Paper III

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cAMP-dependent Protein Kinase Regulates Ubiquitin-Proteasome-mediated Degradation and Subcellular Localization of the Nuclear Receptor Coactivator GRIP1*

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Nuclear receptors and their coactivators are key regulators of numerous physiological functions. GRIP1 (glucocorticoid receptor-interacting protein) is a member of the steroid receptor coactivator family. Here, we show that GRIP1 is regulated by cAMP-dependent protein kinase (PKA) that induces its degradation through the ubiquitin-proteasome pathway. GRIP1 was down-regulated in transiently transfected COS-1 cells after treatment with 8-*para*-chlorophenylthio-cAMP or forskolin and 3-isobutyl-1-methylxanthine and in adrenocortical Y1 cells after incubation with adrenocorticotropic hormone. Pulse-chase experiments with transiently transfected COS-1 cells demonstrated that the half-life of GRIP1 was markedly reduced in cells overexpressing the PKA catalytic subunit, suggesting that activation of PKA increases the turnover of GRIP1 protein. The proteasome inhibitors MG132 and lactacystin abolished the PKA-mediated degradation of GRIP1. Using ts20 cells, a temperature-sensitive cell line that contains a thermolabile ubiquitin-activating E1 enzyme, it was confirmed that PKA-mediated degradation of GRIP1 is dependent upon the ubiquitin-proteasome pathway. Coimmunoprecipitation studies of COS-1 cells transfected with expression vectors encoding GRIP1 and ubiquitin using anti-GRIP1 and anti-ubiquitin antibodies showed that the ubiquitination of GRIP1 was increased by overexpression of PKA. Finally, we show that PKA regulates the intracellular distribution pattern of green fluorescent protein-GRIP1 and stimulates recruitment of GRIP1 to subnuclear foci that are colocalized with the proteasome. Taken together, these data demonstrate that GRIP1 is ubiquitinated and degraded through activation of the PKA pathway. This may represent a novel regulatory mechanism whereby hormones down-regulate a nuclear receptor coactivator.

Nuclear receptors (NRs)[1] represent a large family of transcriptional regulators that play pivotal roles in the regulation of a variety of physiological and developmental functions. Many nuclear receptor coactivator proteins have been identified the past few years. These transcription factors interact with NRs in a ligand-dependent manner and enhance NR-mediated gene transcription (1, 2). Coactivators contain enzymatic activities including histone acetyltransferase (3, 4), methyl transferase (5, 6), and ubiquitin conjugation and ubiquitin ligase activities (7, 8).

The steroid receptor coactivator (SRC/NcoA (nuclear receptor coactivator)/p160) family contains three genetically distinct but structurally and functionally related members: (i) the SRC-1 (steroid receptor coactivator 1) (9), (ii) TIF2 (transcription intermediary factor 2) and its mouse homologue GRIP1 (10, 11), and (iii) the p300/CREB-binding protein (CBP) cointegrator protein (12) and its human homologues: AIB1 (amplified in breast cancer-1) (13), ACTR (activator of the thyroid and retinoic acid receptor) (4), TRAM1 (thyroid hormone receptor activator molecule 1) (14), and RAC3 (receptor-associated coactivator 3) (15). All have similar structural and functional domains, including an N-terminal basic helix-loop-helix-Per-Arnt-Sim domain, C-terminal activation domains 1 and 2 (AD1 and AD2), and several NR interaction modules containing the conserved sequence L*XX*LL (16). Intense studies have focused on the mechanism by which SRCs potentiate transcription activity of NRs. It has been established that the coactivators enhance NR-dependent gene transcription by acting as bridging molecules that interact directly with the NR activation function 2 in the ligand binding domains of various NRs (4, 12, 17, 18). The SRCs interact with the histone acetyltransferase CBP and its homologue p300 in an AD1-dependent manner (3, 12, 19) as well as other proteins such as the coactivator-associated arginine methyltransferase (CARM1) through the AD2 domain (5). Recently, the basic helix-loop-helix-Per-Arnt-Sim domain of GRIP1 was also demonstrated to function as an activation domain by recruiting a secondary coactivator, coiled-coil coactivator (20). CBP, p300, and CARM1 contribute to the gene transcription potentiation through histone acetylation and methylation, respectively (3, 21). It has been also sug-

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[1] The abbreviations used are: NR, nuclear receptor; SRC, steroid receptor coactivator; PKA, protein kinase A; 8-CPT-cAMP, 8-*para*-chlorophenylthio-cAMP; IBMX, 3-isobutyl-1-methylxanthine; ACTH, adrenocorticotropic hormone; GFP, green fluorescent protein; AD1, activation domain 1; AD2, activation domain 2; CBP, cAMP-response element-binding protein-binding protein; CARM1, coactivator-associated arginine methyltransferase 1; SF-1, steroidogenic factor-1; UBA, ubiquitin-activating enzyme; UBC, ubiquitin-conjugating enzyme; ER, estrogen receptor; GR, glucocorticoid receptor; GRE, GR-response element; MSC-1, mouse Sertoli cells; PMSF, phenylmethylsulfonyl fluoride; PBS, phosphate-buffered saline; PBS-T, phosphate buffer saline-Tween; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; PML, promyelocytic leukemia; CRS, cAMP-responsive sequence; CHX, cyclohexamide; Ub, ubiquitin; PA28α, proteasome activator 28α-subunit; E1, ubiquitin-activating enzyme; E2, ubiquitin carrier protein; E3, ubiquitin-protein isopeptide ligase; aa, amino acid(s); HA, hemagglutinin.

gested that CBP and CARM1 may modify other components of the transcriptional complex (22, 23).

GRIP1 is widely expressed, but the levels of expression differ between cell types and organs (11, 12). Mice lacking functional GRIP1 exhibit nearly normal somatic growth, but the fertility is significantly reduced in both the male and female (24). Interestingly, it has recently been demonstrated that a high fat diet induces GRIP1 expression in white and brown adipose tissues. Moreover, it has been shown that GRIP1 plays an important role in lipid metabolism through its interaction with peroxisome proliferator-activated receptor γ, as evidenced by the fact that GRIP1 −/− mice exhibit higher body temperature, less fat accumulation, lower levels of both triglyceride and fasting glycemia, and higher insulin sensitivity (25).

Our knowledge of the molecular mechanisms that regulate the functions of SRC proteins is limited. Covalent modifications, such as phosphorylation, acetylation, and sumoylation (26–29), have been demonstrated. Interestingly, it has been shown that the transcriptional coactivator function of GRIP1 was induced through phosphorylation at Ser-736 by the extracellular signal-regulated kinase (27). We reported previously that stimulation of the PKA-dependent pathway down-regulates the activity of GRIP1 by reducing its protein level (30), whereas this stimulation further potentiates p300/CBP cointegrator protein-mediated coactivation of the NR steroidogenic factor-1 (SF-1). This finding suggests that extracellular signals may modulate the transcriptional activity of NRs via regulation of coactivator level and availability. The major system for selective degradation of proteins in eukaryotic cells is the ubiquitin-proteasome degradation pathway, where target proteins are covalently attached with one or several molecules of the highly conserved 76-aa ubiquitin protein and subsequently degraded by the 26 S proteasome complex (31). Conjugation of ubiquitin to target proteins proceeds via a three-step cascade mechanism: (i) the ubiquitin-activating enzyme E1 (UBA) activates ubiquitin in an ATP-dependent manner to generate a high energy thiol ester intermediate E1-S~ubiquitin; (ii) one of several ubiquitin carrier or ubiquitin-conjugating enzymes E2 (UBCs) transfers the ubiquitin moiety from E1 to target proteins that are specifically bound to E3, a member of the ubiquitin-protein ligase family; and (iii) E3 finally catalyzes covalent attachment of ubiquitin to the substrate (31). The ubiquitin-proteasome degradation system is implied to be a key component in the regulation of transcriptional activity for several nuclear hormone receptors including estrogen receptor (ER) (32), glucocorticoid receptor (GR) (33, 34) and thyroid receptor (35). A recent investigation has also demonstrated that the SRCs are targets of the ubiquitin-proteasome degradation pathway and that the process depends on UBA and specific UBCs (36). However, the signaling mechanisms that control this regulated degradation are still elusive. Here, we demonstrate that stimulation of the PKA pathway leads to down-regulation of GRIP1 protein via targeting it to ubiquitination and proteasome-mediated degradation.

EXPERIMENTAL PROCEDURES

Plasmid Constructs—The expression plasmid pCMV5-SF-1, and the luciferase reporter plasmid pT81–4CRS2-luc (4CRS2-luc) that contains four copies of the SF-1 binding site from the proximal promoter region of the bovine *cyp17* gene, have been previously described (37). The expression plasmids pSG5-HA-GRIP1 encoding wild type GRIP1 and pM-GRIP1 encoding GAL4-GRIP1 were generously supplied by Dr. M. R. Stallcup (Los Angeles, CA). Deletions of potential PEST sequences of GRIP1 in the pSG5-HA-GRIP1 plasmid were performed using the XL QuikChange™ site-directed mutagenesis kit (Stratagene), resulting in pSG5-HA-GRIP1 ΔPEST1 (Δ aa 648–666), ΔPEST2 (Δ aa 713–731), and ΔPEST3 (Δ aa 788–826). The pG5-luc reporter plasmid for GAL4 was purchased from Promega. The reporter construct MMTV-luc-GRE, which contains the mouse mammary tumor virus long terminal repeat with four GR-response elements (GREs), and the pSG5-hGR expression plasmid were kindly provided by Dr. E. Treuter (Stockholm, Sweden). The pCMV5-Cα expressing the catalytic subunit of PKA was a gift from Dr. G. S. McKnight (Seattle, WA). The expression plasmid for ubiquitin pCW7-Ub was purchased from LGC Promochem (Sweden). The pSG5-GFP-GRIP1 expression plasmid encoding GRIP1 as a fusion with green fluorescent protein (GFP) at its N terminus, was provided by Dr. Fred Schaufele (San Francisco, CA).

Cell Culture and Transfection Experiments—COS-1 African monkey kidney cells, Y1 mouse adrenocortical tumor cells, and MSC-1 mouse Sertoli cells were cultured in Dulbecco's modified Eagle's medium (Invitrogen) supplemented with 10% fetal calf serum, 100 units of penicillin and 100 μg of streptomycin per ml. Chinese hamster lung cells (E36) and its derived temperature-sensitive cell line (ts20) were grown in Eagle's minimal essential medium, minimal essential medium-α (Invitrogen), supplemented with 10% fetal bovine serum, 4.5 g/liter glucose, and antibiotics as described above, at 30 °C in 5% CO_2. For transfections, the cells were seeded into 12 or 6-well plates or 60-mm Petri dishes 1 day before. COS-1, ts20, and E36 cells were transiently transfected using SuperFect (Qiagen) according to the manufacturer's recommendations. Y1 cells were transfected by the calcium phosphate precipitation method as described previously (30). MSC-1 cells were transfected using LipofectAMINE (Invitrogen) following the manufacturer's protocol. Transfected cells were washed once with PBS and harvested 48 h (COS-1, E-36, ts20, and MSC-1) or 24 h (Y1) post-transfection. Luciferase assays were performed using the luciferase assay kit (BIOThema AB, Sweden) or the dual luciferase reporter assay system (Promega). All experiments were performed in triplicate. The data shown are representative of 3–5 independent experiments. To ensure that the transfection experiments carried out at different times were reproducible, cell cultures were cotransfected with the reporter construct pRL-TK (Promega), and the *Renilla* luciferase activity was used as a reference for normalizing the firefly luciferase activity produced by the *luc* reporter constructs.

Western Blotting—Cells were lysed in a buffer containing 50 mM Tris-HCl, pH 7.5, 200 mM NaCl, 5 mM EDTA, 1% Nonidet P-40, 1 μg/ml aprotinin, 5 mM *N*-ethylmaleimide, 100 nM sodium orthovanadate, and 0.2 mM PMSF, supplemented with Complete Mini EDTA-free protease inhibitor tablets (Roche Applied Science). Subsequently, the cell lysates were analyzed by 6 or 10% SDS-PAGE and transferred to nitrocellulose membranes (Amersham Biosciences). Immunoblotting was performed by first blocking the membrane with PBS-T containing 5% (w/v) dried skimmed milk for 1 h. After several washes, the membrane was incubated with primary antibody for an additional 1 h and secondary antibodies (goat anti-rabbit IgG or donkey anti-mouse IgG conjugated to horseradish peroxidase) for 30 min. Visualization of proteins was achieved by using the enhanced SuperSignal® West Pico Chemiluminescent Substrates (Pierce) and by exposure to Hyperfilm™ ECL (Amersham Biosciences). The primary antibodies used were rabbit anti-HA (Zymed Laboratories Inc., South San Francisco, CA), mouse anti-GRIP1 monoclonal antibody (NeoMarkers, Fremont, CA), mouse anti-GAPDH monoclonal antibody (Chemicon International, Temecula, CA), and mouse anti-β-actin (Abcam, Cambridge, UK). Quantification of GRIP1 protein expression levels was performed using the NIH Image software (available on the World Wide Web at rsb.info.nih.gov/nih-image/). Relative GRIP1 expression was calculated either as density percentages of corresponding GRIP1 protein bands over the control GRIP1 band or as ratios between GRIP1 and the corresponding GAPDH or β-actin band densities and expressed relative to controls, as indicated in the figures.

Pulse-Chase Analysis—COS-1 cells were seeded at a density of 3.3×10^5 cells/60-mm Petri dish and transfected with pSG5-HA-GRIP1 (3.0 μg) and pCMV5-SF-1 (0.25 μg). After 24 h, the cells were washed twice with PBS and incubated with methionine-free Dulbecco's modified Eagle's medium containing 5% fetal bovine serum for 30 min. The cells were then pulse-labeled with [^{35}S]methionine (50 μCi/ml) for 1 h. Thereafter, the medium was rapidly removed, and the cells were washed twice in PBS before being incubated in a chase medium of Dulbecco's modified Eagle's medium containing a 50× excess of methionine and either the vehicle Me_2SO or the proteasome inhibitor MG132 (10 μM) for the desired chase times. After 1 h, the cells were treated with forskolin (10 μM), IBMX (50 μM), and 8-CPT-cAMP (500 μM), as indicated. At each desired time point, the cells were washed twice with PBS and lysed in an immunoprecipitation buffer containing 50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 2 mM EDTA, 0.5% (w/v) SDS, 1.0% Triton X-100, 1 μg/ml aprotinin, 5 mM *N*-ethylmaleimide, 0.2 mM PMSF, and Complete Mini EDTA-free protease inhibitors (Roche Applied Science). The cell lysates were subjected to a coimmunoprecipitation procedure, as described below, using mouse anti-GRIP1 antibody (NeoMarkers). The

resulting GRIP1-bound protein G-Sepharose beads were resolved by 6% SDS-PAGE, followed by autoradiography using BioMax film (Eastman Kodak Co.) for detection of ^{35}S-labeled GRIP1 protein. GRIP1 expression levels were quantified as described above.

Coimmunoprecipitation—The procedure was used for detection of ubiquitinated GRIP1 protein and during the pulse-chase analysis of GRIP1. Cell lysates in the immunoprecipitation buffer were supplemented with four volumes of a buffer containing 50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 2 mM EDTA, 1 μg/ml aprotinin, 5 mM *N*-ethylmaleimide, 0.2 mM PMSF, and Complete Mini protease inhibitors and then incubated with 30 μl of protein G-Sepharose (Amersham Biosciences) for 1 h at 4 °C on a rotating wheel. Thereafter, the samples were briefly centrifuged. The supernatants were incubated with 25 μl of mouse anti-GRIP1 antibody (NeoMarkers) overnight and subsequently incubated with 50 μl of protein G-Sepharose for 2 h. The precipitated protein G-Sepharose beads were washed five times with an ice-cold washing buffer containing 50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 2 mM EDTA, 0.1% (w/v) SDS, 0.2% Triton X-100, 1 μg/ml aprotinin, 5 mM *N*-ethylmaleimide, 0.2 mM PMSF, and Complete Mini protease inhibitors. The immunoprecipitates were then heated in 30 μl of 2× SDS-loading buffer (100 mM Tris-HCl, pH 6.8, 4% SDS, 0.2% bromphenol blue, 20% glycerol, and 200 mM mercaptoethanol) at 95 °C for 5 min and resolved by SDS-PAGE and Western blotting.

Confocal Microscopy, Immunofluorescence, and Image Analysis—COS-1 cells were plated onto microscopic coverglasses in 35-mm Petri dishes and transfected with expression plasmids encoding GFP-GRIP1 (400 ng), SF-1 (100 ng), and PKA-Cα (100 ng) as described above. After 24 h, the cells were washed with PBS, fixed in 4% paraformaldehyde, and permeabilized with PBS containing 0.5% Triton X-100. The cells were subsequently exposed to a blocking buffer of 0.5% bovine serum albumin in PBS for 1 h, followed by 1-h incubations with the primary and then the secondary antibody at 37 °C. Rabbit polyclonal anti-PA28α (Affiniti Research Products, Devon, UK) and mouse monoclonal anti-PML (Santa Cruz Biotechnology, Inc., Santa Cruz, CA) were used as primary antibodies, together with Texas Red-labeled anti-rabbit (Abcam) and anti-mouse (Southern Biotech) antibodies as secondary antibodies. For each experiment, the intracellular distribution of GFP-GRIP1 was examined in more than 250 cells. Microscopy was performed by using a Leica TCS SP2 AOBS confocal laser-scanning microscope (Leica Microsystems, Heidelberg GmbH). Green fluorescence was monitored by excitation with the 488-nm line from an argon/argon krypton laser, whereas red fluorescence was monitored with the 496-nm line from a helium/neon II laser. Emissions for green and red fluorescence were viewed through a 490–580- and a 596–700-nm band pass filter, respectively.

The percentage of cells containing nuclear foci was determined by counting the number of foci-positive and -negative cells within a defined area of the cell monolayers. The green fluorescence intensity of the cells was measured by using the Leica Confocal Software quantifying program. Area-corrected fluorescence intensity of the GFP-GRIP1-expressing nucleus and a random chosen background area outside the cell was calculated. The background intensity was then subtracted from the fluorescence intensity of the nucleus.

RESULTS

Down-regulation of GRIP1 by Activation of PKA—It has previously been demonstrated that GRIP1 possesses intrinsic activation function when linked to GAL4 (11). Using COS-1 cells transiently transfected with GAL4-GRIP1 expression plasmid and a GAL4-responsive reporter plasmid (pG5-luc), we observed that cotransfection with an expression plasmid encoding the catalytic subunit of PKA (PKA-Cα) resulted in a marked decrease in GRIP1 activation function (Fig. 1*A*). To further study the role of PKA in regulation of GRIP1 function, COS-1 cells were cotransfected with expression vectors encoding GRIP1 and GR together with the reporter construct MMTV-luc-GRE, which contains the mouse mammary tumor virus long terminal repeat with four GREs (38). Since GRIP1-mediated coactivation of GR was very low in the absence of ligand, the transfected cultures were treated with dexamethasone. As expected, GRIP1 strongly potentiated GR-dependent transcription, whereas coexpression of PKA-Cα resulted in a marked decrease of luciferase activity (Fig. 1*B*). Consistent with our previous report (30), Western blot analysis demonstrated that

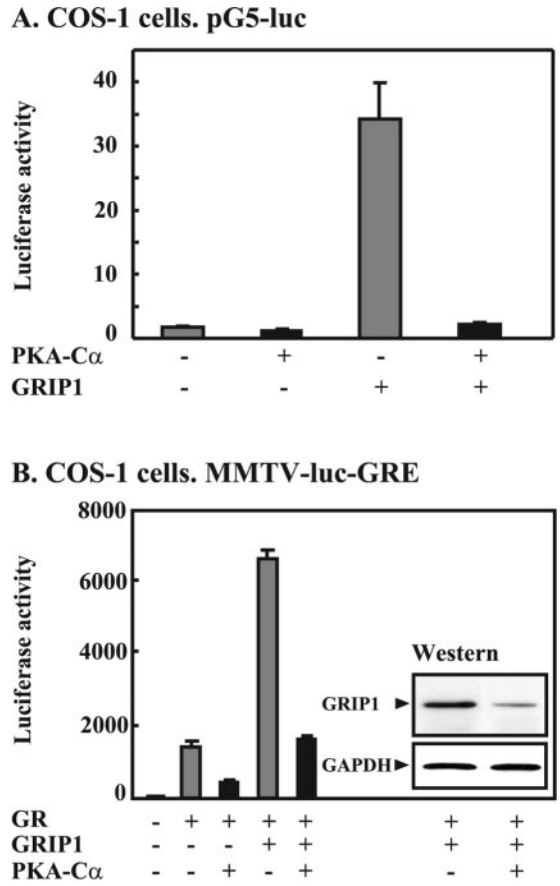

FIG. 1. **PKA inhibits GRIP1-mediated coactivation function through down-regulation.** *A*, COS-1 cells were transfected with the GAL4-responsive reporter construct pG5-luc (1.1 μg) and expression vectors encoding GAL4-GRIP1 (1.5 μg) and PKA-Cα (0.1 μg). *B*, COS-1 cells were transfected with expression vectors encoding GR (0.1 μg), HA-GRIP1 (1.5 μg), and PKA-Cα (0.1 μg) together with the MMTV-luc-GRE reporter construct (1.1 μg) as indicated in the figure and treated with dexamethasone (0.1 μM) after 24 h. Luciferase assays were performed 48 h after transfection. The figures show the mean ± S.D. of triplicate transfections from representative experiments. Cotransfection with a plasmid encoding *Renilla* luciferase was performed to control for transfection efficiencies. *Inset B*, cell lysates from COS-1 cells described in *B* were subjected to Western blotting. GRIP1 and GAPDH were detected using anti-HA and anti-GAPDH antibodies, respectively. The results presented are representative of three independent experiments.

overexpression of PKA-Cα led to a decreased level of GRIP1 protein (Fig. 1*B*, *inset*). GRIP1 down-regulation appeared to be unaffected by overexpression of GR, since we observed no change in GRIP1 protein levels in the presence or absence of coexpressed GR (Fig. 1*B*) (30) (data not shown).

GRIP1 has been reported to have an essential role in mouse reproduction function, and one major expression site is apparently the Sertoli cells (24). Therefore, mouse Sertoli cells (MSC-1) were employed in this study. MSC-1 cells were cotransfected with expression vectors encoding GRIP1 and SF-1 together with the reporter construct pT81–4CRS2-luc, which contains a minimal thymidine kinase promoter and four copies of the SF-1 response element (CRS2) (37). As expected, overexpression of PKA-Cα also inhibited GRIP1-mediated coactivation of SF-1 function and led to down-regulation of GRIP1 protein level in transiently transfected MSC-1 cells (Fig. 2*A*). To assess whether endogenous GRIP1 is regulated by PKA, nontransfected MSC-1 cells were lysed and subjected to Western blot analyses after being treated with 8-CPT-cAMP and the cAMP-elevating agents forskolin and IBMX. As shown in Fig. 2*B*, this treatment reduced the protein level of endogenous

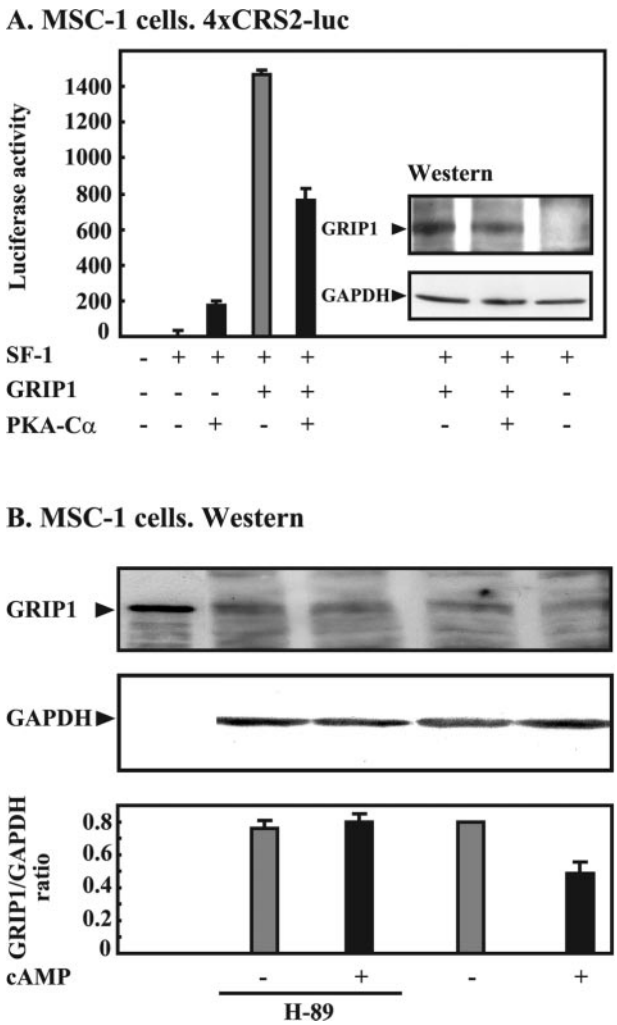

FIG. 2. **GRIP1 protein level is reduced by PKA in MSC-1 cells.** *A*, MSC-1 cells were transfected with expression vectors encoding SF-1 (0.1 μg), HA-GRIP1 (1.5 μg), and PKA-Cα (0.1 μg) together with the CRS2-luc reporter construct (1.1 μg). Luciferase assays were performed 48 h after transfection. Cotransfection with a plasmid encoding *Renilla* luciferase was performed to control for transfection efficiencies. Cell lysates were also subjected to Western blotting using anti-HA and anti-GAPDH antibodies (*inset*). Shown is the mean ± S.D. of triplicate transfections from a representative experiment. The results presented are representative of three independent experiments. *B*, MSC-1 cells were transfected with GRIP1 expression vector (*lane 1*) or left untransfected (*lanes 2–5*). The cells were treated with 10 μM forskolin, 50 μM IBMX, and 500 μM 8-CPT-cAMP (cAMP) for 17 h (*lanes 3* and *5*). H-89 (10 μM) was added to the cell cultures 1 h before cAMP elevation (*lanes 2* and *3*). Western blotting using anti-GRIP-1 and anti-GAPDH antibodies were performed. Relative endogenous GRIP1 expression levels are shown in the *lower panel* as ratios between GRIP1 and the corresponding GAPDH band densities as described under "Experimental Procedures." The results presented are the mean ± S.D. of three independent experiments.

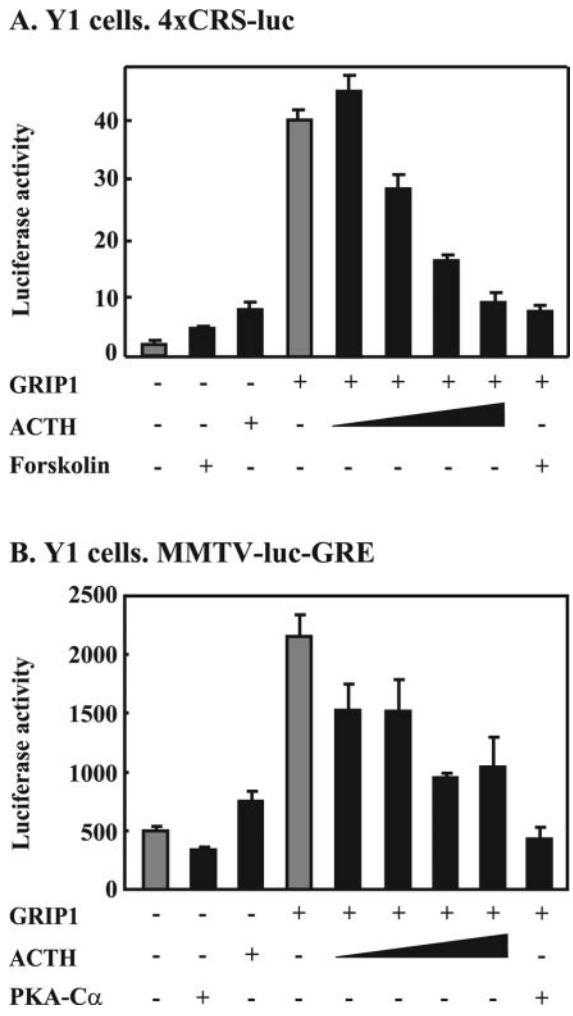

FIG. 3. **ACTH inhibits GRIP1-mediated potentiation of SF-1 and GR transactivation in adrenocortical Y1 cells.** *A*, Y1 cells expressing endogenous SF-1 were transfected with expression vector encoding GRIP1 (1.5 μg) together with the CRS2-luc reporter construct (1.4 μg). One hour after transfection, the cells were treated with ACTH (0.1–1.0 μM) and forskolin (10 μM) as indicated in the figure. Control cultures that were not transfected with GRIP1 expression plasmid were also treated with ACTH (1.0 μM). *B*, Y1 cells were transfected with the MMTV-luc-GRE reporter construct (1.4 μg) and expression vectors encoding GRIP1 (1.5 μg), GR (0.1 μg), and PKA-Cα (0.1 μg) or treated with ACTH as indicated and as described in *A*. Luciferase assays were performed 24 h after transfection. Cotransfection with a plasmid encoding *Renilla* luciferase was performed to control for transfection efficiencies. The figures show the mean ± S.D. of triplicate transfections and are representative of three independent experiments.

GRIP1, whereas pretreatment of the cells with H-89, an inhibitor of PKA, counteracted the down-regulation of GRIP1 protein in MSC-1 cells.

Regulation of GRIP1 Coactivator Function by ACTH—Several peptide hormones act through the cAMP/PKA pathway. Thus, it was of interest to test whether treatment of cell cultures with a natural hormone that increases the intracellular level of cAMP could modulate GRIP1 coactivator function. Since MSC-1 cells do not express the follicle-stimulating hormone receptors (39), a mouse adrenocortical cell line (Y1) that expresses endogenous ACTH receptors was employed. Treatment of Y1 cells with ACTH leads to activation of PKA (40). Thus, first, Y1 cells were transfected with the expression vector encoding GRIP1 together with the CRS2 reporter construct. Expression of GRIP1 strongly potentiated the transcription from the CRS2 reporter gene, but the luciferase activity was markedly impaired in cells treated with ACTH or forskolin (Fig. 3*A*). These findings suggest that continued treatment with ACTH, a known stimulator of steroid hormone biosynthesis, may also lead to reduced CRS2-dependent transcription through down-regulation of GRIP1 coactivator function. Next, Y1 cells were cotransfected with expression vectors encoding GRIP1 and GR together with the reporter construct MMTV-luc-GRE and subsequently exposed to ACTH. As shown in Fig. 3*B*, treatment of Y1 cells with ACTH also inhibited GRIP1-mediated coactivation of GR-dependent transcriptional activity. We noted that in cells with moderately elevated PKA activity induced by cAMP analog (data not shown) or ACTH, GR-dependent transcriptional activity was stimulated also in the absence of overexpressed GRIP1 (Figs. 1*B* and 3*B*). This is

in accordance with the synergism between glucocorticoids and cAMP that has been reported by others (41). However, a presumed strong activation of the PKA signaling pathway mediated by overexpression of PKA-Cα inhibited the MMTV-luc-GRE luciferase activity even in the absence of GRIP1 overexpression. This may be explained by a degradation of endogenous GRIP1 and subsequently reduced GR coactivation. Taken together, these results demonstrate that ACTH, an extracellular hormone that activates the PKA pathway, can modulate the transcriptional activity of nuclear receptors through down-regulation of the nuclear receptor coactivator GRIP1.

GRIP1 Is a Short Lived Protein That Is Degraded after PKA Activation—We have previously reported that stimulation of PKA does not lead to changes in GRIP1/TIF2 mRNA (30), suggesting that the decrease in GRIP1 protein is at the post-transcriptional level. To test whether the PKA-mediated decrease in GRIP1 protein is a process that requires *de novo* protein synthesis, COS-1 cells were treated with cyclohexamide (CHX) after transfection with expression vectors encoding GRIP1 and PKA-Cα. We observed that CHX reduced the level of GRIP1 protein in transfected COS-1 cells, whereas the level of endogenous GAPDH was not affected after treatment with CHX for 28 h. Of note, overexpression of PKA-Cα resulted in a reduction of GRIP1 protein both in the presence and absence of CHX (Fig. 4*A*). We also observed that pretreatment with CHX did not affect GRIP1 down-regulation in transfected COS-1 cells after treatment with forskolin, IBMX, and 8-CPT-cAMP (data not shown). These results suggest that cAMP/PKA-mediated down-regulation of GRIP1 does not require *de novo* protein synthesis.

To examine the effect of continuous stimulation of cAMP/PKA pathway on GRIP1 protein in more detail, COS-1 cells were transfected with GRIP1 expression plasmid and treated with 10 μM forskolin, 50 μM IBMX, and 500 μM 8-CPT-cAMP 24 h after transfection. As shown in Fig. 4*B*, Western blot analysis demonstrated that activation of the cAMP/PKA pathway induced a time-dependent decrease in the level of GRIP1 protein. A major reduction in GRIP1 protein level was noted after 15 h of continuous stimulation of PKA activity. Taken together, these results suggest that PKA mediates a decrease in GRIP1 protein by a mechanism that involves GRIP1 turnover or stability.

cAMP Stimulates Proteasome-mediated Degradation of GRIP1—To study the stability of GRIP1 protein, pulse-chase experiments were performed in COS-1 cells transiently transfected with the pSG5-GRIP1 expression plasmid. The cells were subsequently labeled with [^{35}S]methionine for 1 h, and GRIP1 protein was immunoprecipitated after a chase of 0, 6, 8, 10, 20, and 24 h in the presence of vehicle (Fig. 5*A*) or 10 μM forskolin, 50 μM IBMX, and 500 μM 8-CPT-cAMP (Fig. 5*B*). GRIP1 protein was rapidly degraded in COS-1 cells that were treated with cAMP analog and cAMP-elevating agents. After 8 h of chase, a very low amount of [^{35}S]methionine-labeled GRIP1 was observed, and after 10 h of chase, GRIP1 protein was barely detected. On the other hand, in cells that were not treated with agents that activate the PKA pathway, a slower decrease in level of [^{35}S]methionine-labeled GRIP1 was observed, and GRIP1 protein was detected even after 20 h of chase. The half-life of GRIP1 in transiently transfected COS-1 cells was estimated to be 12 h (Fig. 5*A*). After cAMP elevation, the turnover of GRIP1 increased, and the half-life was reduced to ~7 h (Fig. 5*B*).

The ubiquitin-proteasome pathway is known to regulate the stability of many proteins (31). To address the mechanisms by which GRIP1 is degraded, pulse-chase experiments were performed as described above but in the presence of the proteasome inhibitor MG132. As shown in Fig. 5, *C* and *D*, treatment with MG132 abolished the down-regulation of [^{35}S]methionine-labeled GRIP1 protein both in the presence and absence of PKA-activating agents. To further examine the role of the ubiquitin-proteasome pathway in the regulation of GRIP1 turnover, COS-1 cells that were transfected with GRIP1 expression plasmid were treated with the proteasome inhibitors lactacystin and MG132 for 6 h and analyzed by Western blotting. As shown in Fig. 6*A* and previously reported by others (36), the levels of GRIP1 were increased after treatment with lactacystin and MG132, confirming that the proteasome is in-

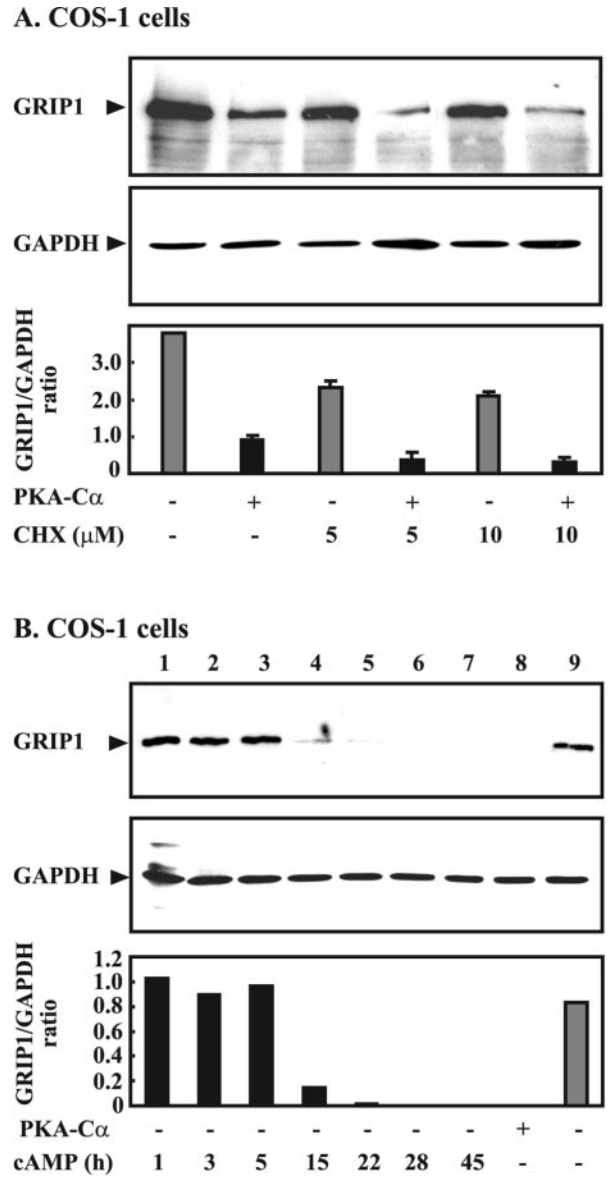

FIG. 4. **GRIP1 protein is subjected to degradation after PKA activation.** *A*, COS-1 cells were transfected with expression vectors encoding HA-GRIP1 (1.5 μg) and PKA-Cα (0.1 μg). Twenty hours after transfection, cells were treated with 5 and 10 μM CHX for a further 28 h as indicated in the figure. *B*, expression vectors encoding HA-GRIP1 (3.0 μg) and SF-1 (0.25 μg) were transfected into COS-1 cells. The cells were subsequently treated with 10 μM forskolin, 50 μM IBMX, and 500 μM 8-CPT-cAMP (*cAMP*) for the indicated periods of time (*h*). As controls, cells that were untreated or cotransfected with HA-GRIP1, SF-1, and PKA-Cα (0.25-μg) expression vectors were also harvested after 48 h (*lanes 8* and *9*). Western blotting was performed using anti-HA and anti-GAPDH antibodies. Relative expression levels of GRIP1 are presented as described under "Experimental Procedures." The results presented in *A* are the mean ± S.D. of three independent experiments, and the data in *B* are representative of two independent experiments.

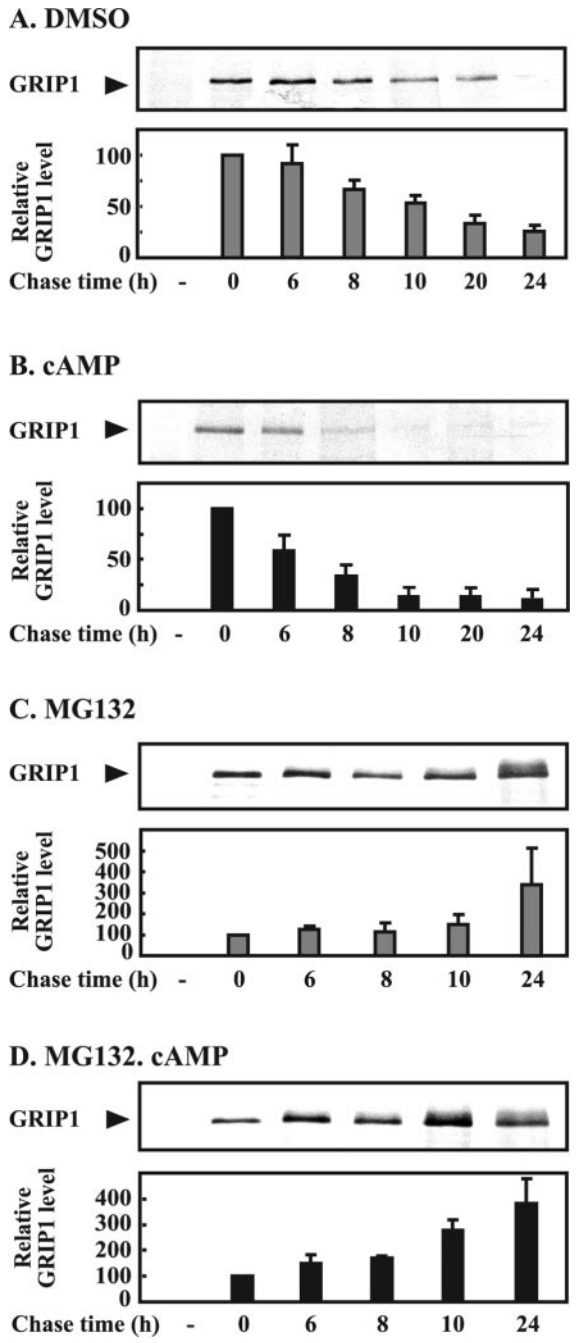

FIG. 5. **PKA decreases the half-life of GRIP1.** COS-1 cells were transfected with expression vectors encoding GRIP1 (3.0 μg) and SF-1 (0.25 μg). After 24 h, the cells were incubated with methionine-free medium for 30 min, pulse-labeled with [^{35}S]methionine for 1 h, and chased for the indicated periods of time (0, 6, 8, 10, 20, and 24 h). One hour after radiolabeling, the cells were treated with vehicle Me_2SO (*A*), with 10 μM forskolin, 50 μM IBMX, and 500 μM 8-CPT-cAMP (*cAMP*) (*B*), with 10 μM MG132 (*C*), or with MG132 and 10 μM forskolin, 50 μM IBMX, and 500 μM 8-CPT-cAMP (*cAMP*) (*D*). GRIP1 was immunoprecipitated using the mouse anti-GRIP1 antibody and analyzed by SDS-PAGE and autoradiography. The results in the *upper panels* are from a representative experiment. The relative amounts of GRIP1 (*lower panels*) were measured as described under "Experimental Procedures" and are the mean ± S.D. of three independent experiments.

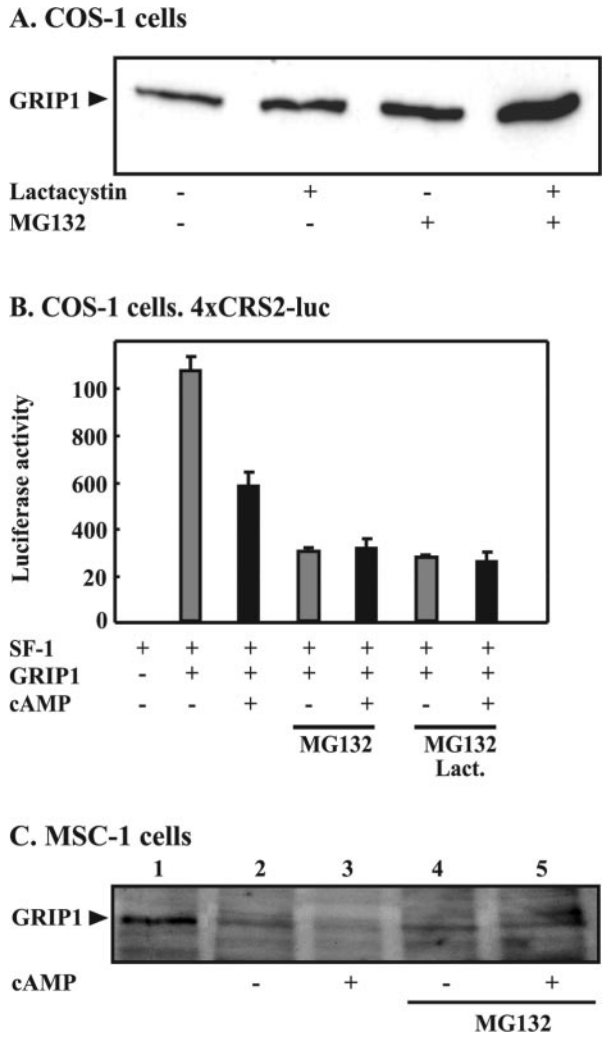

FIG. 6. **The proteasome is implicated in PKA-mediated GRIP1 degradation.** *A*, COS-1 cells were transfected with expression vector encoding HA-GRIP1 (1.5 μg). Twenty-four hours after transfection, the cells were treated with lactacystin (10 μM) and MG132 (10 μM) as indicated for 6 h. Western blotting analysis using anti-HA antibody was performed. *B*, COS-1 cells were transfected with expression vectors encoding SF-1 (0.1 μg) and GRIP1 (1.5 μg), together with the CRS2-luc reporter construct (1.1 μg). Thirty-one hours after transfection, the cells were treated with 10 μM forskolin, 50 μM IBMX, and 500 μM 8-CPT-cAMP (*cAMP*) as indicated. Treatment of the cells with MG132 (10 μM) and lactacystin (10 μM) was performed 1 h prior to cAMP elevation. Luciferase assays were performed 48 h after transfection. The figures show the mean ± S.D. of triplicate transfections from representative experiments. Cotransfection with a plasmid encoding *Renilla* luciferase was performed to control for transfection efficiencies. *C*, MSC-1 cells were left untreated (*lane 2*) or treated with 10 μM forskolin, 50 μM IBMX, and 500 μM 8-CPT-cAMP (*cAMP*) for 17 h (*lanes 3* and *5*). MG132 (10 μM) was added to the cell cultures 1 h before cAMP elevation (*lanes 4* and *5*). MSC-1 cells transfected with GRIP1 expression vector (*lane 1*) was used as control. The cell lysates were subjected to Western blotting using anti-GRIP-1.

volved in the turnover of GRIP1. The importance of the proteasome in PKA-mediated down-regulation of GRIP1 was also investigated using COS-1 cells transfected with expression plasmids encoding SF-1 and GRIP1, together with the CRS2 reporter plasmid. The cells were treated with PKA-activating agents for 16 h after pretreatment with MG132 and lactacystin. It has recently been reported that inhibition of ubiquitin-dependent proteasomal degradation inhibits nuclear receptor-mediated transcription (42) and that proteasome inhibition may have deleterious effects on luciferase activity (43). In this study, we also observed that exposure of the cells to proteasome inhibitors decreased the CRS2-dependent luciferase activity. However, treatment with MG132 and lactacystin also blocked the inhibitory effect of cAMP on GRIP1-mediated coactivation of SF-1 (Fig. 6*B*). Western blot analysis confirmed that MG132 counteracted cAMP-mediated degradation of endogenous GRIP1 in MSC-1 cells (Fig. 6*C*). Taken together, these results

suggest that the normal turnover of GRIP1 protein is mediated by the proteasome. In addition, our data clearly demonstrate that PKA-induced degradation of GRIP1 is counteracted by inhibition of the proteasome.

Ubiquitination Is Required for PKA-mediated Degradation of GRIP1—The mutant Chinese hamster lung cell line ts20 has a thermolabile ubiquitin-activating enzyme E1. The cells have a functional E1 enzyme when being grown at the permissive temperature 30 °C. However, the enzyme is irreversibly inactivated after 60 min at the nonpermissive temperature 40 °C (44). ts20 and the wild-type cell line from which ts20 is derived, E36, were transiently transfected with expression vectors encoding GRIP1 and PKA-Cα. The cells were incubated for 48 h at either 30 or 40 °C and subsequently analyzed for expression of GRIP1 protein by Western blotting of the cell lysates (Fig. 7). Incubation of ts20 cells at 30 °C resulted in degradation of GRIP1 after coexpression of PKA-Cα, confirming the results from COS-1 and MSC-1 cells described above. On the contrary, we observed no effects of PKA-Cα on the level of GRIP1 protein in ts20 cells that were incubated at 40 °C (Fig. 7*A*). This indicates that disruption of the ubiquitination through inactivation of the E1 enzyme blocked PKA-mediated degradation of GRIP1 in transfected ts20 cells. Similar changes were not observed in wild-type E36 control cells (Fig. 7*B*). In these cells, activation of PKA led to down-regulation of GRIP1 protein after incubation at the permissive temperature, 30 °C, as well as at the nonpermissive temperature, 40 °C. These studies suggest that a functional ubiquitin pathway is required for the PKA-mediated degradation of GRIP1.

To determine whether GRIP1 is subjected to ubiquitination after stimulation of the PKA pathway, COS-1 cells were transiently transfected with expression vectors encoding GRIP1 and PKA-Cα. Some cell cultures were also cotransfected with the expression vector encoding wild type ubiquitin, pCW7-Ub. In order to inhibit the degradation of ubiquitin-conjugated proteins, cells were treated post-transfection with the proteasome inhibitor MG132. GRIP1 was immunoprecipitated with an anti-GRIP1 antibody, and ubiquitinated GRIP1 protein was detected using an anti-ubiquitin antibody. As shown in Fig. 8*A*, high molecular weight bands representing ubiquitinated GRIP1 were detected in cells that were transfected with PKA-Cα expression plasmid (*lanes 5* and *6*), and overexpression of ubiquitin further increased the amount of ubiquitinated GRIP1 (*lane 6*). On the other hand, only weak bands corresponding to ubiquitinated GRIP1 were detected in the absence of overexpressed PKA-Cα (*lanes 3* and *4*). These results demonstrate that GRIP1 is ubiquitinated and that activation of PKA increases ubiquitination of GRIP1 protein. As a control of transfection efficiency, the corresponding cell lysates were also analyzed by Western blotting using an anti-GRIP1 antibody (Fig. 8*B*). As noted, the GRIP1 protein levels were increased in cells treated with MG132, which confirms our findings showing that exposure of cells to proteasome inhibitors stabilizes GRIP1 protein.

Analysis of Putative PEST Motifs in GRIP1/TIF2—Ubiquitination of proteins generally requires a short hydrophilic stretch of at least 12 amino acids enriched in proline, glutamic acid, serine, and threonine, termed a PEST motif (45). Analysis of the GRIP1 and TIF2 protein sequences performed by others (36, 46) and us has identified three common potential PEST sequences. Two of them are located in the nuclear receptor interaction domain (aa 648–666 and 713–731), and the other encompasses aa 788–826 (Fig. 9*A*). Therefore, we decided to examine whether these three predicted PEST motifs are necessary for the PKA-mediated degradation of GRIP1. COS-1 cells were transiently transfected with expression vectors encoding GRIP1 deleted at each of the three PEST sites, together with the CRS2 reporter plasmid and expression vectors encoding PKA-Cα and SF-1. All GRIP1 PEST deletion mutants that were examined potentiated SF-1-dependent transcription, and, similar to wild-type GRIP1, coexpression of PKA-Cα inhibited the ability of the GRIP1 deletion mutants to coactivate SF-1 (data not shown). Furthermore, Western blot analysis demonstrated that deletions of the three PEST sites did not block the PKA-mediated degradation of GRIP1 (Fig. 9*B*). Our observations demonstrate that the putative PEST motifs located within and close to the nuclear receptor-interacting domain of GRIP1 are not implicated in the PKA-stimulated ubiquitin-proteasome-mediated degradation of GRIP1.

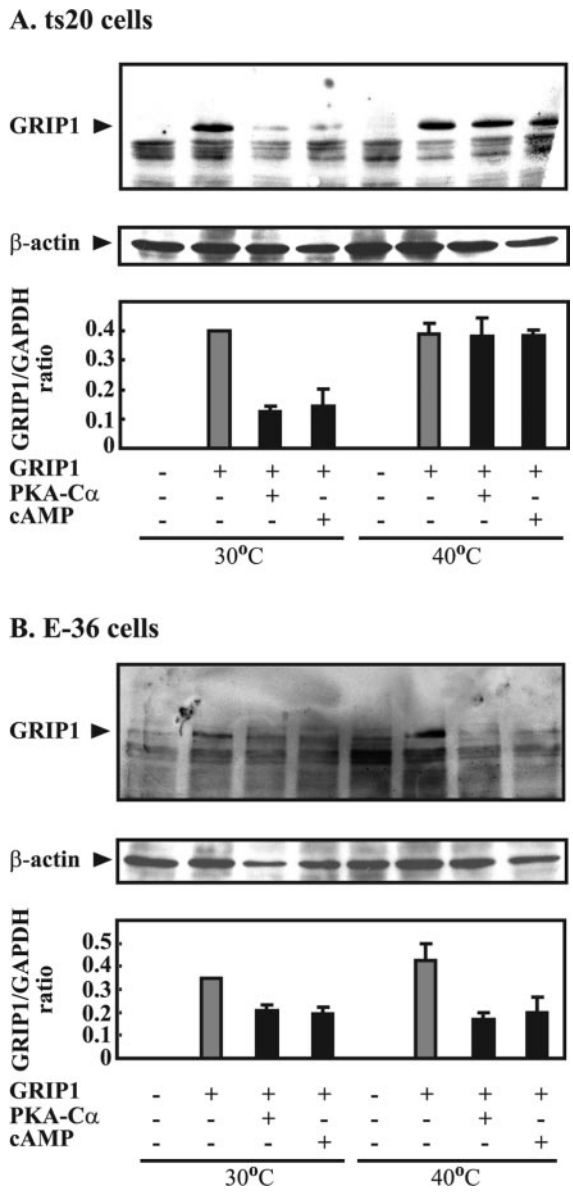

FIG. 7. **Ubiquitination is required for PKA-mediated degradation of GRIP1.** ts20 cells (*A*) and E-36 cells (*B*) were transfected with expression vectors encoding HA-GRIP1 (3.0 μg) and PKA-Cα (0.25 μg). Thirty-one hours after transfection, the cells were treated with 10 μM forskolin, 50 μM IBMX, and 500 μM 8-CPT-cAMP (*cAMP*) as indicated. Incubation of the cells was carried out at either 30 or 40 °C. Forty-eight hours after transfection, the cells were harvested and subjected to Western blot analysis using anti-HA and anti-β-actin antibodies. The relative expression level of GRIP1 is presented as ratios between GRIP1 and corresponding β-actin protein band densities as described under "Experimental Procedures," and the results are the mean ± S.D. of three or four independent experiments.

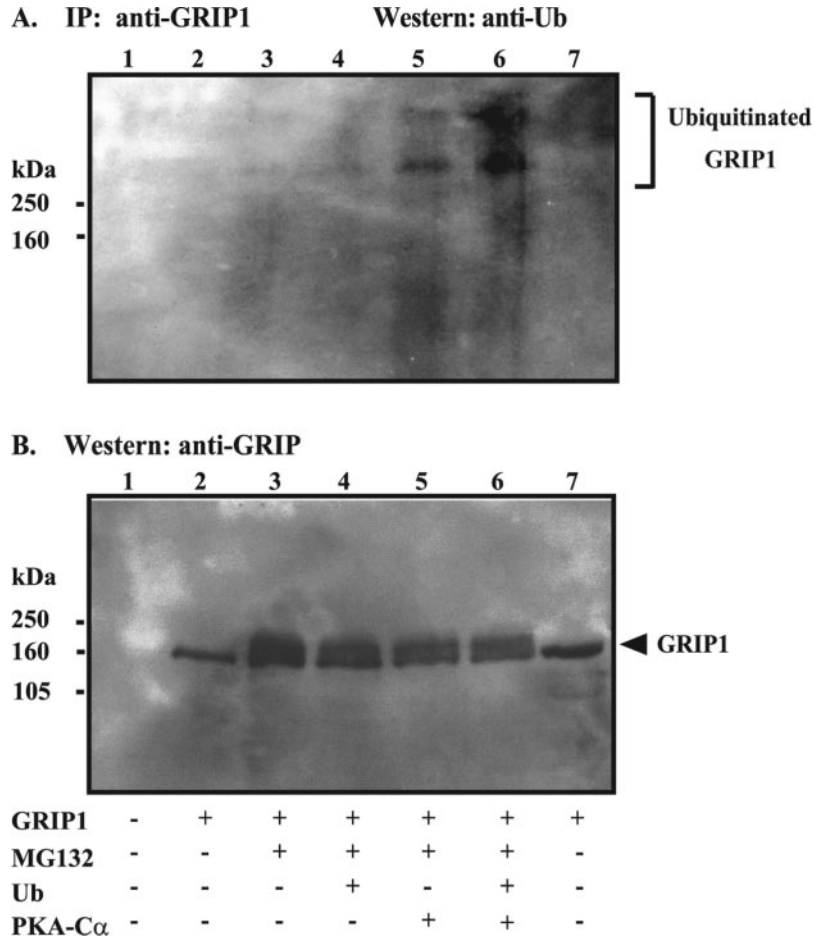

FIG. 8. **Activation of the PKA pathway enhances *in vivo* ubiquitination of GRIP1.** *A*, COS-1 cells were transfected with expression vectors encoding SF-1 (0.25 μg), GRIP1 (3.0 μg), Ub (3.0 μg), and PKA-Cα (0.25 μg). Twenty-four hours after transfection, the cells were treated with MG132 (10 μM) as indicated and incubated for a further 24 h. The cell lysates were subjected to coimmunoprecipitation as described under "Experimental Procedures" using an anti-GRIP1 antibody. The immunoprecipitates were subjected to Western blot analysis using an anti-ubiquitin antibody. *B*, cell lysates from the transfection experiments in *A* were analyzed by Western blotting using an anti-GRIP1 antibody. The results are representative of three independent experiments.

Activation of PKA Leads to Changes in the Intranuclear Distribution of GRIP1 and Recruitment of Proteasome and PML—To examine whether PKA could regulate the intracellular distribution of GRIP1, COS-1 cells were transfected with an expression vector encoding GFP-GRIP1 fusion protein and studied by confocal microscopy. Surprisingly, we did not observe a reduction in the total GFP fluorescence in cells that were cotransfected with PKA-Cα expression plasmid as would be expected from the results above. Interestingly, microscopic images showed that overexpression of PKA-Cα led to a substantial change in the intranuclear distribution pattern of GRIP1 (Fig. 10 and Table I). As reported previously by others (46), we observed both diffuse and focal accumulation of GFP-GRIP1 in the nuclei (Fig. 10). In cell cultures without PKA-Cα overexpression, ~18% of the cells formed discrete intranuclear foci, whereas the remaining cells appeared to have a diffuse nucleoplasmic distribution of GFP-GRIP1. However, in cell cultures that overexpressed PKA-Cα, the number of cells that contained nuclear foci of GFP-GRIP1 was doubled (Table I). There was a great variation in the number of foci within each cell, ranging from 8–10 to even over 200. Transfection with different concentrations of GFP-GRIP1 expression plasmid did not change the number of foci-containing cells. The total fluorescence intensity of cell nuclei containing foci was higher than that of the nuclei without foci (data not shown). Careful analysis of the nuclear fluorescence intensity revealed that cell cultures subjected to PKA-Cα overexpression had a marked decrease in the fluorescence intensity in the nucleoplasm surrounding the foci. The extrafocal nuclear fluorescence intensity was reduced by ~45% (Table I). This is in accordance with the finding that GFP-GRIP1 was recruited from the nucleoplasm to foci after overexpression of PKA-Cα.

To investigate whether the GFP-GRIP1 foci were associated with components of the proteasome, COS-1 cells were transfected with GFP-GRIP1 and PKA-Cα expression plasmids and immunostained with anti-PA28α antibody. PA28α is an activator of the 20 S proteasome and a subunit of the 11 S regulator (47). The experiments revealed that most of the nuclear GFP-GRIP1 foci colocalized with this proteasome component (Fig. 10*A*). Additional support for the association between foci formation and proteasome-mediated degradation was found in experiments with the proteasome inhibitor lactacystin. Treatment of transfected COS-1 cells with 1.0 μM lactacystin for 20 h resulted in a marked decrease in the number of GFP-GRIP1 foci-containing cells as well as inhibition of the PKA-induced redistribution of GRIP1 (Table I). The GRIP1 foci were further characterized by immunofluorescence experiments using an antibody against the promyelocytic leukemia (PML) protein. PML is known to be organized in a speckled pattern in the nucleus, and it has been found to be involved in proteasomal degradation of ubiquitinated proteins (46, 48, 49). We noted that most of the nuclear GRIP1 foci that were detected after overexpression of PKA-Cα also colocalized with the PML bodies (Fig. 10*B*). Together, these findings suggest that activation of PKA induces recruitment of GRIP1 to subnuclear foci that are colocalized with the proteasome and PML.

DISCUSSION

Nuclear receptors interact with multiple coactivator proteins. However, the knowledge of the mechanisms that control the intracellular availability of these coactivators is limited, and modulation of coactivator levels may represent a biological mechanism by which hormones modulate nuclear receptor activity. In this work, it is demonstrated that stimulation of the

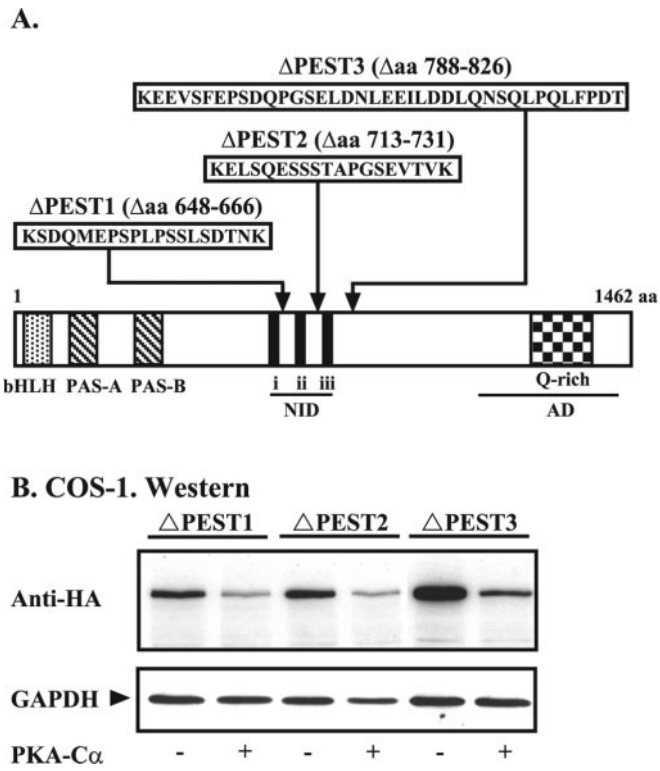

FIG. 9. **Analysis of putative PEST sequences in GRIP1.** *A*, schematic illustration of GRIP1 protein. The *arrows* indicate locations of the putative PEST sequences that were subjected to deletion by site-directed mutagenesis. Both positions and aa sequences of the PEST motifs are presented. *B*, COS-1 cells were transfected with expression vectors encoding HA-GRIP1 that contains deletions of the putative PEST motifs (GRIP1 ΔPEST1, GRIP1 ΔPEST2, and GRIP1 ΔPEST3), together with PKA-Cα expression vector, as indicated. The cells were harvested 48 h after transfection and analyzed by Western blotting using anti-HA and anti-GAPDH antibodies. The results are representative of two independent experiments.

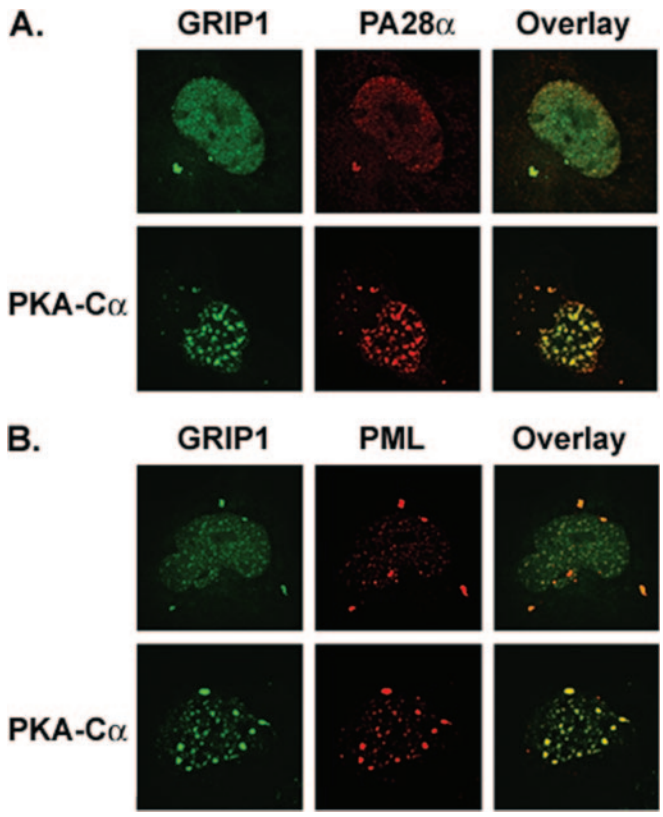

FIG. 10. **Redistribution of GFP-GRIP1 after PKA activation.** COS-1 cells were transfected with expression vectors encoding GFP-GRIP1 and PKA-Cα. *A*, representative confocal images of the intranuclear localization of GFP-GRIP1 and PA28α without (*upper panels*) and with coexpressed PKA-Cα (*lower panels*). *B*, representative images of the intranuclear localization of GFP-GRIP1 and PML without (*upper panels*) and with coexpressed PKA-Cα (*lower panels*). The cells were fixed 24 h after transfection. Immunofluorescence was performed using rabbit polyclonal anti-PA28α and mouse monoclonal anti-PML antibodies, together with Texas Red-labeled anti-rabbit and anti-mouse secondary antibodies, respectively. The overlays of the images are shown on the *right*.

cAMP/PKA pathway, which mediates the intracellular effects of a number of peptide hormones, leads to ubiquitination and proteasomal degradation of the nuclear receptor coactivator GRIP1. We also show that this process involves changes in the intracellular distribution pattern with accumulation of GRIP1 in subnuclear foci that are colocalized with the proteasome.

It has been reported that the SRCs are subjected to ubiquitin-proteasome mediated degradation (36, 50). Degradation of the SRCs is promoted by UBA and specific UBCs. GRIP1/TIF2 degradation depends on UBC5, UBC7, and UBC8, whereas SRC-1 is degraded by UBC2, and p300/CBP cointegrator protein/RAC-3 is degraded predominantly by UBC2, UBC3, UBC4, or UBC5 (36). Coactivator proteins are important modulators of gene transcription, and different coactivators interact with NRs in a competitive and exclusive manner (51). Thus, it is conceivable that the amount and availability of these proteins are subjected to specific regulation and that degradation and exchange of coactivators may facilitate transcription. Targeted degradation of specific coactivators via the ubiquitin-proteasome pathway may represent an important regulatory mechanism that controls the remodeling of the components associated with the nuclear receptors. Previous reports have shown that coactivator activity is regulated by signals that lead to phosphorylation by protein kinases (27, 28). However, as far as we know, this is the first report showing that a signaling pathway regulates the intracellular level of a nuclear receptor coactivator of the SRC family through the ubiquitin-proteasome pathway. Our hypothesis is that this may represent a mechanism by which peptide hormones indirectly modulate the transcriptional activation of specific NRs. GRIP1 has been shown to interact with a number of NRs in overexpression experiments (52). However, various NRs seem to interact with distinct coactivators (53, 54), and a recent study using human breast cancer cells demonstrated that GR interacts preferentially with GRIP1, whereas progesterone receptor preferentially interacts with SRC-1 (55) On the other hand, since the SRC family members seem to be partly available to compensate for each other (56), selective down-regulation of GRIP1 may also result in the recruitment of other coactivators to specific NRs instead of GRIP1. If the transcriptional function of NRs is mediated by a limited number of common coactivators (57), such a competition may also affect the transcriptional activation by other NRs.

The functional effects of PKA-mediated regulation of GRIP1 on the transcriptional activity of specific NRs *in vivo* remains to be examined. This includes the possible biological role of PKA in GRIP1-mediated coactivation of GR. The interaction between the glucocorticoid and cAMP signaling pathways has been extensively studied (58). Although several reports have shown that glucocorticoids and cAMP function in a synergistic or additive manner (59, 60), differential effects have been demonstrated, depending on the promoter context (41). There may also be cell type-specific components that interfere with the cross-talk between the cAMP signaling pathway and GR. The results described in this paper are based on overexpression experiments with COS-1, Y1, and MSC-1 cells and have been

TABLE I
Recruitment of GRIP1 to subnuclear foci

COS-1 cells were transfected with expression vectors encoding GFP-GRIP1. The right column represents cells treated with lactacystin (1 μM) 4 h post-transfection. After 24 h, the intranuclear distribution of GFP-GRIP1 was analyzed. Total fluorescence intensity was measured only in cells that contained foci. The fluorescence intensity in the nucleoplasm surrounding the foci (extrafocal) was determined. In each experiment (n), 250–300 cells were examined. Values presented are the mean ± S.E., $n = 4–8$.

	Cells with foci	Total fluorescence intensity	Extrafocal nuclear fluorescence intensity	Size of foci	Lactacystin, cells with foci
	%			μm^2	%
GFP-GRIP1	17.9 ± 1.26	54.6 ± 6.85	42.6 ± 6.86	0.78 ± 0.20	12.0 ± 1.28
GFP-GRIP1 + PKA-Cα	38.0 ± 1.51	46.1 ± 7.14	23.3 ± 4.60	0.73 ± 0.14	17.6 ± 1.78

confirmed in other cell systems as well (H295R and MCF-7 cells; data not shown). Additionally, PKA-induced degradation of endogenous GRIP1 was observed in MSC-1 cells. Whether down-regulation of GRIP1 by PKA plays an important role for differential coregulator recruitment to endogenous promoters regulated by GR and other NRs in various cell types is presently unknown.

It has been suggested that ubiquitination of proteins often requires a PEST sequence (61). However, deletion of the three highly potential PEST sequences did not counteract the PKA-mediated degradation of GRIP1. Thus, other motifs might act as degradation signals, and we are currently investigating this in more detail. Deletion of the C-terminal activation domain (AD2) of GRIP1 resulted in reduced degradation by PKA (30). Recent data also show increased expression levels of this mutant as compared with wild type GRIP1 in transient transfected cells that are not subjected to PKA stimulation (data not shown). This may indicate that the GRIP1 AD2 deletion mutant is more resistant to degradation than wild type GRIP1, and in accordance with previous findings by others, the AD2 domain appears to be of importance for GRIP1-degradation (46).

One of the important findings of this study is the recruitment of GRIP1 to subnuclear foci after stimulation of the PKA signaling pathway. The nuclear distribution of GRIP1 was heterogenous in COS-1 cells. In the same cell cultures, the distribution of GRIP1 varied from uniform to highly concentrated in subnuclear foci. Several coregulators, including members of the SRC family (62), localize to subnuclear domains. It has been shown that both endogenous and overexpressed GRIP1/TIF2 form subnuclear foci (11, 46, 63). However, it is not known whether the different SRCs are present in the same subnuclear foci. Colocalization of GRIP1 has been demonstrated for several NRs including GR, ER_α, androgen receptor, thyroid receptor, and retinoic acid receptor (RAR_α) (63–65). Interestingly, a recent report has shown that androgen receptor changes the distribution of GRIP1 from subnuclear foci to a more uniform cytoplasmic distribution, and it has been suggested that the subnuclear foci are sites where NRs interact with coactivators prior to transcription (66). Transcription factors such as CBP and p300/CBP-associated factor also appear to be recruited to the same foci as GRIP1/TIF2 (46, 66, 67). Others and we have shown a colocalization of GRIP1 with components of the proteasome (46). In this study, a correlation between this colocalization and PKA-mediated degradation is demonstrated.

In summary, we have shown that GRIP1 coactivator function and protein level are down-regulated by the cAMP signaling pathway. The effect is mediated by PKA and not through the guanine nucleotide exchange factors, since it can be induced by the overexpression of PKA-Cα. Our results provide evidence that GRIP1 is ubiquitinated and degraded by the proteasome and that PKA stimulates the recruitment of GRIP1 to subnuclear foci that are associated with the proteasome. We believe that this is an important regulatory mechanism, and further studies should clarify the biological role of PKA-mediated regulation of GRIP1.

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