Coregulator Small Nuclear RING Finger Protein (SNURF) Enhances Sp1- and Steroid Receptor-mediated Transcription by Different Mechanisms*

(Received for publication, July 21, 1999, and in revised form, September 29, 1999)

Hetti Poukkal‡, Piia Aarnisalo‡, Henriikki Santt‡, Olli A. Jänne‡§, and Jorma J. Palvimo‡¶

From the §Department of Physiology, Institute of Biomedicine and the ¶Department of Clinical Chemistry, University of Helsinki, FIN-00014 Helsinki, Finland

The small nuclear RING finger protein SNURF is not only a coactivator in steroid receptor-dependent transcription but also activates transcription from steroid-independent promoters. In this work, we show that SNURF, via the RING finger domain, enhances protein binding to Sp1 elements/GC boxes and interacts and cooperates with Sp1 in transcriptional activation. The activation of androgen receptor (AR) function requires regions other than the RING finger of SNURF, and SNURF cooperates with the AF-1 region of AR to bind to cognate DNA elements. The zinc finger region (ZFR) together with the hinge region of AR are sufficient for contacting SNURF. The nuclear localization signal in the boundary between ZFR and the hinge region participates in the association of AR with SNURF, and a receptor mutant lacking the C-terminal part of the bipartite nuclear localization signal shows attenuated response to coexpressed SNURF. Some AR ZFR point mutations observed in patients with partial androgen insensitivity syndrome or male breast cancer impair the interaction of AR with SNURF and also render AR refractory to the transcription-activating effect of SNURF. Collectively, SNURF modulates the transcriptional activities of androgen receptor and Sp1 via different domains, and it may act as a functional link between steroid- and Sp1-regulated transcription.

The androgen receptor (AR), 1 a member of the steroid receptor family, acts as a hormone-regulated transcription factor. The N-terminal region contains a powerful ligand-independent transcription activation function-1 (AF-1). The second but weaker activation function (AF-2) localizes to the ligand binding domain; it requires hormone for activation and perhaps also an intramolecular interaction with the AF-1 region (1–6). The apo-ligand binding domains of steroid receptors interact with transcriptional corepressors and heat-shock proteins, and the ligand-induced conformation in the ligand binding domain enable interactions between several coactivators and AF-2 (7–12). The steroid receptor zinc finger region (ZFR) consists of two zinc finger (ZF) structures (13). The first ZF is responsible for contacting the specific hormone response element (HRE), whereas the second ZF stabilizes the receptor-DNA interactions and participates in homodimer formation (13–16). The bipartite nuclear localization signal (NLS) begins immediately C-terminal to the second ZF and continues through the first amino acid residues of the hinge region (17, 18). Besides binding to HREs and mediating the dimerization, the ZFRs of steroid receptors have also other less well characterized but nevertheless important functions. Unlike glucocorticoid receptor (GR) null mice, animals with a GR mutation that prevents dimerization and efficient DNA binding are viable, attesting to the importance in vivo of those GR activities that are independent of DNA binding (19). In human males, mutations in the AR ZFR can lead to either complete or partial androgen insensitivity syndrome (3, 20, 21). AR ZFR is important in transcriptional repression of AP1- and NF-kB-activated genes, and it is able to interact with the coactivator cAMP-response element-binding protein (CREB)-binding protein (22–25). In support to the role of ZFR/hinge as an interaction interface for heterologous proteins, several proteins capable of associating with ZFR and hinge region of nuclear receptors have been identified over the last few years (26–33).

Steroid receptors act together with other transcription factors in the regulation of target genes. Promoter specificity protein 1 (Sp1) is a ubiquitously expressed transcription factor that frequently works in concert with other sequence-specific transactivating proteins to control inducible promoters (34, 35). Functional and physical interactions between Sp1 and factors such as SREBP-1a (36), Stat1 (37), GATA-1 (38), estrogen receptor (39), progesterone receptor (40), and helicase-like transcription factor (41) often result in synergistic activation of specific target promoters, whereas association with promyelocytic leukemia gene product inhibits Sp1-mediated transcription (42). The Sp family of proteins, including Sp2, Sp3, and Sp4 in addition to Sp1, recognize GC-rich promoter sequences, the GC boxes, via their highly homologous C-terminal zinc finger regions (34, 35, 43–45). The N-terminal regions of Sp proteins contain transactivation domains rich in glutamine and serine/threonine residues, and an additional transactivation region in the C terminus (the D domain) is involved in Sp1 synergistic function (46). Sp3 also contains an inhibitory domain, the function of which is promoter- and cell line-dependent.

We recently identified a novel coactivator of AR-dependent transcription, a small nuclear RING finger protein termed SNURF, which interacts with the AR ZFR (27). SNURF also associates with some other steroid receptors and modulates...
their transacting functions. Besides steroid receptor-dependent promoters, SNURF also activates transcription from promoters driven by Sp1 elements/GC boxes. Interestingly, SNURF is able to bind to the TATA-binding protein, and it could thus act as a bridging factor between steroid receptors or other sequence-specific transcription factors and the general transcription machinery (27). To gain a better understanding of the functional domains of SNURF, we have examined the ability of mutated SNURF forms to act as transcriptional coactivators in the context of both androgen- and Sp1-regulated promoters. The requirements for interactions between SNURF and AR were investigated by using a panel of mutated AR forms.

**EXPERIMENTAL PROCEDURES**

**Materials**—Protease inhibitors aprotonin, leupeptin, pepstatin, and phenylmethylsulfonyl fluoride were obtained from Sigma. Testosterone was purchased from Makor Chemicals. Mouse monoclonal M2 anti-Flag antibody, mouse monoclonal anti-Lex antibody, rabbit polyclonal anti-Sp1 antibody, and horse radish peroxidase-conjugated anti-mouse IgG were obtained from Eastman Kodak Co., CLONTECH, Santa Cruz, and Zymed Laboratories Inc., respectively. AP1 and Sp1 oligonucleotides were from Promega.

**Plasmid Constructs**—To construct SNURF deletion mutants, a sequence encoding the Flag epitope was first inserted into pcDNA3.1 (+) vector, after which polymerase chain reaction-generated N-terminal and C-terminal SNURF fragments were ligated C-terminal to the Flag-tag. SNURF point mutants were generated by the overlapping polymerase chain reaction mutagenesis strategy and inserted into the same vector. pSG5-rAR expression vectors for rat (r) and human (h) AR have been described (23, 47). The AR deletion mutants Δ641–902, Δ46–408/Δ641–902, Δ149–295/Δ641–902, Δ40–147/Δ641–902, and rAR-C562G have been described previously (5, 48). The AR expression constructs R600G/K601A, ΔRRK (corresponding to the deletion of amino acids 629–633 in hAR and 612–616 in rAR sequence) and R600G/K601A/ΔRRK are pSOG derivatives of mutants 28.3, 28.1, and 28.31, which were kindly provided by Dr. A. O. Brinkmann (Erasmus University, Rotterdam, The Netherlands). The mutants rAR-G551V, rAR-C562G, rAR-K568A, rAR-A597T, rAR-R590Q, rAR-R597G, rAR-R590Q/ΔRRK, and rAR-R590Q/Y603C were generated by polymerase chain reaction. pCMV β and pBECMV were purchased from CLONTECH and Invitrogen, respectively. The minimal reporter constructs Sp12-TATA-LUC, pARE2-TATA-LUC, pARE2-Δ285-LUC, and the rat probasin promoter construct pPB-28S/12+329-LUC have been described (23, 27). Mammalian expression plasmids pEVr2-Sp1 and pReCMV-Sp3 were kindly provided by Dr. G. Suske (University of Marburg, Germany). Yeast vectors pVP16 for expressing the transactivation domain of herpes simplex virus VP 16 protein and pLexN-a for expressing bacterial Lex-a with the nuclear localization signal of the SV40 large T antigen were kind gifts from Dr. S. M. Hollenberg (Oregon Health Sciences University, Portland, OR). LexN-a AR constructs were generated by inserting polymerase chain reaction-amplified fragments coding for amino acids 537–627 of rat AR into the BamHl/SalI site of pLexN-a, and GST-ZFR fusion proteins were created by inserting the same fragments into BamHl/SalI site of pGEX-5X-1.

**Cell Culture and Transfections**—COS-1 and CV-1 cells were obtained from American Type Culture Collection and were maintained in Dulbecco’s modified Eagle’s medium containing 10% fetal bovine serum and 25 units/ml streptomycin and penicillin. For transient transfections using FuGene reagent (Roche Molecular Biochemicals), 3–3.5 × 10⁴ cells were seeded on 12-well plates 24 h before transfections. For transfections by the calcium phosphate precipitation method (47, 48), 1.5 × 10⁵ cells were plated on 10-cm dishes. Four h before the addition of DNA, the cells received fresh medium with 10% charcoal-stripped fetal bovine serum. After 18 h, the medium was changed to Dulbecco’s modified Eagle’s medium supplied with 2% charcoal-stripped fetal bovine serum and 100 µM testosterone or vehicle. Luciferase and β-galactosidase activities and the concentration of soluble cell proteins were assayed as described previously (47, 49). Statistical analyses were carried out with two-tailed Student’s t test.

**Immunoprecipitation and Immunoblotting**—COS-1 cell extracts were prepared in modified radioimmune precipitation buffer (50 mM Tris-HCl, pH 7.8, 150 mM NaCl, 5 mM EDTA, 0.5% Triton X-100, 0.5% Nonidet P-40, 0.1% sodium deoxycholate, 10 µg/ml aprotinin, 5 µg/ml leupeptin, 5 µg/ml pepstatin, and 0.5 mM phenylmethylsulfonyl fluoride). Immunoprecipitation with mouse monoclonal anti-Flag antibody was performed as described (27), and the samples were resolved on 7.5% SDS-polyacrylamide gel under denaturing conditions and subjected to immunoblotting with monoclonal M2 anti-Flag antibody. The samples are wild-type SNURF (lane 1), Δ31–65 (lane 2), Δ66–98 (lane 3), Δ69–118 (lane 4), Δ121–143 (lane 5), Δ121–143 (lane 6), Δ178–194 (lane 7), Δ157–194 (lane 8), SNURF-CS1 (lane 9), CS2 (lane 10), CS3 (lane 11), CS1Δ31–65 (lane 12), CS1Δ66–98 (lane 13), CS1Δ31–118 (lane 14), CS1Δ121–20 (lane 15), CS1Δ178–194 (lane 16), and CS1Δ157–194 (lane 17).

**FIG. 1. Activation of the Sp1 element-driven transcription is dependent on the RING finger structure of SNURF.** A, schematic structure of SNURF and its deletion mutants used in this study. A consensus bipartite NLS, the domain interacting with AR, and a C3HC4 zinc finger motif (RING) are depicted. The regions rich in positively and negatively charged amino acids are identified by + and −, respectively. Arrowheads depict the locations of cysteine to serine mutations in the RING finger. In SNURF-CS1, the first two cysteines (136 and 139) of the RING finger are substituted with serine; in SNURF-CS2, the last two cysteines (177 and 180) of the RING finger are substituted; and in SNURF-CS3, CS1 and CS2 mutations are combined. B, COS-1 cells on 12-well plates were transfected using FuGene reagent with 165 ng of Sp1-TATA-LUC reporter, 65 ng of pCMV β, and 300 ng of a SNURF expression plasmid. The reporter gene activity in the absence of ectopic SNURF expression was set as 1. The mean ± S.E. values of at least six experiments are shown. C, immunoblot analysis of SNURF mutants. COS-1 cells were transfected with 500 ng of expression plasmid and 50 ng of pCMV β (to control for transfection efficiency) using FuGene reagent. Twenty µg of cell extracts were resolved on 10% polyacrylamide gel under denaturing conditions and subjected to immunoblotting with monoclonal M2 anti-Flag antibody. The samples are wild-type SNURF (lane 1), Δ31–65 (lane 2), Δ66–98 (lane 3), Δ69–118 (lane 4), Δ121–143 (lane 5), Δ121–143 (lane 6), Δ178–194 (lane 7), Δ157–194 (lane 8), SNURF-CS1 (lane 9), CS2 (lane 10), CS3 (lane 11), CS1Δ31–65 (lane 12), CS1Δ66–98 (lane 13), CS1Δ31–118 (lane 14), CS1Δ121–20 (lane 15), CS1Δ178–194 (lane 16), and CS1Δ157–194 (lane 17).
Protein-Protein Interaction in Vitro—GST and GST-ZFR mutants expressed in E. coli were purified by affinity chromatography on glutathione-Sepharose 4B (Amersham Pharmacia Biotech) essentially as described (22) in a buffer containing 50 mM Tris-HCl, pH 7.8, 150 mM KCl, 10% glycerol, 0.5 mM EDTA, 0.1% Nonidet P-40, 5 mM MgCl₂, 50 μM ZnCl₂, 0.5 mM phenylmethylsulfonfyl fluoride, 2 μg/ml aprotinin, and 3 μg/ml pepstatin A. Fusion proteins (5 μg/20 μl glutathione-Sepharose 4B beads) were incubated with 5 μl of [³²P]methionine-labeled SNURF or luciferase synthesized by translation in vitro for 60 min at 4 °C in 500 μl of binding buffer (50 μM Tris-HCl, pH 7.8, 50 mM KCl, 10% glycerol, 0.5 mM EDTA, 0.4% Nonidet P-40, 0.1% Triton X-100, 5 mM MgCl₂, and 50 μM ZnCl₂) in the presence of protease inhibitors. The beads were then washed four times with 1 ml of binding buffer. Bound proteins were released in an SDS-polyacrylamide gel electrophoresis sample buffer at 95 °C for 4 min, resolved by electrophoresis under denaturing conditions, and visualized by fluorography.

Electrophoretic Mobility Shift Assay (EMSA) — EMSAs were performed as previously outlined (50). Briefly, whole cell extracts from COS-1 cells transfected transiently or by electroporation or using a vector (lanes 1–3) contained SNURF (lanes 7–9) or SNURF-CS1 (lanes 12–14) were incubated with 32P-labeled oligonucleotide containing C3(1)-ARE and analyzed by EMSA. Before adding the probe, a 200-fold molar excess of unlabeled AP1 oligonucleotide (lane 10) or Sp1 oligonucleotide (lane 11) was added. To identify the Sp1-DNA complex, ERK-1 antibody (NS ab, lane 12) or Sp1 antibody (lane 13) was included in the preincubation reaction. The asterisk depicts the Sp1 complex that disappeared upon the addition of anti-Sp1 antibody, and the supershifted Sp1-DNA antibody complex is indicated by an arrow. wild type. B, ectopic expression of SNURF does not influence the amount of immunoreactive Sp1 protein in COS-1 cells. Indicated amounts of cell extracts used in the EMSA were subjected to immunoblotting by an anti-Sp1 antibody.

**RESULTS**

The RING Finger Region of SNURF Is Necessary for Stimulation of Steroid-independent Minimal Promoters — To map the functional domains of SNURF, a series of deletion and point mutants was generated (Fig. 1A). Cotransfection of COS-1 cells with a construct encoding wild-type SNURF along with the reporter Sp1-2-TATA-LUC, which contains two binding sites for Sp1 in front of the TATA box, activated the promoter by 40-fold (Fig. 1B). Deletion of the N-terminal 20 amino acids containing a part of the consensus bipartite NLS (mutant A1–20) did not prevent SNURF from entering the nucleus (data not shown) but blunted its ability to stimulate reporter gene transcription (p < 0.01). Deletion of amino acids 66–98 encompassing the supershifted Sp1-DNA antibody complex is indicated by an arrow.

wt, wild type. A, indicated amounts (μg) of proteins from extracts of COS-1 cells transfected with an empty expression vector (lanes 1–3, and 10–13) or vectors encoding SNURF (lanes 4–6) or SNURF-CS1 (lanes 7–9) were incubated with 32P-labeled double-stranded oligonucleotide containing a consensus Sp1 element and analyzed by EMSA. Before adding the probe, a 200-fold molar excess of unlabeled AP1 oligonucleotide (lane 10) or Sp1 oligonucleotide (lane 11) was added. To identify the Sp1-DNA complex, ERK-1 antibody (NS ab, lane 12) or Sp1 antibody (lane 13) was included in the preincubation reaction. The asterisk depicts the Sp1 complex that disappeared upon the addition of anti-Sp1 antibody, and polycrylamide gels under denaturing conditions. Proteins were transferred onto Hybond ECL nitrocellulose membrane (Amersham Pharmacia Biotech) and visualized by using the ECL detection reagents (Amersham Pharmacia Biotech) according to the manufacturer’s instructions. Immunoblots on yeast cell extracts were performed as described previously (27).
ing a region with positively charged residues also severely attenuated the activation of the reporter gene (p < 0.01), whereas deletions of negatively charged regions (∆31–65 and ∆99–118) only weakly influenced the function of SNURF. Any insult in the RING finger domain totally abrogated the ability of SNURF to stimulate transcription from the Sp12-TATA-LUC reporter (mutants ∆121–143, ∆178–194, ∆157–194, CS1 (C136S/C139S), CS2 (C177S/C180S), and CS3 (C136S/C139S/ C177S/C180S)). Similar results were obtained with the AP1-Sp1-TATA-LUC reporter (27).2

SNURF Enhances Protein Binding to GC Box Motifs—To elucidate the mechanism(s) underlying SNURF-mediated stimulation of the Sp1-regulated reporter, we examined by using EMSA the possibility that SNURF influences protein binding to Sp1 elements. Extracts from COS-1 cells transfected with an empty expression vector or plasmids encoding SNURF or the RING finger-deficient SNURF-CS1 mutant were incubated with 32P-labeled double-stranded Sp1 oligonucleotide. Extracts from cells overexpressing wild-type SNURF but not the SNURF-CS1 mutant showed significantly increased binding of proteins to the Sp1 element (Fig. 2A, lanes 1–9). All detected protein-DNA complexes were specific for the Sp1 element, as they were fully competed for by a 200-fold molar excess of cold Sp1 oligonucleotide but not by the same amount of AP1 oligonucleotide (lanes 10 and 11). Immunoblotting of the same cell extracts with an Sp1-specific antibody ruled out the possibility that expression of SNURF would have increased the amount of cellular Sp1 protein (Fig. 2B). To identify Sp1 protein among the protein-DNA complexes, the cell extract was preincubated with an anti-Sp1 antibody, which resulted in the disappearance of the uppermost band and appearance of a supershifted complex (Fig. 2A, lane 12). The bands unaffected by the Sp1 antibody probably represent other GC box-binding proteins such as Sp3 and/or Sp4. SNURF stabilized DNA binding of these proteins as well, but not to the same extent as that of Sp1 (cf. Fig. 2A, lanes 5 and 6 versus lane 3). The effect of SNURF appeared to be specific for GC box-binding proteins, as SNURF overexpression did not influence AR-ARE interaction or the amount of proteins binding to an AP1 element (Fig. 2, C and D).

SNURF Associates with Sp1 in COS-1 Cells—We performed coimmunoprecipitation assays on extracts from COS-1 cells expressing Flag-tagged SNURF or SNURF-CS1 to assess whether SNURF and Sp1 interact physically. Protein complexes precipitated by a monoclonal anti-Flag antibody were subjected to immunoblotting with an anti-Sp1 antibody. The same extracts were subjected to immunoblotting with anti-Sp1 antibody (B) and with M2 anti-Flag antibody (C). WT, wild type.

SNURF Cooperates with Sp1—To study the role of SNURF in Sp1-mediated transcription further, COS-1 cells were cotransfected with the Sp12-TATA-LUC reporter along with Sp1 and/or SNURF expression vectors. Coexpressed Sp1 stimulated the reporter gene activity only minimally over the activity seen with endogenous Sp1 (Fig. 4). However, a strong synergistic activation was observed when SNURF was cotransfected with Sp1 (p < 0.05). It is of note that the amount of SNURF expression plasmid used in this experiment (100 ng/well) yielded only ∼10% of the maximal activity obtained with a saturating SNURF concentration (300 ng/well, see Fig. 1B). Ectopic expression of Sp3, an inhibitory member of the Sp1 family (44), slightly diminished the reporter activity achieved with SNURF or SNURF together with Sp1, and no synergism between Sp3 and SNURF was observed (Fig. 4). The RING finger of SNURF was necessary for the synergistic activation to occur, as the SNURF-CS1 mutant was unable to cooperate with coexpressed Sp1. The enhancement of reporter gene activity was dependent on the Sp1-binding sites on the promoter, since a reporter construct devoid of these sites did not respond to ectopically expressed Sp1.2

SNURF Domains in the Stimulation of AR-mediated Transcription and Cooperation between AR and Sp1—Wild-type SNURF activates AR-mediated transcription more efficiently than the CS1 mutant, but it also enhances the activity of reporter genes in the absence of hormone. SNURF-CS1 exhibits ∼50–80% of the wild-type SNURF activity without influencing the basal activity of androgen-regulated promoters (27). To examine the importance of various SNURF regions in the enhancement of AR-dependent transactivation, we transfected COS-1 cells with pARE2-TATA-LUC and pSG5-rAR along with vectors encoding SNURF deletion mutants (Fig. 5A). To eliminate the influence of SNURF on basal reporter activity, the CS1 mutant was used as a reference, and the CS1 mutation

2 H. Poukka, P. Aarnisalo, J. J. Palvimo, and O. A. Jänne, unpublished observations.
Structural and Functional Analysis of SNURF

Fig. 5. RING finger-mutated SNURF proteins function as coregulators of AR-mediated transcription. A, COS-1 cells were transfected by using FuGene reagent with 165 ng of pARE₂-TATA-LUC, 65 ng of pCMVβ, 15 ng of pSG5-RAR, 45 ng of SNURF-CS1 deletion mutants, and 270 ng of empty pcDNA3.1(+) vector. The reporter gene activity measured with the expression of AR in the presence of 100 nM testosterone (T) was set as 100. B, the experimental conditions were as in panel A, except that CV-1 cells were used. C, COS-1 cells were transfected with 150 ng of pARE₂-tk-LUC reporter, 50 ng of pCMVβ, and 10 ng of pSG5-hAR. Sp1, SNURF, and SNURF-CS1 expression vectors (150 ng each) were cotransfected as depicted, and the total DNA amount was adjusted to 510 ng with the corresponding empty plasmids when appropriate. The cells were treated with 100 nM testosterone as indicated. The reporter gene activity in the absence of testosterone and without SNURF and Sp1 expression vectors was set as 100. The mean ± S.E. values of at least five experiments are shown. D, the experimental conditions were as in panel C, except that CV-1 cells were used.

(C1365/C1398) was included in all the deletion constructs shown in Fig. 5. None of the mutants studied activated the reporter gene in the absence of AR or testosterone (data not shown). Interestingly, the loss of the N-terminal 20 amino acids, a region rich in positively charged residues, and deletion of amino acids 31–65, a negatively charged region, abolished the ability of SNURF to enhance AR-dependent transactivation (p < 0.01). Deletion of another region containing a stretch of positively charged residues (CS1Δ66–98) also resulted in a marked reduction in the coactivator function (p < 0.01), whereas the more C-terminal stretch of negatively charged residues (CS1Δ99–118) had a smaller effect on AR function. When the same N-terminal deletions of SNURF were studied in CV-1 cells, a similar pattern was seen (Fig. 5B). Deletions affecting the RING finger structure tended to diminish the activity of SNURF in both cell lines, whereas point mutations of the two most C-terminal cysteines (CS2) were tolerated in CV-1 cells but, surprisingly, not in COS-1 cells (Fig. 5), suggesting that cell-specific factors are involved in the RING finger interactions.

To investigate the ability of SNURF to integrate AR- and Sp1-mediated transcriptional responses, pARE₂-tk-LUC reporter containing two AREs in front of the herpes simplex virus thymidine kinase proximal promoter (−105 to +51) was transfected to COS-1 cells (Fig. 5C). This portion of herpes simplex virus thymidine kinase promoter contains two GC/GT box sites that are separated by 50 nucleotides. The experiments were carried out in the presence of AR expression, both without and with 100 nM testosterone. Ectopic expression of Sp1 stimulated the reporter gene activity almost 2-fold, and wild-type SNURF and the addition of androgen increased it by 3.8- and 3.3-fold, respectively. There was a more than additive response of pARE₂-tk-LUC to coexpression Sp1 with SNURF in the presence of AR without androgen (−6.7-fold compared with AR alone, p < 0.01). In the presence of androgen, ectopic expression of Sp1 more than doubled the activity of the reporter over that achieved by ligand-activated AR alone. Likewise, coexpression of AR and Sp1 together with SNURF resulted in a greater reporter gene activation than could be produced if one of the factors was omitted, suggesting that SNURF is able to enhance the synergistic activity of AR and Sp1. Furthermore, with the SNURF-CS1 mutant, the functional cooperativity was significantly impaired (p < 0.05) from that with wild-type SNURF, supporting a role for the RING finger in the synergistic response. Very similar results were obtained when CV-1 cells instead of COS-1 cells were used (Fig. 5D).

Mutations in the ZFR and the Hinge Region of AR Weaken the Interaction with SNURF—To identify the residues in the zinc finger and hinge regions of AR important in forming the interaction interface for SNURF, we performed yeast two-hybrid assays by using a panel of AR ZFR/hinge mutants fused to the Lex DNA binding domain together with SNURF (amino acid residues 21–177) tethered to the transcriptional activation domain of VP16 protein. This region of SNURF has previously been shown to encompass the interface for AR interaction (27). The schematic structure of AR ZFR and the mutations studied are illustrated in Fig. 6A. Point mutations in the first and second ZF had varying effects on the recognition of the ZFR by SNURF, and several of the mutations did not markedly impair the interaction. The human AR G568V substitution at the tip of the first ZF causes a partial androgen insensitivity syndrome (51), and the corresponding rat mutation (G551V) rendered the ZF/hinge unresponsive to SNURF (Fig. 6B). A point mutation R590Q (R607Q in hAR) in the tip of the second ZF, associated with male breast cancer and partial androgen insensitivity syndrome (52–54), also weakened the SNURF-AR interaction (p < 0.01). When an additional Y603C substitution was introduced into the sequence (R590Q/Y603C), further attenuation in the interaction was observed. The mutation of the most C-terminal zinc-coordinating cysteine of the second zinc finger (C597G) completely abolished the SNURF-AR interaction. Deletion of the C-terminal part of the bipartite NLS in the mutant...
AR ZFR Mutations Attenuate the Transcriptional Response to SNURF—

To elucidate the importance of the mutations in AR ZFR and hinge region analysis, GST pull-down experiments were performed to corroborate the yeast two-hybrid data. As shown in Fig. 7A, in vitro translated SNURF bound efficiently to wild-type AR ZFR. The binding was specific, as SNURF was not retained by GST alone, and control protein (luciferase) did not bind to GST-AR ZFR. In contrast to wild-type ZFR, the mutants ΔRKLKK and R600G/K601A/ΔRKLKK showed very weak affinity for SNURF in vitro. Also point mutations in the first (G551V) and the second (R590Q) ZF clearly reduced SNURF binding. These results are in line with the yeast two-hybrid data, supporting the notion that the yeast two-hybrid assay reflects direct physical interaction between AR ZFR and SNURF. Collectively, our results indicate that certain AR ZF and hinge region mutations can considerably weaken SNURF-ZFR interaction.

AR ZFR Mutations Attenuate the Transcriptional Response to SNURF—

To elucidate the importance of the mutations in SNURF with selected AR ZFR mutants were performed to corroborate the yeast two-hybrid data. As shown in Fig. 7A, in vitro translated SNURF bound efficiently to wild-type AR ZFR. The binding was specific, as SNURF was not retained by GST alone, and control protein (luciferase) did not bind to GST-AR ZFR. In contrast to wild-type ZFR, the mutants ΔRKLKK and R600G/K601A/ΔRKLKK showed very weak affinity for SNURF in vitro. Also point mutations in the first (G551V) and the second (R590Q) ZF clearly reduced SNURF binding. These results are in line with the yeast two-hybrid data, supporting the notion that the yeast two-hybrid assay reflects direct physical interaction between AR ZFR and SNURF. Collectively, our results indicate that certain AR ZF and hinge region mutations can considerably weaken SNURF-ZFR interaction.

Fig. 6. Interaction of SNURF and AR in yeast is affected by mutations in the AR ZFR and hinge region. A, schematic representation of the rat AR ZFR including 20 residues of the hinge region (amino acids 537–627) used as the target sequence in yeast two-hybrid assays. The P-box involved in sequence-specific DNA binding is indicated as light gray box, and the D-box participating in the dimerization is depicted by cross-hatching. The bipartite NLS is underlined. Mutations analyzed in this study are indicated by arrowheads, and the substituting amino acids are shown. TAD, transcription activation domain. B, two-hybrid analysis of the ZFR and the hinge region with SNURF (amino acids 21–177) in yeast cells. The AR mutants were cloned into LexN-a fusion protein expression vector and transformed into S. cerevisiae strain L40 together with plasmids encoding for VP16 AD or VP16 AD fused to SNURF. b-Galactosidase activities were assayed from liquid cultures in three separate experiments, and the activity of yeast transformed with wild-type AR ZFR/hinge construct (LexN-a-ZFR) together with VP16-SNURF was set as 100. The numbers in parentheses depict fold enhancement of SNURF interactions; the activity of SNURF binding in the presence of LexN-a and VP16-SNURF was set as 1. With VP16 alone, the mutated ARs exhibited the same background activity as wild-type AR. C, immunoblot analysis of LexN-a fusion protein constructs was performed as described under “Experimental Procedures.” The samples are LexN-a (lane 1), ZFR (lane 2), G551V (lane 3), C562G (lane 4), R563A (lane 5), A579T (lane 6), C597G (lane 7), R590Q (lane 8), R600G/K601A/ΔRKLKK (lane 9), R600G (lane 10), ΔRKLKK (lane 11), and R590Q/R600G (lane 12).
mediated transactivation and, even more efficiently, transcription from promoters driven by Sp1 elements/GC box motifs. However, SNURF does not appear to possess an intrinsic transcription activation function (27). The C-terminal region of SNURF harbors a consensus sequence for a C3HC4-type zinc finger, the so-called RING finger, which is present in several transcriptional regulatory proteins and has been proposed to participate in protein-protein interactions (55). Interestingly, the RING finger region of MSL2 protein has been shown to possess multiple interaction surfaces, as mutations in the coordination sites for the first (CS1) and the second zinc (CS2) atoms have distinct effects on its interactions and functions (56). Our results suggest that similar to MSL2, the RING finger region of SNURF may not act as a single functional unit, as mutations in the first and the second zinc coordination sites had dissimilar consequences in COS-1 and CV-1 cells.

An interaction between SNURF and Sp1 was detected by communoprecipitation, and this association was strictly dependent on the intact RING finger. SNURF enhanced the transcriptional activity of Sp1 in a synergistic and RING finger-dependent fashion. In accordance with this, SNURF, but not its RING finger mutated form, enhanced the DNA binding activity of GC box-recognizing proteins, especially that of Sp1. This effect was selective for proteins interacting with GC box motifs, as binding of AP1 components or AR was not affected. However, when purified recombinant Sp1 and SNURF proteins were examined by EMSA using an Sp1-binding site oligonucleotide, no clear stimulation of Sp1 DNA binding by SNURF was observed, suggesting that a third partner present in the cell lysate or a post-synthetic modification is involved in the process.

SNURF is highly hydrophilic, and about 30% of its residues are charged. These residues are asymmetrically distributed, possessing potential for strong electrostatic interactions. These features resemble the electrostatic properties of Ubc9, another relatively small nuclear protein that it capable of modulating AR function via interacting with the ZFR/hinge region (57). On the Sp1-regulated minimal promoter, the role of the N-terminal negatively charged regions of SNURF appears to be weakly modulatory, as deletions of these stretches (∆31–65 and ∆99–118) did not cause profound effects. By contrast, the loss of the first 20 amino acids harboring most of the bipartite putative NLS or the deletion of another cluster of basic residues in the ∆66–98 mutant resulted in severely impaired transcriptional responses.

In contrast to Sp1-dependent transcription, stimulation of AR-mediated transactivation was not dependent on the N-terminal part of RING finger domain in SNURF. Loss of a cluster of negatively amino acid residues (CS1∆31–65) was a destructive deletion for the AR coactivator function, whereas this acidic region appeared to have only a small effect on Sp1 function. However, similar to Sp1, deletion of positively charged clusters in SNURF sequence (mutants CS1∆1–20 and CS1∆66–98) also strongly compromised the activity of SNURF on AR. Even though the AR ZFR and the hinge region are sufficient for the interaction of SNURF with AR (27), other regions of the receptor have auxiliary roles; deletion of the core AF-1 region of AR (residues 149–295) attenuates the response to SNURF, and a larger deletion (∆46–408) abolishes it completely. Thus, SNURF cannot substitute for the principal N-terminal transactivation region of AR.

Certain point mutations in both the first and the second ZF, such as G551V and C597G, severely impaired the interaction of

---

**DISCUSSION**

We have investigated the structural and functional characteristics of a recently identified nuclear protein, SNURF. The protein was discovered in a yeast two-hybrid screen by virtue of its ability to interact with the AR ZFR/hinge region (27). Ectopically expressed SNURF stimulates both steroid receptor-
AR with SNURF. The G551V substitution, situated in the nonhelical region of ZFR and next to the residues making contact with the phosphate backbone, as predicted for the structure of AR ZFR from the crystal structure of GR ZFR (14), results in impaired DNA binding and transactivation by the receptor (25). The R590Q substitution, situated in the beginning of the second α-helix of ZFR and in a close proximity of the dimer interface, impaired the AR-SNURF interaction and attenuated the response to cotransfected SNURF in mammalian cells. Also the C-terminal part of the bipartite nuclear localization signal in the hinge region (deleted in the mutant ΔRRLLK), which corresponds to a disordered area in the GR ZFR crystal structure, appears to be important for AR-SNURF interaction. The latter mutation also weakened the stimulation of AR transactivation by SNURF in the context of minimal ARE-containing promoter and abolished the response entirely when the natural AR-regulated rat probasin promoter was used. Even though the bipartite NLS region of AR is involved in AR-SNURF interaction, the coregulatory role of SNURF is probably not linked to nuclear transport of AR, since ectopic SNURF expression does not increase the amount of nuclear AR protein in mammalian cells (27).

The DNA binding-independent role of steroid receptor ZFRs in transcriptional regulation has gained more attention during the last few years, as these domains have been shown to exhibit the potential for multiple protein interactions. Concomitantly, the importance of the adjacent hinge region has evolved from a passive spacer element to an active participant in making contacts with auxiliary proteins. The ZFR and hinge region are involved in interactions with corepressors SMRT and N-CoR (26, 58), as well as in contacts with octamer transcription factors 1 and 2 (Oct-1/2), Brm-3a/3b, and several novel coregulators (26–33, 57). The histone acetylase PCAF that associates with coactivators CREB-binding protein and p300 was demonstrated to bind directly to the ZFRs of nuclear receptors (59), suggesting that coactivators interacting with distinct domains of nuclear receptors can participate in the formation of common protein complexes. Interestingly, N-CoR binding to the aporetinoid acid receptor a hinge region prevents PCAF association, suggesting that this coactivator either sterically blocks the access of PCAF to the ZFR or induces a conformation that does not allow the ZFR-PCAФ contacts. SNURF may play an analogous role in that it may act in concert with other coactivators and activate steroid receptor-mediated transcription by preventing corepressor binding to the hinge region. Recent results have demonstrated that nuclear receptors and Sp1 can utilize common coactivator complexes (60–63).

In conclusion, the results of the present work indicate that SNURF, a coregulator recognizing the ZFR/hinge region of AR and other steroid receptors (27), participates in the Sp1-dependent transcription and cooperates with AR and Sp1, suggesting a novel transcriptional link between the function of Sp1 and steroid receptors. Identification of other interaction partners of SNURF and genetic studies are, however, needed for better understanding the role of this protein in transcriptional regulation.

Acknowledgments—Excellent technical assistance of Kati Saastamoine, Leena Pietilä, and Pirjo Kilpiö is gratefully acknowledged. We thank Drs. A. O. Brinkmann, G. Suske, and S. M. Hombell for plasmids and materials, Karolina Halmesmaki for her help in the construction of SNURF deletion mutants, and Dr. Anu-Maarit Moilanen for collaboration in the initial phase of the project.

REFERENCES

1. Mangelsdorf, D. J., Thommler, C., Beato, M., Herrlich, P., Schultz, G., Umesono, K., Blumberg, B., Kastner, P., Mark, M., Chambon, P., and Evans, R. M. (1995) Cell 83, 835–839
2. Beato, M., Herrlich, P., and Schultz, G. (1995) Cell 83, 851–857
3. Truss, M., and Beato, M. (1993) Endocrinol. Rev. 14, 459–479
54. Weidemann, W., Peters, B., Romalo, G., Spindler, K.-D., and Schweikert, H.-U. (1998) J. Clin. Endocrinol. Metab. 3, 1173–1176
55. Saurin, A. J., Borden, K. L. B., and Boddy, M. N., and Freemont, P. S. (1996) Trends Biochem. Sci. 21, 208–214
56. Copps, K., Richman, R., Lyman, L. M., Kimberly, A. C., Rampersad-Ammons, J., and Kuroda, M. I. (1996) EMBO J. 17, 5409–5417
57. Poukka, H., Aarnisalo, P., Karvonen, U., Palvimo, J. J., and Ja¨nne O. A. (1999) J. Biol. Chem. 274, 19441–19446
58. Safer, J. D., Cohen, R. N., Hollenberg, A. N., and Wondisford, F. E. (1998) J. Biol. Chem. 273, 30175–30182
59. Blanco, J. C. G., Minucci, S., Lu, J., Yang, X.-J., Walker, K. K., Chen, H., Evans, R. M., Nakatani, Y., and Ozato, K. (1998) Genes Dev. 12, 1638–1651
60. Ito, M., Yuan, C. X., Malik, S., Gu, W., Fondell, J. D., Yamamura, S., Fu, Z. Y., Zhang, X., Qin, J., and Roeder, R. G. (1999) Mol. Cell 3, 361–370
61. Na¨ar, A. M., Beaurang, P. A., Zhou, S., Abraham, S., Solomon, W., and Tjian, R. (1999) Nature 398, 828–832
62. Rachez, C., Lemon, B. D., Saldan, Z., Bromleigh, V., Gamble, M., Na¨ar, A. M., Erdjument-Bromage, H., Tempst, P., and Freedman, L. P. (1999) Nature 398, 824–828
63. Ryu, S., Zhou, S., Ladurner, A. G., and Tjian, R. (1999) Nature 397, 446–450