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Retinoid X Receptors and Retinoid Response in Neuroblastoma Cells

Birju Rana,[1] Gareth J. Veal,[2] Andrew D.J. Pearson,[3] and Christopher P.F. Redfern[1]*

[1]Department of Endocrinology, Medical Molecular Biology Group, Medical School, University of Newcastle, Newcastle upon Tyne NE2 4HH, UK
[2]Department of Oncology, Medical Molecular Biology Group, Medical School, University of Newcastle, Newcastle upon Tyne NE2 4HH, UK
[3]Department(tm) of Child Health, Medical Molecular Biology Group, Medical School, University of Newcastle, Newcastle upon Tyne NE2 4HH, UK

Abstract Retinoic acid (RA) modulates differentiation and apoptosis of neural cells via RA receptors (RARs) and retinoid X receptors (RXRs). Neuroblastoma cells are potentially useful models for elucidating the molecular mechanisms of RA in neural cells, and responses to different isomers of RA have been interpreted in terms of differential homo- and heterodimerization of RXRs. The aim of this study was to identify the RXR types expressed in neuroblast and substrate-adherent neuroblastoma cells, and to study the participation of these RXRs in RAR heterodimers. RXRβ was the predominant RXR type in N-type SH SY 5Y cells and S-type SH EP cells. Gel shift and supershift assays demonstrated that RARβ and RARγ predominantly heterodimerize with RXRβ. In SH SY 5Y cells, RARγ/RXRβ was the predominant heterodimer binding to the DR5 RARE in the absence of 9-*cis* RA (9C), whereas the balance shifted in favor of RARβ/RXRβ in the presence of ligand. There was a marked difference between the N- and S-type neuroblastoma cells in retinoid receptor–DNA interactions, and this may underlie the differential effects of retinoids in these neuroblastoma cell types. There was no evidence to indicate that 9C functions via RXR homodimers in either SH SY 5Y or SH EP neuroblastoma cells. The results of this study suggest that interactions between retinoid receptors and other nuclear proteins may be critical determinants of retinoid responses in neural cells. J. Cell. Biochem. 86: 67–78, 2002.

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Retinoic acid (RA) is one of the main biologically active derivatives of retinol (vitamin A) and has important roles during embryonic development and in the maintenance of normal cellular function [Morris-Kay, 1997; Redfern, 1997]. The physiological effects of RA are mediated, at least in part, via two classes of nuclear receptors: RA receptors (RARs: RARα, -β, -γ), whose ligands include the stereoisomers all-*trans* and 9-*cis* RA (9C), and retinoid X receptors (RXRs: RXRα, -β, -γ), which bind 9C [Mangelsdorf and Evans, 1992]. RARs function as ligand-dependent transcription factors in the context of RAR/RXR heterodimers, which bind to RA response elements (RAREs); these recognition sequences predominantly consist of two direct repeats (DR) or half sites separated by five base pairs, and referred to as a DR5 RARE [de Thé et al., 1990; Stunnenberg, 1993]. As RAR heterodimer partners, RXRs mediate RA effects on gene expression. RXRs also affect multiple physiological pathways since they form heterodimers with other nuclear receptors, including those for thyroid hormone, peroxisome-proliferator activators, vitamin D3, and xenobiotics [DiRenzo et al., 1997; Wan et al., 2000]. Furthermore, RXR homodimers may be induced by 9C [Zhang et al., 1992] and regulate gene expression via a DR1 RARE.

RARs are differentially expressed during embryonic development and in adult tissues. In mouse embryos, neural-crest cells express RARα and RARβ, with RARγ being more

*Correspondence to: Dr. Christopher P.F. Redfern, Medical Molecular Biology Group, Medical School, University of Newcastle, Newcastle upon Tyne NE2 4HH, UK.
E-mail: chris.redfern@ncl.ac.uk
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restricted [Ruberte et al., 1991]. Despite extensive investigation of RARs, few studies have investigated the significance of tissue-specific RXR expression. In mice, RXRβ is the most predominant type expressed in the foetal central nervous system, whereas RXRα expression is low and RXRγ is absent [Mangelsdorf et al., 1992]. The status of the peripheral nervous system with respect to RXR expression is unclear, but migrating neural crest cells in chick embryos at least express RXRγ [Rowe and Brickell, 1995, 1997]. Neuroblastoma cells are derived from the developing peripheral nervous system and show marked differentiation responses to RA [Sidell et al., 1983]. The N-type (neuroblastic) neuroblastoma cells have properties of embryonic sympathoblasts, whereas the S-type (substrate-adherent) cells are similar to embryonic Schwann/glial/melanocytic cells of the neural crest [Ross et al., 1995]. Since both neuroblastoma cells and neuronal cells increase the number and length of neurites in response to all-*trans* RA (AT) [Lovat et al., 1993; Maden et al., 1998], neuroblastoma cells are valuable models for elucidating the molecular mechanisms of RA in neural cells.

Since the discovery of RXRs and the identification of 9C as an RXR ligand, studies on neuroblastoma and other cell types have shown that 9C can be much more effective at inducing differentiation and gene expression than AT [Lovat et al., 1994; Redfern et al., 1994; Wan et al., 1995]. This has been interpreted in terms of differential homo- and heterodimerization characteristics of RXRs in response to 9C, but definitive data on RXR expression and behavior is lacking. SH SY 5Y cells, an N-type line originally derived from the mixed-phenotype SK N SH cell line [Biedler et al., 1973], express RARα and RARγ, with RARβ mRNA being markedly induced in response to both 9C and AT [Lovat et al., 1994; Redfern et al., 1994]. In contrast, the expression and functional significance of RXRs in these cells is not well understood.

The aim of this study was to identify the RXR types expressed in N-type SH SY 5Y cells and their participation in RXR/RAR heterodimers. In addition, S-type SH EP cells, derived from the same mixed-phenotype parental line [Biedler et al., 1973], were compared with SH SY 5Y cells to determine whether or not RXR expression and function is specific to the neuronal phenotype.

MATERIALS AND METHODS

Cell Culture and Treatment With RA Isomers

SH SY 5Y and SH EP neuroblastoma cells, SK23 melanoma cells, and HEP G2 hepatoblastoma cells were grown in Dulbecco's Modified Eagle's Medium (Hyclone-Europe, Tyne & Wear, UK) supplemented with 10% fetal calf serum (FCS; Sera Labs, Crawley, UK) in a humidified atmosphere of 5% CO_2 in air. Prior to treatment with 9C or AT (Sigma, Poole, UK), 8×10^6 cells were seeded into 75-cm^3 flasks (Costar, Bucks, UK) and cultured overnight. 9C or AT were added in absolute ethanol, with an equal volume of ethanol being used to treat control cells. Cells were incubated with retinoids for 6–24 h prior to extraction of RNA or preparation of nuclear extracts.

RNA Extraction and Northern Blotting

Cells were detached from the culture flasks and lysed as described by Wilkinson [1988]. Nuclei were removed by centrifugation at 6,000*g* for 30 s and cytoplasmic RNA was purified from the supernatant by extraction with phenol/chloroform [Redfern and Wilson, 1993]. RNA samples (20 μg/track) were size-fractionated by electrophoresis through 1.2% agarose–formaldehyde gels, transferred to nylon membranes (Amersham International, Aylesbury, UK) and probed with ^{32}P-labeled cDNA probes for human RAR-β [Brand et al., 1988], human cellular RA binding protein II (CRABPII) [Redfern and Wilson, 1993] and rat glyceraldehyde-3-phosphate dehydrogenase (GAPDH) [Fort et al., 1985] as described previously [Redfern and Todd, 1992]. Blots were exposed to phosphor storage screens and scanned in a Molecular Dynamics phosphorimager.

Preparation of RXR cDNA for Riboprobes

Probes for RNase protection assays were generated using T7 or T3 RNA polymerase to give protected fragments of 401, 570, and 420 bp when hybridized with RXRα, RXRβ, and RXRγ mRNA, respectively. Plasmid pSKXR3-1 containing RXRα cDNA [Mangelsdorf et al., 1990] was linearized with StuI to prepare a RXRα 3′ end probe. The RXRβ probe was prepared by sub-cloning full length hRXRβ from pTL1-RXRβ [Leid et al., 1992] into Bluescript KS and linearizing with Bam H1. The RXRγ probe was prepared using HinfI to linearize human RXRγ

cDNA, cloned into Bluescript [Kumarendran et al., 1994]. As a positive control for hRXRβ, sense transcripts were prepared from XbaI-linearized plasmid to give a 440-bp protected fragment when hybridized to the antisense RXRβ probe.

RNase Protection Assays

RNase protection assays were performed using the Ambion RNase protection assay kit (Ambion, AMS Biotechnology, Oxford, UK) according to manufacturer's instructions and 1 μg linearized RXRα, -β, -γ plasmid cDNA [Lovat et al., 1999]. Three independent experiments were carried out for each probe, and a standard amount of labeled RNA marker was run on each RNase protection gel. Sizes of protected fragments were calculated from a linear regression of distance vs. log (molecular weight).

Preparation of Nuclear Extracts

Confluent cells in 162-cm^3 flasks were washed once in 10 ml of ice-cold phosphate buffered saline (PBS, Sigma), scraped into 1 ml of ice-cold PBS and centrifuged at 13,000*g* for 5 min at 4°C. Cells were lysed by re-suspension in 100 μl of ice-cold lysis buffer (20 mM HEPES, pH 7.9, 1 mM EDTA, 0.2% NP40, 1 mM dithiothreitol (DTT), 1 mM protease inhibitor E64, 1 mM benzamidine, 1 mM leupeptine, and 1 mM freshly-added phenylmethylsulphonyl fluoride (PMSF)) and centrifuged at 5,000*g* for 5 min at 4°C. The nuclear pellet was re-suspended in 100 μl of high salt buffer (as for lysis buffer, but without 0.2% NP40, and with 20% glycerol and 420 mM NaCl) and incubated for 2 h on ice before centrifugation at 13,000*g* for 2 min. The resulting supernatant (nuclear extract) was frozen rapidly and stored at −80°C. Protein concentration was determined using the Bio-Rad protein assay (Bio-Rad, Hemel Hempstead, Herts).

Electrophoretic Mobility Shift Assay (Gel Shift Assay)

Eight micrograms of nuclear extract was incubated at room temperature for 30 min with 5 μl of 5 × binding buffer (250 mM Tris-HCl, pH 8.0, 40 mM NaCl, 10 mM EDTA, 2.5 mM DTT, 10 mM spermidine, 25% glycerol), 2 μl of poly dI−dC (1 μg/μl), 1 μl of calf thymus DNA (5 μg/μl), and 1 μl of labeled oligonucleotide probe (4×10^5 cpm). DNA−protein complexes were separated by electrophoresis through 5% polyacrylamide gels in 0.25 × Tris-borate/EDTA (TBE) (10 × stock: 0.9 M Tris base, 0.9 M orthoboric acid, and 0.5 M EDTA, pH 8.0). The gels were dried and exposed to a phosphor screen overnight. The oligonucleotide probes used in this study were synthetic double-stranded RARE probes prepared by annealing complementary strands (1 mg each strand) (Table I), for 2 h at 37°C. Oligonucleotide probes were labeled with (α^{32}P) dCTP (Amersham) by fill-in reactions with (α^{32}P) dCTP (3,000 Ci/mol, 10 mCi/ml) and Klenow exo-enzyme (10 U/μl) (Stratagene, The Netherlands) and 2 μl of 10 × Klenow buffer (0.5 M Tris-HCl, pH 7.5, 0.1 M $MgCl_2$, 10 mM DTT, and 0.5 mg/ml bovine serum albumin (BSA)). For competition analysis, nuclear extracts were pre-incubated for 15 min on ice with a 10-fold excess of unlabeled DR5 or DR1 RARE as appropriate, or with an unrelated double-stranded oligonucleotide (Table I) before adding the labeled oligonucleotide probe. Gel Shift analysis using in vitro-translated RXRβ protein (Applied Biosystems, Cambridge Biosciences, Cambridge, UK) was carried out by incubating 3 μg of RXRβ protein with 20 μl of binding buffer (150 mM NaCl, 20 mM Tris-HCl, 0.2 mM EDTA, 0.5 mM DTT, 5% glycerol, and 2 μg poly dI−dC) on ice for 10 min, prior to addition of the appropriate labeled oligonucleotide probe.

For "supershift" analysis, nuclear extracts were incubated with a 1 in 40 dilution of an

TABLE I. Oligonucleotides Consisting of DR1 and DR5 Response Elements Used in Gel and Supershift Reactions

RARβ DR5 RARE	CAGCCCGGGTAGGGTTCACCGAAAGTTCACTCGCAT and CGACCCATGCGAGTGAACTTTCGGTGAACCCTACCC
CRABP II DR1 RARE	CCCACTGGATCCAGTTCAGGGTTCAATGGAGCTAGG and ACCTGGCCTAGCTCCATTGAACCCTGAACTGGATCC
CRBP II DR1 RARE	GATCCAGGTCACAGGTCACAGGTCACAGTT and GATCTTGAACTGTGACCTGTGACCTGTGAC
Random sequence	CACGGAGATGTATTCTTGCTCCGTGTGGAATAGTGT and ACACTATTCCACACGGAGCAAGAATACATCTCCGTG

appropriate mouse monoclonal antibody (generously provided by Prof. P. Chambon).

Western Blotting

For Western blotting, 20 μg of nuclear protein was separated by electrophoresis through a 10% sodium dodecyl sulfate–polyacrylamide gel (SDS–PAGE) at 100 V and transferred to nitrocellulose (Bio-Rad) membrane at 4°C and 30 V overnight. Membranes were blocked with blocking solution (5% w/v skimmed powdered milk, in Tris-buffered saline (TBS) with 0.05% Tween 20, pH 8.0), for 1 h at room temperature with agitation, and then immediately probed with an appropriate antibody diluted in blocking solution. Membranes were subsequently washed in TBS/Tween for 30 min, and then incubated for 1 h with biotinylated-anti-rabbit IgG (Amersham Pharmacia), diluted 1 in 500 in blocking solution. After a final 30 min wash, membranes were agitated for 1 h in a 1 in 500 dilution of streptavidin-biotinylated-horseradish peroxidase complex (Amersham Pharmacia) and developed using an enhanced chemiluminescence (ECL) detection kit (Amersham Pharmacia) and Fuji X-ray film. RAR and RXRβ proteins were identified on the basis of apparent molecular weight and cross reactivity with antibodies.

Antibodies

The ascites-fluid antibodies used were: RARα (F region) Ab9α(F) (9α-9A6), RARβ (F region) Ab8β(F)2 (8β-10B2), RARγ (hF region) Ab4γ(F) (4γ-7A11), pan RXR (m-α,β,γ) (E region or D region) 4RX-1D12, RXRα (full protein) 4RX3A2 and RXRβ (83-106-PC13) 16RX3E8. In addition, some supershift analyses were carried out using a commercial RARα antibody formulated for supershift experiments (Santa Cruz Biotechnology, CA). Control supershift reactions were carried out with the appropriate antibody being replaced by the same volume of ascites fluid for an IgG2a ovine lactogen monoclonal antibody. The protein concentrations for all antibody stocks ranged from 23–32 mg/ml.

The RAR antibodies used in Western blot experiments (all from Santa Cruz Technologies) were specific for a number of isoforms within a sub-type, for example, RARβ antibody was described as being specific to the -β1 and -β2 isoforms; the RXRβ antibody used was specific for RXRβ2.

RESULTS

Constitutive Expression of RXRs in SH SY 5Y and SH EP Cells

Previous Northern blot data on RXR expression in neuroblastoma cells [Carpentier et al., 1997] have been inconclusive, and in the past, we have had poor results with human RXRβ cDNA probes on Northern blots [Kumarendran et al., 1994]. Therefore, we used RNase protection assays to identify RXR types expressed in SH SY 5Y and SH EP neuroblastoma cells. Cells were treated with 1 μM of 9C or AT for 6 and 24 h to investigate both early and late effects of RA on RXR expression.

In RNase protection assays, a 401-bp protected fragment corresponding to RXRα was weakly detected in RNA extracted from both SH SY 5Y and SH EP cells (Fig. 1), and in HepG2

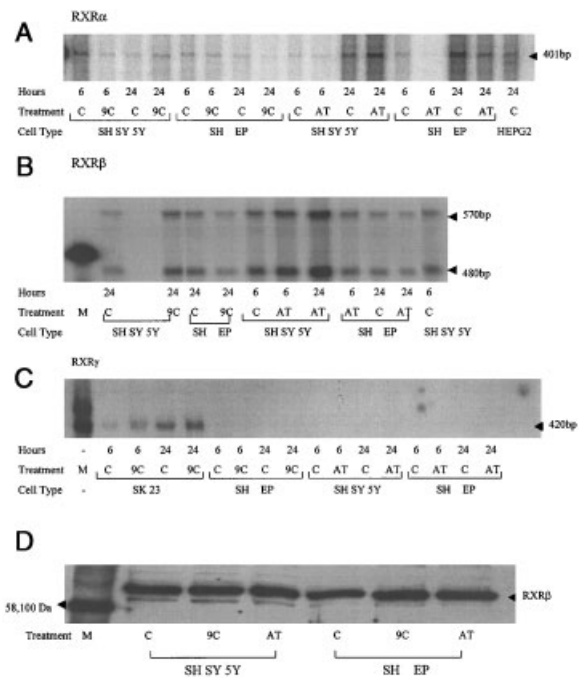

Fig. 1. RXR expression in SH SY 5Y and SH EP neuroblastoma cell lines detected by RNase protection assay. RNA (10 μg) was extracted from cells treated for 6 and 24 h with 1 μM AT, 9C or control ethanol (C) and hybridized with (^{32}P)-labeled complementary riboprobes for RXRα (**Panel A**: 401-bp protected fragment), RXRβ (**Panel B**: 570-bp protected fragment), and RXRγ (**Panel C**: 420-bp protected fragment). Lanes labeled (**M**) represent RNA size markers. RNA from SK23 melanoma cells was used as a positive control for RXRγ. **Panel D**: Western blot analysis to determine RXRβ expression. Twenty micrograms of nuclear extract from cells treated with either 9C, AT, or C was blotted onto a membrane, which was incubated with an RXRβ2 polyclonal antibody. No significant induction of RXRβ protein was observed after treatment with RA.

cells included as a positive control [Nomura et al., 1999]. There was no apparent change in abundance of RXRα message for either SH SY 5Y or SH EP cells after treatment with either 9C or AT for 6 or 24 h. Conversely, for RXRβ, a strong signal corresponding to the expected 570-bp protected fragment was obtained for both neuroblastoma cell lines (Fig. 1). Relative to the 400-bp RNA marker, this RXRβ protected fragment in both cell types was 2.4-fold greater in intensity than RXRα (mean intensities relative to 400 bp marker were 0.056 and 0.0232 for the 570 and 401 bp fragments, respectively, Student's $t_8 = -4.9$, $P < 0.002$). This difference was significant after allowing for the difference in probe length and relative 'C' content between the two probes. For RXRβ, in addition to the predicted 570-bp fragment, a more-intense second protected fragment of approximately 480 bp, corresponding to an alternatively spliced RXRβ transcript [Rana et al., 2001], was expressed in both SH SY 5Y and SH EP cells (Fig. 1). Relative to control cells, there was an apparent 1.9-fold (median) increase in intensity of the RXRβ fragments after treatment of SH SY 5Y cells with 9C or AT for up to 24 h (range 1.2–3.1-fold in three independent experiments); however, there was no apparent increase in RXRβ protein detectable by Western blotting (Fig. 1). Induction of RXRβ mRNA was not observed in SH EP cells.

An RNA probe corresponding to the 3′ end of RXRγ mRNA detected the predicted 420-bp fragment in RNA from SK 23 melanoma cells used as a positive control [Kumarendran et al., 1994], but not in RNA from SH SY 5Y or SH EP cells. RXRγ was not induced by RA in neuroblastoma cells although both 9C and AT clearly induced RXRγ message in SK 23 cells (Fig. 1). These data demonstrate that only two of the three RXR genes, RXRα and RXRβ, were expressed in these neuroblastoma cells, and suggest that mRNA for RXRβ was the most abundant RXR message.

RXR Heterodimers Binding to a DR5 Probe in Retinoid Signaling

Two major oligonucleotide–protein complexes were observed as bands on 5% polyacrylamide gels after incubating a DR5 RARE probe with nuclear extract from SH SY 5Y and SH EP cells (Fig. 2). These were not observed when the same oligonucleotide was incubated with the same amount of cytoplasmic protein (Fig. 2C).

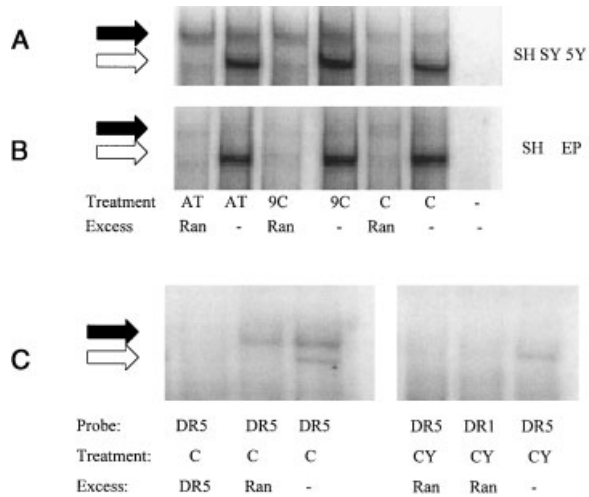

Fig. 2. Gel shift analysis of DR5 RARE binding complexes. Nuclear extract from SH SY 5Y (**Panel A**) and SH EP (**Panel B**) was isolated from cells cultured for 18 h with 1 μM 9C, AT, or an equivalent volume of ethanol (C). Specific complexes in 8 μg of nuclear protein were verified using a 10-fold excess of random oligonucleotide (Ran) for both cell types, or a 10-fold excess of unlabeled DR5 RARE probe (DR5). **Panel C** shows that 8 μg of cytoplasmic protein (CY) from the same cells (only shown for SH SY 5Y) did not show evidence of a specific complex with either the DR5 or DR1 response element. Specific complexes are indicated by a filled arrow and non-specific complexes with an open arrow.

Only the top band was specific to the DR5-probe sequence; although both complexes were abolished by a 10-fold excess of unlabeled DR5 RARE oligonucleotide, the lower band was abolished with a 10-fold excess of random-sequence oligonucleotide (Fig. 2C). Specific DR5 binding activity was present in nuclear extracts from control and RA-treated SH SY 5Y and SH EP cells, but was more prominent with the SH SY 5Y cells (Fig. 2A,B).

A pan-RXR antibody and specific antibodies to RXRα and RXRβ were used to identify RXRs involved in binding to the DR5 RARE probe. The pan-RXR and RXRβ antibodies demonstrated a clear supershift with nuclear extract from SH SY 5Y cells, and this was increased in intensity in nuclear extracts from RA-treated cells (Fig. 3A). Conversely, a very weak supershift was detected with the RXRα antibody. These data suggest that in SH SY 5Y neuroblastoma cells, RXRβ is the major component of complexes binding to a DR5 RARE probe.

RARs in RXR Heterodimers

With antibodies to RARα, no supershifted complex was observed in nuclear extract from

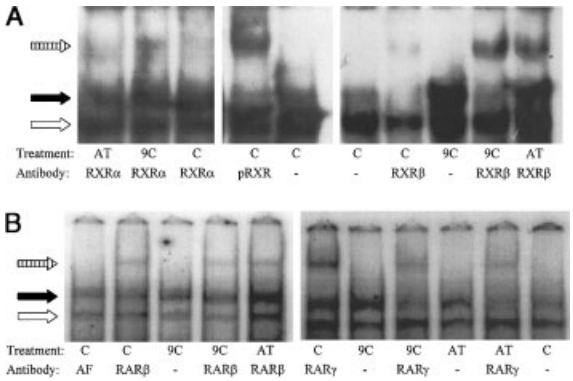

Fig. 3. Supershift experiments to identify RXRs and RARs binding to the DR5 RARE probe. Prior to electrophoresis, nuclear extracts from SH SY 5Y cells treated with 1 μM 9C, AT, or control ethanol (C) for 18 h were incubated with RXR antibodies: **Panel A**: RXRα antibody, Pan RXR antibody (pRXR), and RXRβ antibody. Supershifted complexes were observed with pRXR, RXRα, and RXRβ antibodies. **Panel B**: shows supershifted complexes with RARβ and RARγ antibody. Supershifted complexes are indicated with a vertical striped arrow, specific complexes with filled arrow and non-specific complexes with an open arrow. AF is control ascities fluid.

SH SY 5Y cells with either of the RARα antibodies used (9α-9A6 and a commercial RARα antibody from Santa Cruz) (data not shown). Conversely, an RARβ antibody gave a supershifted complex with nuclear extract from control SH SY 5Y cells and SH SY 5Y cells treated for 18 h with 1 μM 9C or AT (Fig. 3B). Specific supershifted complexes were observed with the RARγ antibody with nuclear extract from SH SY 5Y cells, but these were consistently less pronounced in nuclear extract from RA-treated cells (Fig. 3B). Double-supershift experiments were performed using RARβ and RARγ antibodies to ask whether the participation of RARs in DR5-RARE-binding complexes changes in relation to RA treatment. Incubation of nuclear extracts with both RARγ and RXRβ antibodies (and to a lesser extent RXRα) produced a double-shifted complex in untreated SH SY 5Y cells. Conversely, incubation with RARβ and RXRβ antibodies produced a prominent double-shifted complex in nuclear extracts from cells treated for 18 h with 9C, but to a slightly lesser extent in control, untreated cells (Fig. 4).

Pan-RXR-antibody supershifts using nuclear extracts from SH EP cells gave weak supershifts with the DR5 probe that were barely detectable by comparison to those observed with SH SY 5Y cell nuclear extracts (Fig. 5A). Furthermore, nuclear extracts from SH EP cells treated for

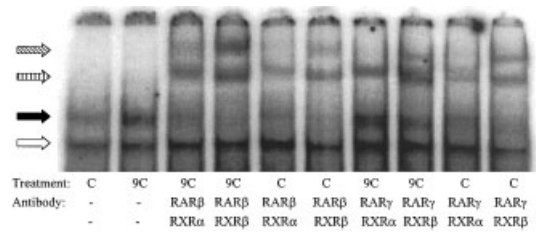

Fig. 4. Double-supershift experiments to identify RAR and RXR partners binding to DR5 RARE before and after RA treatment. Prior to electrophoresis, nuclear extracts from SH SY 5Y cells treated with 1 μM 9C or control ethanol (C) for 18 h were incubated with combinations of RAR and RXR antibodies as indicated. Supershifted complexes are indicated with a vertical striped arrow, double supershifted complexes are indicated by a horizontal hashed arrow, specific complexes with a filled arrow, and non-specific complexes with an open arrow.

18 h with 1 μM 9C failed to produce a clear supershift with the RARβ antibody (Fig. 5B).

Differences in RA Responses Between SH SY 5Y and SH EP Cells

It has been shown previously [Redfern et al., 1994] that RARβ and CRABP II are induced by

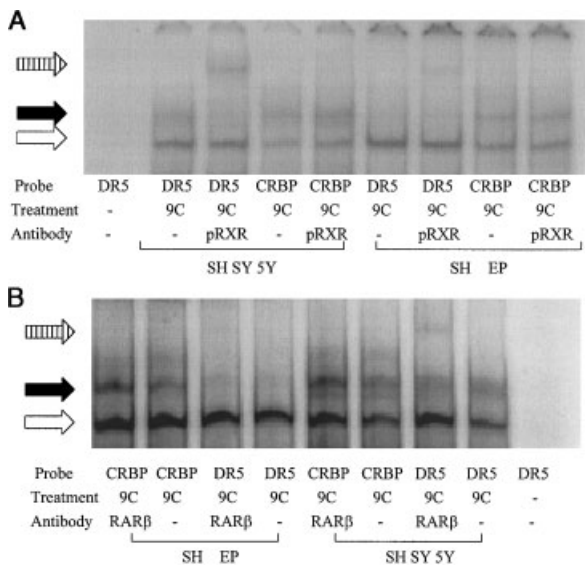

Fig. 5. Supershift analysis (using pan RXR and RARβ antibodies) of DR5 RARE and CRBP II DR1 RXRE binding. Eight micrograms of nuclear extract from SH SY 5Y and SH EP cells were isolated from cells cultured for 18 h with 1 μM 9C. Prior to electrophoresis, nuclear extract was incubated with a pan RXR (pRXR) antibody, **panel A**, along with either a DR5 or DR1 (CRBP) probe, or with an RARβ antibody, **panel B**, along with either a DR5 or DR1 (CRBP) probe. Supershifted complexes are indicated with a vertical striped arrow, specific complexes with a filled arrow, and non-specific complexes with an open arrow. A DR1 (CRBP) probe refers to the CRBP II DR1 RXRE.

RA in SH SY 5Y neuroblastoma cells. Differences in retinoid responses between SH SY 5Y and SH EP cells were investigated by comparing the induction of RARβ and CRABP II in response to 6 h treatment with AT at doses ranging from 0.01 to 10 μM. RARβ mRNA was expressed in both cell types but was more abundant in SH EP. RARβ was induced by AT in SH SY 5Y cells, but not in SH EP cells. Unlike SH SY 5Y cells, CRABP II message was not expressed or induced by RA in SH EP cells (Fig. 6).

Determination of Retinoid Receptor Protein Levels in Neuroblastoma Cells

Western blots were used to compare RAR receptor levels in both SH SY 5Y and SH EP cells before and after RA treatment. RARα, -β, and -γ were detected in both SH SY 5Y and SH EP cells, with similar levels in the two cell types (Fig. 7). There were no marked differences in the levels of RARα, -β, or -γ in nuclear extracts from 9C or AT-treated SH SY 5Y and SH EP cells.

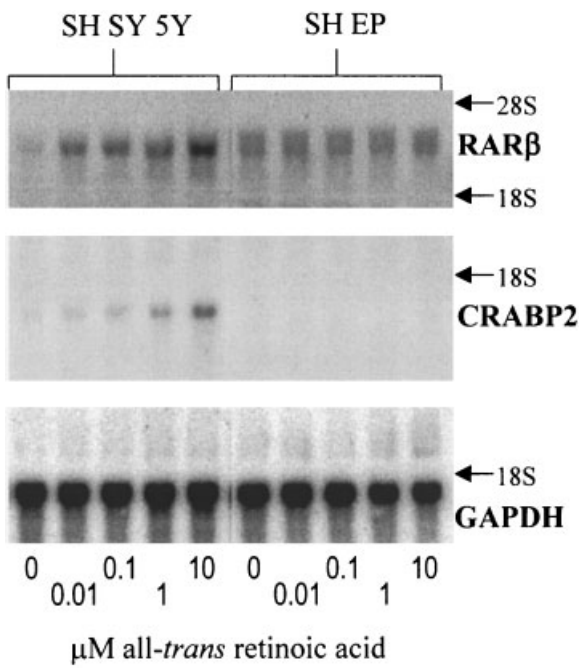

Fig. 6. Differences in retinoid responses between SH SY 5Y and SH EP neuroblastoma cells. Expression of RARβ and CRABP II (CRABP2) in SH SY 5Y and SH EP cells relative to GAPDH (loading control), in response to treatment with AT at concentrations ranging from 0.01 to 10 μM. Arrows mark the positions of the 28S and 18S rRNA.

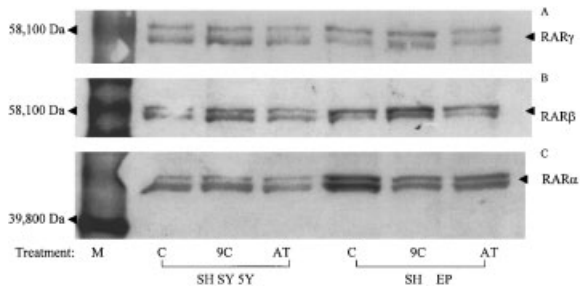

Fig. 7. Western blot analysis to determine RAR expression in SH SY 5Y and SH EP neuroblastoma cells. RARα, RARβ, and RARγ protein in SH SY 5Y and SH EP cells. Twenty micrograms of nuclear extract from cells treated with either 9C, AT, or control ethanol (C) were blotted onto a membrane, which was incubated with a polyclonal antibody for each of the receptor subtypes. No significant induction of protein levels was observed for any of the RAR subtypes after treatment with RA. The differences in band intensity observed with SH EP nuclear extract are due to loading variation. Two bands were detected for each RAR subtype since the antibodies used were not specific for individual isoforms within each RAR subtype. M, marker track.

RXR Homodimers in Response to 9C

Since 9C dependent RXR homodimers are reported to bind to DR1 elements, differences in utilization of DR1 and DR5 elements by 9C and AT could account for the different biological effects of 9C in neuroblastoma cells. In addition, 9C induces CRABP II to a greater extent than AT in neuroblastoma cells [Redfern et al., 1994] and could be acting through a DR1 CRABP II response element. To test this hypothesis, gel shift assays on neuroblastoma cell nuclear extracts were carried out using oligonucleotides representing CRABP II DR1 and a CRBP II DR1 RXRE. Two different response elements were used since the sequence motif of the RXRE is important for RXR homodimer binding and not just the spacing separating the two half sites [Zhang et al., 1992; Subauste et al., 1994].

In gel shift assays using the CRBP II DR1 RXRE and nuclear extracts from untreated SH SY 5Y and SH EP cells, there were two pronounced shifted complexes (Fig. 8). The lower band was competed out with a 10-fold excess of random oligonucleotide, indicating that only the upper band represented specific binding in both cell types. A second, faint, specific band was also observed in these gel-shift experiments with a CRBP II DR1 RXRE (Fig. 8), although this specific band was not investigated further. Compared to a positive control consisting of a

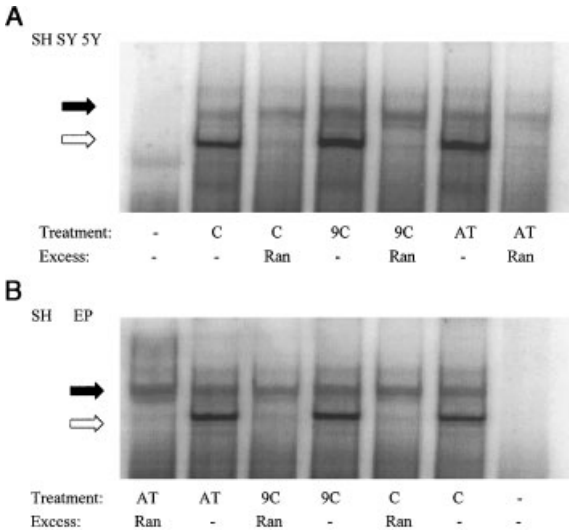

Fig. 8. Gel shift analysis of CRBPII DR1 RXRE binding complexes. Eight micrograms of nuclear extract from SH SY 5Y and SH EP cells were isolated from cells cultured for 18 h with 1 μM 9C, AT, or an equivalent volume of ethanol (C). Specific complexes are indicated by a filled arrow and non-specific complexes are shown by an open arrow. Ran, random oligonucleotide used in excess.

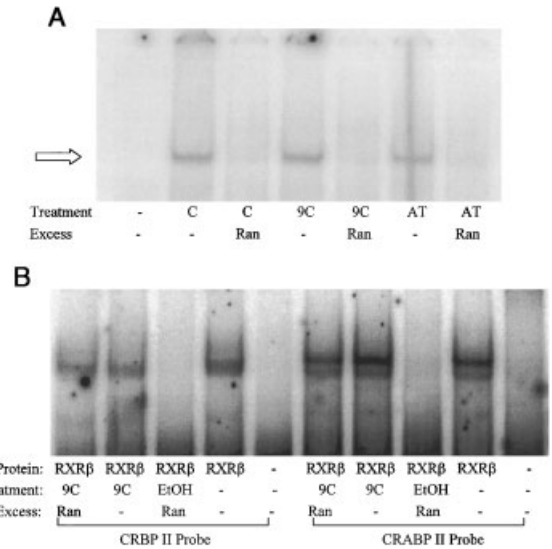

Fig. 9. Gel shift analysis of CRABP II and CRBP II DR1 RXRE binding complexes using nuclear and in vitro translated protein. **A**: Eight micrograms of nuclear extract from SH EP cells were isolated from cells cultured for 18 h with 1 μM 9C, AT, or an equivalent volume of ethanol (C). No specific complexes were observed. Non-specific complexes competed out by an excess of random oligonucleotide are shown by an open arrow. Similar results were obtained for SH SY 5Y cells. **B**: Gel shift analysis of DR1 RXRE binding complexes using in vitro translated RXRβ protein. The protein was either incubated with control ethanol (EtOH) or 0.1 μM 9C for 40 min, or incubated with both 0.1 μM 9C/EtOH and Random (Ran) excess sequence for 40 min prior to electrophoresis. In the absence of 9C RA, no specific bands were observed, however, in the presence of 9C, two specific bands were detected with the CRABP II probe and a single specific band with the CRBP II probe.

DR5 RARE probe and pan-RXR antibody, no pan-RXR antibody supershift was observed with nuclear extract from either SH SY 5Y or SH EP cells using the CRBP II DR1 RXRE probe, indicating that RXRs were not components of the specific complex (Fig. 5). Supershift experiments using 9C-treated nuclear extract, CRBP II DR1 RXRE and an RARβ antibody also failed to produce a supershift with nuclear extract from either cell type. There was no evidence for the appearance of new bands after the treatment of cells with 9C nor was there any apparent difference in specific binding between control, 9C or AT-treated nuclear extract for either cell types (Fig. 8).

Gel shifts using the CRABP II DR1 RXRE probe and nuclear extract from untreated SH SY 5Y and SH EP cells only gave a single pronounced shifted complex (Fig. 9), which was competed out by a 10-fold excess of random oligonucleotide. Furthermore, there was no evidence for the appearance of new bands after the treatment of cells with 1 μM 9C or AT for 18 h (Fig. 9). As an additional positive control, the CRABP II DR1 and in vitro transcribed and translated RXRβ protein were used in gel shift assays. Two pronounced bands that were both competed out by a 10-fold excess of an unrelated oligonucleotide were detected. However, when the RXRβ protein was incubated with 0.1 μM 9C for 40 min prior to gel shift analysis, the same two pronounced bands were not competed out by a 10-fold excess of unrelated oligonucleotide (Fig. 9), indicating that both complexes were specific. Similar results were obtained using the CRBP II DR1 RXRE, except that only one specific complex was observed.

DISCUSSION

Expression of RXRs

The expression of RXRs in neuroblastoma has been investigated in two previous studies; Carpentier et al. [1997] and Giannini et al. [1997] both reported the detection of RXRα by RT-PCR in SMS-KCNR, SH SY 5Y, and LAN 5 cells. While we confirm a low level of RXRα expression in SH SY 5Y cells, the present data clearly demonstrate that RXRβ mRNA was expressed in SH SY 5Y cells at a higher level. RXRγ was not expressed or induced by RA in SH

SY 5Y or SH EP neuroblastoma cells, but the expression and clear induction by RA of RXRγ in SK23 melanoma cells suggests that this RXR may be important in melanoma cell biology. Previous studies have focused on RXRα as the RXR involved in heterodimer formation, but these results raise the possibility that the expression of RXRβ and RXRγ may be more important in retinoid responses of neural crest derivatives than has been previously acknowledged.

RAR/RXR Heterodimers Involved in DR5 Binding in Neuroblastoma Cells

Evidence from supershift assays suggest that the majority of receptor dimers or oligomers binding to the DR5 RARE probe in SH SY 5Y cells included RXRβ and that RXRα represented only a minor component of the binding activity. Although there was an apparent increase in RXRβ protein binding to the DR5 probe when nuclear extract from RA-treated cells was used in supershift assays, there was no increase in RXRβ protein levels after treatment with either 9C or AT; this implies that any increase in DR5 binding activity was due to an increase in the availability of RXRβ, or to its ability to bind to the DR5 probe. Numerous studies have shown that the expression of RARβ mRNA is induced by RA in neuroblastoma cells; [Lovat et al., 1993, 1997b,c; Redfern et al., 1994, 1995; Carpentier et al., 1997; Giannini et al., 1997]. However, as also found by Reboul et al. [1995], there was no evidence from Western blots that RARβ protein increased after treatment with 9C in SH SY 5Y or SH EP cells.

Since 9C increased binding specificity of RXRβ for DR1 RXREs, and both 9C and AT apparently increased the binding of RXRβ to the DR5 RARE probe in vitro, it is possible that ligand may induce conformational changes increasing RXRβ binding as a result of increases in specificity or affinity for an RARE, or increase the nuclear availability of RXRβ. Increased nuclear availability of RXRβ may result from the disassociation of RXR tetramers, according to the tetramer model of Kersten et al. [1995a,b,c] resulting in an increased RXR pool available for heterodimerization [Chen et al., 1994, 1998; Kersten et al., 1995a,b, 1998; Dong and Noy, 1998], or from direct or indirect ligand-dependent alterations in the equilibrium of dimers between RXRs and other nuclear receptors. In addition, an increase in RXRβ binding to a DR5 RARE in RA-treated cells might occur as a result of the downregulation of factors such as COUP-TF, TAK1, and TR4 that can also compete for RARE (DR1 or DR5) binding [Tran et al., 1992; Hirose et al., 1995; Lee et al., 1998], although the expression levels of these receptors have not been established in neuroblastoma cells.

Double supershift experiments suggested that in the absence of 9C, RARγ/RXRβ was the predominant DR5-bound heterodimer, whereas the balance was altered in favor of RARβ/RXRβ heterodimers in the presence of ligand. Therefore, 9C may enable RARβ protein to be more accessible for DR5 binding, possibly by inducing specific co-regulators, with a concomitant reduction in the RARγ/RXRβ heterodimers binding to the DR5 RARE. Husmann et al. [1991] have reported that $RAR\gamma_1$ can inhibit the activity of RARβ on the RARβ2 RARE. They suggest that $RAR\gamma_1$-mediated inhibition of other RARs may involve competition for the actual response element as well as direct interaction with other receptors [Husmann et al., 1991].

Despite similar levels of RARα, RARβ, and RARγ protein, gel shift analysis demonstrated a lower level of specific binding to the RARβ2 DR5 RARE in SH EP cells compared to SH SY 5Y cells. The marked differences observed between SH SY 5Y and SH EP cells in their response to RA, with respect to the induction of RARβ, the expression of CRABP II and of DR5 RARE binding, suggests that the differential expression of co-regulators or other factors may be the primary mechanism determining retinoid response by affecting the binding of RAR receptors to their response elements [Liu et al., 1998]. Identifying the factors which control retinoid responses in differentiated cells arising from a common precursor cell would represent a significant advance in understanding of the mechanisms regulating cell phenotype.

RXR Homodimers

9C is a more potent retinoid than AT in neuroblastoma cells, particularly in the induction of CRABP II [Lovat et al., 1997a]. Results from the gel shift experiments do not support the idea that 9C induces the formation of RXR homodimers capable of binding to a CRABP II DR1 RARE or a CRBP II RXRE. Furthermore, the fact that no specific binding was observed to the CRABP II DR1 RXRE suggests that CRABP II induction in response to 9C is not

modulated through this response element but is induced via the DR5 response element situated further upstream [Astrom et al., 1992]. Thus, the existence and role of RXR homodimers in vivo is questionable since all the evidence to date for their existence is based on in vitro, cell-free experiments [Zhang et al., 1992; Medin et al., 1994]. It has been suggested [Chen et al., 1994; Kyakumoto et al., 1997] that the evidence for RXR homodimer formation may be a result of improperly folded and conformationally stabilized protein synthesized in vitro. Gel shift data presented in this report, using in vitro transcribed/translated RXRβ protein suggest that 9C induced a conformational change of RXRβ protein that increased oligonucleotide specificity of RXR monomers or homodimers and also showed that both these DR1 RXREs would bind RXRβ specifically in the presence of 9C. In addition, RXR selective ligands have also been shown to be unable to elicit morphological differentiation, growth arrest, and gene expression on their own in neuroblastoma cells, suggesting that RXR homodimers do not contribute to the biological responses of these cells [Giannini et al., 1997]. An alternative explanation for the differential effects of 9C and AT isomers may be due to their differing ability to displace or induce co-repressors (e.g., SUG 1 and Trip 3) from the RAR receptor [Hong and Privalsky, 1999; Lovat et al., 1999].

A specific complex was detected on the CRBP II RXRE when nuclear extract from both SH SY 5Y and SH EP cells was used in gel shift experiments. Since no supershift was observed with either pan RXR antibodies or RAR antibodies, this implied that RAR or RXR receptors were not major components of this complex. Previous reports have suggested that a number of receptor types, (e.g., COUP-TF, TAK-1), compete with RARs and RXRs for specific DNA binding sites. The specific complex detected on the CRBP II RXRE is probably due to such receptors.

This study suggests that competition or interactions between nuclear proteins and other RARs may be important determinants of retinoid response in neural cells. Identifying these proteins and the way in which differential expression and sub-nuclear localization controls transcriptional activation will be a major step forward in understanding the molecular biology of retinoid responses in neural cells.

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