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Ubiquitin/Proteasome Pathway Regulates Levels of Retinoic Acid Receptor γ and Retinoid X Receptor α in Human Keratinocytes[1]

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ABSTRACT

Repeated exposure of human skin to solar UV radiation leads to premature aging (photoaging) and skin cancer. UV-induced skin damage can be ameliorated by all-*trans* retinoic acid treatment. The actions of retinoic acid in skin keratinocytes are mediated primarily by nuclear retinoic acid receptor γ (RARγ) and retinoid X receptor α (RXRα). We found that exposure of cultured primary human keratinocytes to UV irradiation (30 mJ/cm^2) substantially reduced (50–90%) RARγ and RXRα mRNA and protein within 8 h. The rates of disappearance of RARγ and RXRα proteins after UV exposure or treatment with the protein synthesis inhibitor cycloheximide were similar. UV irradiation did not increase the rate of breakdown of RARγ or RXRα but rather reduced their rate of synthesis. The addition of proteasome inhibitors MG132 and LLvL, but not the lysosomal inhibitor E64, prevented loss of RARγ and RXRα proteins after exposure of keratinocytes to either UV radiation or cycloheximide. Soluble extracts from nonirradiated or UV-irradiated keratinocytes possessed similar levels of proteasome activity that degraded RARγ and RXRα proteins *in vitro*. Furthermore, RARγ and RXRα were polyubiquitinated in intact cells. RXRα was found to contain two proline, glutamate/aspartate, serine, and threonine (PEST) motifs, which confer rapid turnover of many short-lived regulatory proteins that are degraded by the ubiquitin/proteasome pathway. However, the PEST motifs in RXRα did not function to regulate its stability, because deletion of the PEST motifs individually or together did not alter ubiquitination or proteasome-mediated degradation of RXRα. These results demonstrate that loss of RARγ and RXRα proteins after UV irradiation results from degradation via the ubiquitin/proteasome pathway. Taken together, the data here indicate that ubiquitin/proteasome-mediated breakdown is an important mechanism regulating the levels of nuclear retinoid receptors.

INTRODUCTION

UV radiation from the sun induces profound biological changes in human skin and is believed to be the major cause of skin cancer and premature skin aging (photoaging; Refs. 1 and 2). The vitamin A metabolite all-*trans* retinoic acid and related synthetic retinoids are widely used to treat skin disorders that result from UV irradiation, such as photodamage and certain epithelial malignancies (1, 3). All-*trans* retinoic acid and its synthetic analogues elicit their biological effects by binding to and activating members of two nuclear receptor gene families, RARs[3] and RXRs. Each family is composed of three members, α, β, and γ (4). In both cultured keratinocytes and human skin *in vivo*, RARγ and RXRα are the predominant retinoid receptor isoforms (5). Upon ligand binding, these receptors up-regulate transcription of genes containing retinoic acid response elements (RAREs). In addition, once liganded, both RARs and RXRs can inhibit expression of certain genes by antagonizing the transcriptional activity of the activator protein-1 complex (c-jun/c-fos; Ref. 4).

We have shown previously that exposure of human skin *in vivo* to relatively low levels of UV irradiation causes substantial reduction of RARγ and RXRα mRNA and protein (6). Loss of retinoid receptors after UV irradiation was associated with loss of retinoid-responsive gene expression in human skin. In essence, UV caused a functional retinoid deficiency.

In the current study, we have investigated the mechanisms of UV irradiation-induced loss of RARγ and RXRα in cultured human keratinocytes. We find that UV irradiation inhibits synthesis of RARγ and RXRα proteins. RARγ and RXRα are degraded with a half-life of ~4 h in both UV-irradiated and nonirradiated cells. Reduction of RARγ and RXRα after UV irradiation is blocked by inhibitors of proteasome activity. Furthermore, we demonstrate that RARγ and RXRα are substrates for ubiquitination and proteasome-mediated breakdown. These data reveal a novel mechanism for regulation of retinoid receptor-dependent signal transduction through the ubiquitin/proteasome pathway.

MATERIALS AND METHODS

Expression Plasmids. pCMV-His-myc-Ub plasmid was provided by Dr. R. Kopito (Department of Biological Sciences, Stanford University, Stanford, CA). Expression vectors for human RARα, mouse RXRα, His-tagged RARγ, and 36B4 cDNA were generously provided by Professor P. Chambon (Institut de Genetique et de Biologie Moleculaire et Cellulaire, Strasbourg, France). His-tagged c-Jun expression vector was kindly provided by Professor D. Bohmann (European Molecular Biology Laboratory, Heidelberg, Germany). The pCMV-Flag-ubiquitin expression vector was constructed by inserting the ubiquitin cDNA excised from a pCMV-His-myc-Ub plasmid (7) into a pCMV-Flag expression vector. The pSG5-His-tagged RXRα expression vector was generated by replacing the RARγ cDNA in pSG5-His-RARγ with RXRα cDNA excised from pSG5-RXRα. pSG5-His-RXRα was used as a template in the PCR reaction to generate four pSG5-His-RXRα deletion mutants: His-RXRα Δ80–115, His-RXRα Δ215–235, His-RXRα Δ80–115/Δ220–235, and His-RXRα Δ235–467.

Cell Culture. Primary human keratinocytes were prepared from skin samples taken from normal adult volunteers, as described previously (8). Cells were grown as monolayer cultures in serum-free, low-calcium MCDB 153 medium in a humidified incubator with 5% CO_2 at 37°C. For UV irradiation, cells were seeded in 10-cm dishes, grown to ~80% confluence, and then exposed to UV while submerged in 6 ml of Dulbecco's PBS. Media were then replaced, and plates were returned to the incubator for the indicated times. Protease inhibitors (see below) were dissolved in DMSO and added to the cultures immediately after exposure to UV. HeLa cells were grown in DMEM containing 10% fetal bovine serum.

UV Source and Irradiation. Cultured human keratinocytes were irradiated with 30 mJ/cm^2 UV using an Ultralite Panelite lamp containing six FS24T12 UVB-HO bulbs. A Kodacel filter was used to eliminate wavelengths <290 nm (UVC). The irradiation intensity was monitored with an I1443 phototherapy radiometer and a SED240/UVB/W photodetector (International Light, Newbury, MA).

Preparation of Whole-Cell Extracts and Western Blot Analysis. After treatment, cultured keratinocytes were harvested in PBS by scraping and pelleted by centrifugation at 500 × *g* for 5 min at 4°C. Cells were homogenized in 150 μl of extraction buffer [10 mM Tris (pH 7.4), 300 mM NaCl, 1 mM EDTA, 10 mM $MgCl_2$, 2 mM DTT, 5 mM phenylmethysulfonyl fluoride, 10

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[3] The abbreviations used are: RAR, retinoic acid receptor; RXR, retinoid X receptor; AP-1, activator protein-1; AMC, 7-amino-4-methylcoumarin; PEST, proline, glutamate/aspartate, serine, and threonine.

μg/ml aprotinin, 10 μg/ml leupeptin, and 0.5% NP40]. The homogenate was centrifuged at 14,000 × *g* for 15 min, the supernatant was collected, and protein concentrations were measured using a commercial Bio-Rad assay.

Equal amounts of whole-cell extract proteins were subjected to 10% SDS-PAGE, transferred to polyvinylidene difluoride nitrocellulose membrane, and probed with specific antibodies. Polyclonal antibodies specific for RARγ and RXRα and monoclonal antibodies for β-actin were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Immunoreactive proteins were visualized by enhanced chemiluminescence detection and quantified by laser densitometry. RARγ and RXRα that were overexpressed in HeLa cells were used as standards.

Pulse-Chase Labeling. Keratinocytes at 80% confluency were preincubated in cysteine/methionine-free MEM for 2 h and then pulse-labeled with 50 μCi/ml [^{35}S]methionine/cysteine (1175 Ci/mmol) for 1 or 2 h. Cells were washed with PBS twice and then either exposed or not exposed to UV (30 mJ/cm^2). In some experiments, cells were UV irradiated in PBS before the addition of [^{35}S]methionine/cysteine. Labeled cells were placed in serum-free, low-calcium MCDB 153 containing 300 μg/ml methionine/cysteine, harvested 1–24 h after exposure to UV, and washed with PBS, and whole-cell extracts were prepared, as described above. Whole-cell extracts (200 μg) were incubated at 4°C with 5 μl of anti-RARγ or anti-RXRα antibodies (Santa Cruz Biotechnology, Santa Cruz, CA). Samples were rotated overnight in 150 μl of immunoprecipitation buffer [10 mM Tris (pH 7.4), 1 mM EDTA, 150 mM NaCl, 10 mM $MgCl_2$, 2 mM DTT, 5 mM phenylmethylsulfonyl fluoride, 10 μg/ml aprotinin, 10 μg/ml leupeptin, and 0.5% NP40]. Protein G agarose beads (30 μl) were added, and samples were rotated for 2 h at 4°C. Beads were washed three times with immunoprecipitation buffer and then subjected to 12% SDS-PAGE. Gels were dried, visualized, and quantified by STORM PhosphorImager (Molecular Dynamics, Sunnyvale, CA).

Proteasome Activity Assay. Proteasome activity was measured as described by Craiu *et al.* (9) and Dick *et al.* (10). Whole-cell extracts (50–100 μg) prepared from keratinocytes were placed in 200 μl of assay buffer [20 mM HEPES, 0.5 mM EDTA (pH 8.0), and 100 nM ATP] containing 50 μM of one of the following peptide substrates: Suc-Leu-Leu-Tyr-AMC, Boc-Leu-Arg-Arg-AMC, or FITC-casein (Sigma Chemical Co., St. Louis, MO). These substrates specifically measure the chymotryptic, tryptic, and protein hydrolysis activities, respectively, of proteasomes (9, 10). After incubation for 2 h at 37°C, reactions were halted by adding 2.5 ml of cold ethanol. Proteasome activity was monitored by measuring the fluorescence of released AMC at excitation wavelength 380 nm and emission wavelength 460 nm or FITC at excitation wavelength 490 nm and emission wavelength 520 nm.

Transient Transfection and Purification of His-tagged Proteins. HeLa cells were transfected with expression vectors for His-tagged RARγ, RXRα, and c-Jun and Flag-tagged ubiquitin using Superfect (Qiagen, Chatsworth, CA), according to the manufacturer's instructions. For purification of expressed His-tagged proteins, the proteasome inhibitor MG132 was added to the media (final concentration, 50 μM) 36 h after transfection. Eight h later, cells were lysed in 3–4 ml of 6 M guanidinium-HCl, 0.1 M Na_2HPO_4/NaH_2PO_4 (pH 8.0) containing 5 mM imidazol per 100-mm dish. His-RARγ, His-RXRα, and His-c-Jun were purified using Ni^{2+}-NTA-agarose (Qiagen, Chatsworth, CA), as described (11). Ubiquitinated His-RARγ, His-RXRα, and His-c-Jun were detected by Western analysis using anti-Flag monoclonal antibody (Sigma Chemical Co., St. Louis, MO). His-RARγ, His-RXRα, and His-c-Jun (antibody from Transduction Laboratories, Lexington, KY) levels were determined by Western analysis.

Northern Blot Analysis of Retinoid Receptors. Total RNA was isolated from UV-irradiated and nonirradiated cultured human keratinocytes by guanidine hydrochloride lysis and ultracentrifugation, as described (12). Northern analysis of total RNA with randomly primed [^{32}P]cDNA probes for human RARγ and RXRα and 36B4 (a ribosomal protein used as an internal control) were performed as described (13).

***In Vitro* Protein Translation and Degradation Assay.** RARγ and RXRα proteins were translated *in vitro* in the presence of [^{35}S]methionine by TNT T7 reticulocyte lysate as described by the manufacturer Promega Corp. (Madison, WI), using pSG5-RARγ, pSG5-RXRα, His-RXRα Δ80–115, His-RXRα Δ220–235, and His-RXRα Δ80–115/Δ220–235 plasmids as templates. ^{35}S-labeled RARγ or RXRα protein (5 μl) was incubated with keratinocyte whole-cell extract (50 μg), prepared as described above, but in the absence of any protease inhibitor, in a total volume of 50 μl 20 mM Tris (pH 7.4), 50 mM NaCl, and 0.2 mM DTT. Reaction mixtures were incubated at 37°C for 2 h and then resolved on 12% SDS-PAGE. Dried gels were visualized and quantified by STORM PhosphorImager (Molecular Dynamics).

RESULTS AND DISCUSSION

UV Irradiation Reduces RARγ and RXRα mRNA and Protein in Human Keratinocytes. Exposure of human keratinocytes to UV (30 mJ/cm^2) caused substantial decreases in the levels of RARγ and RXRα mRNA (Fig. 1*A*) and proteins (Fig. 1*B*). RARγ mRNA began to decline 8 h after UV and was reduced 60% 24 h after irradiation. RXRα mRNA began to decline 4 h after UV irradiation and was further reduced 80% 24 h after UV. Quantitative analyses of Western blots (shown in Fig. 1*B*) revealed that both RARγ and RXRα proteins

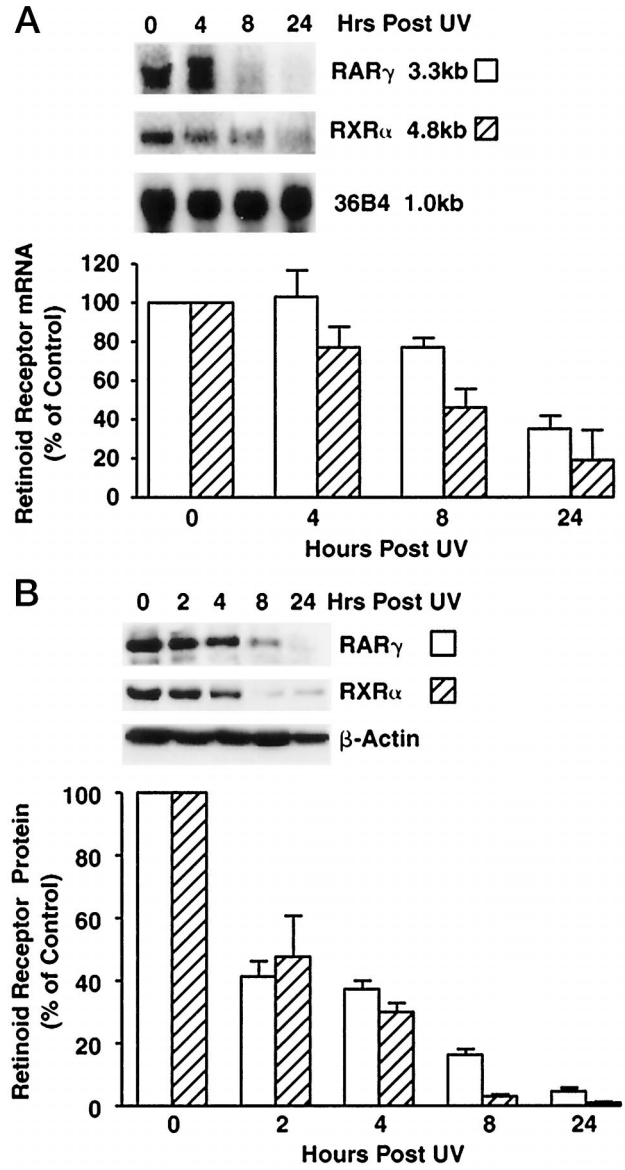

Fig. 1. UV irradiation reduces RARγ and RXRα mRNA and protein in human keratinocytes. *A*, time course of reduction of RARγ (□) and RXRα (▨) mRNA in cultured keratinocytes after UV irradiation (30 mJ/cm^2). *Inset*, representative Northern blots for RARγ, RXRα, and 36B4 (internal control). *B*, time course of reduction of RARγ (□) and RXRα (▨) proteins in cultured keratinocytes after UV irradiation (30 mJ/cm^2). *Inset*, a representative Western blot for RARγ, RXRα, and β-actin (internal control). RARγ and RXRα protein levels were quantified by STORM PhosphorImager. Data are means for three experiments; *bars*, SE.

were reduced ~50% within 2 h after UV exposure and continued to decrease for at least 24 h (Fig. 1*B*).

Loss of RARγ and RXRα proteins after UV exposure was not attributable to reduced cell viability or general protein loss. Cell viability, determined by trypan blue exclusion, was >90% 24 h after UV treatment (data not shown). β-actin protein levels, used as a control, were not affected by UV (Fig. 1*B, inset*). In addition, c-jun and c-fos protein levels were increased within 2 h after UV irradiation and remained elevated for at least 16 h (data not shown).

RARγ and RXRα Turnover Is Rapid in Cultured Keratinocytes and Not Altered by UV. The preceding data demonstrate that UV irradiation reduces both RARγ and RXRα mRNA and protein in a time-dependent manner. The decrease in RARγ and RXRα mRNA after UV exposure could be the result of inhibition of RARγ and RXRα gene transcription and/or increased RNA degradation. Loss of RARγ and RXRα proteins preceded loss of their transcripts, indicating that initial loss of retinoid receptor proteins occurred via a posttranscriptional mechanism. The initial loss of retinoid receptor proteins could result from reduced retinoid receptor protein synthesis and/or accelerated retinoid receptor degradation. To investigate these possibilities, we first determined the rate of RARγ and RXRα protein turnover in nonirradiated keratinocytes. Keratinocytes were treated with cycloheximide to prevent new protein synthesis, and RARγ and RXRα protein levels were determined by Western analysis.

Both RARγ and RXRα protein levels were reduced 40% within 2 h of addition of cycloheximide, compared with their levels in untreated control keratinocytes (data not shown). Levels of both retinoid receptors continued to decline at similar rates, with 90% reduction after 10 h. The rate of RARγ and RXRα protein breakdown after cycloheximide treatment was similar to the rate of RARγ and RXRα loss after UV exposure. These data suggest that UV irradiation inhibits RARγ and RXRα synthesis rather than accelerates their breakdown.

To investigate this possibility, we performed pulse-chase experiments with [^{35}S]methionine/cysteine to metabolically label RARγ and RXRα proteins in keratinocytes. Cells were UV irradiated or left untreated, then pulsed for 2 h, and chased for 0–8 h. Synthesis of RARγ (Fig. 2*A*) and RXRα (Fig. 2*B*), as assessed by incorporation of label during the 2-h pulse, was 50% less in UV-irradiated keratinocytes, as compared with nonirradiated keratinocytes. During the chase period, the levels of labeled RARγ and RXRα declined at similar rates (Fig. 2). These data indicate that UV irradiation inhibits synthesis of RARγ and RXRα proteins, without altering the rate of their breakdown. To further substantiate this latter conclusion, keratinocytes were pulsed with [^{35}S]methionine/cysteine for 1 h, then exposed to UV irradiation or left untreated, and chased for 1–24 h. The rates of loss of labeled RARγ (Fig. 3*A*) and RXRα (Fig. 3*B*) were similar in UV irradiated and nonirradiated keratinocytes. The half-lives of both retinoid receptors were between 4 and 8 h, regardless of whether cells had been UV irradiated (Fig. 3). Taken together, the above data indicate that loss of retinoid receptors in human keratinocytes after UV irradiation results from inhibition of their protein synthesis through a posttranscriptional mechanism, coupled with inherent (*i.e.*, not altered by UV irradiation) breakdown.

RARγ and RXRα Proteins Are Degraded by the Proteasome Pathway in Human Keratinocytes. Because proteolysis is ultimately responsible for loss of retinoid receptors, we next investigated the pathway responsible for proteolytic degradation of RARγ and RXRα in human keratinocytes. Keratinocytes were UV irradiated or treated with cycloheximide and then cultured for 10 h in the presence of inhibitors specific for lysosomal proteases [E64, *trans*-epoxysuccinyl-L-leucylamido-(4-guanidino) butane; Ref. 14], or proteasome activity [MG132 (Z-Leu-Leu-Leu-H) and LLvL (N-CBZ-Leu-Leu-Norvalinal); Ref. 15], or in the presence of vehicle (DMSO). As

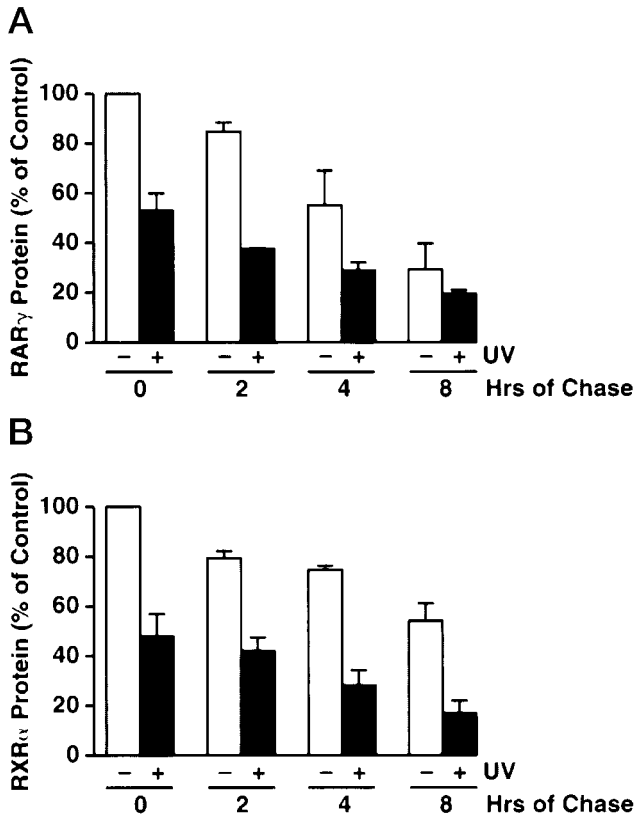

Fig. 2. UV irradiation inhibits synthesis of RARγ and RXRα in human keratinocytes. Keratinocytes were left untreated (□) or UV-irradiated (■) prior to pulse with [^{35}S]methionine/cysteine for 2 h. Cells were then washed and placed in media containing unlabeled methionine/cysteine (300 μg/ml each) for the indicated times. ^{35}S-labeled RARγ (*A*) and RXRα (*B*) proteins were immunoprecipitated and then subjected to 12% SDS-PAGE. Results are means for two experiments; *bars*, SE.

expected, RARγ and RXRα were reduced substantially (75–90%) in vehicle-treated keratinocytes exposed to UV irradiation (Fig. 4*A*) or incubated with cycloheximide (Fig. 4*B*). Treatment of cells with MG132 and LLvL, but not E64, largely prevented this loss of retinoid receptors in both UV-irradiated and cycloheximide-treated cells. In UV-irradiated keratinocytes, MG132 and LLvL reduced loss of RARγ to <30% and completely prevented loss of RXRα (Fig. 4*A*). In cycloheximide-treated cells, addition of MG132 and LLvL maintained RARγ and RXRα levels at or above their respective levels in untreated control cells (Fig. 4*B*). The proteasome inhibitor lactacystin also inhibited loss of RARγ and RXRα in both UV-irradiated and cycloheximide-treated keratinocytes (data not shown). MG132 and LLvL also maintained RARγ and RXRα at their initial levels for at least 10 h in pulse chase experiments in both UV-irradiated and nonirradiated keratinocytes (data not shown).

Keratinocytes Possess Proteasome Activity That Degrades RARγ and RXRα. The above results indicate that the proteasome degradation pathway is involved in regulating the level of RARγ and RXRα in human keratinocytes. To further support this conclusion, we measured proteasome activity in whole-cell extracts prepared from irradiated and nonirradiated human keratinocytes using synthetic fluorescent peptides, specific for chymotryptic and tryptic hydrolyzing activities of proteasomes (16). Keratinocyte extracts exhibited both chymotrypic and tryptic proteasomal activities. Chymotryptic and tryptic activities were similar in whole-cell extracts from irradiated and nonirradiated keratinocytes and were inhibited by the proteasome inhibitor MG132 but not by the calpain inhibitors I and II (data not shown).

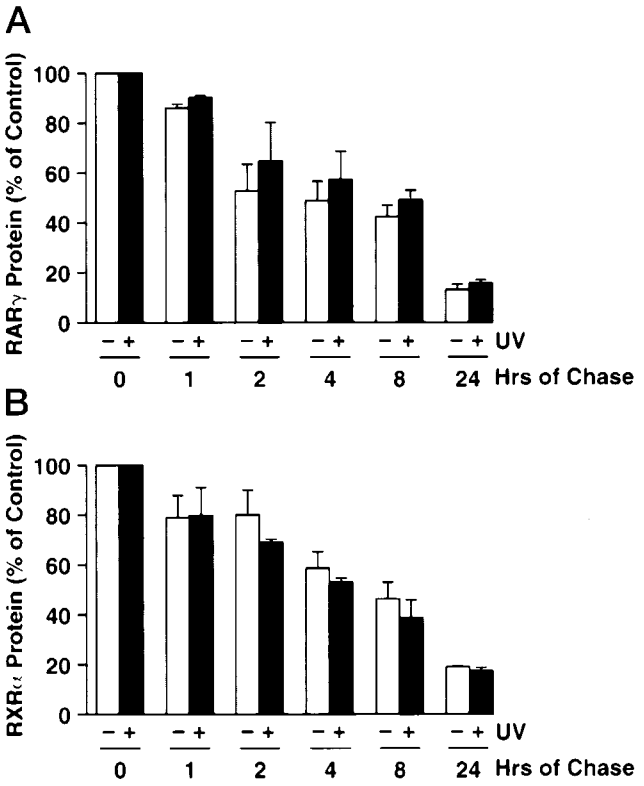

Fig. 3. UV irradiation does not alter the rate of RARγ or RXRα degradation. Keratinocytes were pulsed with [^{35}S]methionine/cysteine for 1 h. Cells were then washed with PBS, left untreated (□) or UV irradiated (■) and placed in media containing unlabeled methionine/cysteine (300 μg/ml each) for the indicated times. ^{35}S-labeled RARγ (*A*) and RXRα (*B*) proteins were immunoprecipitated and then subjected to 12% SDS-PAGE. Results are means for three experiments; *bars*, SE.

Because the primary function of the proteasome is to hydrolyze proteins into oligopeptides (17), we also used FITC-conjugated casein as a substrate. This protein substrate is degraded in an ATP-dependent reaction by the 26S complex, without ubiquitination (9). Whole-cell extracts from both UV-irradiated and nonirradiated keratinocytes cleaved FITC-casein to similar extents. This activity was blocked by MG132 but not by the calpain I and II inhibitors (data not shown).

We next used *in vitro*-translated ^{35}S-labeled RARγ and ^{35}S-labeled RXRα as substrates for proteasome activity in whole-cell extracts from either UV-irradiated or nonirradiated keratinocytes. Whole-cell extracts from nonirradiated keratinocytes degraded 48% and 63% of added RARγ and RXRα, respectively (Fig. 5). Degradation of RARγ and RXRα was reduced to 2 and 24%, respectively, by addition of the proteasome inhibitor MG132. Calpain I and II inhibitors did not prevent degradation of either retinoid receptor. Similar results were obtained using whole-cell extracts from UV-irradiated keratinocytes and untreated or UV-irradiated human skin (data not shown). Taken together, the above data demonstrate that keratinocytes possess functional proteasome activity that degrades RARγ and RXRα and that is unaltered by UV irradiation.

RARγ and RXRα Are Ubiquitinated. Ubiquitin, a 76-amino acid protein, is covalently attached to protein lysine residues through the sequential actions of three families of enzymes (18). Polyubiquitination targets proteins for degradation by proteasomes. Therefore, we next investigated whether RARγ and RXRα could be ubiquitinated in intact cells. HeLa cells were transfected with His-tagged RARγ or RXRα or c-Jun expression plasmids together with Flag or Flag-ubiquitin expression vectors. c-Jun is known to be ubiquitinated (11) and therefore served as a positive control. We used HeLa cells for these experiments because of their high transfection efficiency, compared with keratinocytes, which is necessary for purification of expressed His-tagged retinoid receptors. The rate of turnover of RARγ and RXRα after UV irradiation or addition of cycloheximide in HeLa cells is similar to that observed for keratinocytes (data not shown). Additionally, loss of RARγ and RXRα proteins in either UV-irradiated or cycloheximide-treated HeLa cells is prevented by MG132 and LLvL but not E64 (data not shown). Therefore, proteasome-mediated degradation of RARγ and RXRα appears to be similar in keratino-

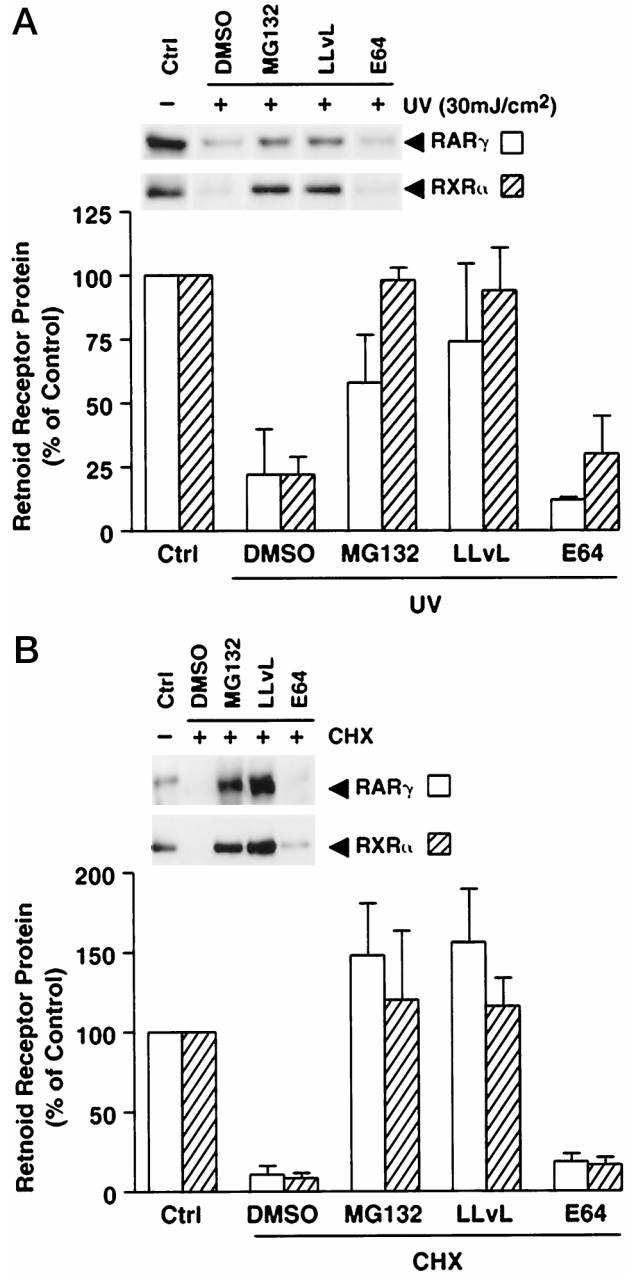

Fig. 4. Proteasome inhibitors block loss of RARγ and RXRα proteins in UV-irradiated and cycloheximide (*CHX*)-treated keratinocytes. *A*, keratinocytes were UV irradiated (30 mJ/cm^2) and cultured in the presence of the indicated protease inhibitors or vehicle (DMSO) for 10 h. Whole-cell extracts were prepared, and RARγ (□) and RXRα (▨) were determined by Western analyses. *Inset*, representative Western blots for RARγ and RXRα. *B*, keratinocytes were cultured in the presence of cycloheximide (50 μg/ml) and the indicated protease inhibitors or vehicle (DMSO) for 10 h. Whole-cell extracts were prepared, and RARγ (□) and RXRα (▨) were determined by Western analyses. *Inset*, representative Western blots for RARγ and RXRα. Data are means for three experiments; *bars*, SE.

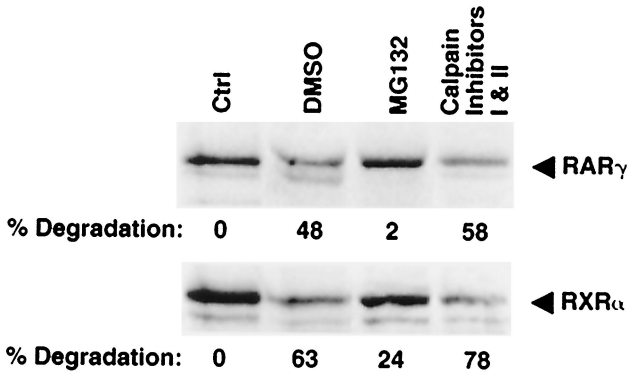

Fig. 5. Keratinocyte proteasome activity degrades RARγ and RXRα *in vitro*. *In vitro* translated ^{35}S-labeled RARγ and ^{35}S-labeled RXRα were incubated with nonirradiated keratinocyte whole-cell extracts in the presence of vehicle (DMSO), MG132, or calpain I and II inhibitors (25 μM each) for 2 h. Samples were subjected to SDS-PAGE, and RARγ and RXRα proteins were quantified by STORM PhosphorImager. Data are presented as percentage of degradation of RARγ and RXRα protein relative to control (*Ctrl*) incubations in buffer alone, without whole-cell extracts. Results are representative of three experiments.

cytes and HeLa cells. After transfections, equivalent amounts of cell lysates were used to purify His-tagged retinoid receptors or His-tagged c-Jun by Ni^{+2}-NTA chromatography. Retinoid receptors and c-jun, in column eluates, that were ubiquitinated were detected by Western analysis using anti-Flag antibody.

In column eluates from cells transfected with His-RARγ and Flag-ubiquitin, there was a broad ladder of bands that migrated with apparent molecular weights larger than RARγ (Fig. 6, *left*), indicating formation of polyubiquitinated RARγ. These higher molecular weight forms of RARγ were only present in cells transfected with RARγ and Flag-ubiquitin; they were absent in cells transfected with His-tagged RARγ or Flag-ubiquitin alone. Similarly, His-RXRα was polyubiquitinated in HeLa cells (Fig. 6, *middle panel*). The appearance of the polyubiquitination ladder that was observed for RARγ and RXRα was similar to that observed for the positive control c-Jun (Fig. 6, *right panel*). Western analysis confirmed that His-RARγ, His-RXRα, and His-c-Jun had been efficiently expressed and recovered by Ni^{+2}-NTA chromatography from transfected cells (data not shown).

PEST Motifs in RXRα Are Not Required for Ubiquitination. A feature commonly found in proteins with short half-lives that are degraded by the ubiquitin/proteasome pathway is regions rich in PEST sequences (19). Using the PEST-FIND computer program (20), we identified two PEST motifs in RXRα. The first was located within the A/B domain (amino acids 80–113 in human and 75–108 in mouse), and the second was located within the hinge D domain (amino acids 215–233). To further investigate regulation of RXRα ubiquitination, we determined the ability of RXRα mutant proteins lacking one or both PEST motifs to be ubiquitinated and degraded by proteasomes. His-tagged mutant RXRα constructs were cotransfected with Flag-ubiquitin and analyzed for ubiquitination as described above for wild-type RXRα. Ubiquitination of RXRα proteins lacking the A/B domain PEST motif (Δ80–115; Fig. 7), the D domain PEST motif (Δ200–235; Fig. 7), or both PEST motifs (Δ80–115/Δ220–235; Fig. 7) was readily detectable. The COOH-terminal half of RXRα (Δ235–467, Fig. 7), which does not contain any PEST sequences, was also ubiquitinated. The levels of expression and ubiquitination of all three mutant proteins were comparable with those of wild-type RXRα (Fig. 7). These data indicate that the PEST motifs in RXRα are not required for ubiquitination.

We next determined whether RXRα PEST motifs are required for proteasome-mediated degradation. Each of the three PEST deletion mutant RXRα proteins was translated *in vitro* and incubated with proteasome-containing extracts from human skin. The three mutant RXRα proteins were substantially degraded within 2 h, and this degradation was blocked by proteasome inhibitors MG132 and LLvL (data not shown). Taken together, the above data indicate that the PEST motifs in RXRα do not function to regulate degradation through the ubiqitin/proteasome pathway, as has been described for other proteins (16, 21–24).

We also identified PEST motifs located within the A/B domain (amino acids 56–85) and hinge D domain (amino acids 172–192) of RARγ. In addition, a third PEST motif was localized within the COOH-terminal of the ligand-binding domain (amino acids 413–

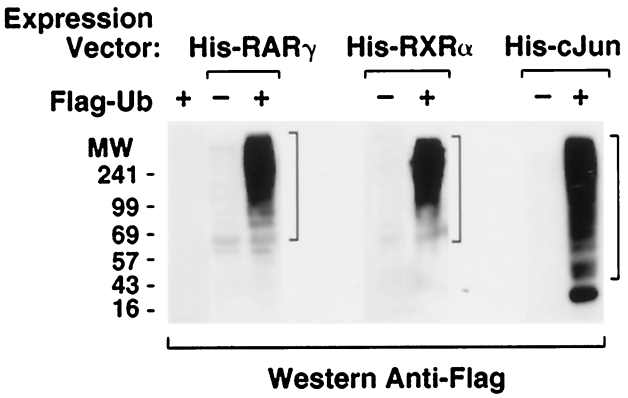

Fig. 6. RARγ and RXRα proteins are ubiquitinated in cells. HeLa cells were transfected with His-tagged RARγ (*left*), His-tagged RXRα (*middle*), or His-tagged c-Jun (*right*), alone or with Flag-ubiquitin (*Flag-Ub*), as indicated. His-tagged proteins were purified from lysates of the transfected cells and analyzed for ubiquitination by Western blot with anti-Flag antibody. Ubiquitinated RARγ, RXRα, and c-Jun appear as slower migrating proteins (in *brackets*).

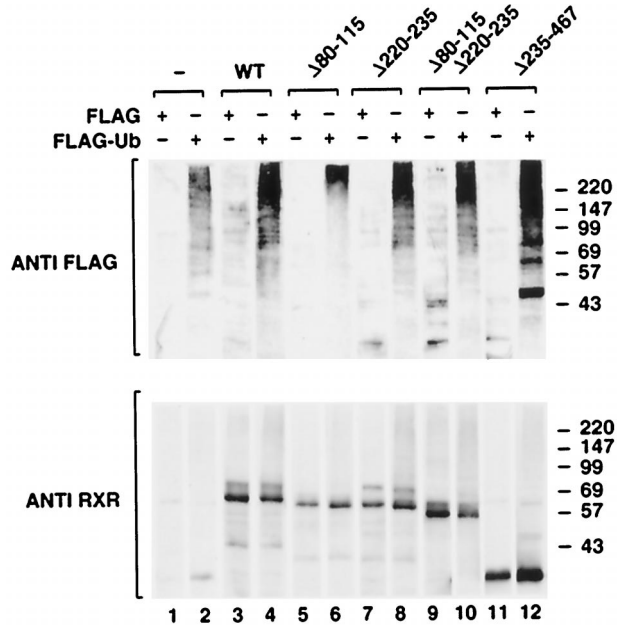

Fig. 7. RXRα PEST motifs are not required for ubiquitination. Wild-type (*WT*) and mutant His-tagged RXRα expression vectors lacking the A/B domain PEST motif (Δ80–115), the D domain PEST motif (Δ220–235), both PEST motifs RXRα (Δ80–115/Δ220–235), or N-terminal sequences (Δ235–467) were cotransfected with Flag or Flag-ubiquitin (*Flag-Ub*) expression vectors into HeLa cells. His-tagged RXRα proteins were purified from lysates of transfected cells by nickel chelate chromatography. Purified RXRα proteins were analyzed for ubiquitination by Western blot with anti-Flag antibody (*upper*) and for protein levels by Western blot with anti-RXRα antibody (*lower*). *Upper panel*, ubiquitinated RXRα proteins appear as multiple higher molecular weight bands. Results are representative of three experiments.

427). The functional role of these PEST motifs in the turnover of RARγ remains to be determined.

The above data describe a novel mechanism for regulation of retinoid signaling through ubiquitin/proteasome-mediated degradation of retinoid receptors. Recent data indicate that the vitamin D receptor is a substrate for degradation by proteasomes (25). We have found recently that the vitamin D receptor is ubiquitinated and degraded by proteasomes in human keratinocytes (26). In addition, Nawaz *et al.* (22) reported that estrogen receptor levels, but not progesterone receptor or thyroid hormone receptor levels, are regulated by proteasome degradation, although estrogen receptor ubiquitination has not been demonstrated. These data raise the possibility that ubiquitin/proteasome-mediated breakdown participates in the regulation of the levels of some, but not all, members of the nuclear receptor superfamily. Future research should be directed toward determining which nuclear receptor members are ubiquitinated and toward identification and characterization of the substrate specificity of the ubiquitinating enzymes.

Ubiquitination of RARγ and RXRα, and their subsequent degradation by the proteasome, likely functions to terminate the transcriptional activity of both receptors. The identity and regulation of the enzymes that ubiquitinate RARγ and RXRα remain to be determined. In addition, it is possible that ubiquitination of RARγ and RXRα serves not only to target the receptors for degradation but may also serve to regulate their activities.

Additionally, the role of retinoic acid in regulating retinoid receptor ubiquitination and turnover has yet to be determined. We found that pretreatment of keratinocytes with retinoic acid prior to UV or cycloheximide exposure did not prevent loss of RARγ and RXRα (data not shown). This lack of effect of retinoic acid on degradation of RARγ and RXRα is in contrast to the effects of ligands on vitamin D and estrogen receptor turnover. Vitamin D stabilizes the vitamin D receptor by inhibiting its ubiquitination and subsequent proteasomal degradation (26). In contrast, estradiol induces proteasome degradation of the estrogen receptor (22, 27, 28). The possible role of retinoic acid in the turnover of RARγ and RXRα proteins warrants further investigation.

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