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Involvement of all-*trans*-retinoic acid in the breakdown of retinoic acid receptors α and γ through proteasomes in MCF-7 human breast cancer cells

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Abstract

Most studies have reported an up-regulation of retinoic acid receptor (RAR) mRNA expression by all-*trans* retinoic acid (RA). We aimed to study the effect of RA on RAR protein levels in MCF-7 human breast cancer cells. Incubation of these cells with 10^{-6} M RA induced a rapid breakdown of both RARα and RARγ in spite of the accumulation of their mRNAs. Proteasome specific inhibitors blocked the RA-induced breakdown of RARs. Furthermore, RA enhanced the formation of the complex between RARα and ubiquitin in a concentration- and time-dependent manner, suggesting the involvement of ubiquitin and proteasome in this reaction. Retinoid X receptor α (RXRα) was also decreased, albeit to a lesser extent, in RA-treated cells. Use of synthetic receptor agonists and antagonists clearly showed that the effect of the retinoid on the breakdown of the retinoid receptors is receptor-ligand agonist-dependent and blunted by the antagonist. An electrophoretic mobility shift assay, using nuclear extracts from RA-treated cells, showed that a reduction in complex formation with hormone response elements correlated with the reduction of RAR and RXR protein. These data suggest that RA induces the breakdown of RARs through a process involving ubiquitination and that this phenomenon causes a reduction in the formation of DNA-receptor complexes.

Keywords: Retinoic acid receptor; Proteasomes; Ubiquitination; Retinoic acid; Proteolysis

1. Introduction

RA plays a pivotal role in the development, cellular differentiation, and control of cell division [1,2]. The physiological actions of RA are mediated through two distinct nuclear receptor families [3], the retinoic acid receptors (RARα, β, and γ), each of which binds both RA or 9-*cis*-RA, and the retinoid X receptors (RXR-α, β, and γ), which preferentially bind 9-*cis*-RA. RARs and RXRs bind to a specific DNA response element (RARE) in the 5′-flanking region of target genes as homodimers or heterodimers, thereby promoting gene transcription [4]. Up-regulation of RAR mRNA by RA treatment has been demonstrated among a number of different types of cells, and RAREs have been identified in the 5′-flanking region of the RAR genes themselves [5,6], suggesting that they are first-order targets of RA action [1]. Retinoids enter the cytosol by free diffusion [7] through the cell membrane and interact with cellular retinoic acid binding proteins (CRABP type I and II) [8]. Retinoids also can access the nuclei and bind to receptor proteins such as RAR/RXR and RXR/RXR [9]. It is assumed that these receptors remain in the nucleus until they are degraded; however, little is known about the half-life of these receptor proteins, and how and where they are degraded. The problem is compounded by the fact that measurements of RAR regulation are usually preferentially made on the mRNA, and little has been done on the proteins themselves. We have reported previously that RA induces the degradation of RARα in NIH-3T3 cells [10], which are receptor-dependent for RA-induced inhibition of growth [11]. In human breast carcinoma cells we have also reported that RA inhibits cell growth and causes a reduction of p21, and Rb, as well as cyclin D3 and CDK4 expression [12].

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Abbreviations: RA, all-*trans* retinoic acid; RAR, retinoic acid receptor; RXR, retinoid X receptor; SUMO-1, small ubiquitin-related modifier; 4HPR, 4-hydroxyphenyl retinamide; EMSA, electrophoretic mobility shift assay; SSC buffer, 150 mM NaCl and 15 mM sodium citrate, pH 7.0; DTT, dithiothreitol; and PMSF, phenylmethylsulfonyl fluoride.

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These down-regulatory effects of RA may be due to proteolysis of the cyclins. Eukaryotic cells contain multiple proteolysis systems such as lysosomal protease, which is mainly responsible for degradation in the extracellular milieu, as well as ATP-ubiquitin-dependent or ATP-ubiquitin-independent proteasomes pathways [13]. Ubiquitin is a highly conserved small (76 amino acids) polypeptide that plays a pivotal role in determining protein turnover. Ubiquitin is activated by ATP to a high energy thiol-ester intermediate by ubiquitin-activating enzyme (E1). Ubiquitin-conjugated enzyme (E2) transfers activated ubiquitin molecules from E1 to the target protein substrate, which is bound to a ubiquitin-protein ligase (E3) [13], and then the polyubiquitinated protein is degraded by 26S proteasome. Therefore, we were interested in determining whether RA also reduces RARs in growth-inhibited MCF-7 breast cancer cells, as we had previously observed in an NIH-3T3 cell system [10], and in studying the mechanism of proteolysis. In this study, we report that RARα and RARγ were degraded rapidly after RA treatment through a ubiquitin-mediated pathway.

2. Materials and methods

2.1. Reagents

RA was obtained from the Sigma Chemical Co. A 10^{-2} M stock solution was prepared in DMSO, and used for cell culture at 10^{-6} M [final DMSO concentration of 0.01% v/v]. Proteasome inhibitors were purchased from Calbiochem. We obtained synthetic retinoid receptor selectives from Dr. Roshantha A. S. Chandraratna, Allergan.

2.2. Antibodies

Rabbit anti-RARα and -RARγ antibody and goat anti-actin antibody were obtained from Santa Cruz Biotechnology, and were used for western blotting at a dilution of 1:1000. The RARα antibody (RPα(F)), used for immunoprecipitation was a gift from Dr. Pierre Chambon. Mouse anti-ubiquitin and SUMO-1 antibodies were purchased from Zymed.

2.3. Cell culture

MCF-7 cells were obtained from the ATCC, and were maintained in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum in a humidified atmosphere supplemented with 5% CO_2 at 37°. The subconfluent cells were trypsinized and seeded onto 10-cm-diameter dishes at 10^6 cells/dishes. Twenty hours after seeding, cells were treated with DMEM containing 10^{-6} M RA or the same volume of DMSO supplemented with 2% fetal bovine serum for different times as indicated in the text.

2.4. Western blotting

After treatment, cells were washed twice with ice-cold PBS, and scraped gently into ice-cold PBS containing protease inhibitors (Roche). The cell pellets were lysed in Laemmli buffer without reducing agent and bromophenol blue. Lysates were boiled for 5 min and centrifuged at 30,000 *g* for 10 min to remove cellular debris. Protein concentration was determined by the bicinchoninic acid method (Pierce Science). Thirty micrograms of total protein from each sample was loaded onto 10% polyacrylamide gels and then transferred to PVDF membranes. Blots were stained with Ponceau S in order to check for loading. Next, blots were blocked with blocking solution for 1 hr at room temperature. They were exposed to anti-RARα or -RARγ for 1 hr. Following three washes in TBS-T, species-specific HRP-conjugated antibodies were added and the blots were incubated for 0.5 hr at room temperature. Proteins were visualized on X-ray film following enhanced chemiluminescence.

2.5. Northern blotting

Isolation of total RNA was performed by the RNeasy Kit (Qiagen). Ten micrograms of total RNA was denatured with 2.2 M formaldehyde, fractionated by electrophoresis on 1.0% agarose gels, and blotted overnight onto nitrocellulose membranes. RARα cDNA [14] was labeled with [α-^{32}P]dCTP (3000 Ci/mmol, ICN Biochemicals), using the random primer method. The membranes were probed with radiolabeled RARα cDNA overnight at 42°. The membranes were washed twice with 2-x SSC, 0.1% SDS at 42°, once with 1-x SSC, 0.1% SDS, at room temperature, and then twice with 0.1x SSC, 0.1% SDS at room temperature. mRNA was visualized on X-ray film.

2.6. Immunoprecipitation

To detect ubiquitin conjugation, cells were washed twice with ice-cold PBS and lysed in lysis buffer [50 mM Tris–HCl (pH 7.5), 150 mM NaCl, 1% Nonidet P-40, 0.1% SDS] supplemented with protease inhibitors (Roche). The lysates were vortexed and cleared by centrifugation at 15,000 *g* for 10 min. Supernatants were adsorbed with pre-immune control sera in lysis buffer with constant agitation at 4° for 1 hr. Then 30 μL of Protein-A-Sepharose was added to the cell lysate and incubated for an additional 4 hr at 4°. After a brief centrifugation at 15,000 *g*, the supernatants were adsorbed for an additional 4 hr under similar conditions. The appropriate antibodies, 3 μL of rabbit anti-RARα (F) [15] or 8 μg of mouse anti-ubiquitin antibody, were added, followed by a 2-hr incubation. The resulting antigen-antibody complexes were bound to Protein-A-Sepharose and washed twice with lysis buffer, twice with buffer containing 50 mM Tris-HCl, 500 mM NaCl, 0.1% Nonidet P-40, and 0.05% SDS, and once with buffer containing 50 mM Tris-

HCl, 0.1% Nonidet P-40, and 0.05% SDS. Antigen-antibody complexes were eluted in Laemmli buffer by denaturation at 100° for 10 min, fractionated by SDS-PAGE, and subjected to western blot analysis with rabbit anti-RARα (diluted 1:1000) or mouse anti-ubiquitin (diluted 1:200) antibodies.

2.7. Preparation of nuclear protein

Nuclear proteins were prepared as described by Schreiber *et al*. [16]. Cells were washed and pelleted by low-speed centrifugation (5000 *g*) and then suspended in buffer A [10 mM HEPES (pH.7.9), 10 mM KCl, 0.1 mM EDTA, 0.1 mM EGTA, 1 mM DTT, 0.5 mM PMSF, 200 μM Na_3 VO_4, 2 mM NaF]. After brief centrifugation (5000 *g*), the cell pellet was resuspended in buffer A containing 0.3% Nonidet P-40 and lysed on ice. Crude nuclei were centrifuged at 6000 *g* for 5 min at 4° and washed with buffer A. The nuclear pellet was resuspended in nuclear extraction buffer [10 mM HEPES (pH 7.9), 420 mM NaCl, 1.5 mM $MgCl_2$, 25% glycerol, 0.1 mM EDTA, 0.1 mM EGTA, 1 mM DTT, 0.5 mM PMSF, 200 μM Na_3 VO_4, 2 mM NaF] and ultracentrifuged at 75,000 *g* for 10 min at 4°. Protein concentration was determined by the bicinchoninic acid method (Pierce Science). The supernatants were stored at −70°. All the buffers used for nuclear protein extraction contained protease inhibitors.

2.8. EMSA

Double-stranded oligonucleotides used for EMSA (DR1; 5′-AGGTTCAGGTCAGAGGTCAGAGAGCT-3′), (DR2; 5′-GGTAAGAACAAGAGAACACTCGCC-3′), (DR4; 5′-AGGTTCAGGTCACAGGAGGTCAGAGAGCT-3′), (DR5; 5′-TCGAGGGTAGGGTTCACCGAAAGT-TCACTCG-3^{32}P]ATP using T4 PNK. Equal amounts of nuclear proteins (3–5 μg for DR1, DR2, DR4, and DR5) were incubated in binding buffer [20 mM HEPES (pH 7.9), 50 mM KCl, 2.5 mM $MgCL_2$, 10% glycerol, 0.1 mM EDTA, 1 mM DTT] for 20 min at room temperature in the presence of oligonucleotide probes. The DNA and nuclear protein mixture were separated on a 6% 45 mM Tris-borate, 1 mM EDTA (TBE) acrylamide gel in TBE buffer at 100 V at room temperature. The gel was dried under vacuum and exposed to a PhosphoImager screen.

3. Results

3.1. RA-dependent degradation of RARα and RARγ

MCF-7 cells treated with RA ranging from 10^{-9} to 10^{-5} M for 24 hr showed an RA concentration-dependent decrease in RARα and RARγ (Fig. 1A). A 50% reduction of RARα and RARγ was observed with 10^{-7} and 10^{-6} M RA, respectively (Fig. 1B). To investigate the time-dependency of the effect of RA on RAR degradation, MCF-7 cells were incubated with 10^{-6} M RA for the indicated times (Fig. 1, C and D). A decline of RARα was observed 60 min after RA addition, in the absence of an effect on the level of actin protein.

To explore the mechanisms responsible for the rapid decline of RARα, northern blot analysis was performed, using total RNA extracted from MCF-7 cells treated with 10^{-6} M RA for different times as described in Fig. 2. Interestingly, RARα mRNA levels were elevated markedly in cells treated with 10^{-6} M RA. However, RARγ mRNA showed only a slight increase (Fig. 2, A and B). Other retinoid compounds, such as 9-*cis*-RA and 4HPR (10^{-6} M), were also able to down-regulate the level of RARα and γ protein (Fig.3A). Since estrogen is known to up-regulate RAR expression [17], as a negative control, 10^{-8} M estrogen was added to the culture medium, instead of RA. As expected, the RARα and RARγ proteins were not degraded in the presence of estrogen (Fig. 3A). This implies that the decline of RARα and RARγ was induced in a retinoid-dependent manner. To see if this down-regulation is ligand binding-dependent, cells were treated with 10^{-6} M RAR- or RXR-selective agonists and an RAR-selective antagonist (Fig. 3B). Down-regulation of RARα was induced by all RAR isoform-selective agonists (RARα, AG193836; RARβ, AG193174; and RARγ, AG194433). On the other hand, RARγ down-regulation was RARγ agonist (AG194433) specific. Interestingly, the RAR antagonist AG193109 failed to induce the breakdown of either RARα or RARγ (Fig. 3B). Interestingly, the RXR-specific agonist AG194204 induced the reduction of RXRα, as well as RARα and γ (Fig. 3B). These results suggest that RARα and γ are modified post-transcriptionally in a time- and RA concentration-dependent manner. Moreover, this down-regulation presumably occurs in a ligand-specific manner.

3.2. Effect of proteasome inhibitors on degradation of RARα and γ

To determine whether the degradation of RARα and γ mediated by RA is 26S proteasome-dependent, MCF-7 cells were incubated with proteasome-specific or non-specific protease inhibitors. As shown in Fig. 4, ALLN (Calpain inhibitor I), which inhibits proteasomal proteolysis as well as calpains and cathepsins, effectively reduced RA-induced RAR degradation. ALLM (Calpain inhibitor II), which is a potent inhibitor of calpains and cathepsins, but does not inhibit proteasomes, was also used as a negative control. ALLM was unable to inhibit RA-mediated RAR degradation (Fig. 4). Potent cell permeable proteasome inhibitors, such as MG132 and PS1, inhibited the degradation of RARα and γ. On the other hand, E64, widely used as a cysteine protease inhibitor, did not show any effect on this degradation by RA. Thus, these results suggest that rapid degradation of RARα and γ mediated by RA treatment was

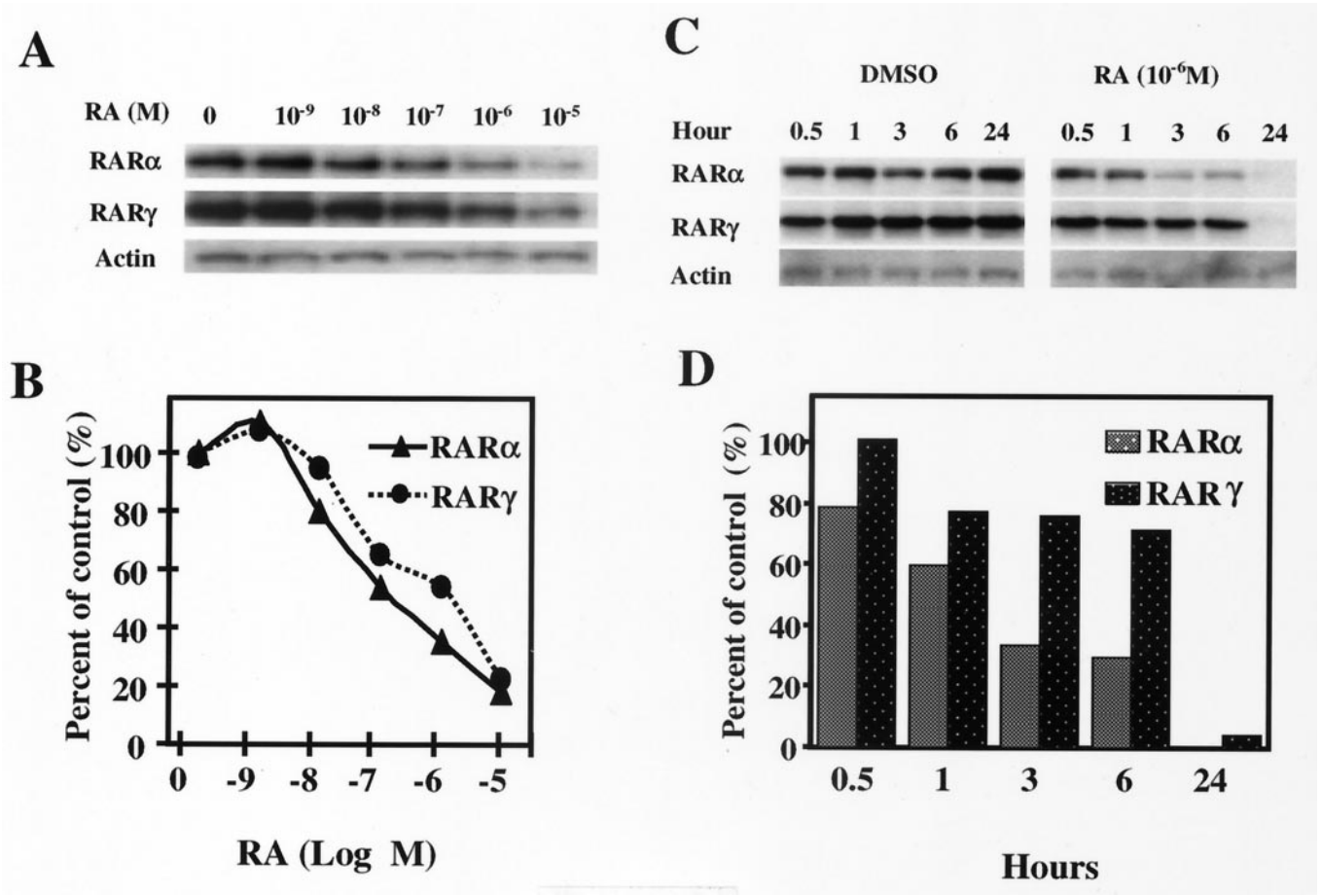

Fig. 1. Concentration- and time-dependent effects of RA on RAR proteins in MCF-7 cells. (A) Concentration-dependent effect of RA on RARα and RARγ. Cells were treated with RA ranging from 10^{-9} M to 10^{-5} M for 24 hr. Cells were lysed into Laemmli buffer. 30 μg of total cell lysate was subjected to western blot, and total cell protein was collected as described in "Materials and methods". (B) Densitometric analysis of the data shown in panel A, normalized for actin. (C) Time-dependent effect of RA on the RARα and RARγ protein level. Cells were treated with 10^{-6} M RA for the time indicated up to 24 hr. (D) Densitometric analysis of the data shown in panel C, normalized for actin.

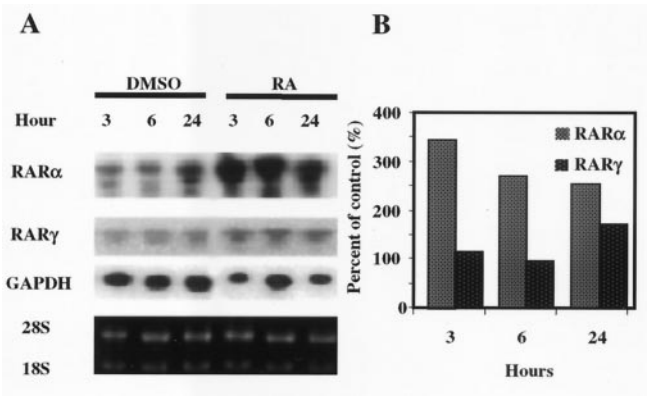

Fig. 2. Effect of RA on the mRNA of RARs in MCF-7 cells. (A) Northern blot analysis of total RNA (10 μg) extracted from MCF-7 cells treated with 10^{-6} M RA for the times indicated above. The cDNA was labeled by a random primer method and hybridized overnight at 42°. (B) Densitometry results are standardized on the basis of glyceraldehyde-3-phosphate dehydrogenase (GAPDH). Data are expressed as percent of the DMSO control value at the same time.

taking place in a proteasome-dependent way, and presumably via ubiquitination.

3.3. RA induction of the formation of RARα/ubiquitin conjugate

Since the degradation of RARα and γ was proteasome-dependent, we attempted to detect the RARα/ubiquitin conjugates by immunoprecipitation. Cells were treated with 10^{-8} to 10^{-6} M RA, for 1 hr, and extracted with lysis buffer. The cellular extracts were immunoprecipitated with either rabbit anti-RARα polyclonal antibody or mouse anti-ubiquitin monoclonal antibody. Blots were probed with mouse anti-ubiquitin or rabbit anti-RARα, respectively. Extracts from RA-treated cells immunoprecipitated with anti-RARα showed discrete higher (than RARα) molecular weight bands, which correspond to a presumptive RARα/ubiquitin conjugate. The formation of these bands was highly RA-concentration-dependent (Fig. 5). RA concentration-dependent higher molecular weight bands were also detected in reciprocal experiments where extracts were im-

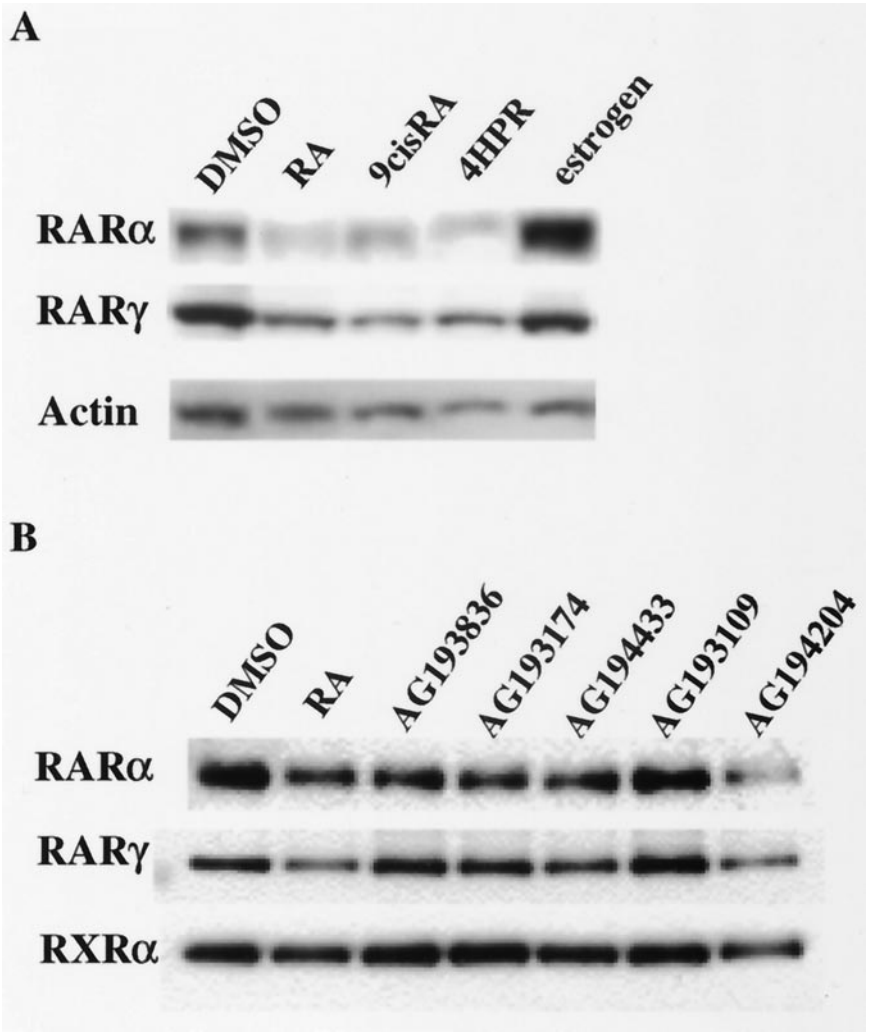

Fig. 3. (A) Retinoid-dependency of RARα and RARγ. RA, derivatives, and estrogen were added into the culture medium, and the cells were cultured in their presence at 37° for 24 hr. Thirty micrograms of total cell protein was subjected to western blot analysis. The same blot was probed for RARα, RARγ, and actin. (B) Effects of receptor-selective agonists on RAR and RXR. Agonists specific for RARα (AG193836), RARβ (AG193174), RARγ (AG194433), and an RAR antagonist (AG193109), as well as an RXR agonist (AG194204), were included in the culture medium at a final concentration of 10^{-6} M, and cells were incubated for 24 hr. Nuclear protein was extracted as described in the text. Five micrograms of nuclear protein was analyzed by western blot, and the same blot was probed for RARα, RARγ, and RXRα.

munoprecipitated with mouse anti-ubiquitin antibody. The immunoprecipitates were run on a polyacrylamide gel, blotted, and probed with rabbit anti-RARα antibody, (Fig. 5).

MCF-7 cells were treated with 10^{-6} M RA for different times to study the time-dependency of RARα/ubiquitin conjugation. Cellular extracts were immunoprecipitated with an anti-ubiquitin antibody. The immunoprecipitates were run on a gel, blotted, and probed with RARα. A higher molecular weight band was prominent at 60 min of RA treatment (Fig. 6A). This band was nearly absent by 3 hr. To confirm that the slower migrating band represents a ubiquitin conjugate, the proteasome inhibitor ALLN was added to the culture medium and incubated for 1 hr under similar experimental conditions. Fig. 6B shows an accumulation of the conjugate in the presence of ALLN. These data suggest that RA induces the formation of an RARα/ubiquitin conjugate.

3.4. DNA binding to hormone response elements in nuclear protein extracts from RA-treated MCF-7 cells

To study the biological relevance of this breakdown, nuclear proteins extracted from MCF-7 cells treated with or without RA were subjected to EMSA. DNA binding to the hormone response elements including DR1, 2, 4, and 5, which are target DNA binding sites of RAR and RXR, was reduced in nuclear extracts from cells treated with RA (Fig. 7A). Analysis by western blot was performed, using the same nuclear extracts, in order to confirm the amount of protein. RARα, RARγ, and RXRα protein levels were reduced in nuclei as well as in whole cell lysates. This down-regulation correlated with the reduction of DNA binding to hormone response elements (HREs).

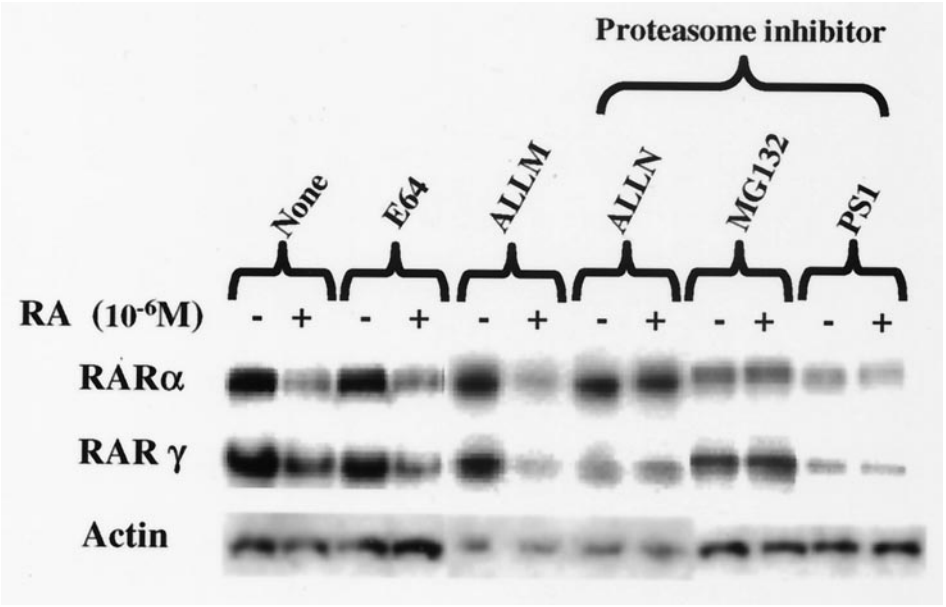

Fig. 4. Study of the effect of proteasome inhibitors on the RA-mediated decline of RARs. Cells were pre-treated with protease inhibitors for 30 min, and RA or DMSO was added to the culture medium. Cells were maintained for an additional 2 hr at 37°. One hundred micromolar E64, 25 μM ALLM, 25 μM ALLN, 100 μM MG132, and 100 μM PS1 were added directly into the culture medium. Fifteen to thirty micrograms of total cell lysate protein was analyzed by western blot, and probed with RARα and RARγ.

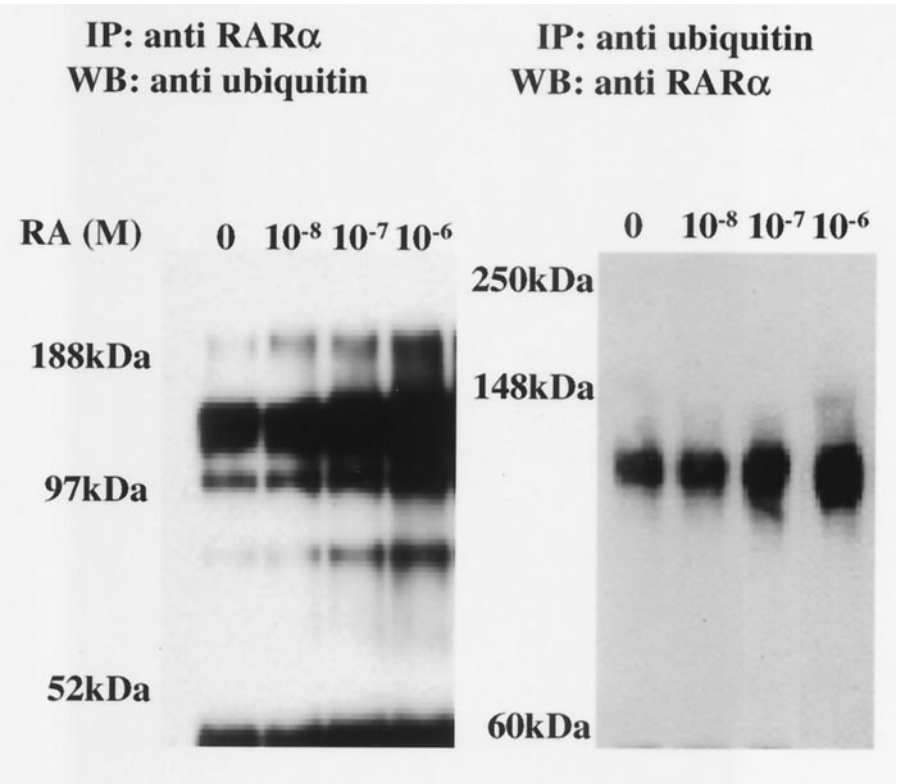

Fig. 5. RA-dependent ubiquitination of RARα. (A) Cellular extracts (50 μg) from MCF-7 cells treated with different concentrations of RA (from 10^{-8} M to 10^{-6} M) for 1 hr were immunoprecipitated with anti-RARα-(F) antibody. Immunoprecipitates were subjected to SDS-PAGE (10% acrylamide), and probed with anti-ubiquitin antibodies. (B) Cellular extracts (250 μg) from cells treated as above were immunoprecipitated with anti-ubiquitin antibody. Immunoprecipitates were subjected to SDS-PAGE (6% acrylamide), blotted, and probed for RARα.

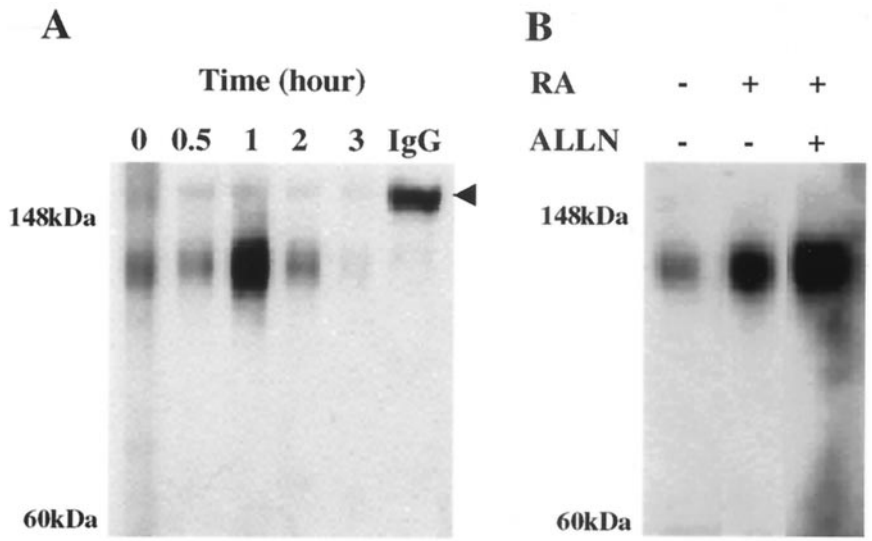

Fig. 6. Time-dependence of the ubiquitination of RARα. (A) Cellular extracts (250 μg) of MCF-7 cells treated with 10^{-6} M RA for different times were immunoprecipitated with anti-ubiquitin antibodies. Immunoprecipitates were subjected to SDS-PAGE (6% acrylamide), and probed for RARα. The arrowhead indicates a non-specific band. (B) The cells were treated with RA for 1 hr in the presence or absence of 25 μM ALLN. Cellular extracts (250 μg), treated as indicated in section 2, were immunoprecipitated with anti-ubiquitin antibodies. Immunoprecipitates were subjected to SDS-PAGE, blotted, and probed for RARα.

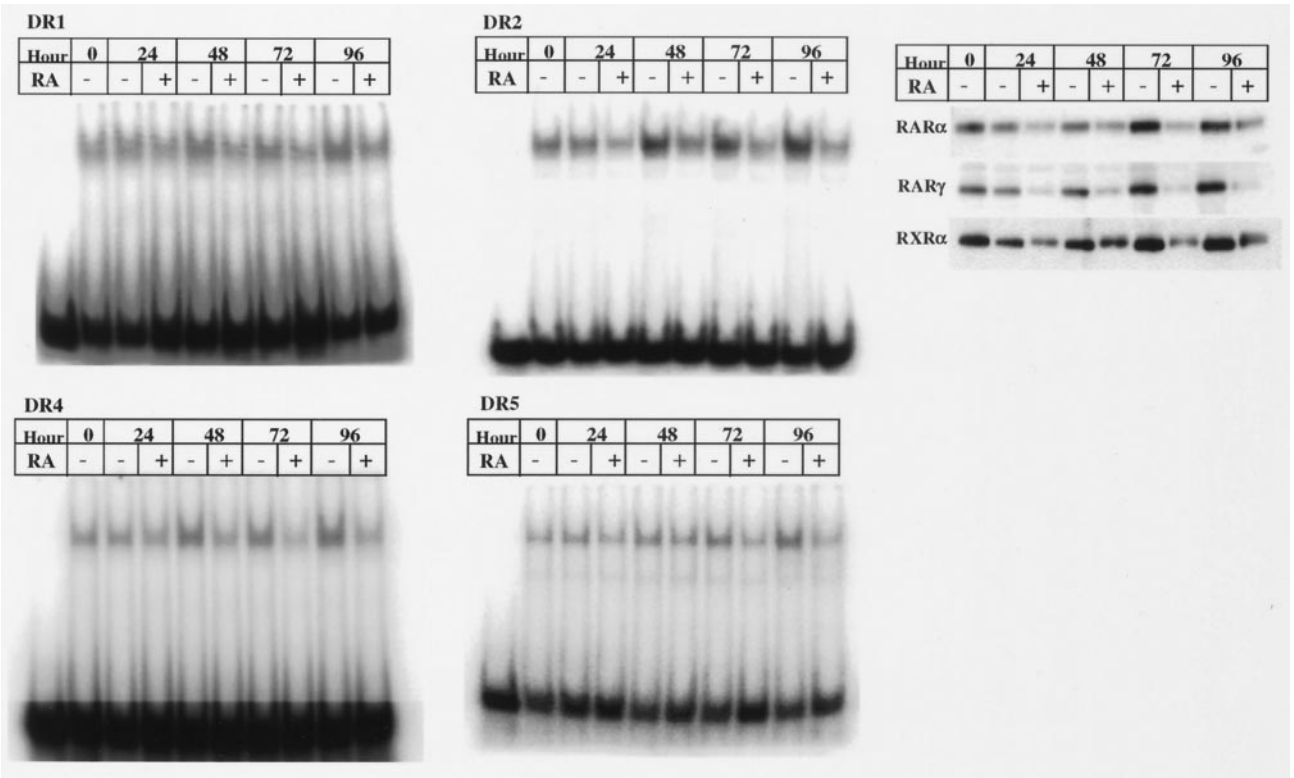

Fig. 7. DNA binding to hormone response elements in nuclear extracts of MCF-7 cells treated with RA. Nuclear extracts (5 μg for HREs) prepared from cells treated with 10^{-6} M RA or DMSO were used for EMSA. End-labeled oligonucleotide (10,000 cpm) was incubated with nuclear extracts for 20 min at room temperature. The reaction mixture was run on the 6% polyacrylamide gel in 0.5$\times$ TBE buffer (left and center panels). The reduction of DNA binding to HRE correlated with the reduction of the amount of nuclear protein. Nuclear extracts (5 μg) used for EMSA were subjected to western blotting (right panel).

4. Discussion

We have demonstrated that RARα and RARγ are degraded rapidly in an RA concentration- and time-dependent manner, that degradation of these proteins is inhibited by proteasome inhibitors, and that an RARα/ubiquitin complex is formed in cultured MCF-7 breast cancer cells. RARβ protein could not be detected under these experimental conditions by western blot analysis. Interestingly, although RA caused down-regulation of the RARα and γ proteins, it up-regulated RARα and γ mRNA. We were unable to detect either smaller molecules, possibly generated through the degradation process, or RARα/ubiquitin conjugates under the experimental conditions used for western blot analysis. However, our immunoprecipitation data suggest that ubiquitin forms covalent complexes with RARα, i.e., multi- or poly-ubiquitin chains were eventually degraded by the 26S proteasome.

The RARα/ubiquitin conjugate was formed efficiently in an RA concentration-dependent manner, with an ED_{50} for RA of approximately 10^{-7} M (Fig. 1, A and B, and Fig. 5). Interestingly, the immunoprecipitation study showed that the RARα/ubiquitin conjugate accumulates for up to 1 hr of RA treatment and then is degraded within 3 hr by proteasome. These immunoprecipitation data are consistent with the decline of RAR observed in the western blot. The RARα/ubiquitin conjugate was formed even in the absence of RA (Fig. 4). We anticipate that alteration of serum concentration might also trigger ubiquitination; RARα tended to be degraded gradually, even in solvent-treated cells, after the serum concentration was changed from 10% to 2% (data not shown). Since we also observed that RARα and γ proteins were degraded by RA treatment in the presence of 10% serum in the culture medium as well as medium containing 2% serum (data not shown), we conclude that RA stimulates RARα/ubiquitin conjugation and that the RARα/ubiquitin complex might be recognized and degraded by proteasomes. RARα and RARγ contain putative PEST sequences rich in Pro, Gln, Ser, and Thr residues, which serve as a signal for rapid proteolysis [18]. The PEST region contains negatively charged residues surrounded by a cluster of basic amino acids and are most likely located on the surface of proteins. The amino acid sequence with the highest PEST score is located in the N-terminus of both RARα and RARγ. The 26S proteasome has been reported to be localized in the cytosol, as well as in nuclei [19]. On the other hand, RARs are predominantly localized in the nucleus, but it is unknown how and where breakdown of RARs occurs.

We also detected a SUMO-1/RARα conjugate, but the formation of the SUMO-1/RARα conjugate was not RA-concentration-dependent (data not shown). SUMO-1 is known to covalently bind Ran-GTPase [20] activating protein (RanGAP1), promyelocytic leukemia (PML) [21], and IκBα [22]. SUMO-1 has been localized in the nuclear pore complex within the nucleus [20]. Modification of RanGAP1 by SUMO-1 does not lead to proteolysis, but rather seems to modulate translocation to the nuclear pore complex or stabilization of the protein. Since many unknown proteins seem to be modified by SUMO conjugation *in vivo*, it would also be interesting to know how SUMO-1 attaches to RAR and what is the subsequent role of this modification. It has been demonstrated that retinoids have chemopreventive activity against tumor development in different epithelial tissues [23]. The central role of retinoids in chemoprevention is understood to inhibit proliferation and induce differentiation through RARs in epithelial cells [24]. An interesting study has been published by Langenfeld *et al*. [25], who reported that RA treatment of BEAS-2B cells reduces their growth and induces proteolysis of cyclin D1 through ubiquitination of this cyclin. Further, Spinella *et al*. [26] reported that RA promotes ubiquitination of cyclin D1 through RARγ and regulates G1-S transition in the human embryonal carcinoma cell line NT2/D1. Sueoka *et al*. [27] also have reported that CDK-4 was degraded in the presence of RA by 26S proteasomes, a finding consistent with down-regulation of CDK4 by RA in MCF-7 cells [28]. RA may possibly also contribute to cell cycle regulation through the breakdown of RARα and RARγ in MCF-7 cells. More recently, Zhu *et al*. [29] reported that RA induces proteasome-dependent breakdown of RARα and PML/RARα. UV light has also been shown to induce RAR and RXR breakdown by ubiquitination in human keratinocytes [30]. Preliminary reports of a similar phenomenon in WEHI-3B human leukemia cells[1] and in MCF-7 cells[2] have appeared. Our findings in MCF-7 breast cancer cells confirm their results and imply that the RAR breakdown through ubiquitination induced by RA is a common event among different types of cells. Further, we emphasize that breakdown through ligand-dependent ubiquitination occurs in all RARα, RARγ, or RXR, suggesting that ligand-inducible breakdown is a common event among hormone receptors. Interestingly, our results showed that RAR binding to hormone response elements was diminished in RA-treated cells, although most published studies have clearly shown that RA enhances transactivation of HRE among various cell types. Zhu *et al*. [29] suggested that RAR/RXR heterodimerization to DNA might have been a trigger for degradation. We speculate that the biological role of RAR and RXR breakdown, if any, might be to limit extended transactivation by RA. Indeed, a transactivation signal may activate the ubiquitination of many transcription factors [31].

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