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Modulation of expression of RA-regulated genes by the oncoprotein v-erbA

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ABSTRACT

Retinoic acid (RA) modulates the expression of genes involved in embryogenesis, development and differentiation processes in vertebrates. The v-erbA oncogene is known to exert a dominant-negative effect on the expression of RA-responsive genes. v-erbA belongs to a superfamily of transcription factors called nuclear receptors, which includes the retinoic acid receptors (RARs) responsible for mediating the effects of retinoic acid. While RA inhibits cell proliferation and promotes cell differentiation and apoptosis in a variety of tissues, v-erbA seems to play a role in oncogenesis, namely in the development of hepatocellular carcinoma (HCC) in a transgenic mouse model.

In order to study the effect of v-erbA on RA-responsive genes, we used microarray analysis to identify genes differentially expressed in murine hepatocytes in culture (AML12 cells) stably transfected with v-erbA and exposed to RA for 3 h or 24 h. We have identified RA-responsive genes that are affected by v-erbA, as well as genes that are regulated by v-erbA alone. We have found that v-erbA can affect gene expression in the presence of RA and at the level of basal transcription. We have also identified a number of v-erbA-responsive genes that are known to be involved in carcinogenesis and which may play a role in the development of HCC.

1. Introduction

As modulator of gene expression, retinoic acid (RA) plays important roles in embryonic development, pattern formation, and in the regulation of cell differentiation and proliferation. RA is the natural ligand for two types of nuclear receptors (hormone-regulated transcription factors), the retinoid acid receptors (RARs) and the retinoic X receptors (RXRs) (Leid et al., 1992). The physiological unit responsible for RA response is a heterodimer with RXR which binds to RA-response elements (RARE) typically located upstream of target genes (Mader et al., 1993).

The mechanisms of transcriptional activation by RARs are relatively well understood and involve recruitment of transcriptional co-repressors and co-activators (reviewed in Weston et al., 2003). In addition, RA is now recognized in ligand-dependent transcriptional repression (Fernandes et al., 2003).

The oncoprotein v-erbA, a mutated version of thyroid hormone receptor α (TRα) has been shown to act as constitutive dominant repressor of transcription of genes regulated by TR and RAR (Damm et al., 1989; Sharif and Privalsky, 1991). The exact mechanism of v-erbA repression has not been determined, but it is believed to involve competition for response elements (Selmi and Samuels, 1991) and co-regulators (Zubkova and Subauste, 2004), as well as sequestration of TR and RXR in the cytoplasm and interference with the translocation of these nuclear receptors to the nucleus (Bonamy and Allison, 2006). The repressor activity of v-erbA is linked to oncogenesis in avian erythroleukemia (AEL) (Rietveld et al., 2001). In addition, transgenic mice over-expressing v-erbA develop hepatocellular carcinoma (HCC) (Barlow et al., 1994). It has been suggested that the mutations acquired by v-erbA may be responsible for its oncogenicity by altering gene transcription (Lee and Privalsky, 2005). However, the identity of the genes affected by v-erbA has not been thoroughly investigated.

Previous microarray analyses in our laboratory have identified RA-responsive genes and analyzed their importance in the context of liver metabolism using the AML12 cell line as a model system (Mamoon et al., 2008). The aim of the present study is to contribute to the understanding of nuclear receptor-mediated transcriptional regulation by investigating the effect of v-erbA on the expression of RA-responsive genes. In addition, this study will investigate the role of v-erbA in the regulation of gene expression in the absence of RA and its relevance to the development of HCC.

Abbreviations: AEL, avian erythroleukemia; Ang, Angiogenin; ApoA-I, apolipoprotein A-I; ApoA-II, apolipoprotein A-II; C1d, nuclear binding protein; Cyp3a41, cytochrome P450, family 3, subfamily a, polypeptide 41; dCHIP, DNA-Chip analyzer; Fgfr3, fibroblast growth factor receptor 3; Fgg, fibroblast growth factor; HCC, hepatocellular carcinoma; Lgals2, galectin 2; Mt1, metallothionein; qPCR, real-time PCR; RA, retinoic acid; RAR, retinoic acid receptor; RARE, retinoic acid response element; RXR, retinoic X receptor; TR, thyroid hormone receptor.

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2. Materials and methods

2.1. Cell culture

AML 12 cells were grown in modified Eagles's/Ham's F-12 medium supplemented with 10% fetal bovine serum (Hyclone, Logan, UT), 100 nM dexamethasone and ITS (insulin, transferrin and selenium, Invitrogen) at 37 °C in an atmosphere with 5% CO_2.

AML12 cells were transfected with the gag-v-erbA oncogene cloned into the pcDNA3.1/H^+ vector using Lipofectamine Plus (Invitrogen). Transfected cells were selected in culture medium supplemented with hygromycin at 100 μg/ml. Stable transfectants selected two weeks after transfection were confirmed by Northern blot analysis using a ^{32}P-labeled gag riboprobe. The V6 clone was selected for further studies based on a strong hybridization signal with the gag probe. Expression of v-erbA protein in the V6 clone was determined by immunoblot using a rabbit antiserum raised against a bacterially-synthesized subdomain of v-erbA (a generous gift from Dr. Martin Privalsky).

In order to investigate the effect of v-erbA on RA-responsive genes, AML12 control cells and cells stably transfected with v-erbA were exposed to 1 μM RA for 3 h and 24 h. Controls for both groups were cells that were not exposed to RA.

2.2. Microarrays

RNA for gene expression analysis was obtained from untreated AML12 cells, AML12 cells exposed to RA, untreated v-erbA-transfected cells, and v-erbA-transfected cells exposed to RA. RNA isolation and preparation for microarray analysis have been previously described (Mamoon et al., 2008). The Affymetrix GeneChip Mouse Genome 430 2.0 array consisting of over 39,000 transcripts representing well-known genes was used for these studies. Assays of triplicate samples were performed at the core facility at the University of Missouri at Columbia. Expression data thus obtained were analyzed by the software DNA-Chip Analyzer (dCHIP) available at www.dchip.org. Microarray expression data was deposited in the Gene Expression Omnibus database (GEO, National Center for Biotechnology Information) under accession numbers GSM296415, GSM296416, GSM296417, GSM297234, GSM297235, GSM297238, and GSM297243.

2.3. Quantitative, real-time PCR (qPCR)

Quantitative PCR reactions were performed in a DNA Engine Opticon 2 System (MJ Research Inc., Waltham, MA) as previously described (Mamoon et al., 2008). Samples were prepared using the DyNAmo™ SYBR® Green qPCR kit from Finnzymes (New England Biolabs, Beverly, MA). Gene-specific primer sequences can be found in Table 1. Gapdh expression was used to normalize the data to correct for possible differences in reverse transcription.

3. Results and discussion

3.1. Expression of v-erbA in AML12 cells

We have previously demonstrated that AML12 cells constitutively express adequate amounts of RARs and RXRs to mediate ligand-dependent activation of RA-responsive genes (Mamoon et al., 2008). In order to study the effect of v-erbA on the regulation of RA-dependent genes in this system, AML12 cells were stably transfected with the gag-v-erbA fusion protein cloned into a pcDNA vector containing the hygromycin-resistance gene. Expression of v-erbA in a number of hygromycin-resistant clones was confirmed by Northern blot with a probe for the gag gene, and a clone that showed high level of expression (clone V6) was selected for subsequent studies (Fig. 1A). Expression of the v-erbA protein in the V6 clone was confirmed both in the presence and absence of RA by immunoblot (Fig. 1B) using an antibody raised against a bacterially-synthesized subdomain of v-erbA.

In order to investigate the effect of v-erbA on RA-responsive genes, AML12 control cells and V6 cells were exposed to 1 μM RA for 3 h or 24 h. RA-regulated genes differentially expressed in the presence of v-erbA were identified using microarray analysis. Although the microarray threshold was set to 1.5, we have elected to concentrate on genes that showed changes in expression ≥2. Microarray results were validated by quantitative, real-time PCR (qPCR) of a sample group of genes using primers specific for those genes. Although there are differences in the sensitivity of the two methods, we found that the microarray data agreed well with the qPCR results (see Supplementary Files).

We found 475 genes up-regulated ≥2-fold at 3 h, and 505 genes down-regulated in the same time period in v-erbA-transfected, RA-treated cells. At 24 h, we found 562 genes up-regulated, and 601 genes down-regulated ≥2-fold under the same conditions.

In addition to the effect of v-erbA on gene expression in RA-treated cells, we observed that genes could be affected by v-erbA alone. We found 454 genes up-regulated and 627 genes down-regulated ≥2-fold at 3 h in untreated, v-erbA-transfected cells. Under the same conditions, 590 genes were up-regulated and 724 genes were down-regulated ≥2-fold at 24 h. Of these, 120 (3 h) and 217 (24 h) genes were also RA-responsive genes.

In the interest of clarity in the discussion of these results, we have selected examples of groups of genes expressed in a coordinated manner across a set of conditions to gain insight into the molecular mechanisms involved in disruption of regulation of RA-responsive genes by v-erbA. The complete comparison analyses of genes affected by the different treatments are available as Supplementary Files.

3.2. Dominant-negative effect of v-erbA on expression of RA-activated genes

The dominant-negative activity of v-erbA on RA-responsive genes is evident from the sample genes in Table 2. In untransfected control cells, expression of these genes is either up- or down-regulated by RA. In transfected cells, v-erbA antagonizes the activity of RA, with the opposite result in terms of gene expression. However, the effects of v-erbA alone suggest two different mechanisms of action. Genes such as Fmo5 (flavin-containing monooxygenase 5), Nr0b2/Shp (nuclear receptor subfamily 0, group b, member 2/small heterodimer partner)

Table 1
Sequence of forward (F) and reverse (R) primers used to determine relative gene expression by qPCR

Gene	Primer sequence
ApoA1	(F) 5′-GTCACCCACACCCTTCAG-3′
	(R) 5′-CGCATCCACATACACATTAGC-3′
Fgfr3	(F) 5′-CACCGACAAGGAGCTAGAGG-3′
	(R) 5′-ACGCAGAGTGATGGGAA AAC-3
Fmo5	(F) 5′-AGTGTGCAGCGTGAAGAAGC-3′
	(R) 5′-GCAGGTGAGCATCC GTGTG-3′
H19	(F) 5′-ACTGAAGGCGAGGATGAC-3′
	(R) 5′-GAACAGACGGCTTCTA CG-3′
Nrip1	(F) 5′-TGGTGAGCAACGAAAGATG-3′
	(R) 5′-AACTCGGGTGCAGA CTAC-3′
Ppara	(F) 5′-CGACCTGAAAGATTCGGAAA-3′
	(R) 5′-AACCATTGGGTCAGCTCTTG-3′
Shp	(F) 5′-CCGTGGAATGGAGTCTGG-3′
	(R) 5′-CTTGCTGGACAG TTAGTAGTG-3′
Sppr1a	(F) 5′-TCCATCACCATACCAGCAGA-3′
	(R) 5′-AGACAGCA GCCTCAGCATCT-3′
Tll1	(F) 5′-GAAGAATTGGCTCTGGCTTG-3′
	(R) 5′-GTAGCAG CTCTGGGAACTCG
Txnip	(F) 5′-CAACAACAACAACAACAACG-3′
	(R) 5′-GTTCCCCGCTGTAGAGACTG-3′