Paper IV

cAMP Response Element Binding Protein (CREB) Interacts with the Nuclear Receptor Coactivator GRIP1 and Mediates its Degradation

[1]TUYEN HOANG, [1]INGVILD S. FENNE, [3]MONA JOHANNESSEN, [1,2]ERNST ASBJØRN LIEN, [3]UGO MOENS, and [1,2§]GUNNAR MELLGREN

[1]Hormone Laboratory, Section for Endocrinology, Institute of Medicine, University of Bergen. [2]Haukeland University Hospital, N-5021 Bergen. [3]Department of Microbiology and Virology, Institute of Medical Biology, University of Tromsø, Norway.

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[§]To whom correspondence should be addressed:

Gunnar Mellgren, MD, PhD

Hormone Laboratory, Section for Endocrinology, Institute of Medicine

University of Bergen, Haukeland University Hospital

N-5021 Bergen, Norway.

Phone: (+47) 55 97 43 66 Fax: (+47) 55 97 58 14

E-mail: gunnar.mellgren@med.uib.no

SUMMARY

The Glucocorticoid Receptor Interacting Protein (GRIP1) is a member of the p160 Steroid Receptor Coactivator (SRC) family, playing essential roles in transcriptional activation mediated by nuclear receptors. In a previous study (Hoang, T., Fenne, I.S., Cook, C., Børud, B., Bakke, M., Lien, E.A., Mellgren, G. (2004) J. Biol. Chem. 279, 49120-30), we have demonstrated that GRIP1 is ubiquitinated and degraded through activation of the cAMP-dependent protein kinase A (PKA) signaling pathway. The cAMP response element binding protein (CREB) is a central mediator in the cAMP/PKA signaling pathway. Here we show that CREB mediates GRIP1 degradation, and is required for the PKA-induced downregulation of GRIP1. CREB overexpression in COS-1 cells reduced both the GRIP1-stimulated transcriptional activity and the protein level of GRIP1. Overexpression of CREB mutants (KCREB and CREBS133A) that are unable to mediate PKA-induced gene transcription also resulted in downregulation of GRIP1 similar to wild type CREB. The mRNA levels of GRIP1 were not affected by CREB and the proteasome inhibitor MG132 abolished CREB-mediated degradation of GRIP1 protein. CREB-specific small interfering RNA and anti-CREB antibodies counteracted cAMP-induced degradation of GRIP1, indicating that CREB is required for the PKA-mediated degradation of GRIP1. We also demonstrate that CREB binds directly to GRIP1 both *in vitro* and *in vivo*. We propose therefore that the transcription factor CREB mediates the cAMP/PKA-stimulated degradation of GRIP1 through direct protein-protein interaction. These results may represent a novel mechanism by which GRIP1 protein stability is regulated by the cAMP/PKA/CREB signaling pathway.

INTRODUCTION

The Steroid receptor coactivator (SRC)[¶] family is a class of transcription-associated proteins that play important roles in transcriptional regulation mediated by nuclear receptors (NR) and other transcription factors. The family consists of three members: (i) SRC-1 (steroid receptor coactivator 1) (1), (ii) TIF2 (transcription intermediary factor 2) or GRIP1 (glucocorticoid receptor-interacting protein) (2,3), and (iii) p/CIP (p300/CBP cointegrator protein) (4) or AIB1 (amplified in breast cancer-1) (5), also known as SRC-3, ACTR (activator of the thyroid and retinoic acid receptor) (6), TRAM 1 (thyroid hormone receptor activator molecule) (7), and RAC3 (receptor-associated coactivator 3) (8). The SRC members share sequence and structural similarity. They interact with and enhance the transcriptional activities of multiple NRs in a ligand-dependent manner (reviewed in (9)). The coactivator proteins have also been reported to interact with and potentiate gene transcription regulated by other transcription factors such as activator protein 1 (10), serum response factor (11), and nuclear factor-κB (12) It has been established that the coactivators are recruited to an enhancer DNA-bound NR in response to a transcription activation signal, and they participate in chromatin remodeling and assembly of the transcription initiation machinery via their intrinsic histone acetyltransferase (HAT) properties (6,13), as well as via recruitments of other transcriptional cofactors including HAT-possessing CBP/p300 and p/CAF (4,6,14,15), and/or histone methyltransferases CARM1 and PRMT1 (16,17). Depending on types of NRs, ligands, and/or promoter context, the coactivators are recruited preferentially and differentially to NRs, resulting in different assemblies of downstream cofactor complexes on target genes, and specific activation of gene transcription (18-23). General models for sequential order of recruitment and the roles of SRCs have been proposed (9,24,25), although the molecular mechanisms that regulate the functions of SRC proteins are not fully understood. Gene ablation studies have identified the importance of GRIP1 in reproduction

and control of energy homeostasis. Mice lacking GRIP1 exhibit reduced fertility as a result of placental hypoplasia in females and abnormal spermatogenesis in males (26). It has also been demonstrated that GRIP1$^{-/-}$ mice display a higher level of adaptive thermogenesis, lower fat accumulation, higher lipolysis, and resistance to obesity (27). In addition, mice subjected to a high fat diet have an increased expression of GRIP1 in both white and brown adipose tissues (27). Moreover, it has recently been reported that ablation of the GRIP1 gene leads to changes in gene expression of several key regulatory enzymes of energy metabolism, strengthening the pivotal roles of GRIP1 in energy homeostasis (28). Several studies have now demonstrated that different cellular signaling pathways regulate transcriptional activities of NRs via modulation of the SRC proteins (29-41). The extracellular signal-regulated kinase (ERK) and p38 of the mitogen activated protein kinase (MAPK) family stimulates GRIP1 coactivation function in estrogen receptor-mediated transcription by phosphorylation of the coactivator protein at Ser736 (31,39). Sumoylation at the NR-interaction domain of GRIP1, which leads to impaired colocalisation of GRIP1 with androgen receptor (AR) and attenuation of AR-dependent transcriptional activity, has also been reported (34). We have examined the role of the cAMP-dependent protein kinase (PKA) in regulation of GRIP1 coactivator function, and demonstrated that activation of PKA downregulates GRIP1 coactivator function via ubiquitin-proteasome mediated degradation (32,38). A key component of several PKA-regulated pathways is the transcription factor cAMP-response element binding protein (CREB). CREB is ubiquitously expressed in cells and has been found to bind to the promoter and activate transcription of a vast number of cAMP responsive genes (42). CREB is activated by PKA and recruits the transcriptional coactivator CBP (CREB binding protein) and its paralog p300 (43). Structural and functional analyses have identified a centrally located domain in CREB (the kinase-inducible domain, KID) which contains a PKA phosphorylation site (Ser-133) as well as phosphorylation sites for other

protein kinases such as protein kinase C (Ser-89 and Ser-121), glycogen synthase kinase GSK-3β (Ser-129) and the Ca(2+)/calmodulin-dependent protein kinase II (Ser-142, 143) (reviewed in ref (44)). The KID-domain is required for the interaction between CREB and CBP. CREB is also comprised of two glutamine-rich regions (Q1 and Q2) functioning as constitutive transactivation domains, and a basic leucine zipper domain (bZIP) which is located at the C-terminus and participates in molecular dimerization and DNA-binding (43). Interestingly, it has been demonstrated that CREB has important biological roles in the hepatic gluconeogenesis, fatty acid oxidation and lipid metabolism programs by indirectly and directly modulating gene expression of the nuclear hormone receptor PPAR-γ and coactivator PGC-1 respectively (45,46). Transgenic mice deficient in CREB exhibit fasting hyperglycemia and have a fatty liver phenotype (46). These findings raise questions whether CREB modulates the transcriptional activity of GRIP1. In this report, we demonstrate that CREB inhibits GRIP1 coactivator function and is required for the PKA-mediated degradation of GRIP1. CREB interacts directly with GRIP1 and the CREB-mediated degradation of GRIP1 is independent of Ser-133 phosphorylation. Based on these findings we suggest that CREB mediates GRIP1 degradation via protein-protein interaction. This may represent a novel mechanism by which CREB regulates the function of a coactivator protein.

EXPERIMENTAL PROCEDURES

Plasmid Constructs- The expression plasmids pSG5-hGR, pCMV5-Cα, pM-GRIP1, pSG5-HA-GRIP1, and the luciferase reporter constructs pG5-luc and MMTV-luc-GRE have been described previously (38). VP16-GRIP1 encoding GRIP1 fused to an activation domain (AD) of the GAL4, were kindly supplied by Dr. M.R. Stallcup (Los Angeles, CA). The expression plasmids pΔM-CREB wild-type (wt) and ΔbZIP were generated from GAL4-CREB wt and ΔbZIP constructs (47), respectively by removing the GAL4-encoding sequence with the *SacI* restriction enzyme after *SacI* sites were introduced upstream and downstream for GAL4 by site directed mutagenesis. *SpeI* sites were introduced upstream and downstream for KID, and pΔM-CREB ΔKID was created by deleting the KID domain of CREB from the pΔM-CREB wt construct by using the *SpeI* restriction enzyme. The pSG5-HA-CREB plasmid was constructed by inserting an *EcoRI* fragment of CREB into the *EcoRI* site of a pSG5-HA vector. VP16-CREB construct was made by inserting a PCR-generated CREB cDNA sequence into a VP16 vector (Clontech, CA) at *EcoRI* and *BamHI* sites. RSV-CREB encoding wild type CREB was supplied by Dr. J. Lund (Bergen, Norway). The expression plasmids for CREB wt and mutant proteins, pCMV5-CREB, pCMV5-KCREB, and pCMV5-CREB133, were purchased from BD Biosciences (Palo Alto, CA). pM- plasmid encoding only GAL4-DBD were purchased from Promega (Madison, WI). The donor plasmid pFastBac-HTa-GRIP1 employed for protein expression in insect cells was created by cloning the gene encoding GRIP1 full-length into the pFastBac-HTa (Invitrogen, CA) at the *EcoRI* site.

Cell Culture and Transfections- COS-1 African monkey kidney cells were cultured in Dulbecco's modified Eagle's medium (Cambrex, Verviers, Belgium) supplemented with 2mM L-glutamine, 10% foetal bovine serum, 100 units penicillin and 100 µg streptomycin per ml. Transient transfections were carried out using SuperFect (Qiagen, CA) as described

previously (38). The cells were generally seeded one day before transfections. Transfected cells were harvested at 48 h post transfection. Luciferase assays were performed using the Luciferase Assay Kit (BIOThema AB, Sweden) or the Dual-Luciferase Reporter Assay System (Promega, Madison, WI). The renilla luciferase reporter construct pRL-TK (Promega, Madison, WI) was cotransfected to ensure the reproducibility of the transfection experiments. The firefly luciferase activity produced by luc-reporter constructs was normalized against the renilla luciferase activity.

RNA Interference and Introduction of Intracellular Antibodies- RNA interference experiments were performed in COS-1 cells using small interfering RNA duplexes (siRNA) that targets specifically against CREB gene (CREB siRNA) or act as a non-specific negative control (NSC siRNA) (Upstate, Lake Placid, NY). Briefly, cells were seeded in growing medium without antibiotics on 12- or 24-well plates one day before transfection. siRNA was transfected using Lipofectamine 2000 (Invitrogen, CA) following the manufacturer's procedures. Cotransfection with expression plasmids and reporter constructs were carried out 3 h after transfection with siRNA. Forty-eight h after siRNA transfection, the cells were harvested and assayed for luciferase activity or analysed by Western blotting. Introduction of intracellular antibodies was carried out using the protein transfection reagent Chariot (ActiveMotif, Rixensart, Belgium) according to the manufacturer's instructions. Shortly, cells seeded on 24-well plates were first transfected with expression plasmids and luciferase reporter constructs using SuperFect as described above. Three hours later, the cells were washed with PBS and transfected with antibodies. Rabbit anti-CREB antibody and normal rabbit serum IgG (normal IgG, Upstate, NY) were used. Assays for luciferase activity were performed 48 h after plasmid transfection. All experiments were performed in triplicate, and the data shown are representative of at least three independent experiments.

Western blotting and Coimmunoprecipitation- Western blotting was performed as described previously (38). The primary antibodies used were rabbit anti-HA (Zymed Laboratories Inc., San Francisco, CA) or rat monoclonal anti-HA-Peroxidase (Roche), mouse anti-GAPDH monoclonal antibody (Chemicon International, Temecula, CA), and anti-CREB polyclonal antibody (Upstate, NY). For coimmunoprecipitation, COS-1 cells grown in 90 mm-petri dishes were transiently transfected with expression plasmids. The cells were lysed after 48 h of incubation in an immunoprecipitation (IP) buffer containing 50 mM Tris-HCl pH 7.5, 150 mM NaCl, 5 mM EDTA, 1% NP-40, 0.1% SDS, 0.5% sodium deoxycholate, 1 µg/ml aprotinin, 5 mM N-ethylmaleimide, 0.2 mM PMSF and the Complete Mini EDTA-free protease inhibitor tablets. The cell lysates were cleared by centrifugation at 13000 x g for 10 min at 4^{o}C. The resulting supernatants were then incubated with 5µg of anti-CREB (Upstate, NY) or anti-NCOA2 (Bethyl, Montgomery, TX) antibody at 4^{o}C overnight on a rotating wheel, and subsequently incubated with 40 µl protein A-sepharose (Amersham Biosciences, Uppsla, Sweden) for 2 h. Normal IgG (Upstate, NY) was used instead of the antibodies as control. The protein A-sepharose beads were precipitated by centrifugation at 2000xg for 1 min, and washed several times with the IP buffer. Proteins were eluted from the beads by heating at 95^{o}C in a 2X sample buffer containing 100 mM Tris-HCl pH 6.8, 4% SDS, 0.2% bromophenol blue, 20% glycerol, and 100mM DTT, and resolved by SDS-PAGE and Western blotting using the anti-HA-Peroxidase or anti-CREB antibodies.

Purification of RNA and Real-Time RT-PCR- Total RNA from COS-1 cells transfected for 48 h with indicated expression plasmids were extracted with Trizol reagent (Invitrogen, CA). The quantitative real-time reverse transcriptase- PCR (RT-PCR) analysis was carried out using a LightCycler rapid thermal cycler system (Roche, Basel Switzerland). The primers used in the RT-PCR reactions were as follows: TIF2, 5'-GAG-TAC-CAA-CAC-AGG-CAC-C-3' (forward) and 5'-AGG-TTG-CTG-ACT-TAT-TCC-GTA-

G-3' (reverse); GRIP1, 5'-AAG-CCT-TTG-CCA-GAT-TCA-G-3' (forward) and 5'-CAA-CGA-GAG-TGC-CAT-CAG-AC-3' (reverse); GAPDH, 5'-ACC-ACA-GTC-CAT-GCC-ATC-AC-3' (forward) and 5'-TCC-ACC-ACC-CTG-TTG-CTG-TA-3' (reverse). Details regarding the PCR reactions and quantification of mRNA have been described previously (32).

Protein-Protein Interaction Assays- Full-length GRIP1 protein fused to a 6x His-containing sequence at its N-terminus ($(His)_6$-GRIP1) was expressed and purified from the sf21 insect cell line. Briefly, the donor plasmid pFastBac-HTa-GRIP1 was transformed into MAX efficiency DH10Bac *E. coli* strain (Invitrogen, CA) to produce recombinant bacmids, which were in turn transfected into sf21 cells to generate recombinant baculoviruses carrying the gene encoding $(His)_6$-GRIP1. The protein was expressed by infecting sf21 cells with the recombinant baculoviruses for 72 h. Purification of $(His)_6$-GRIP1 protein was performed using affinity chromatography with Ni-sepharose beads (Amersham Biosciences, Uppsala, Sweden) following the manufacturer's recommendations.

For His-tag pull-down assays, COS-1 cells were transfected with RSV-CREB or an empty pCMV5 vector. Fourty eight hours after transfection, the cells were washed with PBS and lysed in an interaction lysis buffer containing 20mM Tris-HCl pH 7.5, 100mM NaCl, 20% glycerol, 1.0% Nonidet P-40, 2µg/ml aprotinin, 5mM N-ethylmaleimide, 100nM sodium orthovanate, 30mM imidazole, 0.2mM PMSF, supplemented with the Complete Mini EDTA-free protease inhibitor tablets (Roche Diagnostics, Mannheim, Germany). The lysates were cleared by centrifugation at 13000 x g for 10 min. at 4^oC. For binding assays, $(His)_6$-GRIP1 protein bound to Ni-sepharose or Ni-sepharose beads alone was incubated with equal volumes of the precleared COS-1 lysates for 2 h at 4^oC. After several washings, the retained proteins were eluted with the 2X sample buffer and resolved by SDS-PAGE (10%). Western blotting was performed using the anti-CREB antibody.

For *in vitro* protein-protein interaction, CREB protein was prepared by using the TNT reticulocyte lysate system (Promega, Madison, WI) in the presence of [^{35}S]-Methionine. Ni-sepharose-bound $(His)_6$-GRIP1 protein or Ni-sepharose beads alone was incubated with the [^{35}S]Methionine-labeled CREB protein in an interaction buffer containing 20mM Tris-HCl pH 7.5, 100mM NaCl, 10% glycerol, 0.5% Nonidet P-40, 5μg/ml aprotinin, 20mM imidazole, and 1mM PMSF, for 1 h 30 min at room temperature. After the incubation, the sepharose beads were extensively washed with the same buffer, and subsequently boiled at 95^{o}C for 5 min in the 2X sample buffer. The eluted proteins were resolved by SDS-PAGE followed by autoradiography.

RESULTS

Overexpression of CREB leads to downregulation of GRIP1- We have previously demonstrated that the PKA-signaling pathway regulates GRIP1 through ubiquitin-proteasome-dependent protein degradation (38). Since CREB is implicated as a central downstream mediator of the cAMP/PKA signaling pathway (48-51), we examined whether CREB is involved in the regulation of GRIP1 coactivator function. GAL4-responsive luciferase reporter assays using COS-1 cells transfected with the expression plasmids encoding a GAL4-GRIP1 fusion protein and CREB demonstrated that CREB overexpression led to a dose-dependent decrease in GRIP1 activity (Fig. 1A). To examine whether GRIP1 could affect CREB function, we also studied the effects of GRIP1 overexpression on the transcriptional activity of CREB. GAL4-CREB chimera was coexpressed with full length HA-tagged GRIP1 (HA-GRIP1) along with the GAL4-luciferase reporter. GRIP1 did not inhibit the CREB-stimulated transactivity (Fig. 1B). Similar to our previous report showing that activation of PKA leads to GRIP1 degradation (38), overexpression of CREB also reduced the protein levels of GRIP1 in cotransfected COS-1 cells. By using an expression plasmid encoding HA-GRIP1 and different amounts of CREB expression plasmid, Western blot analysis demonstrated that CREB led to a dose-dependent decrease in the protein levels of GRIP1 in transfected COS-1 cells (Fig. 1C).

In order to confirm that CREB-mediated downregulation of GRIP1 coactivation also affected NR-activated gene transcription, COS-1 cells were transfected with the luciferase reporter construct containing four glucocorticoid receptor (GR) responsive elements or GREs (MMTV-luc) (52), together with expression plasmids encoding GR, HA-GRIP1 and CREB. As expected, GRIP1 strongly stimulated GR-activated transcription in the presence of ligand (dexamethasone). However, coexpression of CREB inhibited GRIP1-mediated coactivation of GR significantly (Fig. 2A). Western blot analyses confirmed that the level of GRIP1

protein was reduced by CREB in COS-1 cells that were cotransfected with GR expression plasmid (Fig 2B). Together, these results suggest that CREB overexpression inhibits GRIP1 coactivator function through downregulation of the GRIP1 level.

GRIP1 is regulated by CREB through proteasome-mediated degradation- Since CREB is a transcription factor that regulates the expression of many human genes (42,53,54), one might speculate whether CREB regulates GRIP1 through transcriptional regulation of the GRIP1 gene. To test this hypothesis, we examined the effects on GRIP1 by two dominant-negative CREB mutants, CREBS133A and killer CREB (KCREB). CREBS133A has a point mutation at residue 133 (Ser → Ala), which is the PKA-phosphorylation site (55), while KCREB contains mutations in the DNA-binding basic domain that abolishes its DNA-binding, but retains its ability to bind to wild-type CREB forming an inactive dimer (56). Both of the CREB mutants are therefore incapable of activating transcription. However, similar to wild type CREB, overexpression of both mutants in COS-1 cells inhibited GRIP1-stimulated GAL4-transcription (Fig. 3A) through downregulation of GRIP1 protein (Fig. 3B, *top panel).* The inhibitory effects of CREB, CREBS133A and KCREB were observed both in the absence and presence of overexpressed PKA-Cα (Fig. 3A, B). Western blot analyses confirmed the expression of all CREB-constructs and the levels of expression were related to the levels of GAPDH using an anti-GAPDH antibody (Fig. 3B). We also analyzed the mRNA levels of endogenous TIF2 as well as the levels of ectopic GRIP1 mRNA in COS-1 cells that were cotransfected with expression plasmids encoding CREB or PKA-Cα. Similar to previous findings on the activation of PKA (32), and consistent with our recent observations with the CREB dominant negative mutants (Fig. 3), overexpression of CREB or PKA did not modulate the mRNA levels of endogenous TIF2 or the levels of ectopically expressed GRIP1 in COS-1 cells. These data indicate that the effect of CREB occurs at the post-transcriptional level.

The ubiquitin-proteasome-mediated protein degradation has been implicated in regulation of GRIP1 (37,38,57). In order to examine whether the proteasome is required for the CREB-induced downregulation of GRIP1 protein, COS-1 cells were transfected with the GAL4-luc reporter and expression plasmids encoding GAL4-GRIP1 and CREB, and subsequently treated with vehicle or the proteasome-inhibitor MG132. Treatment of the cells with MG132 blocked the inhibitory effect of CREB on GRIP1-dependent transactivation of the GAL4-responsive reporter, and we even observed a stimulation in the luciferase activity in the presence of MG132 (Fig. 4A). This may be caused by the enhanced levels of GRIP1 protein that have been observed after treatment with MG132 (37,38). Furthermore, Western blot analyses confirmed that MG132 counteracted CREB-induced downregulation of GRIP1 protein in transfected COS-1 cells (Fig. 4B). Thus, inhibition of the proteasome by MG132 abolished CREB-induced degradation of GRIP1.

CREB is required for the PKA-mediated degradation of GRIP1- In response to activation of the PKA, CREB is phosphorylated on Ser-133 and induces transcription of several genes (42). Ser-133-phosphorylation has been implicated as a major mechanism for activation of CREB by the PKA signaling pathway. However, based on the results described above it appears that CREB induces GRIP1 downregulation independently of the Ser-133 (Fig. 3). To study this in more detail, we employed siRNA against CREB mRNA (CREB siRNA) to reduce the endogenous levels of CREB. As control, we used a proper non-specific control (NSC) siRNA. COS-1 cells were transfected with the GAL4-luc reporter construct and the expression plasmid encoding GAL4-GRIP1. As reported previously (38), treatment of the transfected cells with cAMP-elevating agents (forskolin, IBMX) and cAMP-analog (8-CPT-cAMP) led to a significant downregulation of GRIP1. Transfection of the cells with CREB siRNA abolished the cAMP-stimulated reduction of GRIP1 transcriptional activity, whereas

transfection with NSC-siRNA did not show any diminishing effect (Fig. 5A). The inhibitory effect of CREB siRNA on cAMP-induced downregulation of GRIP1 was further demonstrated by Western blot analyses using an anti-HA antibody against coexpressed GRIP1 protein (Fig. 5B, *top panel*). To confirm that the endogenous CREB level were markedly reduced in cells transfected with CREB siRNA, Western blot assays were performed using anti-CREB antibodies (Fig. 5B, *middle panel*). Western blots were also performed using anti-GAPDH antibodies to ensure that the GRIP1 and CREB protein levels were not affected by differences in loading or cell density (Fig 5B, *bottom panel*). The GRIP1/GAPDH and CREB/GAPDH ratios are shown in Fig. 5C.

In order to confirm these results suggesting that CREB is required for the cAMP-mediated downregulation of GRIP1, we employed an alternative method whereby anti-CREB antibodies were introduced into cells using the protein transfection reagent Chariot. COS-1 cells were cotransfected with the GAL4 reporter and GAL4-GRIP1 expressing plasmid, and subsequently with the anti-CREB antibodies or the corresponding normal serum (IgG) as control. After the transfections the cells were treated with forskolin, IBMX, and 8-CPT-cAMP as described previously or with vehicle. In consistence with the results above, intracellular anti-CREB antibodies inhibited the cAMP-induced downregulation of GRIP1 coactivator function, whereas introduction of the normal IgG had no effects (Fig. 5D). Taken together, these results indicate that CREB is required for the cAMP/PKA-stimulated downregulation of GRIP1.

CREB interacts directly with GRIP1- In order to examine whether CREB modulated GRIP1 coactivator function through protein-protein interaction, we first performed protein interaction assays by using baculovirus-expressed $(His)_6$-GRIP1 fusion protein and [^{35}S]methionine-labeled *in vitro* translated CREB. As shown in Fig. 6A, CREB bound to Ni-

sepharose beads containing $(His)_6$-GRIP1 (*lane 4*), but not to the Ni-sepharose beads alone (*lane 3)*. The interaction was also demonstrated by using a His-tag pull down assay where purified $(His)_6$-GRIP1 protein was incubated with COS-1 lysates containing overexpressed CREB protein (Fig. 6B). Similarly, overexpressed CREB associated with $(His)_6$-GRIP1 bound-Ni sepharose (*lane 4*), but not to the Ni-sepharose beads alone (*lane 3*). Activation of PKA has been reported to increase the amount of phosphorylated CREB, but not the total level of CREB protein (55). Our experiments also demonstrated lack of increase of the total level of CREB protein after stimulation of the cAMP/PKA-pathway (Fig 3B, data not shown). In addition, we observed no differences in the interaction between $(His)_6$-GRIP1 and CREB when the cells were cotransfected with the expression plasmid encoding PKA-Cα (data not shown).

We next examined the interaction *in vivo* by performing reciprocal co-immunoprecipitations of CREB and GRIP1 proteins (Fig. 6C). First, anti-CREB antibodies were employed to precipitate CREB from COS-1 cells that were cotransfected with CREB and HA-GRIP1 expression plasmids (*left panels*). The immunoprecipitates were subsequently analyzed by Western blots. Expression of CREB and HA-GRIP1 were detected in the transfected cells (*lanes 1)*, and the anti-CREB antibody effectively and specifically precipitated CREB protein (*lanes 2 & 3, lower panel)*. Western blots using an anti-HA antibody showed a significant co-immunoprecipitated band for HA-GRIP1 protein when the transfected cell lysates were incubated with the anti-CREB antibody, while no such interaction band was detected after incubation with normal IgG (*lanes 2 & 3, upper panel)*. A reciprocal precipitation employing an antibody against endogenous GRIP1 protein (anti-NCOA2) was then performed with COS-1 cell lysates (*right panels*). TIF2/GRIP1 as well as CREB are expressed ubiquitously in different cells lines. In the monkey kidney cell line COS-1, expression of endogenous CREB was detectable (*lane 4, upper panel*). Although the endogenous level of TIF2/GRIP1

(NCOA2) in COS-1 cells was barely detected (*lane 4, lower panel*), the anti-NCOA2 was sufficiently effective and specific for the precipitation of TIF2/GRIP1 as compared to normal IgG (*lanes 5 & 6, lower panel).* In the Western blots using an anti-CREB antibody (*upper panel*), CREB was well detected from the anti-NCOA2 precipitated immunocomplex (*lane 5*), whereas the normal IgG-precipitated complex showed only a background band (*lane 6).* Finally, to confirm the *in vivo* interaction between CREB and GRIP1 we performed mammalian hybrid assays. The GAL4 and VP16 constructs along with a luciferase reporter (pG5luc) were transfected into COS-1 cells. GAL4-CREB activated the cotransfected GAL4-responsive luciferase reporter (Fig. 6D), which is consistent with the constitutive properties of CREB in activating both basal- and phosphorylation-dependent transcription (58,59). However, the GAL4-CREB transcriptional activity was clearly stimulated by coexpression of VP16-GRIP1 and not by VP16, suggesting that CREB interacts with GRIP1. Taken together, our results indicate that CREB interacts directly with GRIP1.

Deletion of the KID and the bZIP domains of CREB does not attenuate GRIP1 downregulation- The structural components of CREB are schematically presented in Fig. 7A and include two glutamine-rich regions (Q1 and Q2), a kinase inducible domain (KID), and a basic leucine zipper domain (bZIP) (reviewed in (60)). To examine whether the KID or bZIP domain is required for CREB-induced regulation of GRIP1, we generated expression plasmids encoding CREB mutants lacking the KID (ΔKID) and the bZIP (ΔbZIP) domains, respectively. The actions of these CREB deletion mutants on GRIP1 coactivator function were studied by transfection of COS-1 cells with expression plasmids encoding the CREB deletion-mutants or wt CREB along with the GAL4-GRIP1 expression plasmid and the GAL4-luc reporter construct. Similar to wt CREB, overexpression of CREB ΔKID and CREB ΔbZIP led to a decrease in GRIP1-stimulated transactivation (Fig. 7B). Accordingly,

the CREB deletion mutants significantly reduced the protein levels of GRIP1 in transfected COS-1 cells (Fig. 7C). We noticed that the expression levels of the CREB-deletion mutants appeared to be significantly lower than wt CREB as judged by the Western blot analysis. One explanation could be decreased stability of the truncated CREB forms. Alternatively, diminished CREB mutant protein levels could be caused by less efficient binding of the anti-CREB antibodies. The results suggest that the CREB KID and bZIP domains are dispensable for the ability of CREB to downregulate GRIP1.

DISCUSSION

Regulation of coactivators is one of the mechanisms by which the transcriptional activities of NRs can be modulated. Several cellular signaling pathways including the cAMP/PKA pathway have been reported to regulate GRIP1 (31,32,34,38,39,61). In contrast to other SRCs, activation of PKA leads to specific downregulation of GRIP1 coactivator function through ubiquitin-proteasome-mediated degradation (38). However, the downstream signaling pathway that mediates this effect has been elusive. In this study, we demonstrate that the transcription factor CREB is required for cAMP/PKA-mediated degradation of GRIP1. Interestingly, CREB-mediated downregulation of GRIP1 is not dependent on Ser-133 phosphorylation that has been associated with PKA-mediated activation of CREB (60). Our findings demonstrate that CREB overexpression leads to GRIP1 downregulation and that CREB interacts directly with GRIP1.

CREB has been reported to regulate transcription of several genes (43). These CREB target genes generally contain consensus sites for CREB binding (CREs) which occur as eight-base pair palindromes (TGACGTCA) or as half-site motifs (CGTCA) (43). A recent genome-wide analyse has identified approximately 4000 putative human CREB target genes (42), and computational prediction of CREs on the promoter of TIF2/GRIP1 genes by using the CREB-

target gene database (http://natural.salk.edu/CREB/) reveals conserved CRE-half sites without TATA box on the gene promoter. Since CRE-half sites are less active for CREB binding and cAMP responsiveness (62,63), and the presence of TATA box in promoters of CREB target genes has been observed to be important for the induction in gene transcription in response to cAMP/PKA activation (42,53), it is unlikely that CREB regulates transcriptional expression of TIF2/GRIP1 gene. As expected, we did not observe any changes in TIF2/GRIP1 mRNA expression after stimulation of the cAMP/PKA pathway or CREB overexpression. This confirms that CREB downregulates GRIP1 at a posttranslational level. Moreover, the CREB mutants (KCREB, CREBS133A, CREB ΔKID and CREB ΔbZIP) that are unable to mediate gene transcription were still capable of reducing GRIP1 coactivity and protein level, suggesting that CREB does not induce its negative effect on GRIP1 via modulation of gene transcription.

Inhibition of the proteasome abolished cAMP/PKA dependent downregulation of GRIP1 (38). In accordance with this, we observed that the proteasome inhibitor MG132 counteracted CREB-induced downregulation of GRIP1. Moreover, activation of the cAMP/PKA signaling pathway (32), as well as CREB overexpression, did not modulate the TIF2/GRIP1 mRNA expression. Thus, we conclude that CREB-mediated downregulation of GRIP1 is accomplished through protein degradation. Since both siRNA and intracellular antibodies against CREB abolished cAMP-induced degradation of GRIP1, we also propose that the cAMP/PKA-dependent regulation of GRIP1 coactivator function is dependent on CREB.

Stimulation of the cAMP/PKA pathway leads to phosphorylation of CREB at Ser-133 that is located within a transcriptionally important region, the kinase inducible domain (KID) (43). Phosphorylation of CREB at Ser-133 promotes binding of CREB to CRE and recruitment of CBP and p300 (64). However, in this paper, we report that mutation of CREB at Ser-133 (CREBS133A) or deletion of the KID domain does not attenuate CREB-mediated

downregulation of GRIP1. Thus, it is unlikely that CREB mediates the cAMP/PKA-induced downregulation of GRIP1 via Ser-133 phosphorylation. Several putative and novel phosphorylation sites for other protein kinases, such as ERK, p38 MAPK, the protein kinase C and B, and the Ca^{2+}- calmodulin dependent kinase have been identified in the KID domain of CREB (reviewed in ref (44)), and one possibility could be that PKA acts through cross-talk with another signaling pathway such as the MAPK pathway (65,66). Interestingly, it has been reported that GRIP1 is regulated by the ERK and p38 of the MAPK signaling pathway (31,39). One might therefore suspect that PKA mediates its effect on GRIP1 regulation by interacting with other signaling pathways to phosphorylate CREB at other sites than Ser-133. However, our results do not support this hypothesis since deletion of the KID domain did not attenuate the CREB-mediated downregulation of GRIP1. Moreover, we have previously observed that inhibition of MEK/ERK and p38 by the specific inhibitors PD98058 and SB203580 did not counteract PKA-stimulated degradation of GRIP1 (data not shown). We therefore propose that cAMP/PKA and CREB mediate GRIP1 degradation by another mechanism that is not dependent on the MAPK signaling pathway or Ser-133 phosphorylation. Other post-translational modifications of CREB such as acetylation, ubiquitination and sumoylation, as well as glycosylation have been reported to regulate CREB activity (67-69). However, whether any of these CREB modifications are regulated by PKA and/or involved in CREB-mediated GRIP1 degradation remains unknown. Although the precise mechanism by which PKA induces degradation of GRIP1 is not characterized, our report indicates that PKA and CREB act together via a hitherto unknown mechanism that is Ser-133 independent and transcription-independent. This mechanism may involve the direct protein-protein interaction that we observed between CREB and GRIP1 in this study. Protein-protein interaction represents another form of regulation in various cellular pathways. The interaction networks involving CREB and GRIP1 are obviously complicate and their

regulation depends on the cellular environment and extracellular signals. CREB recruits and interacts with CBP/p300 upon activation of the PKA (70), and the interaction with CBP may serve as an important bridge for the interaction between CREB and NRs, as observed in the case of AR during PKA-stimulated potentiation of its transactivation (51). Furthermore, it has been reported that CREB physically interacts with CARM1 in a cAMP-dependent manner in hepatocytes (71). Since GRIP1 can bind to multiple NRs and recruit both CBP/p300 and CARM1 (9), it is not surprising that CREB is associated with GRIP1.

Based on the present and previous (32,38) studies, we propose that CREB mediates cAMP/PKA-induced proteasomal degradation of GRIP1. This may involve the newly discovered CREB-GRIP1 interaction. Although CREB is required for the cAMP/PKA-mediated regulation of GRIP1, our results clearly indicate that phosphorylation of Ser-133 is not required. We believe that this represents a novel mechanism by which the cAMP/PKA signaling pathway and CREB act together to regulate a nuclear receptor coactivator. Further studies should identify the exact CREB and GRIP1 domains that are involved in the interactions and the degradation process. This may also provide novel details regarding the cAMP/PKA-mediated activation of CREB in general.

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[¶] The abbreviations used are:

SRC, steroid receptor coactivator; NR, nuclear receptor; TIF2, transcription intermediary factor 2; GRIP1, glucocorticoid receptor interacting protein 1; NcoA2 (NCOA2), nuclear receptor coactivator 2; HAT, histone acetyltransferase; CBP, cAMP-response element-binding protein-binding protein; CARM1, coactivator-associated arginine methyltransferase 1; PPARγ, peroxisome proliferator-activated receptor γ; PGC-1, PPARγ coactivator-1; GR, glucocorticoid receptor; GRE, GR-response element; AR, androgen receptor; CRE, cAMP responsive element; CREB, CRE binding protein; KID, kinase inducible domain; bZIP, basic leucine zipper; PKA, protein kinase A; PKA-Cα, catalytic subunit of the PKA; 8-CPT-cAMP, 8-para-chlorophenylthio-cAMP; IBMX, 3-Isobutyl-1-methylxanthine; PKI, protein kinase inhibitor; ERK, extracellular signal-regulated kinase; MAPK, mitogen-activated protein kinase; siRNA, small interfering RNA; NSC, non-specific control; IgG, immunoglobulin G; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; HA, hemagglutinin tag; IP, immunoprecipitation; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; DMEM, Dulbecco's modified Eagle's medium; EDTA, ethylenedinitro tetraacetic acid; PMSF, phenyl methyl sulfonyl fluoride; PBS, phosphate buffer saline; PBS-T, phosphate buffer saline-tween; FBS, fetal bovine serum.

FIGURE LEGENDS

FIG. 1. **Overexpression of CREB leads to downregulation of GRIP1.** A, COS-1 cells were transfected with expression plasmids encoding GAL4-GRIP1 (1.0 µg) and CREB (0.01, 0.02, 0.05, 0.1, 0.25, 0.5, 1.0, 2.0 µg) together with a GAL4-responsive luciferase reporter construct (GAL4-luc) (1.1µg). B, COS-1 cells were transfected with expression plasmids encoding GAL4-CREB (1.0 µg), and HA-GRIP1 (0.25, 0.4, 0.5, 0.75, 1.0, 2.0, 3.0 µg) together with the GAL4-luc reporter construct (1.1 µg). Luciferase activities were measured as described in *Experimental Procedures*. The figures show the mean ± S.D of triplicate transfections from representative experiments. C, Western blotting was performed with lysates from COS-1 cells transfected with expression plasmids encoding HA-GRIP1 (2.0 µg) and CREB (0.5, 1.0, 1.5, 2.0 µg). Anti-HA and anti-GAPDH antibodies were used to detect HA-GRIP1 and GAPDH, respectively. The results presented are representative of at least three independent experiments.

FIG. 2. **CREB inhibits GRIP1-mediated coactivation of GR.** A, COS-1 cells were transfected with expression plasmids encoding human GR (hGR, 0.1 µg), HA-GRIP1 (1.0 µg) and CREB (0.02 µg) together with the MMTV-luc GRE reporter construct (1.1 µg). Luciferase assays were performed as described in *Experimental Procedures.* The figure shows the mean ± S.D of triplicate transfections, and is representative of three independent experiments. B, Cell lysates containing HA-GRIP and GR together with or without CREB from A were subjected to Western blotting using anti-HA and anti-GAPDH antibodies.

FIG. 3. **Downregulation of GRIP1 is not regulated through CREB-dependent transcriptional activation.** A, COS-1 cells were transfected with the expression plasmids encoding GAL4-GRIP1 (1.0 µg), PKA-Cα (0.075 µg), CREB wt (0.5 µg), CREBS133A (0.5

μg) and KCREB (0.5 μg), together with the GAL4-Luc reporter construct (1.1 μg). Luciferase assays were performed as described in *Experimental Procedures.* The results are presented as mean ± S.D of triplicate transfections from a representative experiment. B, COS-1 cells were transfected with expression plasmids encoding HA-GRIP1 (3.0 μg), PKA-Cα (0.25 μg) and CREB wt (1.5 μg), S133A (1.5 μg), or KCREB (1.5 μg), and subjected to Western blotting using anti-HA, anti-CREB and anti-GAPDH antibodies. C, RNA was purified from COS-1 cells transfected with expression plasmids encoding HA-GRIP1 (6.0 μg), PKA-Cα (0.3-0.6 μg) and CREB (3.0 μg). The mRNA levels of endogenous TIF2 and overexpressed GRIP1 were quantitated by real time PCR as described in the *Experimental Procedures*. The results are presented as ratios (mean ± S.D) between TIF2 or GRIP1 and GAPDH mRNA. The results presented in this figure are based on three independent experiments.

FIG. 4. **Inhibition of the proteasome abolished CREB-mediated downregulation of GRIP1.** A, COS-1 cells were transfected with expression plasmids encoding GAL4-GRIP1 (1.0 μg) and CREB (0.5 μg) together with the GAL4-luc reporter construct (1.1 μg). Cells were treated with MG132 (1μM) for 16 h as indicated in the figure. Luciferase assays were performed as described in *Experimental Procedures.* The results are presented as mean ± S.D of triplicate transfections from three independent experiments. B, Western blotting was performed on lysates from COS-1 cells transfected with the expression plasmids encoding HA-GRIP1 (3.0 μg) and CREB (1.0 μg) in the presence of MG132 (1μM) for 6h. GRIP1, CREB and GAPDH were detected with anti-HA, anti-CREB and anti-GAPDH antibodies, respectively. The results are representative of three independent experiments.

FIG. 5. **CREB is required for cAMP-mediated degradation of GRIP1.** A, COS-1 cells were grown in 24 well-plates and transfected with the GAL4-GRIP1 expression plasmid (0.7μg) and the GAL4-luc reporter construct (0.6μg), as well as siRNA specific (CREB-siRNA, 50pmol) or non-specific (NSC-siRNA, 50 pmol) to CREB. Cells were treated with forskolin (10μM), IBMX (50μM), and 8-CPT-cAMP (500μM) (cAMP) for 24h before lysis. Luciferase assays were performed as described in *Experimental Procedures.* The figure shows the mean ± S.D of triplicate transfections from three independent experiments. B, COS-1 cells were grown in 12-well plates and transfected with the HA-GRIP1 expression plasmid (1.0μg) and the siRNAs as described above (150pmol each). Cells were treated with forskolin, IBMX and 8-CPT-cAMP (cAMP) as described in A. Cell lysates were subjected to Western blotting using anti-HA (GRIP1), anti-CREB and anti-GAPDH antibodies. C, The relative expression levels of GRIP1 and CREB in B are presented as ratios between GRIP1 or CREB and the corresponding GAPDH protein band densities, respectively. The results are the mean ± S.D of three independent experiments. D, COS-1 cells were grown in 24-well plates and transfected with the GAL4-GRIP1 expression plasmid (0.7 μg), the GAL4-luc reporter (0.6 μg), as well as anti-CREB antibodies (α-CREB) or normal IgG (0.4-1.0 μg) as indicated. Transfections were performed as described in the *Experimental Procedures*. Treatment of the cells with forskolin, IBMX and 8-CPT-cAMP was carried out as in A. Luciferase assays were performed as described in *Experimental Procedures.* The figure shows the mean ± S.E of triplicate transfections from three independent experiments.

FIG. 6. **CREB interacts with GRIP1 *in vitro* and *in vivo*.** A, [^{35}S]Methionine-labeled *in vitro* translated CREB ([^{35}S]-CREB) was incubated with beads containing Ni-sepharose or Ni-sepharose bound to $(His)_6$-GRIP1. Proteins bound to the Ni-sepharose beads were subsequently resolved by SDS-PAGE (10%) and autoradiography. [^{35}S]Methionine-labeled

reticulocyte lysate devoid of [^{35}S]-CREB was used as negative control (*lanes 2, 5*). Three percent of the input reticulocyte lysate containing radiolabeled CREB is shown on the left (*lanes 1*). B, Lysates from COS-1 cells that were transfected with CREB expression plasmid (3.0 µg) were incubated with sepharose beads as described in A. Lysates from unstransfected COS-1 cells were used as negative controls (*lanes 2, 5*). His-tag pull-down was performed as described in *Experimental Procedures*. The beads were then analysed by Western blotting using an anti-CREB antibody. Three percent of the input lysate are shown on the left (*lanes 1, 2*). C, Co-immunoprecipitations were performed on lysates from COS-1 cells transfected with expression plasmids encoding HA-GRIP1 (7.0 µg) and CREB (2.0 µg) or untransfected cells as indicated in the figure. Anti-CREB (*left panels*) and anti-NCOA2 (GRIP1) (*right panels*) antibodies were employed to immunoprecipitate CREB and TIF2/GRIP1, respectively. Normal IgG was used as negative control. Proteins from the immunoprecipitates were subsequently resolved by SDS-PAGE and Western blotting using anti-HA-Peroxidase or anti-NCOA2 (GRIP1) and anti-CREB antibodies as indicated in the figure. Five and 10% of the input cell lysates are shown (*lanes 1*, *4*). D, Mammalian hybrid assays were carried out in COS-1 cells grown in 24-well plates. Cells were transfected with the expression plasmids encoding GAL4 (0.7 µg), GAL4-CREB (0.7 µg), VP16 (0.7 µg) and VP16-GRIP1 (0.7 µg) together with the GAL4-luc reporter construct (0.6 µg), as indicated in the figure. The results are the mean ± S.D. of triplicate transfections and are representative of three independent experiments.

FIG. 7. **Deletions of the CREB-KID and -bZIP domains do not change the CREB induced downregulation of GRIP1.** A, A schematic illustration of CREB with its functional domains. The Q1 and Q2 domains are glutamine-rich regions. KID is the kinase-inducible domain that contains serine 133 and represents the CBP/p300 interaction domain. The bZIP

domain contains the basic leucine zipper motifs and is involved in DNA-binding and dimerisation. B, COS-1 cells were transfected with expression plasmids encoding GAL4-GRIP1 (0.7 µg), CREB wt (0.35 µg) and CREB mutants lacking the bZIP (CREB ΔbZIP) or the KID (CREB ΔKID) domains (0.35 µg) together with the GAL4-luc reporter construct (0.6 µg). Luciferase assays were performed as described in *Experimental Procedures.* The figure shows the mean ± S.D of triplicate transfections and is representative of three independent experiments. C, COS-1 cells were transfected with the expression plasmids encoding CREB wt and mutants as described A (0.75 µg) and HA-GRIP1 (1.5 µg). Equal volumes of cell lysates were analysed by Western blotting using anti-HA (GRIP1), anti-CREB and anti-GAPDH antibodies. The results are representative of three independent experiments.

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FIG. 1

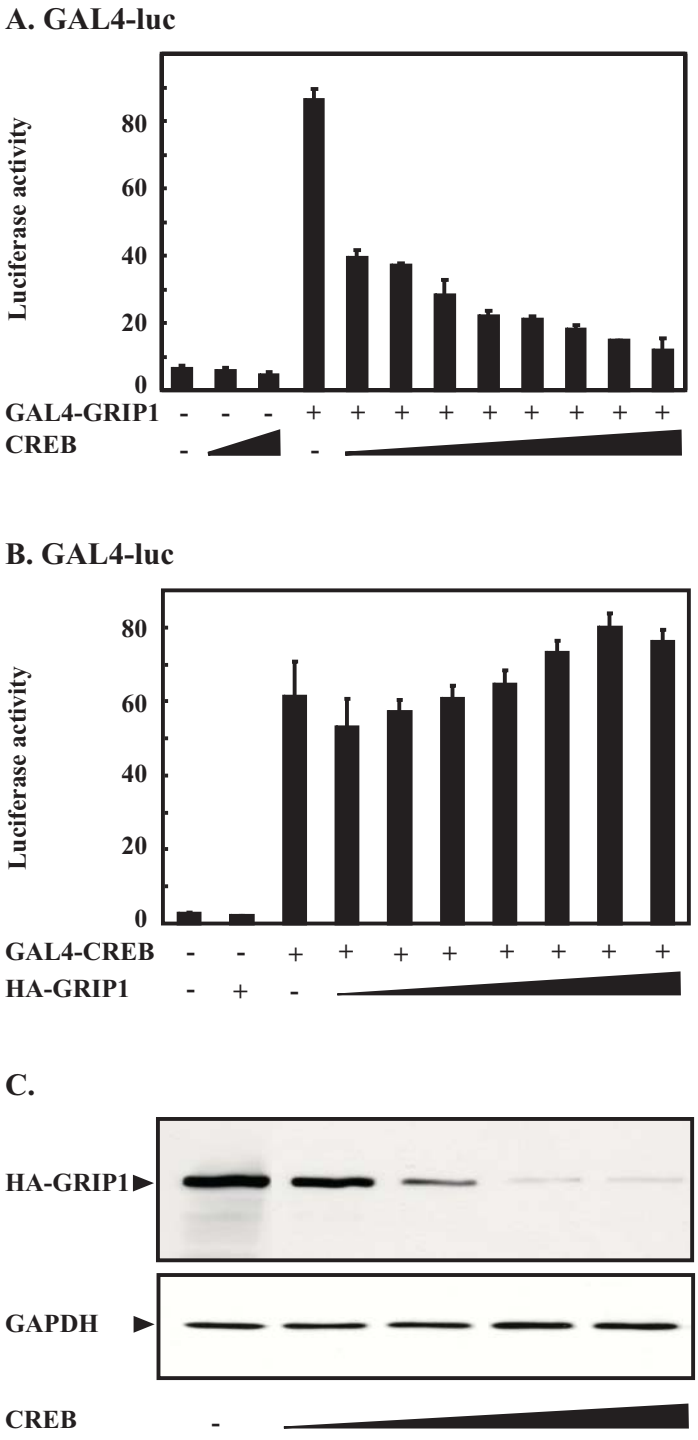

A. GAL4-luc
Luciferase activity
0
20
40
60
80
GAL4-GRIP1 - - - + + + + + + + + +
CREB - - +
B. GAL4-luc
Luciferase activity
0
20
40
60
80
GAL4-CREB - - + + + + + + + +
HA-GRIP1 - + -
C.
HA-GRIP1
GAPDH
CREB -

FIG. 2

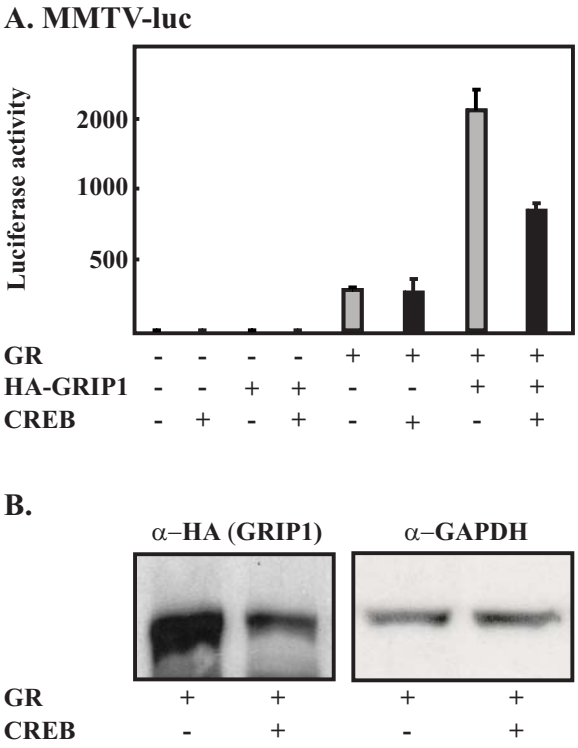

A. MMTV-luc
Luciferase activity
2000
1000
500
GR - - - - + + + +
HA-GRIP1 - - + + - - + +
CREB - + - + - + - +
B.
α–HA (GRIP1)
α–GAPDH
GR + + + +
CREB - + - +

FIG. 3

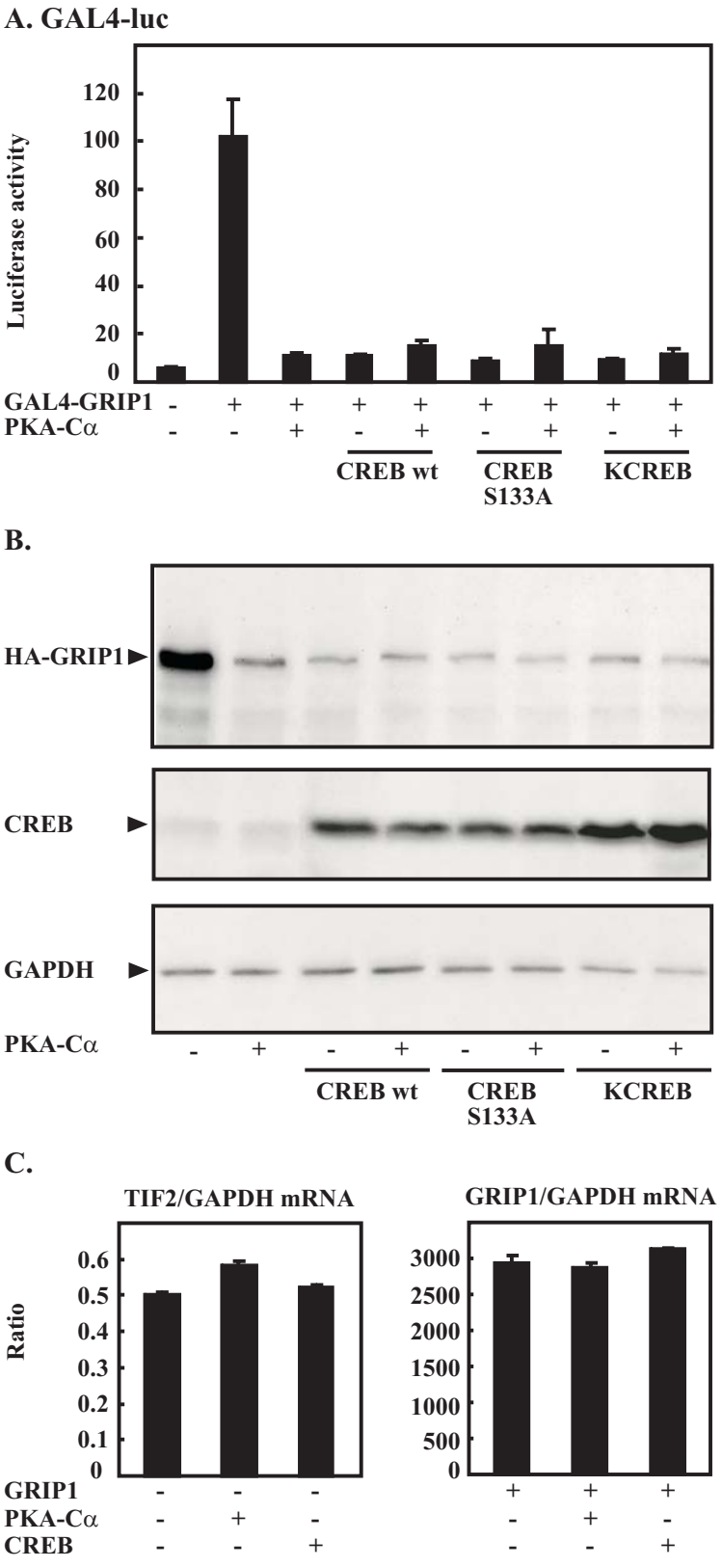

A. GAL4-luc
Luciferase activity
120
100
80
60
40
20
0
GAL4-GRIP1 - + + + + + + + +
PKA-Cα - - + - + - + - +
CREB wt
CREB S133A
KCREB
B.
HA-GRIP1
CREB
GAPDH
PKA-Cα - + - + - + - +
CREB wt
CREB S133A
KCREB
C.
TIF2/GAPDH mRNA
GRIP1/GAPDH mRNA
Ratio
0.6
0.5
0.4
0.3
0.2
0.1
0
3000
2500
2000
1500
1000
500
0
GRIP1 - - - + + +
PKA-Cα - + - - + -
CREB - - + - - +

FIG. 4

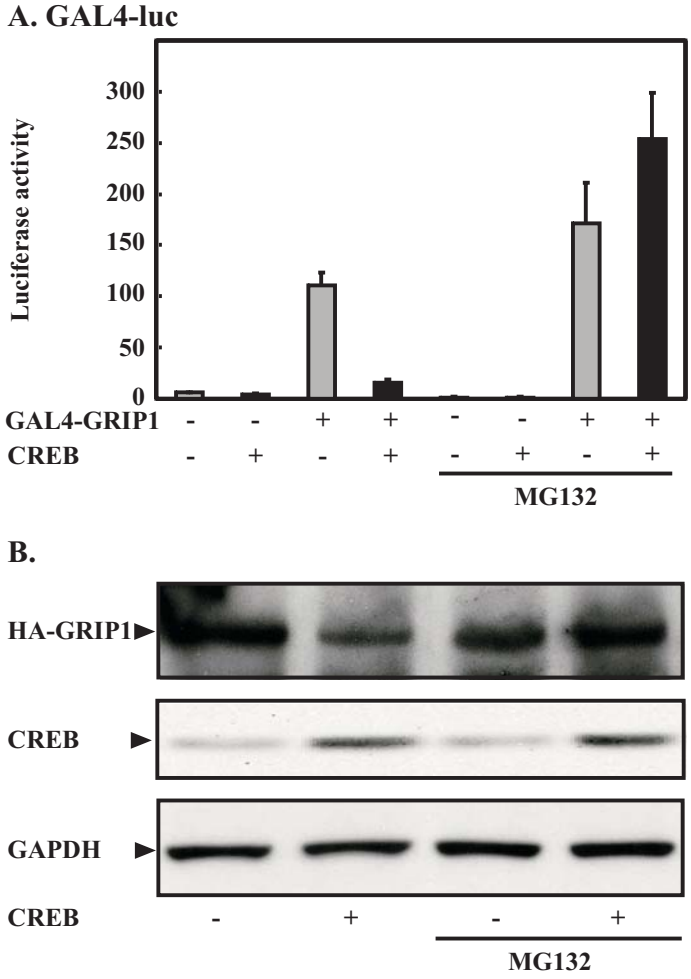

A. GAL4-luc
Luciferase activity
300
250
200
150
100
50
0
GAL4-GRIP1 - - + + - - + +
CREB - + - + - + - +
MG132
B.
HA-GRIP1
CREB
GAPDH
CREB - + - +
MG132

FIG. 5

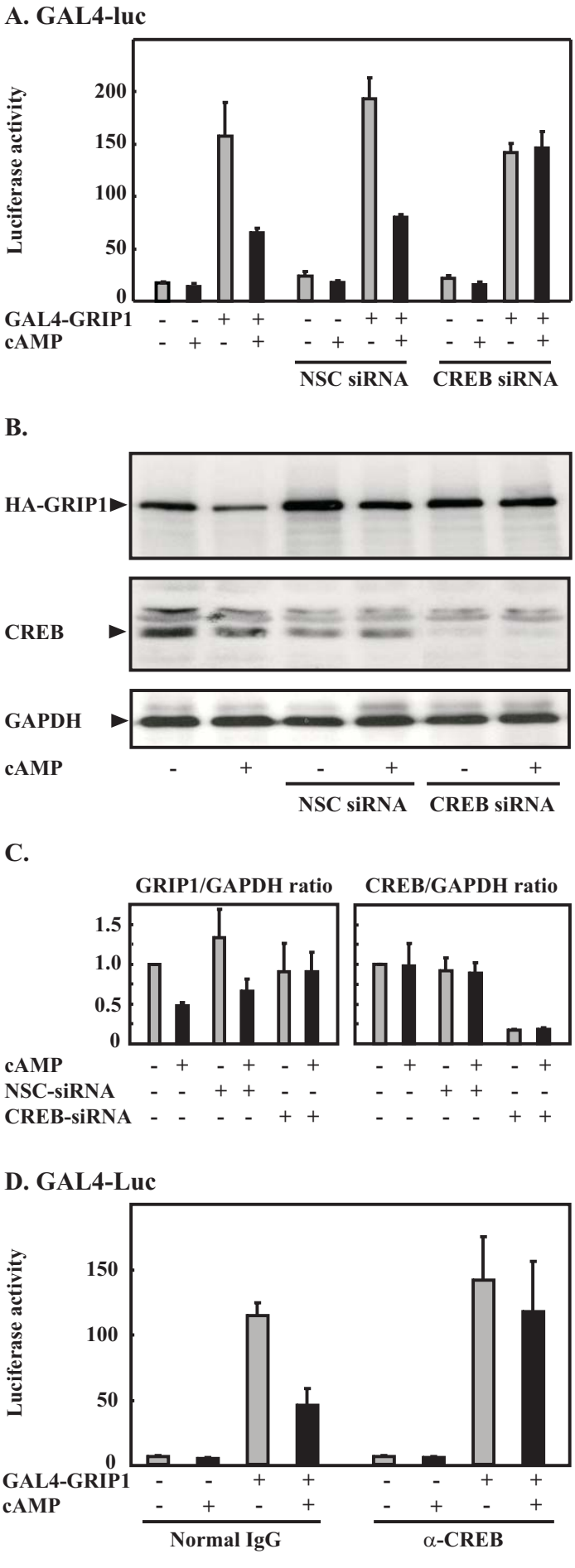

A. GAL4-luc
Luciferase activity
200
150
100
50
0
GAL4-GRIP1 - - + + - - + + - - + +
cAMP - + - + - + - + - + - +
NSC siRNA
CREB siRNA
B.
HA-GRIP1
CREB
GAPDH
cAMP - + - + - +
NSC siRNA
CREB siRNA
C.
GRIP1/GAPDH ratio
CREB/GAPDH ratio
1.5
1.0
0.5
0
cAMP - + - + - + - + - + - +
NSC-siRNA - - + + - - - - + + - -
CREB-siRNA - - - - + + - - - - + +
D. GAL4-Luc
Luciferase activity
150
100
50
0
GAL4-GRIP1 - - + + - - + +
cAMP - + - + - + - +
Normal IgG
α-CREB

FIG. 6

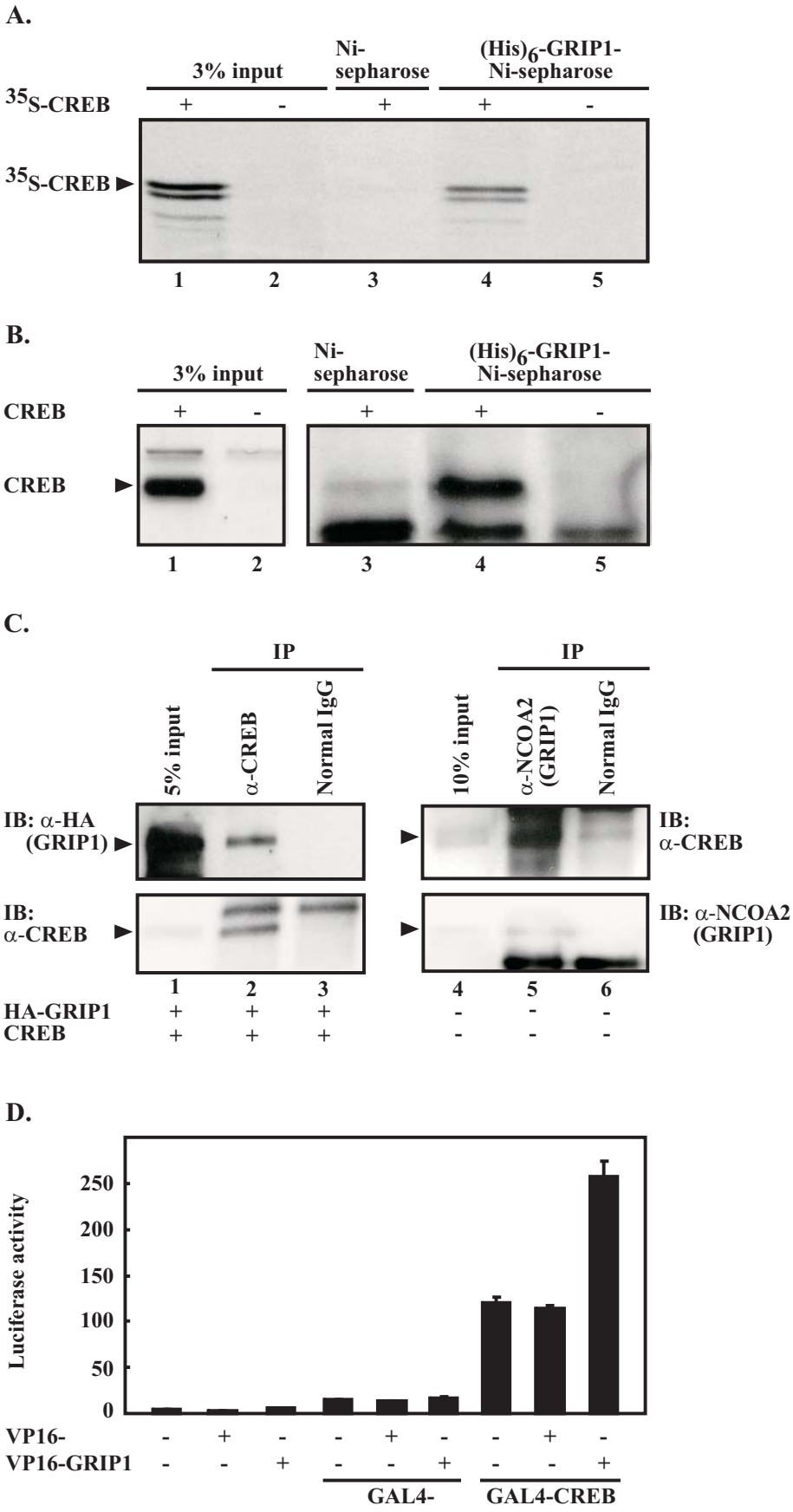
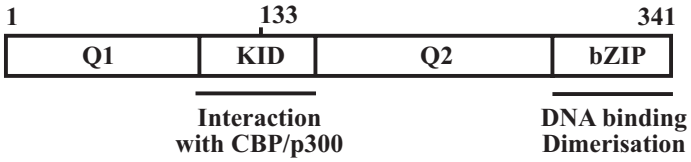
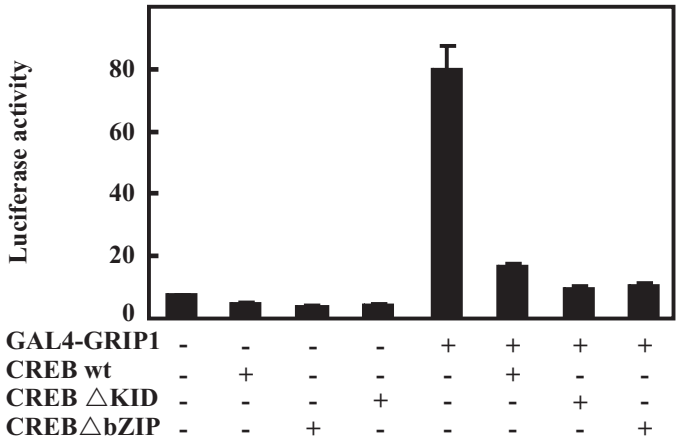

A.
3% input
Ni-sepharose
(His)6-GRIP1-Ni-sepharose
35S-CREB
+ - + + -
35S-CREB
1 2 3 4 5
B.
3% input
Ni-sepharose
(His)6-GRIP1-Ni-sepharose
CREB
+ - + + -
CREB
1 2 3 4 5
C.
IP
IP
5% input
α-CREB
Normal IgG
10% input
α-NCOA2 (GRIP1)
Normal IgG
IB: α-HA (GRIP1)
IB: α-CREB
IB: α-CREB
IB: α-NCOA2 (GRIP1)
1 2 3 4 5 6
HA-GRIP1 + + + - - -
CREB + + + - - -
D.
Luciferase activity
250
200
150
100
50
0
VP16- - + - - + - - + -
VP16-GRIP1 - - + - - + - - +
GAL4-
GAL4-CREB

FIG. 7

A.



B. GAL4-luc



C.