfamily—To date, the AKR superfamily contains more than 40 unique members isolated from bacteria, yeast, plants, invertebrates, and vertebrates (28). The AKRs catalyze the metabolism of a diverse array of substrates, and individual family members have been implicated in human diseases such as diabetes and asthma (28). Our sequence analysis has placed mAKRa into the AKR1C subfamily (30), which includes proteins such as 3α-HSD, chlordecone reductase, 20α-HSD, and prostaglandin F synthase (28, 43). Proteins within this subfamily catalyze the interconversion of weak and potent forms of steroid hormones and prostaglandins among their many functions and have been proposed to act as “molecular switches” by regulating the amount of hormone available to bind to a nuclear hormone receptor (43). Potential physiological substrates for mAKRa include steroids, prostaglandins, and retinoids, and identification of the specific substrate(s) for mAKRa will be critical for determining whether it does indeed serve as “molecular switch” in the regulation of myelopoiesis.

Unfortunately, the substrates for the most closely related AKRs, RAKb, RAKd, and RAKf, are unknown (35). However, structure/function analyses of various other AKRs have begun to identify residues that may play a role in determining whether the enzyme substrate is a sugar or a steroid (28). Position 54 is generally occupied by a leucine or isoleucine residue in HSDs and by a valine in aldehyde reductases (28);
however, it is an alanine in mAKRa and each of the RAKs. Likewise, residue 118 is a tryptophan in aldose reductases, phenylalanine in HSDs, and valine or methionine in 5β-reductases (28), but it is a tyrosine in mAKRa and the RAKs. Therefore, if these residues are indeed substrate-selective, this may indicate that mAKRa and the RAKs utilize similar or identical substrates that differ from the most common substrates of related family members. Biochemical analysis of the activity of recombinant mAKRa is under way to address this question.

Regulation of mAKRa Expression in Hematopoietic Cells: Implications for Function—Our data demonstrate that mAKRa mRNA is present at low levels in murine hematopoietic tissues, including fetal liver and adult bone marrow and thymus, and is expressed robustly in myeloid cell lines, particularly those at the promyelocyte stage. However, the strongest evidence that mAKRa may play a role in hematopoietic differentiation has come from our studies on the EML and MPRO cell lines. We and others have used these cell lines to examine the expression pattern of genes during myeloid differentiation, and, in general, expression patterns agree with those seen in normal hematopoietic cells (17, 24, 25, 44). Therefore, although we have not yet examined the expression of mAKRa in bona fide hematopoietic progenitors, it is likely that our findings in EML and MPRO will be relevant to its role in hematopoiesis.

The initial stages of granulocytic differentiation of multipotent EML cells require the combined action of IL-3 and atRA, whereas the terminal stages of the process occur in the presence of atRA and GM-CSF (22). By examining the relative contributions of each of these factors to the regulation of mAKRa expression, we can begin to predict the likely function of mAKRa during the differentiation process. First, IL-3 was responsible for the initial increase in mAKRa mRNA levels which resulted in peak levels being achieved after just 24 h. The fact that mAKRa mRNA levels began to increase within the first 4 h suggests that mAKRa may be a direct target for an IL-3-dependent signaling pathway and therefore may be critical for mediating the early effects of IL-3. IL-3 was first identified as a soluble factor synthesized by T cells (45) and has since been shown in numerous studies in vitro to be capable of promoting the proliferation and differentiation of hematopoietic progenitor cells of the granulocyte, macrophage, eosinophil, erythroid, and megakaryocytic lineages (46). In the EML cell line, IL-3 promotes the expansion and lineage commitment of many of these same lineages over the first 3–4 days of culture, leading to significant increases in the number of cells responsive to more lineage-specific factors such as erythropoietin and GM-CSF (17). Therefore, mAKRa may function in the production of factors that promote the proliferation of committed myeloid progenitors.

atRA had no apparent effects on mAKRa mRNA during the first 48 h of culture when the function of retinoic acid appears to be to delay the differentiation of primitive hematopoietic progenitors (26). However, atRA appeared to be the critical factor promoting the decrease in mAKRa mRNA levels during the terminal stages of myeloid maturation in EML cells. This effect was most apparent in MPRO cells but was also seen in both EML time course experiments. There is significant evidence that retinoic acid promotes terminal granulocytic differentiation (47). For example, atRA can promote terminal differentiation of human promyelocytic leukemia cell lines such as HL-60 (18). In addition, atRA has proven to be effective in differentiative therapy of patients with acute promyelocytic leukemia, which carries translocations that affect the RARα gene (48–51). Although the dominant negative RARα403 molecule used to establish the EML and MPRO cell lines differs from the PML-RARα chimeric protein associated with the most common form of acute promyelocytic leukemia, it is likely that they act via similar pathways (52). Thus, the identification of atRA-regulated genes in EML and MPRO cell lines may provide important information about the molecular events underlying acute promyelocytic leukemia. The mAKRa expression patterns observed in these cell lines are consistent with mAKRa being associated with proliferation of myeloid precursor cells and the need to extinguish mAKRa expression as terminally differentiated cells exit the cell cycle. Additional experiments will clearly be necessary to determine whether atRA directly affects mAKRa expression levels and whether prolonged expression of mAKRa might delay terminal granulocytic differentiation.

Although the exact function of mAKRa in hematopoietic cells remains to be firmly established, we favor the hypothesis that it functions in the production or catabolism of factors that act in an autocrine or intracrine fashion to regulate hematopoietic development. A similar hypothesis has been developed to explain the ability of non-steroidal anti-inflammatory drugs (NSAIDs) to potentiate the retinoic acid-mediated differentiation of the human HL-60 promyelocytic cell line (53, 54). NSAIDs are capable of inhibiting a broad spectrum of enzymes, including the cyclooxygenases that convert arachidonic acid to biologically active prostanoids (55), and 3α-HSDs (56). However, the effects of the NSAIDs in HL-60 cells do not appear to be mediated by the prostanooid biosynthetic pathway or by a known 3α-HSD (54). Instead the target for NSAIDs is likely to be a member of the A KR family, and a novel 3α-HSD-related, retinoic acid-regulated AKR, HAKRα, has been identified in HL-60 cells (7). In addition, ectopic expression of 3α-HSD in HL-60 cells confers increased resistance to the differentiation effects of retinoic acid (57). Thus, there is growing evidence that AKRs are involved in the regulation of myeloid differentiation, and examination of the functions of mAKRa and other family members may be important for the understanding of normal hematopoiesis and the detection and treatment of certain types of leukemia.

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