Ubiquitinated or sumoylated retinoic acid receptor α determines its characteristic and interacting model with retinoid X receptor α in gastric and breast cancer cells

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Abstract

Retinoic acid receptor α (RARα) plays an important role in mediating all-trans retinoic acid (ATRA) signals. In this study, we found that ATRA up-regulated RARα mRNA and protein expression in gastric cancer BGC-823 cells. However, in breast cancer MCF-7 cells it down-regulated RARα protein expression with no effect on its RARα mRNA. Immunoprecipitation/Western blot analysis showed that, although sumoylated and ubiquitinated RARα existed simultaneously in both cancer cell lines, ATRA exerted different regulatory effects on sumoylation and ubiquitination of RARα. In MCF-7 cells, ATRA treatment enhanced the ubiquitination of RARα and the subsequent degradation of RARα through the ubiquitin/proteasome pathway. This resulted in a reduction in the DNA binding activity of RARα/RXRα heterodimer, the separation of RXRα from RARα and the translocation of RXRα from the nucleus to the cytoplasm. By contrast, in BGC-823 cells, ATRA augmented sumoylation, not ubiquitination, of RARα. The stability of sumoylated RARα was significantly stronger than in non-sumoylated RARα. These results also showed an increase in the DNA binding activity of the RARα/RXRα heterodimer and the stability of nuclear localization of this heterodimer, which normally facilitates the ATRA signal transduction. In conclusion, our results reveal a novel mechanism for the regulation of RARα-dependent signal transduction through the ubiquitin/proteasome pathway in breast cancer cells and the sumoylation pathway in gastric cancer cells.

Journal of Molecular Endocrinology (2004) 32, 595–613

Introduction

Retinoic acid (RA) and its natural and synthetic vitamin A derivatives, retinoids, are known as regulators of a broad range of biological processes and are used in the treatment of epithelial cancer and promyelocytic leukemia (Gudas et al. 1994, Hong & Itri 1994). The physiological function of RA is mediated through its cellular receptors, the retinoic acid receptors (RARα, β, and γ) and the retinoid X receptors (RXRα, β, and γ) (Zhang & Pfahl 1993, Kastner et al. 1995). RARs and RXRs are commonly located in the nucleus and thus are classified as nuclear receptors. RXRs usually form homodimers, RXR/RXR, or interact with RARs to form heterodimers, RAR/RXR (Kliewer et al. 1992, Leid et al. 1992, Marks et al. 1992). RA can pass through the cell membrane and enter the nucleus via free diffusion, thus interacting with and binding to its nuclear receptors RAR/RXR or RXR/RXR. These dimers can bind to and act on RA response elements (RAREs) to control the expression of relevant RA-responsive genes in the presence of RA (Yu et al. 1991, Bugge et al. 1992, Kliwer et al. 1992, Leid et al. 1992, Zhang et al. 1992). The transcriptional activity of retinoid receptors is mainly controlled by ligand binding; however posttranscriptional covalent modifications, such as phosphorylation, ubiquitination and sumoylation, also play an important role in regulation of retinoid receptor activity (Sternsdorf et al. 1999b, Lallemand-Breitenbach et al. 2001, Tanaka et al. 1994)}
2001, Gianni et al. 2002). These posttranslational modifications often elicit fast alterations in the protein–protein interactions of multiprotein complexes and subcellular structures.

Some nuclear receptors are known to be degraded by covalent ligation to ubiquitin, a highly conserved 76-amino acid peptide, via the lysine residues (Li et al. 1999, Nawaz et al. 1999, Gianni et al. 2002). Protein ubiquitination is initiated by multiple enzyme reactions catalyzed by ubiquitin-activating enzyme (E1), ubiquitin-conjugating enzymes (E2s), and ubiquitin-protein ligases (E3s) (Weissman 1997, Wilkinson 2000). Evidence has been found that the fate of the ubiquitinated protein is determined by the types of ubiquitin conjugate formed. For instance, a single ubiquitin tag does not target a protein for proteasomal degradation, whereas a chain of four or more does (Thrower et al. 2000). Therefore, the polyubiquitin chain is essential for the degradation of proteins whose levels are regulated either constitutively or in response to changes in the cellular environment (Tanaka et al. 2001). In addition, ubiquitinated proteins are recognized specifically by a large protease complex, known as the 26S proteasome (Fenteany et al. 1995, Hochstrasser 1996), which may be responsible for 80–90% of protein degradation in the cell (Lee & Goldberg 1998). It is now understood that both ubiquitin and the proteasome can also carry out various non-proteolytic tasks, controlling activities as diverse as receptor internalization (Terrell et al. 1998), ribosome function (Spence et al. 2000) and nucleotide excision repair (Russell et al. 1999).

The small ubiquitin-related modifier (SUMO) modification pathway (sumoylation) resembles that of ubiquitin conjugation, however the enzymes involved in the two processes are distinct (Melchior 2000). SUMO-1, also named Sentrin, Ub1 or, in yeast, Smt3 (Johnson et al. 1997, Kamitani et al. 1997), is 18% identical to ubiquitin and utilizes a similar conjugation pathway. This pathway involves activation by a heterodimeric Aos1-Uba2 activating enzyme (E1) and conjugation to a substrate by Ubc9 (Johnson et al. 1997, Johnson & Blobel 1999), a protein with a strong sequence similarity to ubiquitin-conjugating enzymes (E2s). However, unlike ubiquitin, SUMO-1 does not conjugate to itself and thus is not capable of forming chains similar to polyubiquitin. Recently, the functions of SUMO-1 modification have been promulgated by accumulated reports. It appears to play multiple roles including protein targeting, protein stabilization and transcriptional activation. SUMO-1 conjugation has been found to alter the subcellular localization of its RanGAP1, Sp100 and PML substrates (Mahajan et al. 1997, Duprez et al. 1999, Sternsdorf et al. 1999a). Sumoylation of p53 is believed to increase its transcriptional activity (Gostissa et al. 1999, Rodriguez et al. 1999). However, SUMO-1 has an entirely different effect on IκBa, where it attaches to the major ubiquitination site preventing ubiquitination and protecting sumoylated IκBa from proteasome-dependent proteolysis (Desterro et al. 1998).

Our previous studies have demonstrated that the expression and localization of RARα and RXRα are associated with diverse signaling transduction pathways and their resulting physiological processes in carcinoma cells (Wu et al. 2002b, Ye et al. 2004). In addition, we have found that RARα may regulate the signaling switch from RAR-mediated to RXR-mediated growth inhibition in breast cancer cells (Wu et al. 1997a), however, the regulatory role of retinoic acid on RARα at the posttranscriptional level is still largely unknown. In this study, we have investigated RARα ubiquitination and sumoylation in breast and gastric cancer cell lines and further inquired into the regulatory effect of all-trans retinoic acid (ATRA) on RARα ubiquitination and sumoylation, as well as the resultant functional influences in these cells.

Materials and methods

Cell culture

Cell lines, including MCF-7 (breast cancer) and BGC-823 (gastric cancer) were obtained from the Institute of Cell Biology, Shanghai, China. The MGC80–3 cell line was established by the Cancer Center in Xiamen University, Fujian, China. Cells were maintained in RPMI-1640 medium supplemented with 10% fetal bovine serum, 1 mmol/l glutamine and 100 U/ml penicillin, and cultured in a humidified atmosphere supplemented with 5% CO₂ at 37 °C.

Antibodies and inhibitors

Monoclonal antibodies against SUMO-1 and ubiquitin were purchased from Zymed Laboratories.
Northern blotting

Total RNA was isolated by guanidium thiocyanate followed by ultracentrifugation in CsCl solutions as described previously (Wu et al. 2002a). Twenty micrograms total RNA were denatured with formaldehyde, fractionated by electrophoresis on 1% agarose-formaldehyde gel, and blotted onto nitrocellulose membrane. RARα cDNA was labeled with [$\alpha$-$^{32}$P]dATP and [$\alpha$-$^{32}$P]dCTP, using the random primer method. The membrane was prehybridized at 42 °C for 4 h and then hybridized with radiolabeled RARα cDNA overnight at 42 °C. After hybridization, the nylon filter was washed twice with 2 × SSC and 0·1% SDS at 42 °C and finally washed twice with 1 × SSC and 0·1% SDS at 60 °C. mRNA was visualized in X-ray film at −80 °C overnight. 18S and 28S RNA indicated the amount of total RNA used in each lane. RARα mRNA level was quantified by Densitometer. Data were means ± s.d. for duplicate experiments.

Western blotting

Cell extract was essentially prepared according to the method previously described (Wu et al. 2002c). In brief, lysate was 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). Fifty micrograms total protein were loaded onto an 8–10% denaturing gel and then transferred to a nitrocellulose membrane. The membrane was incubated with appropriate antibody overnight, followed by the corresponding secondary antibody for 3–4 h. Protein was visualized on X-ray film by enhanced chemiluminescence (ECL, Pierce Biotechnology, IL, USA) according to the manufacturer’s instructions. α-Tubulin indicated the amount of protein used in each lane. RARα protein level was quantified by Densitometer. Data were means ± s.e. for three independent experiments.

Immunoprecipitation/Western blot analysis

Cells were washed three times with ice-cold PBS and lysed in 1 ml ELB buffer (140 mM NaCl, 0·5% NP40, 100 mM NaF, 50 mM Tris/HCl pH 8·0), then vortexed and centrifuged at 15 000 g for 10 min to obtain all cell lysate (supernatant). To preclear the cell lysate, it was mixed and incubated with protein A beads for 30 min and centrifuged for 15 min at 1000 g to get preclear lysate (supernatant). The precleared lysate was then incubated with anti-RARα antibody for 2 h. The resulting antigen–antibody complexes were bound to protein A-Sepharose and incubated for an additional 4 h at 4 °C. After a brief centrifugation at 1000 g, the immunoprecipitate was collected and divided into two parts. One was subjected to Western blot analysis with corresponding antibodies, such as ubiquitin, SUMO-1 or RXRa, to indicate their interaction with RARα. The other was analyzed by Western blotting with the anti-RARα antibody to indicate the amount of immunoprecipitated RARα protein used in each lane. About 50~100 µg nuclear extract were applied to ascertain the position of blotted proteins (Input).

Nuclear extract preparation and gel retardation assay

The nuclear extract was essentially prepared according to the method described previously (Wu et al. 1997b). In brief, cells were grown to about
90% confluence, washed with cold PBS and scraped into PBS using a rubber policeman. Cells were pelleted by low-speed centrifugation, then resuspended in a buffer containing 10 mM Tris–HCl (pH 7·4), 3 mM CaCl₂, and 2 mM MgCl₂, then re-pelleted and lysed in buffer containing 1% Nonidet P-40 by 20 strokes using an ice-cold Dounce homogenizer. Immediately after lysis, nuclei were collected by centrifugation at 2000 g and washed once in a buffer containing 10 mM HEPES-KOH (pH 7·9), 1·5 mM MgCl₂, 10 mM KCl, and 0·5 mM dithiothreitol. Nuclear protein was extracted with a high-salt buffer containing 20 mM HEPES-KOH (pH 7·9), 25% glycerol, 420 mM NaCl, 1·5 mM MgCl₂, 0·2 mM EDTA and 0·5 mM dithiothreitol. Protein concentration was determined by the bicinchoninic acid method (Pierce Science). The supernatants were stored at −70 °C. All the buffers used for the procedure contained protease inhibitors, such as PMSF (100 µg/ml), leupeptin (1 µg/ml) and aprotinin (1 µg/ml). The 32P-labeled receptor response element DR5 (oligonucleotide: 5’-TCGAGGG TAGGTTGCACGAAAGTTCACTCG-3’) was used as a probe, as described in the published literature (Tanaka et al. 2001). Five micrograms nuclear extract were incubated first with 1 µl of required antibody at room temperature for 30 min, then with the probe at room temperature for another 30 min prior to gel retardation assay. The gel retardation assay was completed on a nondenaturing polyacrylamide gel at 4 °C, which was then dried under vacuum and exposed at −80 °C overnight.

Laser-scanning confocal microscopy

Cells were immunostained according to the method described previously by Wu et al. (2002b). Briefly, cells were cultured on covered glass overnight and then treated with ATRA or other agents as required. After washing with phosphate-buffered saline (PBS), cells were fixed in 4% paraformaldehyde. To display the RARα or RXRα proteins, cells were incubated with anti-RARα or anti-RXRα antibody respectively, then reacted with the corresponding secondary antibody – Texas-red-conjugated (Molecular Probes, Eugene, OR, USA) or FITC-conjugated anti-IgG (BD Biosciences, Pharmingen, San Diego, CA, USA). Fluorescent images were examined and analyzed under a laser-scanning confocal microscope (Bio-Rad MRC-1024ES).

Pulse-chase experiment

This experiment was completed as described by Osburn (Osburn et al. 2001). Cells were labeled with 35S-methionine (0·1 mCi/ml) in methionine-free DMEM medium for 4 h. After labeling, cells were either directly lysed or chased with the addition of 2 mM cold methionine for the indicated time points in the absence or presence of ATRA. Upon completion of chase, cells were washed with ice-cold PBS three times and collected. Cells were pelleted for 1 min at 14 000 g at 4 °C, and then lysed in 200 µl lysis buffer (50 mM Tris [pH 8·8], 5 mM EDTA, 2% SDS, 10 mM dithiothreitol, 100 µM sodium vanadate, 10 mM sodium fluoride, protease inhibitors). The supernatant was pre-cleared through incubation with protein A/G-agarose for 30 min and then centrifuged to obtain the pre-cleared supernatant. The pre-cleared supernatant was incubated with anti-RARα antibody for 1 h and the resulting antigen–antibody complexes were bound to protein A-Sepharose and further incubated for an additional 4 h at 4 °C. After a brief centrifugation at 1000 g, the immunoprecipitate was collected and washed three times with lysis buffer, separated on 8% SDS-PAGE. Finally, the gel was dried under vacuum and exposed at −80 °C for 7 days. Protein levels were quantified by Densitometer. The bars represent ± S.D from three independent assays.

Results

ATRA induced distinct patterns of alteration on transcriptional activity and protein synthesis of RARα in different cell lines

Northern blot and Western blot showed that in gastric cancer cells BGC-823, ATRA could enhance RARα expression in both mRNA and protein levels in a time-dependent manner (Fig. 1A). The results also showed an enhancement of RARα mRNA (~ fourfold) and protein (~ threefold). This was clearly observed after 24 h of ATRA addition, as compared with the control (Fig. 1B). By contrast, in the breast cancer cells MCF-7, there was not an obvious change in RARα mRNA expression (Fig. 1A) and the cell’s protein level was
significantly inhibited (more than 80% inhibition as compared with the control) at 24 h of treatment with ATRA (Fig. 1A and B). However, the expression of RXRα was not affected by ATRA treatment either at the mRNA or protein level in both gastric and breast cancer cell lines (data not shown). The distinct patterns of ATRA-treated RARα mRNA and protein expression in these two cell lines suggest that RARα may have a functional role in mediating ATRA signaling events in different cell lines.

The interactions between ATRA and its receptors, as well as the resultant functional effect on the transcriptional activity of the reporter, were
further tested by transient transfection assay in both cancer cell lines. Here, a CAT reporter containing βRARE (RA response element) linked with a thymidine kinase promoter (βRARE-tk-CAT) was used. When transfected into both MCF-7 and BGC-823 cells respectively, CAT activity in response to ATRA was observed due to endogenous RARα and RXRα activity (Fig. 1C). More importantly, the ATRA-induced CAT activity in BGC-823 cells cotransfected with both reporter and RARα expression vector, was affected by RARα in a concentration-dependent manner and

![Graph showing CAT activity in MCF-7 and BGC-823 cells with varying concentrations of RARα and ATRA.]

![Western blot images showing RARα and α-tubulin expression in MCF-7 and BGC-823 cells with and without ATRA treatment.]

**Figure 1 (C) and (D).**
was higher than in MCF-7 cells (Fig. 1C). This difference implied that ATRA induction might initiate the distinct behavior of the RA target gene transcription in these two cell lines.

Low RARα protein levels in ATRA-treated MCF-7 cells may be due to the reduced rate of RARα protein synthesis and/or the accelerated rate of its degradation. To explore this question, cycloheximide (CHX) was used to prevent new protein synthesis in Western blot examination. As shown in Fig. 1D, in MCF-7 cells, the RARα protein level in the CHX-treated sample was lower than that of control, indicating the inhibitory effect of CHX on RARα protein synthesis, yet it was higher than the ATRA-treated sample. In addition, in cells treated with CHX plus ATRA, the RARα protein level was significantly lower than after either ATRA or CHX treatment alone (Fig. 1D), indicating that ATRA-related low RARα protein in MCF-7 cells was likely to be due to the accelerated rate of RARα breakdown.

A parallel experiment was carried out in BGC-823 cells and as shown in Fig. 1D, CHX not only inhibited the newly synthetic process of RARα protein, but also abolished the high RARα protein level stimulated by ATRA alone (Fig. 1A and D), indicating that ATRA-related low RARα protein in MCF-7 cells was likely to be due to the accelerated rate of its protein synthesis.

ATRA exerts diverse effects on RARα ubiquitination and sumoylation in different cell lines

An interesting phenomenon appeared when the RARα protein from both MCF-7 and BGC-823 cells was examined by Western blot analysis. Many reacting bands (between 63-8 and 113-7 kDa) were observed in both cell lines (Fig. 2A, modified), suggesting that other molecules, such as SUMO-1 or ubiquitin, may be covalently conjugated to RARα, resulting in the up-shift of RARα onto bands of higher molecular weight. We further analyzed the status of RARα, testing the possibility of its ubiquitination and sumoylation. RARα was immunoprecipitated with an anti-RARα antibody on extracts from both gastric and breast cancer cell lines and these immunoprecipitates were subjected to Western blotting with anti-ubiquitin antibody. As indicated by the molecular marker, only protein of upper-bands could be detected by the anti-ubiquitin antibody in both BGC-823 and MCF-7 cells, implying that the RARα may be in some ubiquitinated form in both cell lines (Fig. 2B). With the extension of ATRA treatment, further accumulation of ubiquitinated-RARα could be seen in the MCF-7 cells even as early as 12 h treatment, but not in BGC-823 cells (Fig. 2B). The molecular weight of ubiquitin has been reported to be about 8·5 kDa (Goldstein et al. 1975), but the bands of interest have a molecular mass within the range of 40–50 kDa (Fig. 2A and B), suggesting that four or more ubiquitin molecules might covalently conjugate with RARα.

A parallel experiment was conducted on anti-RARα immunoprecipitates from the extracts of these two cell lines. The immune complexes were probed with anti-SUMO-1, instead of anti-ubiquitin antibody. The results showed that the RARα–SUMO-1 complex appeared in samples from both MCF-7 and BGC-823 cells and that the intensity increased from 12 h of ATRA treatment. The time-dependent augmented action of the RARα–SUMO-1 complex by sustained ATRA treatment only occurred in the BGC-823 cells, not in MCF-7 cells (Fig. 2C). Therefore, the patterns of RARα ubiquitination and sumoylation stimulated by ATRA were significantly divergent.
Figure 2 Ubiquitination and sumoylation of RARα in response to ATRA in different cell lines. (A) Protein expression of RARα was examined by Western blot. The lowest band is endogenous RARα, and the upper bands are these modified. (B) Whole cell extracts were immunoprecipitated with anti-RARα antibody, then divided into two parts. After the regular SDS-PAGE and blotting procedures, samples were probed with anti-ubiquitin antibody for demonstrating the formation of RARα–ubiquitin complex, or probed with anti-RARα antibody for quantifying the amount of immunoprecipitated RARα protein used in each lane respectively. The same nuclear extract was applied to ascertain the position of protein by Western blotting (Input). (C) The sample was prepared as described in (B), but probed with anti-SUMO-1 antibody. IgG was used as a negative control, no band appeared in the position corresponding to SUMO-1. IP, Immunoprecipitation; WB, Western Blotting.
in the different cells (Fig. 2B and C). In summary, Fig. 2 demonstrates that although both ubiquitination and sumoylation of the RARα protein are normal events in gastric and breast cancer cells, ATRA is able to enhance RARα ubiquitination in MCF-7 cells and RARα sumoylation in BGC-823 cells. This effect may relate to the divergent biological roles of RARα in different cell types.

**Ubiquitin/proteasome pathway mediates RARα degradation**

Based on the results shown in Fig. 2B, it might be suggested that degradation of RARα is associated with the ubiquitination pathway in MCF-7 cells. Since, as in most cases, ubiquitination is closely associated with the protein degradation-related proteasome, the possible involvement of the ubiquitin/proteasome pathway was evaluated by using compounds known to inhibit proteasome activity. In this experiment, proteasome-specific inhibitors, MG132 (potent cell permeable proteasome inhibitor), ALLN (inhibitor for calpains, cathepsins and proteasomal proteolysis), and ALLM (a potential inhibitor for calpains and cathepsins, but not for proteasome) were used (Rock et al. 1994). As shown in Fig. 3A, the addition of MG132 and ALLN completely blocked ATRA-induced degradation of RARα in MCF-7 cells, while ALLM was much less effective. These results indicated that not only the proteasome, but also the ubiquitination pathway might be involved in degradation of RARα by ATRA. We further analyzed the relationship between ubiquitination/proteasome and degradation of RARα using these inhibitors in an immunoprecipitation/Western blot experiment on MCF-7 cells. ALLN increased the intensity of the ubiquitin-containing complex when compared with the control. Treatment with ATRA together with ALLN caused a marked increase in accumulation, not degradation, of the ubiquitinated complex. This complex was even more abundant than that of the ALLN treatment alone (Fig. 3B). The enhancing effect was also found in the MG132 treatment group (data not shown), but not in the ALLM treatment group (Fig. 3B). Clearly, ALLN and MG132, rather than ALLM, block the ATRA-induced RARα degradation pathway and promote the accumulation of ubiquitinated RARα. This indicates the existence of a close correlation between the ubiquitination/proteasome pathway and RARα degradation.

**RARα ubiquitination/degradation decreases its heterodimerization with RXRα**

Next, we investigated whether the ubiquitination and sumoylation of RARα may affect RARα–RXRα heterodimer interaction. The cellular localization of RARα and RXRα in MGC-823 and MCF-7 cells with and without ATRA treatment was first examined. RARα and RXRα proteins were immunostained using relevant antibodies and fluorescent secondary antibodies, and then examined under a laser scanning confocal microscope. The result demonstrated that RARα and RXRα occupy the same nuclear location in both MCF-7 and BGC-823 cells, shown by a unique yellow color due to the overlapping of red RARα with green RXRα (Fig. 4A). In response to ATRA treatment, most of the RXRα protein showed a clear translocation from the nucleus to the cytoplasm in MCF-7 cells (Fig. 4A). In contrast, RXRα remained in the nucleus of BGC-823 cells, even after 24 h of ATRA treatment (Fig. 4A). Results from the experiments on RARα showed it remained in the nucleus and there was no ATRA-induced translocation found in either BGC-823 or MCF-7 cells (Fig. 4A). These results infer an association of RARα with RXRα in BGC-823 cells and a disassociation accompanied by RXRα nucleocytoplasmic translocation in MCF-7 cells in response to ATRA.

The heterodimerization status of RARα with RXRα was then analyzed by gel retardation assay and further confirmed by immunoprecipitation/Western blot. Nuclear proteins were prepared from both MCF-7 and BGC-823 cells for gel retardation assay. When receptor response element DR-5, an idealized direct repeat RA-specific response element with 5-bp spacer (Umesono et al. 1991), was used as the probe, a binding complex was detected in the absence of ATRA in both cancer cell lines (Fig. 4B). After the nuclear protein was separately incubated with anti-RXRα or anti-RARα antibody (represented in Fig. 4B with aRXRα or aRARα respectively), the formation of the binding complex was significantly repressed in each case (Fig. 4B). This indicates that the complex was mainly composed of RARα and RXRα protein. However, we noted that the binding complex formation could
Figure 3 Effect of proteasome inhibitors ALLN (25 µmol/l), MG132 (10 µmol/l) and the calpain inhibitor ALLM (25 µmol/l), on ATRA-mediated RARα degradation and RARα ubiquitin interaction. (A) Effect of different inhibitors on ATRA-induced RARα degradation. Different inhibitors were added directly, as required, into cell cultures for 2 h, followed by ATRA treatment for 12 h. Fifty micrograms protein were analyzed by Western blot, probed with anti-RARα antibody. (B) Effect of proteasome inhibitors, ALLN and ALLM, on RARα–ubiquitin conjugation. Cells were treated with ALLN, ALLM and ATRA as described above, and then were lysed. Whole cell extract was immunoprecipitated with anti-RARα antibody. Protein bound to the beads was eluted, and the ubiquitinated protein level was examined with anti-ubiquitin antibody. Quantification of immunoprecipitated protein was made as described in Fig. 2B. IP, Immunoprecipitation; WB, Western Blotting.
be augmented in BGC-823 cells and decreased in MCF-7 cells under ATRA treatment of not less than 24 h (Fig. 4B). Immunoprecipitation/Western blot analysis further supported the view that endogenous RARα was presented in a complex with RXRα in both MCF-7 and BGC-823 cells. The presence of this complex was clearly increased in BGC-823 cells and decreased in MCF-7 cells by 24 h of ATRA treatment (Fig. 4C). Good agreement in each panel of Fig. 4B and C supports the view that the RARα/RXRα heterodimer would be disassociated in MCF-7 cells and associated in BGC-823 cells in response to ATRA.
With the disassociation of RARα and RXRα in ATRA-treated MCF-7 cells in mind, we supposed that the ubiquitination and degradation of RARα may be the main cause for this disassociation. To investigate this by immunoprecipitation/Western blot analysis, ALLN was used to treat MCF-7 cells. The intensity of the RARα/RXRα complex was not affected by ATRA (Fig. 4D) due to inhibition of the ubiquitination (Fig. 3B) and degradation pathways (Fig. 3A) of RARα by ALLN. Thus,
ubiquitination and degradation of RARα were proved to be responsible for the disassociation of RARα with RXRα. In addition, the confocal microscopic observation that ALLN blocked the ATRA-induced RXRα translocation from the nucleus to the cytoplasm (Fig. 4A) also strongly supports the blockage of the RARα degradation pathway preventing its separation from RXRα in MCF-7 cells.

Sumoylation increases the stability of RARα and facilitates heterodimerization of RARα with RXRα

The stability of sumoylated RARα was then investigated. We measured the half-life of endogenous and sumoylated RARα by pulse-chase experiment. In BGC-823 cells, the location of endogenous and sumoylated RARα was indicated by immunoprecipitation/Western blot (Fig. 5A, right and middle panels). Pulse-chase analysis revealed two major bands in the gel, corresponding to the endogenous RARα and sumoylated RARα respectively (Fig. 5A, left panel). In the absence of ATRA, the half-life of the endogenous RARα was less than 2 h and between 2 and 4 h for sumoylated RARα (Fig. 5A, left panel and 5B, open bars), indicating that conjugation to SUMO-1 may stabilize RARα. Moreover, the half-life of sumoylated RARα is clearly extended (after 6 h) in the presence of ATRA (Fig. 5A, left panel and 5B, solid bars), demonstrating that ATRA could further stabilize sumoylated RARα. In addition, the relative level of RARα protein synthesized was higher in ATRA-treated (Fig. 5B solid bars) than in untreated BGC-823 cells (Fig. 5B open bars).

Taking Fig. 1D and Fig. 2C into account, it becomes clear that ATRA may accelerate the biosynthesis rate, increase the cellular content of RARα protein and prolong its half-life via the sumoylation pathway.

The effects of ATRA on the behavioral aspects of RARα as described above, were further examined by the same experimental approaches in another gastric cancer cell line MGC80–3. The results from MGC80–3 cells confirmed that ATRA can up-regulate RARα protein synthesis (Fig. 5C), promote its sumoylation (Fig. 5D, right panel) and its heterodimerization with RXRα (Fig. 5D, left panel). The results also demonstrated that the nuclear location of both RARα and RXRα was stable regardless of the presence or absence of ATRA (Fig. 4A) (refer to legend of relevant Figure for details). Clearly, these data are consistent with the experiments in the BGC-823 cell line and are further confirmation that in these gastric cancer cell lines, sumoylation of RARα facilitates the formation of the RARα/RXRα heterodimer and restricts it to its original nuclear location. Thus, sumoylation is advantageous for the RARα/RXRα heterodimer binding to its response element in signal transduction.

Discussion

In the present study, we have demonstrated that RARα is covalently modified by both SUMO-1 and ubiquitin simultaneously in breast and gastric cancer cells. Interestingly, ATRA has directly the opposite effect on the sumoylation and ubiquitination of RARα in cells of different cancer type, i.e. enhancing RARα sumoylation in gastric cancer

Figure 4 RARα/RXRα heterodimer is decreased by ubiquitination/degradation of RARα. (A) Effect of ALLN (pre-treatment for 2 h) and ATRA (treatment for 24 h) on translocation of RXRα and RARα in different cancer cell lines. Cells were treated with different agents as indicated, and then immunostained with anti-RXRα or anti-RARα antibody followed by the corresponding FITC-conjugated (for RXRα) or Texas-red-conjugated (for RARα) secondary antibody to show the location of RXRα and RARα protein respectively. The fluorescent images were visualized under a laser-scanning confocal microscope. (B) RARα/RXRα heterodimerization in both MCF-7 and BGC-823 cells. Heterodimerization of RARα with RXRα was analyzed by gel retardation assay using 32P-labeled DR5 as a probe. To determine the formation of heterodimer, antibodies specific for RARα and RXRα (designated as aRARα and aRXRα) were separately incubated with nuclear protein for 30 min at room temperature prior to the gel retardation assay. (C) Effect of ATRA on association of RARα with RXRα. Cell extract was immunoprecipitated with anti-RARα antibody and subjected to SDS-PAGE, blotting and was then probed with anti-RXRα antibody. No band appeared at the position corresponding to RXRα in the negative control where IgG was used. (D) The effect of ALLN on RARα/RXRα complex formation in the presence of ATRA in breast cancer MCF-7 cells, analyzed by immunoprecipitation/Western blotting. No specific band for IgG could be seen. The immunoprecipitated RARα used in each lane was quantified as described in Fig 2B. IP, Immunoprecipitation; WB, Western Blotting.
cells BGC-823 while restricting ubiquitination in breast cancer cells MCF-7. These regulatory effects of ATRA might be dependent on the distinct nature of cells from different tissue origins. Our findings may provide insight into a novel mechanism of ATRA on RARα regulation through the dynamic balance between sumoylation and ubiquitination.

The ubiquitin/proteasome pathway is the major system in eukaryotic cells for the selective degradation of short-lived regulatory proteins and transcription factors (Haas & Siepmann 1997, Pickart 1997). Covalent attachment of polyubiquitin is required for the efficient degradation of protein by the 26S proteasome complex (Hershko & Ciechanover 1998). In breast cancer MCF-7

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Figure 5 (A) and (B).
cells, the inhibitory effect of ATRA on RARα protein expression (Fig. 1A and B) could be blocked by proteasome-specific inhibitors (Fig. 3A), also indicating the involvement of the proteasome. In addition, polyubiquitin conjugating to RARα promoted ATRA-induced RARα ubiquitination in
a time-dependent manner (Fig. 2B), whereas proteasome inhibition led to enhanced accumulation of polyubiquitin conjugates of RARα even in the presence of ATRA (Fig. 3B). These results demonstrate that RARα is specifically subjected to ubiquitination in an ATRA-regulated manner. It has been verified that a sequence known as PEST serves as a signal for proteolytic degradation (Rechsteiner & Rogers 1996). RARα contains a putative PEST sequence EELIPSPSPPLPR (Swiss prot., No. P10276, Rogers et al. 1986), which is rich in Pro and Ser residues. Thus, the RARα molecule meets this biological requirement for its degradation.

Consistent with our finding, the degradation of RARs through the ubiquitin/proteasome pathway has also been reported by other authors in other cell systems (Zhu et al. 1999, Boudjelal et al. 2000, Tanaka et al. 2001). However, what is significant about our results is that once RARα has been degraded by ATRA in MCF-7 cells, it prevents the heterodimerization of RARα/RXRα and its binding to DNA (Fig. 4B). In addition, RXRα becomes disassociated from RARα (Fig. 4C) and is translocated to the cytoplasm (Fig. 4A). It is well known that the RAR/RXR heterodimer is a functional unit that preferentially transduces the retinoic acid signal in vivo and in vitro (Zhang et al. 1992, Kastner et al. 1995, Clifford et al. 1996, Chiba et al. 1997a,b, Wu et al. 1997a).

Recently, a number of publications have shown that the binding of RAR/RXR heterodimers to receptor response elements not only leads to positive transactivation but also signals a rapid destruction of active retinoid receptors (Zhu et al. 1999, Kopf et al. 2000, Osburn et al. 2001). For example, the RA-induced degradation of RARα1 and RARγ2 in transfected COS-1 cells depends strictly on its heterodimerization with RXRα (Kopf et al. 2000). Binding of the RAR/RXR heterodimer to DNA might be a triggering event for eliciting degradation (Zhu et al. 1999). Obviously, this evidence does not correspond to our observations. We have demonstrated that in MCF-7 cells, RARα is degraded through the ubiquitin/proteasome pathway within 12 h of ATRA treatment (Fig. 1A and Fig. 2B) and that at least 24 h of treatment is needed for ATRA to repress the binding activity of RARα/RXRα heterodimer to its response element (Fig. 4B). These data suggest that RARα degradation by ATRA might be an early event, which consequently caused repression of the extended transactivation of RARα/RXRα–DNA binding. We therefore propose that the ubiquitination of RARα and the subsequent degradation by the proteasome might function by terminating the transcriptional activity of RARα and RXRα.

The result of the transient transfection assay in MCF-7 cells (Fig. 1C) further verifies that RARα is not the main retinoic acid receptor engaged in transcriptional activity affected by ATRA in breast cancer cells. Thus, ubiquitination of RARα serves as a process for degradation and inactivation of the receptor in breast cancer cells. In this situation, RXRα becomes isolated from the RARα/RXRα heterodimer and translocates from the nucleus to the cytoplasm after 24 h of ATRA treatment. Accordingly, the augmented RARα ubiquitination and the disassociation of RARα from RXRα are likely to explain why our previous research shows that RARα is incapable of exerting its function to mediate ATRA signals in MCF-7 cells (Ye et al. 2004). Certainly, further studies are necessary to establish if and how these differences may reflect

Figure 5 Sumoylation increases the stability of RARα and facilitates RARα/RXRα heterodimerization. (A) Half-life of endogenous and sumoylated RARα in BGC-823 cells. Cells were divided into two groups: cells in the first group were pulsed with [35S]-methionine for 4 h and chased in the absence or presence of ATRA for different times as indicated; cells in the remaining group were harvested for immunoprecipitation analysis. Protein samples, prepared as described in Materials and methods, were loaded onto 8% SDS-PAGE gel simultaneously. The sample-loaded gel was cut into two parts: isotope-labeled gel was dried and exposed at −80°C for 5 days (left panel, from lane 1 to 8); non-isotope-labeled gel was transferred to a nitrocellulose membrane, then subjected to Western blotting with corresponding antibodies as indicated (right and middle panels). As a control, lane 1 indicated total cell lysate. (B) Endogenous and sumoylated RARα in a pulse-chase experiment were quantified by Densitometer. Data were means±S.D. of three independent experimental results. (C) Gastric cancer MGC80-3 cells were treated with ATRA for the indicated times. Expression of RARα was examined by Western blot and quantified by Densitometer. (D) Effect of ATRA on interaction of RARα with RXRα (left panel) or with SUMO-1 (right panel) in MGC80-3 cells, revealed by immunoprecipitation/Western blot. The duration of ATRA treatment is indicated. IP, Immunoprecipitation; WB, Western Blotting.
specific functions of RARα in retinoic acid signaling under physiological conditions.

In addition to ubiquitination, an increasing number of cellular proteins have been found to covalently conjugate with SUMO-1 in eukaryotic cells. Although the molecular modification pathway of sumoylation resembles that of ubiquitination, their resultant functional consequences are different (Muller et al. 2001, Seeler & Dejean 2001, Wilson & Rangasamy 2001). In some cases, SUMO-1 antagonizes ubiquitin by conjugating onto the same lysine residue of target substrates, resulting in the stabilization of sumoylated proteins (Desterro et al. 1998, Hochstrasser 2000). A well-known example is the SUMO-1 modification of Mdm2 which prevents self-ubiquitination and increases Mdm2’s ability to ubiquitinate p53 (Buschmann et al. 2000).

In the present study, we demonstrated that both ubiquitinated and sumoylated RARα are endogenously present in breast and gastric cancer cells (Fig. 2B and C). A typical consensus sequence of a protein sumoylation site is RVTILKMEIPGSM (Swiss prot., No. P10276, Johnson & Blobel 1999, Sternsdorf et al. 1999a). The specific Lys residue found in RARα molecule, provides the possibility of RARα sumoylation.

In the present study, RARα sumoylation was enhanced by ATRA in a time-dependent manner in BGC-823 cells (Fig. 2C), although ubiquitination of RARα was still detectable (Fig. 2B). This suggests a possible dynamic balance mechanism between sumoylation and ubiquitination. When ATRA induced additional RARα sumoylation in BGC-823 cells, the RARα molecule became stabilized and its half-life increased by at least 2 h (Fig. 5A and B). This indicates that protein sumoylation may result in multiple biological outcomes, varying with the nature of the protein sumoylated. Sumoylation of the RARα promoted binding of the RARα/RXRα heterodimer to the receptor response element (Fig. 4B) and enhanced its transcriptional activity (Fig. 1C). In addition to enhancing the transcriptional activity of the transfected reporter by cotransfection of RARα in BGC-823 cells, the results also indicate the involvement of RARα in ATRA signal transduction in relation to transcriptional activity in gastric cancer cells. Such a correlation between protein stability and transcriptional activity is in accordance with other reports (Kim & Maniatis 1996, Molinari et al. 1999, Salghetti et al. 2000). When considered together, our observations confirm that SUMO-1 conjugation could stabilize RARα, keep the RARα/RXRα heterodimer fixed in the nucleus, as well as stimulate the binding of RARα/RXRα to DNA, promoting its transcriptional activity. Thus SUMO-1 conjugation is a critical step of functional significance in the pathway of ATRA signal transduction.

In summary, RARα may be subjected to either ubiquitination or sumoylation. In breast cancer MCF-7 cells, degradation of RARα by ATRA is through the ubiquitin/proteasome pathway, which consequently leads to inhibition of the RARα/RXRα heterodimer binding to DNA. Under these circumstances, RXRα disassociates with RARα, and then translocates from the nucleus to the cytoplasm. By contrast, in gastric cancer BGC-823 cells ATRA-induced up-regulation of RARα is closely associated with its sumoylation. ATRA-enhanced RARα sumoylation promotes the stability of RARα and the steady nuclear localization of RARα/RXRα, which is then capable of serving as a functional unit in ATRA signal transduction. These results reveal a novel mechanism: in its sumoylated form, RARα is more efficient in mediating ATRA signals than ubiquitinated RARα.

Acknowledgements

This work was supported by the National Natural Science Foundation of China (No. 30170477), the National Outstanding Youth Science Foundation of China (No. 39825502), and the National Natural Science Foundation of Fujian Province (C0110004). We thank Dr Xiao-kun Zhang (Burnham Institute, La Jolla, CA, USA) for the kind gifts of RARα and RXRα probes.

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Received in final form 9 January 2004
Accepted 3 February 2004
Made available online as an Accepted Preprint