Androgen receptor-interacting protein 3 and other PIAS proteins cooperate with glucocorticoid receptor-interacting protein 1 in steroid receptor-dependent signaling

Received for publication, July 9, 2001, and in revised form, February 12, 2002
Published, JBC Papers in Press, March 13, 2002, DOI 10.1074/jbc.M106354200

Noora Kotaja‡, Marianne Vihinen‡, Jorma J. Palvimo‡§, and Olli A. Jänne‡¶

From the ‡Biomedicum Helsinki, Institute of Biomedicine (Physiology), Institute of Biotechnology, and the ¶Department of Clinical Chemistry, University of Helsinki and Helsinki University Central Hospital, Helsinki FIN-00014, Finland

Androgen receptor (AR)-interacting protein 3 (ARIP3/PIASxα) is a coregulator capable of modulating transcriptional activity of various steroid receptors. We have characterized functional regions of ARIP3 and studied its interaction with the glucocorticoid receptor (GR)-interacting protein 1 (GRIP1). We find that the potential zinc-binding domain is critical for ARIP3 to function as a coactivator; the deletion of amino acids 347–418 or the mutation of the conserved cysteines 385 and 388 to serines converts ARIP3 to a transcriptional repressor from AR-dependent minimal promoters and abolishes its ability to activate GR. By contrast, mutations in the two LXxxL motifs of ARIP3 have relatively minor effects on its ability to regulate AR or GR function. ARIP3 is able to interact with different regions of GRIP1, but the strongest interaction is detected with the C-terminal region (amino acids 1122–1462) of GRIP1. The interaction of ARIP3 with the latter GRIP1 domain or full-length GRIP1 and the ability of ARIP3 to cooperate with GRIP1 in the regulation of AR- or GR-dependent transcription are dependent on the ARIP3 zinc-binding region. We also find a strong synergism between GRIP1 and two other PIAS family members, Miz1 and PIAS1. Taken together, our results suggest that PIAS proteins and GRIP1 interact functionally in transcriptional regulation.

The steroid, retinoic acid, and thyroid hormone receptors belong to a large family of nuclear receptors. These receptors are ligand-inducible transcription factors that, after binding of the ligand, dimerize and bind to their cognate DNA response elements, thereby regulating the transcription of specific genes (1). In addition to the nuclear receptor, a large number of coregulator proteins are involved in the regulation of transcription. These proteins have usually no specific DNA binding activity, but they interact with nuclear receptors and play essential roles in mediating transcriptional regulation by the receptors.

Androgen receptor-interacting protein 3 (ARIP3) (2) belongs to the PIAS family of proteins, which also includes Miz1 (Max-interacting zinc finger) (probably a splicing variant of the ARIP3 gene), Gu/RNA helicase II-binding protein, protein inhibitor of activated Stat1 (PIAS1), PIAS3 (2–6), and PIASy/γ (7). PIAS proteins share a high sequence homology, with some regions exhibiting amino acid sequence identities of 60–80%, and these proteins are not restricted to vertebrates, since the Drosophila gene zimp encodes a homolog of the PIAS proteins (8). PIAS proteins contain two LXxxL motifs in their sequences. These motifs have been shown to be involved in recognition of several coregulators via the activation function 2 region of nuclear receptor ligand-binding domains (9–11). There are also four highly conserved cysteines and one histidine residue in the sequence of PIAS proteins, which are predicted to form a zinc-binding structure (3). PIAS proteins have been found to interact with and modulate the activities of various transcription factors. As predicted from their high sequence homology, PIAS proteins share functional properties and are all able to modulate transactivation capacity of different steroid receptors in a promoter- and cell-specific fashion (12–14).

The p160 coactivator family comprises a well established group of proteins involved in the activation of nuclear receptor function (15). It includes SRC-1/NCoA-1 (16), TIF2/GRIP1/NCoA-2 (SRC-2) (17, 18), and p/CIP/ACTR/AIB1/RAC3/TRAM-1 (SRC-3) (19–23). The members of the p160 family share ~40% amino acid sequence identity. They consist of an amino-terminal basic helix-loop-helix region, a period/aryl hydrocarbon receptor/single-minded domain, a serine/threonine-rich region, and a C-terminal glutamine-rich region/activation domain 2 (AD2). Gene targeting in mice has confirmed the physiological relevance of the SRC-1 and SRC-3 in steroid hormone-dependent signaling (24, 25). In addition to nuclear receptors, many other proteins involved in steroid receptor-dependent transcription have been shown to interact with p160 family members. Activation domain 1 (AD1) of GRIP1, located in the C-terminal region between amino acids 1040–1120 (17, 26), mediates the activating signal via interacting with CREB-binding protein or p300, two related signal integrators also capable of acting as nuclear receptor coactivators (19, 27). The histone acetyltransferase activity-possessing coactivator p/CAF interacts, in turn, with CREB-binding protein/p300, p160 coactivators. *
tors, and nuclear receptors, and these associations result in the formation of a multimeric transcription activation complex (21, 28, 29).

In this study, we have examined the functional regions of the PIAS family member ARIP3 and searched for interactions between ARIP3 and nuclear receptor coactivator proteins. We show that ARIP3 is able to interact functionally with GRIP1 in a fashion that is mediated by the putative zinc-binding region of ARIP3 and the AD2 of the glucocorticoid receptor-interacting protein 1 (GRIP1).

**EXPERIMENTAL PROCEDURES**

**Materials**—pARE, TATA-LUC reporter containing two AREs and TATA sequence and pPB–(285/+32)–LUC containing nucleotides –258 to +32 of the rat probasin promoter in front of the luciferase gene have been described previously (30, 31). Mouse mammary tumor virus promoter LUC construct (pHH-LUC, containing region –203/+105 of the promoter) was obtained from the American Type Culture Collection (ATCC; Manassas, VA). pG5-LUC has five Gal4-binding sites in front of the minimal TATA box sequence driving the LUC gene (Promega). pG5-LUC has five Gal4-binding sites in front of the pBK-CMV-LUC containing nucleotides 325–285 of the rat probasin promoter (ATCC; Manassas, VA). pG5-LUC has five Gal4-binding sites in front of the pBK-CMV-LUC containing nucleotides 325–285 of the rat probasin promoter (ATCC; Manassas, VA). pG5-LUC has five Gal4-binding sites in front of the pBK-CMV-LUC containing nucleotides 325–285 of the rat probasin promoter (ATCC; Manassas, VA).

-pM-GRIP1-(1121), and pM-GRIP1-(1122–1462) were gifts from Dr. M. R. Stallcup. cDNAs were from Dr. K. Shuai, and Miz1 cDNA was assembled as described previously (30, 31). Mouse mammary tumor virus promoter LUC construct (pHH-LUC, containing region –203/+105 of the promoter) was obtained from the American Type Culture Collection (ATCC; Manassas, VA). pG5-LUC has five Gal4-binding sites in front of the minimal TATA box sequence driving the LUC gene (Promega). pG5-RAR expression vector was constructed as previously described (32). pG5-hGR was created as described (30). PIAS1 and PIAS3 cDNA were from Dr. K. Shuai, and Miz1 cDNA was assembled as described (12). pSG5-GRIP1, pGRIP1-(5–765), pGRIP1-(563–1121), and pGRIP1-(1122–1462) were gifts from Dr. M. R. Stallcup. The following mammalian two-hybrid vectors were used: pM-GRIP1, p53 reporter, and p53 reporter containing wild type p53 (Promega) and pM-GRIP1-(1122–1462) bound to glutathione-Sepharose 4B (Amersham Biosciences). Immunoprecipitation was performed with mouse monoclonal anti-FLAG antibody (Sigma). Bound proteins were released in 2% SDS sample buffer, resolved on SDS-PAGE gels (35). Lysis buffer containing 50 mM Tris-HCl (pH 7.8), 150 mM NaCl, 5 mM EDTA, 15 mM MgCl2, 0.5% Nonidet P-40, 0.3% Triton X-100, 1 mM dithiothreitol, 1.200 protease inhibitor mixture (Sigma), and 10 mM N-ethylmaleimide. Immunoprecipitation was performed with mouse monoclonal anti-FLAG antibody (Sigma). Bound proteins were released in 2% SDS sample buffer, resolved on SDS-PAGE gels, and transferred onto Hybrid enhanced chemiluminescence nitrocellulose membrane, and GRIP1 was detected with mouse monoclonal GRIP1 antibody (gift from Dr. M. Brown). AR and GR were detected with polyclonal antibodies (PIAS family member ARIP3 and the AD2 of the glucocorticoid receptor-interacting protein 1 (GRIP1).

**Immunoblotting and Immunoprecipitation—**Whole cell extracts from HeLa cells were resolved by electrophoresis on 12% polyacrylamide gels under denaturing conditions (SDS-PAGE). Proteins were electrophoretically transferred onto Hybond ECL membrane (Amersham Biosciences). Membranes were incubated with M2 monoclonal antibody against FLAG epitope (Sigma) or monoclonal antibodies against VP16 or GAL4 DBD (Santa Cruz Biotechnology, Inc., Santa Cruz, CA). Horseradish peroxidase-conjugated goat anti-mouse immunoglobulin G antibody (Zymed Laboratories Inc.) was used as the secondary antibody. Immunocomplexes were detected by horseradish peroxidase-conjugated goat anti-mouse immunoglobulin G (Sigma). Bound proteins were released in 2% SDS sample buffer, resolved on SDS-PAGE, and transferred onto Hybond enhanced chemiluminescence nitrocellulose membrane, and GRIP1 was detected with mouse monoclonal GRIP1 antibody (gift from Dr. M. Brown). AR and GR were detected with polyclonal antibodies (PIAS family member ARIP3 and the AD2 of the glucocorticoid receptor-interacting protein 1 (GRIP1).

**RESULTS**

**ARIP3 Regions Important for the Regulation of AR Function—**To map the ARIP3 domains important in transcriptional regulation, different regions of the protein were deleted, or selected amino acid residues were mutated (Fig. 1A), and the effects of the deletions were studied by cotransfecting HeLa cells with pCMV-AR and either expression vectors along with an androgen receptor (AR)-encoding vector and a reporter construct. FLAG-tagged wild-type ARIP3, ARIP3Δ347–418, and ARIP3Δ3467–547 were expressed to comparable levels, whereas the expression levels of ARIP3Δ1–102, ARIP3Δ347–418, and ARIP3Δ3467–547 were expressed to comparable levels, whereas the expression levels of ARIP3Δ1–102, ARIP3Δ347–418, and ARIP3Δ3467–547 were expressed to comparable levels, whereas the expression levels of ARIP3Δ1–102, ARIP3Δ347–418, and ARIP3Δ3467–547 were expressed to comparable levels, whereas the expression levels of ARIP3Δ1–102, ARIP3Δ347–418, and ARIP3Δ3467–547 were expressed to comparable levels, whereas the expression levels of ARIP3Δ1–102, ARIP3Δ347–418, and ARIP3Δ3467–547 were expressed to comparable levels, whereas the expression levels of ARIP3Δ1–102, ARIP3Δ347–418, and ARIP3Δ3467–547 were expressed to comparable levels, whereas the expression levels of ARIP3Δ1–102, ARIP3Δ347–418, and ARIP3Δ3467–547 were expressed to comparable levels.
part of this structure to serines. The double mutant ARIP3(C385S,C388S) behaved in a fashion similar to ARIP3Δ347–418, in that it repressed AR-mediated transcription from both the minimal ARE2-TATA promoter and the pro-basin promoter, albeit somewhat less efficiently than ARIP3Δ347–418 (Fig. 2, C and D). ARIP3(C385S,C388S) was expressed to levels lower than ARIP3Δ347–418 in HeLa cells (Fig. 1B), which may, at least in part, explain the decreased activity of ARIP3(C385S,C388S) (cf. Fig. 2, A and C). Collectively, these results suggest that the cysteines/histidine in the ARIP3 sequence form a zinc finger structure and that the disruption of this structure leads to altered protein function.

To determine whether the ARIP3 mutants maintained their ability to interact with AR, AR and FLAG-tagged ARIP3 or ARIP3 mutants were ectopically expressed in COS-1 cells, and the cell extracts were subjected to immunoprecipitation with anti-FLAG antibody followed by immunoblotting with anti-AR antibody. Full-length ARIP3 and ARIP3 mutants Δ1–102, Δ467–547, L23A, L304A, and C385S,C388S displayed interactions with AR that did not differ much from each other, especially when related to their somewhat dissimilar expression levels, whereas the interaction of ARIP3Δ347–418 with AR was clearly weaker (Fig. 2E). In view of the finding that region 467–547 is sufficient for the interaction of ARIP3 with the zinc finger region of AR in yeast (10), these results indicate that ARIP3 interacts with AR via more than one domain. Since ARIP3(C385S,C388S) mutant is capable of being communoprecipitated with AR, the loss of its coactivation function must derive from reasons other than poor interaction with AR. The inability of ARIP3Δ347–418 to associate with AR under the coimmunoprecipitation conditions may also derive from deletion-induced alterations in the folding of the adjacent ARIP3 region 467–547.

**ARIP3 Regions Important for the Regulation of GR Function**—The influence of wild-type ARIP3, ARIP3Δ41–102, ARIP3Δ347–418, and ARIP3Δ467–547 on GR-dependent transcription was studied using two different promoters. With the minimal ARE2-TATA promoter, ARIP3 enhanced GR-dependent transcription up to 40-fold (Fig. 3A). When amino acids 347–418 or 467–547 of ARIP3 were deleted, most of the GR-activating function was abolished, in that ARIP3Δ347–418 and ARIP3Δ467–547 stimulated the GR-dependent transcription only by 2–3-fold. In contrast to the two latter mutations, deletion of 1–102 of ARIP3 had a smaller effect, and this mutant was still capable of stimulating the activity of GR by 20-fold. With the mouse mammary tumor virus promoter-containing reporter (HH-LUC), ARIP3 repressed the transcription, as did ARIP3Δ347–418 and ARIP3Δ467–547 (Fig. 3B). Again, the strongest repression (to 25% of the control level) was seen when ARIP3Δ347–418 was cotransfected with GR. The double mutant ARIP3(C385S,C388S) exhibited diminished activity on the function of GR on the minimal promoter, although not as much as the mutant devoid of the zinc-binding region (ARIP3Δ347–418; Fig. 3C) and repressed GR-mediated transactivation of HH-LUC reporter in a fashion not much different from that of wild-type ARIP3 (Fig. 3D). As was the case with AR, ARIP3Δ347–418 interacted very poorly with GR in communoprecipitation experiments (Fig. 3E). The behavior of the double mutant ARIP3(C385S,C388S) was equivocal, in that in repeated experiments and with multiple plasmid preparations, ARIP3(C385S,C388S) was expressed to very low levels in the presence of concomitant GR expression (Fig. 3E). The reason for this phenomenon is elusive; however, it did not occur to the same extent with simultaneously expressed AR (Fig. 2) or GRIP1.

**The Role of LXXLL Motifs in ARIP3 Function**—LXXLL mo-

---

**Fig. 1. ARIP3 constructs used.** A, schematic structure of the wild-type ARIP3 together with the deletion and point mutants used. B, immunoblot analysis of proteins encoded by the indicated expression vectors in HeLa cells. The cells were transfected by using FuGene with the expression vectors (200 ng of DNA/well, 12-well plate) and cultured for 48 h. Whole cell extracts were resolved by SDS-PAGE and immuno-

---

2 N. Kotaja, O. A. Jänne, and J. J. Palvimo, unpublished observations.

---

---
FIG. 2. Effects of ARIP3 mutations on AR-dependent transactivation. A, modulation of transactivation from the minimal ARE2TATA promoter. HeLa cells cultured on 12-well plates were transfected with 200 ng of pARE2TATA-LUC, 20 ng of pSG5-rAR, 20 ng of pCMVβ, and increasing amounts (2, 10, and 20 ng) of expression vectors encoding wild-type ARIP3 (WT) or the indicated ARIP3 mutants in the presence (+) or absence (−) of 100 nM testosterone (T). The total amount of DNA was balanced by empty pFLAG-CMV2 as needed. After normalization for transfection efficiency using β-galactosidase activity, reporter gene activities are expressed relative to those of rAR + T without a coregulator (1.0). B, modulation of transactivation from the natural probasin promoter. The experimental conditions were the same as in A, except that pPB(−285/+32)-LUC reporter was used. C and D, influence of ARIP3(C385S,C388S) on AR-dependent transactivation from the minimal ARE2TATA promoter (C) and from the natural probasin promoter (D). The experimental conditions were the same as in A and B. The values represent means ± S.D. from 3–6 independent experiments. E, COS-1 cells were transfected with 0.7 μg of empty pFLAG-CMV2 vector or pFLAG-ARIP3 constructs and 0.9 μg of pSG5 or pSG5-AR as indicated. The cells were collected 48 h after transfection and lysed in radiimmune precipitation buffer. Portions of the lysates (5%, input) were immunoblotted with the anti-AR antibody (α-AR) or anti-FLAG antibody (α-FLAG), and the rest of the sample was subjected to immunoprecipitation (IP) with anti-FLAG antibody. Immunoprecipitates were resolved by SDS-PAGE and blotted (WB) with anti-AR antibody.

FIG. 3. Ability of ARIP3 mutants to modulate GR-dependent transactivation. A, effect of ectopic expression of wild-type and mutant ARIP3 proteins together with GR (pSG5-hGR) on ARE2TATA promoter activity in HeLa cells. Experimental conditions were same as those in Fig. 2A, except that GR-dependent transcription was activated by the exposure to 100 nM dexamethasone (DEX). B, the same experiment was performed in HeLa cells using pHH-LUC reporter containing region −203/+105 of the mouse mammary tumor virus promoter in front of the LUC gene. C and D, influence of ARIP3(C385S,C388S) on GR-dependent transactivation from the minimal ARE2TATA promoter (C) and from the natural probasin promoter (D). The experimental conditions were the same as in A and B. The values represent means ± S.D. from 3–6 independent experiments. E, COS-1 cells were transfected with 0.7 μg of empty pFLAG-CMV2 vector or pFLAG-ARIP3 constructs and 0.9 μg of pSG5 or pSG5-GR as indicated. The cells were collected 48 h after transfection and lysed in radiimmune precipitation buffer. Portions of the lysates (5%, input) were immunoblotted with the anti-GR antibody (α-GR) or anti-FLAG antibody (α-FLAG), and the rest of the sample was subjected to immunoprecipitation (IP) with anti-FLAG antibody. Immunoprecipitates were resolved by SDS-PAGE and blotted (WB) with anti-GR antibody.
tifs have been shown to be important in mediating the interaction between many coactivator proteins and nuclear receptors. ARIP3 sequence contains two LXXLL motifs starting at residues 19 and 304. To examine the importance of these motifs in the coregulator function of ARIP3, the last leucine residue of each motif (Leu23 and Leu304) was mutated to alanine (LXXLL→LXXLA). Similar mutations have been shown to be sufficient to disrupt the interaction between the LXXLL motifs of RIP140 and estrogen receptor ligand-binding domain (9). ARIP3 with two mutated LXXLL motifs (ARIP3(L23A,L304A)) was unable to enhance AR-dependent transactivation on ARE2TATA-LUC (Fig. 2A), but it behaved the same way as wild-type ARIP3 on the probasin promoter (Fig. 2B). In the case of GR, the maximal enhancement caused by ARIP3(L23A,L304A) coexpression was somewhat lower than that by wild-type ARIP3 (30 versus 40-fold) on the ARE2TATA promoter (Fig. 3A). ARIP3(L23A,L304A) is expressed to a slightly lower level than wild-type ARIP3 (Fig. 1B), which may be, at least in part, the reason for its weaker effect in transactivation assays. ARIP3(L23A,L304A) was also capable of repressing the GR-dependent transcription from the mouse mammary tumor promoter, albeit to a somewhat lesser extent than the wild-type ARIP3 (Fig. 3B). In agreement with the preceding transactivation results, the ability of ARIP3(L23A,L304A) to interact with either AR or GR in communoprecipitation experiments was not significantly attenuated (Figs. 2E and 3E).

**ARIP3 and GRIP1 Interact in Mammalian Cells**—Because ARIP3 does not possess an intrinsic transcription activating function when fused to a heterologous DNA-binding domain (12), a plausible mechanism underlying ARIP3 action in steroid receptor-dependent transcription is that it is exerted via other proteins, such as steroid receptor coactivators. GRIP1 is a well-established steroid receptor coactivator belonging to the p160 family (15, 18). To analyze whether ARIP3 interacts with GRIP1, full-length ARIP3 fused to VP16 activation domain (VP16-ARIP3) and different regions of GRIP1 linked to Gal4 DNA-binding domain (Gal4) were cotransfected with the Gal4-regulated pG5-LUC reporter into HeLa cells (Fig. 4A). Cotransfection of VP16-ARIP3 with Gal4-GRIP1A (1–5–765) (Gal4-GRIP1A) resulted in a 15-fold enhancement of reporter gene activity compared with the VP16 control containing polyoma virus coat protein (VP16-CP). Gal4-GRIP1B (563–1121) (Gal4-GRIP1B) harbors a strong transcription activating function and, therefore, activated the reporter gene even with VP16-CP. However, coexpressed VP16-ARIP3 was capable of enhancing further the transcription by 7-fold. When Gal4-GRIP1A (1122–1462) (Gal4-GRIP1C) was coexpressed with VP16-ARIP3, transcription was induced up to ~200-fold over that seen with VP16-CP. Thus, ARIP3 is able to interact with different regions of GRIP1, either directly or indirectly, but the strongest interaction is detected with the C-terminal region of GRIP1 (Fig. 4A).

The C-terminally extended variant of ARIP3, Miz1, also interacted with the C-terminal domain of GRIP1 (Fig. 4A). VP16-Miz1 enhanced Gal4-GRIP1C-mediated transcription somewhat less than VP16-ARIP3 (195 versus 120-fold). However, Miz1 was expressed to a lower level than the corresponding ARIP3 construct. Interestingly, VP16-Miz1 exhibited a rather poor interaction with Gal4-GRIP1B as assessed by the two-hybrid system (Fig. 4A).

To elucidate the role of the conserved cysteine-rich region of ARIP3 in the binding to GRIP1, VP16-ARIP3,347–418, and VP16-ARIP3(385S,388S) were cotransfected with Gal4-ARIP3 constructs and pG5-LUC reporter. As shown in Fig. 4A, the activity of VP16-ARIP3,347–418 and VP16-ARIP3(385S,388S) was about one-half of that of full-length ARIP3 when cotransfected with Gal4-ARIP1B, but importantly, these two ARIP3 mutants displayed interactions with the C-terminal domain of GRIP1 that were only less than one-tenth of that of wild-type ARIP3 (Fig. 4A). These results indicate that the putative zinc-binding structure of ARIP3 is important for the interaction with GRIP1.

Interaction of ARIP3 with GRIP1 was also examined by performing communoprecipitations in COS-1 cells that were transfected with expression vectors encoding GRIP1 and FLAG-tagged ARIP3 or ARIP3 mutants. As shown in Fig. 4B, full-length GRIP1 associated poorly to ARIP3 mutants with a deleted or mutated zinc-binding region (ARIP3,347–418 and ARIP3(385S,388S)) as revealed by immunoprecipitation of cell extracts with monoclonal anti-FLAG antibody followed by immunoblotting with anti-GRIP1 antibody.
with both the middle part and the C-terminal region of GRIP1 in GST pull-down assays, and the deletion of amino acids 347–418 resulted in attenuated interaction of ARIP3 with the C-terminal region of GRIP1. ARIP3 is devoid of intrinsic transcription activating function (12). However, when wild-type ARIP3 without the VP16 trans-activation domain was provided in trans, it was able to enhance transcriptional activity of Gal4-GRIP1B and Gal4-GRIP1C in HeLa cells by 20- and 46-fold, respectively (Fig. 5). Ectopic expression of Miz1 yielded very similar results in this modified one-hybrid assay. Deletion of the zinc-binding region (ARIP3/H9004347–418) or mutation of two cysteines in this region to serines (ARIP3(C385S,C388S)) abolished the activation of Gal4-GRIP1C. Deletion of the zinc-binding region also abolished the activation of Gal4-GRIP1B; however, the action of ARIP3(C385S,C388S) on Gal4-GRIP1B did not differ from that of wild-type ARIP3 (Fig. 5). The reason for this latter result is unknown at present. In any event, these data imply that GRIP1 and ARIP3 do not only interact with each other but that the interaction of ARIP3 with GRIP1 modulates the activity of the latter protein, most likely through recruitment of other proteins to the complex.

**ARIP3 and GRIP1 Act Synergistically to Activate AR- and GR-dependent Transcription**—Since both ARIP3 and GRIP1 are able to modulate AR activity, we examined whether they also cooperate in steroid receptor-dependent transcription. Reporter gene assays were performed using the ARE2TATA promoter to compare the effects of either ARIP3 or GRIP1 alone to those of their combinations. When GRIP1 alone was cotransfected with AR, androgen-dependent transcription in HeLa cells was enhanced by 3-fold (Fig. 6A). ARIP3 (5 ng) enhanced the transcription by 4-fold, and cotransfection of GRIP1 with ARIP3 resulted in a more than additive 10-fold enhancement.

When a higher dose of ARIP3 (20 ng) was cotransfected with GRIP1 and AR, the synergism between ARIP3 and GRIP1 was abolished, probably due to a squelching effect. ARIP3Δ347–418 and ARIP3(C385S,C388S) mutants blunted AR-dependent transactivation also with ectopically expressed GRIP1, attesting to the importance of the putative zinc-binding region for the function of ARIP3 (Fig. 6A).

Miz1 and PIAS1 are under many experimental conditions stronger AR coactivators than ARIP3 (12). Miz1 (20 ng) enhanced the AR-dependent transcription by 11-fold, and a 31-fold increase in androgen-dependent transactivation was observed when Miz1 was coexpressed with GRIP1. This is almost 3 times the sum of their separate effects. Also PIAS1 functioned synergistically with GRIP1, whereas no synergism was observed between PIAS3 and GRIP1 (Fig. 6A). Immunoblot analysis indicated that the expression levels of AR were not influenced by coexpressed ARIP3 or GRIP1; nor did ARIP3 affect the amount of immunoreactive GRIP1 in HeLa cells.2 ARIP3 and other PIAS proteins expressed together with GRIP1 in HeLa cells in the absence of AR and androgen activated ARE2TATA-LUC reporter marginally, to a level that was 1–2%
of that in the presence of AR and androgen. This marginal activation was independent of the presence of apo-AR.2

ARIP3 and Miz1 exhibited a strong synergism with GRIP1 on GR-dependent transcription (Fig. 6B). ARIP3 (5 ng) enhanced the activity of GR by 5-fold and GRIP1 alone by 3-fold, but together these two proteins stimulated GR-dependent transcription by 19-fold. Miz1, which is a less potent coactivator of GR than ARIP3, also acted with GRIP1 in a synergistic fashion (Fig. 6B). Taken together, all PIAS proteins but PIAS3 are able to cooperate with GRIP1 in steroid receptor-dependent transcription, and the AD2-containing C-terminal region of GRIP1 seems to function as a downstream signaling domain for the PIAS proteins.

**DISCUSSION**

The most visible structural elements of PIAS proteins are the two LXXLL motifs starting at amino acid residues 19 and 304 (in the ARIP3 sequence) and conserved cysteines and a histidine between amino acids 346 and 425. The latter region is likely to form a zinc-binding structure. It has also been recently predicted that PIAS proteins harbor a putative DNA-binding motif, the SAF-A/B, Acinus, and PIAS (SAP) module, at their N termini (residues 11–45 in ARIP3) (36). Our initial in vitro DNA-binding assays using GST-ARIP3 have failed to show high affinity DNA binding, but the N-terminally fused GST may impair the function of the putative SAP module of ARIP3.

The C-terminal region of ARIP3 (amino acids 443–547) encompassing a serine-rich acidic domain (amino acids 475–483) was first shown to interact with AR in yeast. In line with this finding, the deletion of amino acids 467–547 abolished the ability of ARIP3 to modulate AR-dependent transcription both on minimal and more complex promoters. Likewise, the C-terminal region of PIAS1 (amino acids 392–650) has been shown to be involved in the interaction with Stat1 and required for the inhibition of Stat1-dependent gene activation (37). However, these results do not rule out the possibility that also other regions of ARIP3 and PIAS1 interact with AR and Stat1, respectively.

Because the LXXLL motifs have been shown to be involved in the interactions between many coactivators and nuclear receptor ligand-binding domains (9, 10) and the N-terminal LXXLL motif of PIASy has been reported to be essential for its ability to repress Stat1-dependent transactivation (38), it was of interest to examine whether these motifs have a role in ARIP3 function. Mutation of the last Leu in the LXXLL motif to Ala weakened the activity of ARIP3 on the minimal ARE1/TATA promoter but did not influence its activity on the probasin promoter. Moreover, the mutant was only slightly less active than wild-type ARIP3 on GR-dependent transcription. Thus, the two LXXLL motifs may be needed for ARIP3 function in certain promoter contexts, but the interactions between ARIP3 and AR or GR do not rely solely on these motifs.

The conserved region between amino acids 347 and 418 of ARIP3 is of special interest, since it contains a probable zinc-binding structure. Zinc fingers typically form interfaces for protein contacts. Deletion of this region converted ARIP3 to a strong dominant negative regulator of AR function on the ARE1/TATA promoter and abolished almost completely its ability to activate GR-dependent transcription from the same promoter. On more complex promoters, ARIP3Δ347–418 behaved as a stronger repressor than the wild-type protein. Point mutations of the conserved cysteines 385 and 388 to serines caused effects similar to those of the deletion mutant, strongly suggesting that this region in PIAS proteins indeed forms a zinc-coordinated structure. In this context, it is of interest to note that our own studies and results from other laboratories have recently revealed that an intact zinc-binding structure of PIAS proteins is required for the ability of these proteins to function as E3-type SUMO-1 ligases (39).3

The mechanism(s) underlying the ability of PIAS proteins to modulate steroid receptor-dependent transcription is not known. One potential mechanism is that PIAS proteins elicit their actions through interacting with other nuclear receptor coactivators. Sumoylation may also play an important role in these interactions, and the deletion of the zinc-binding region important for the E3-type SUMO-1 ligase activity2 attenuates the interaction of ARIP3 with GRIP1. ARIP3 interacts both in vitro and in mammalian cells with the p160 family member GRIP1. Interestingly, the strongest interaction between ARIP3 and GRIP1 in mammalian one- and two-hybrid experiments was observed with the C-terminal region of GRIP1 containing the AD2. To date, only two other AD2-interacting proteins have been described; the coactivator-associated arginine methyltransferase 1 and mouse Zac1 were both found in yeast two-hybrid screens using amino acids 1121–1462 of GRIP1 as the bait (40, 41). In contrast to PIAS proteins, coactivator-associated arginine methyltransferase 1 enhances the transcriptional activity of nuclear receptors only in the presence of coexpressed GRIP1 (40). Zac1 also interacts with CREB-binding protein/p300 and nuclear receptors themselves and thereby functions as a powerful coactivator for hormone-dependent transcription (41). Besides the C-terminal region of GRIP1, ARIP3 also interacts with the central part of GRIP1 (amino acids 563–1121). This latter GRIP1 region contains the nuclear receptor interaction domain that harbors three LXXLL motifs (9–11, 17) and the AD1 (17, 26). Mutations in the putative zinc-binding domain of ARIP3 completely abolished the functional interactions with the C-terminal region of GRIP1 in intact cells, whereas those with the central GRIP1 region were influenced to a lesser extent.

Like coactivator-associated arginine methyltransferase 1 and Zac1, ARIP3 is capable of acting in a synergistic fashion with GRIP1 on steroid-dependent transcription. In agreement with their more robust activity as AR coactivators, Miz1 and PIAS1 showed a more pronounced synergism with GRIP1 than other PIAS proteins. The cooperation between ARIP3 and GRIP1 on GR-dependent transcription was stronger than that on AR-mediated transcription, which agrees with the finding that ARIP3 is a more efficient coactivator of GR than AR (12). Interestingly, ARIP3Δ347–418, which invariably acted as a negative regulator of AR function, also blocked totally the coactivation of AR by GRIP1. The mutant ARIP3Δ385–388 (C385S, C388S) behaved in a fashion similar to ARIP3Δ347–418, and importantly, ARIP3Δ385S,388S was able to interact with AR under coimmunoprecipitation conditions. Although ARIP3Δ347–418, devoid of the putative zinc-binding structure, failed to coimmunoprecipitate with AR or GR and GRIP1, it is still possible that it interacts in vivo with AR and the central domain of GRIP1 through other regions. These interactions would, in turn, block the activity of AR and the ability of GRIP1 to activate receptor function. However, it is equally likely that the interactions of ARIP3 with AR and GRIP1 in intact cells are stabilized/mediated by some other, currently unknown protein(s) (perhaps those involved in sumoylation) existing in the same complex.

According to Baumann et al. (42), localization of GRIP1 in a subpopulation of cells into discrete intranuclear foci is dependent on the same C-terminal AD2-containing region of GRIP1 that interacts strongly with ARIP3. In contrast to ARIP3 (2),

---

3 Kotaja, N., Karvonen, U., Janne, O. A., and Palvimo, J. J. (2002) *Mol. Cell. Biol.* in press.
both coactivator-associated arginine methyltransferase 1 and mouse Zac1 show diffuse nuclear distribution without focal accumulations (42). Determination of whether functionally active PIAS proteins target to the same nuclear bodies containing the promyelocytic leukemia gene product and associated factors, to which also a subpopulation of GRIP1 colocalizes (42), will require further studies.

Acknowledgments—We thank Kati Saastamoinen, Saija Kotola, and Leena Pietila for technical assistance; Michael Stallcup for providing the GRIP1 expression vector; and Myles Brown for the GRIP1 antibody.

REFERENCES
1. Beato, M., Herrlich, P., and Schu (1995) Cell 83, 851–887
2. Moilanen, A-M., Karvonen, U., Poukka, H., Yan, W., Toppuri, J., Janne, O. A., Palvimo, J. J. (1999) J. Biol. Chem. 274, 3760–3764
3. Wu, L., Wu, H., Land, S., Sangiorgi, F., Wu, N., Bell, J. R., Lyons, G. E., Maxson, R. (1997) Mech. Dev. 65, 3–17
4. Valdez, B. C., Henning, D., Perlaky, L., Busch, R. K., Busch, H. (1997) Biochim. Biophys. Res. Commun. 234, 335–340
5. Liu, B., Liao, J., Xiaoqing, R., Kushner, S. A., Chang, C. D., Chang, D. D., Shuai, K. (1998) Proc. Natl. Acad. Sci. U. S. A. 95, 10626–10631
6. Chang, C. D., Liao, J., Liu, B., Tan, X., Jay, P., Berta, P., Shuai, K. (1997) Science 278, 1803–1805
7. Sturm, S., Koch, M., White, P. A. (2000) J. Mol. Neurosci. 14, 107–121
8. Mohr, S. E., Bowell, R. E. (1999) Gene (Amst.) 229, 109–116
9. Heery, D. M., Kalkhoven, E., Hoare, S., and Parker, M. G. (1997) Nature 387, 733–736
10. Darmont, B. D., Wagner, R. L., Apriletti, J. W., Stallcup, M. R., Kushner, P. J., Baxter, J. D., Fletterick, R. J., and Yamamoto, K. R. (1998) Genes Dev. 12, 3343–3356
11. Ding, X. F., Anderson, C. M., Ma, H., Hong, H., Ueh, R. M., Kushner, P. J., and Stallcup, M. R. (1998) Mol. Endocrinol. 12, 302–313
12. Kotaja, N., Aistomaki, S., Siiveno, O., Palvimo, J., and Janne, O. A. (2000) Mol. Endocrinol. 14, 1886–1900
13. Tan, J.-A., Hall, S. H., Hamil, K. G., Grossman, G., Petrusz, P., Liao, J., Shuai, K., and French, F. S. (2000) Mol. Endocrinol. 14, 14–26
14. Junichro, M., Matsuda, T., Yamamoto, T., Kishi, H., Korkmaz, K., Saactioglu, F., Fuse, H., and Muraguchi, A. (2000) Biochim. Biophys. Res. Commun. 278, 9–13
15. Leo, C., and Chen, J. D. (2000) Gene (Amst.) 245, 1–11
16. Oni, S. A., Bounyaratanakornkit, V., Spencer, T. E., Tsai, S. Y., Tsai, M.-J., Edwards, D. P., and O’Malley, B. W. (1998) J. Biol. Chem. 273, 12101–12108
17. Voegel, J. J., Heine, M. J. S., Tini, M., Vivat, V., Chambon, P., and Gronemeyer, H. (1998) EMBO J. 17, 507–519
18. Hong, H., Kohli, K., Trivedi, A., Johnson, D. L., and Stallcup, M. R. (1996) Proc. Natl. Acad. Sci. U. S. A. 93, 4948–4952
19. Takviv, J., Rose, D. H., Inoet, J., Kamei, Y., Westin, S., Glass, C. K., and Rosenfeld, M. G. (1997) Nature 387, 677–684
20. Anzick, S. L., Kononen, J., Walker, R. L., Azorsa, D. O., Tanner, M. M., Guan, X. Y., Sauter, G., Kallioniemi, O. P., Trent, J. M., and Meltzer, P. S. (1997) Science 277, 965–968
21. Chen, H., Lin, R. J., Shultz, R. L., Chakravarti, D., Nash, A., Nagy, L., Privalsky, M. L., Nakatani, Y., and Evans, R. M. (1997) Cell 90, 569–580
22. Li, H., Gomes, P. J., and Chen, J. D. (1997) Proc. Natl. Acad. Sci. U. S. A. 94, 8479–8484
23. Takeda, H., Cardona, G. R., Kibuchi, N., Suen, C. S., and Chiu, W. W. (1997) J. Biol. Chem. 272, 27629–27634
24. Xu, J., Qiu, Y., DeMayo, F. J., Tsai, S. Y., Tsai, M. J., and O’Malley, B. W. (1998) Science 279, 1922–1925
25. Xu, J., Liao, L., Ning, G., Yoshida-Komiya, H., Deng, C., and O’Malley, B. W. (2000) Proc. Natl. Acad. Sci. U. S. A. 97, 6379–6384
26. Hong, H., Darmont, B. D., Ma, H., Yang, L., Yamamoto, K. R., and Stallcup, M. R. (1999) J. Biol. Chem. 274, 3496–3502
27. McKenna, N. J., Lanz, R. B., and O’Malley, B. W. (1999) Endocr. Rev. 20, 321–344
28. Blanco, J. C. G., Minucci, S., Lu, J. M., Yang, X. J., Walker, K. K., Chen, H. W., Evans, R. M., Nakatani, Y., and Ozato, K. (1998) Genes Dev. 12, 1638–1651
29. Korzus, E., Tercia, J., Rose, D. W., Xu, L., Kurokawa, R., McNeary, E. M., Mullen, T. M., Glass, C. K., and Rosenfeld, M. G. (1998) Science 273, 703–707
30. Moilanen, A-M., Poukka, H., Karvonen, U., Hakli, M., Janne, O. A., and Palvimo, J. J. (1999) Mol. Cell. Biol. 19, 5128–5139
31. Palvimo, J. J., Reinikainen, P., Ikonen, T., Kallio, P. J., Moilanen, A., and Janne, O. A. (1996) J. Biol. Chem. 271, 24151–24156
32. Palvimo, J. J., Kallio, P. J., Ikonen, T., Mehto, M., and Janne, O. A. (1993) Mol. Endocrinol. 7, 1399–1407
33. Ikonen, T., Palvimo, J. J., and Janne, O. A. (1997) J. Biol. Chem. 272, 29821–29829
34. Rosenthal, N. (1987) Methods Enzymol. 152, 704–720
35. Kallio, P. J., Poukka, H., Moilanen, A., Janne, O. A., and Palvimo, J. J. (1995) Mol. Endocrinol. 9, 1017–1028
36. Aravind, L., and Koonin, E. V. (2000) Trends Biochem. Sci. 25, 112–114
37. Lian, J., Fu, Y., and Shuai, K. (2000) Proc. Natl. Acad. Sci. U. S. A. 97, 5267–5272
38. Liu, B., Gross, M., ten Hoeve, J., and Shuai, K. (2001) Proc. Natl. Acad. Sci. U. S. A. 98, 3203–3207
39. Kahyo, T., Nishida, T., and Yasuda, H. (2001) Mol. Cell 8, 713–718
40. Chen, H., Ma, H., Hong, H., Koh, S. S., Huang, S.-M., Schurter, B. T., Arwas, D. W., and Stallcup, M. R. (1999) Science 284, 2174–2177
41. Huang, S.-M., and Stallcup, M. R. (2000) Mol. Cell. Biol. 20, 1855–1867
42. Baumann, C. T., Ma, H., Woldorf, R., Reyes, J. C., Marvada, P., Lim, C., Yen, P. M., Stallcup, M. R., and Hager, G. L. (2001) Mol. Endocrinol. 15, 485–500
Androgen Receptor-interacting Protein 3 and Other PIAS Proteins Cooperate with Glucocorticoid Receptor-interacting Protein 1 in Steroid Receptor-dependent Signaling

Noora Kotaja, Marianne Vihinen, Jorma J. Palvimo and Olli A. Jänne

J. Biol. Chem. 2002, 277:17781-17788.
doi: 10.1074/jbc.M106354200 originally published online March 13, 2002

Access the most updated version of this article at doi: 10.1074/jbc.M106354200

Alerts:
- When this article is cited
- When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts

This article cites 42 references, 22 of which can be accessed free at http://www.jbc.org/content/277/20/17781.full.html#ref-list-1