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GRIP1, a Transcriptional Coactivator for the AF-2 Transactivation Domain of Steroid, Thyroid, Retinoid, and Vitamin D Receptors

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After binding to enhancer elements, transcription factors require transcriptional coactivator proteins to mediate their stimulation of transcription initiation. A search for possible coactivators for steroid hormone receptors resulted in identification of glucocorticoid receptor interacting protein 1 (GRIP1). The complete coding sequence for GRIP1, isolated from a mouse brain cDNA library, contains an open reading frame of 1,462 codons. GRIP1 is the probable ortholog of the subsequently identified human protein transcription intermediary factor 2 (TIF2) and is also partially homologous to steroid receptor coactivator 1 (SRC-1). The full-length GRIP1 interacted with the hormone binding domains (HBDs) of all five steroid receptors in a hormone-dependent manner and also with HBDs of class II nuclear receptors, including thyroid receptor α, vitamin D receptor, retinoic acid receptor α, and retinoid X receptor α. In contrast to agonists, glucocorticoid antagonists did not promote interaction between the glucocorticoid receptor and GRIP1. In yeast cells, GRIP1 dramatically enhanced the transcriptional activation function of proteins containing the HBDs of any of the above-named receptors fused to the GAL4 DNA binding domain and thus served as a transcriptional coactivator for them. This finding contrasts with previous reports of TIF2 and SRC-1, which in mammalian cells enhanced the transactivation activities of only a subset of the steroid and nuclear receptors that they physically interacted with. GRIP1 also enhanced the hormone-dependent transactivation activity of intact glucocorticoid receptor, estrogen receptor, and mineralocorticoid receptor. Experiments with glucocorticoid receptor truncation and point mutants indicated that GRIP1 interacted with and enhanced the activity of the C-terminal AF-2 but not the N-terminal AF-1 transactivation domain of the glucocorticoid receptor. These results demonstrate directly that AF-1 and AF-2 domains accomplish their transactivation activities through different mechanisms: AF-2 requires GRIP1 as a coactivator, but AF-1 does not.

Nuclear hormone receptors (NRs) are conditional transcription factors that play important roles in various aspects of cell growth, development, and homeostasis by controlling expression of specific genes (4, 15, 29, 43). Members of the NR superfamily, which includes the five steroid receptors (SRs) as well as the receptors for thyroid hormone (TR), retinoic acid (RAR), and vitamin D (VDR), are structurally characterized by three distinct domains: an N-terminal transcriptional activation domain (AD), a central DNA binding domain (DBD), and a C-terminal hormone binding domain (HBD). Before the binding of hormone, SRs, which are sometimes called class I of the NR family, remain inactive in a complex with hsp90 and other stress family proteins. The binding of hormone induces critical conformational changes in SRs that cause them to dissociate from the inhibitory complex, bind as homodimers to specific DNA enhancer elements associated with target genes, and modulate their transcription (43). Thyroid, retinoid, and vitamin D receptors, which belong to class II of the NR family, do not associate stably with the heat shock protein complex in the absence of their cognate ligands; rather, they are already associated with their cognate hormone response elements, either as homodimers or as heterodimers with retinoid X receptors (RXR), and in this unliganded state they may cause transcriptional repression. Binding of cognate ligands induces conformational changes that usually convert the NR dimer to a transcriptional activator (29). The mechanism by which NRs regulate the efficiency of transcriptional initiation is currently under intensive investigation. The ligand-activated, enhancer-bound NRs may stabilize or promote formation of the preinitiation complex of basal transcription factors for RNA polymerase II on the nearby promoter (43). These effects may be transmitted partly by direct interactions between NRs and basal transcription factors; such interactions have been demonstrated (3, 22, 38, 41), but their functional relevance is still under investigation. In addition, NR effects on the transcription initiation complex may be transmitted through indirect interactions, mediated by intermediary proteins called transcriptional coactivators.

In addition to RNA polymerase II, seven basal transcription factors are required to form a preinitiation complex: TFIIA, -B, -E, -F, -H, and -J and TATA binding protein, which is the DNA binding subunit of TFIID. While the basal transcription factors are sufficient for basal-level transcription, enhancement of transcription by transcriptional activator proteins bound to enhancer elements requires the presence of additional mediator proteins, known as transcriptional coactivators (18). A coactivator is neither a member of the basal transcription apparatus nor a transcriptional activator by itself, since it lacks a DBD. Coactivators are defined functionally by their ability to enhance selectively the stimulatory activity of specific subsets of enhancer binding transcriptional activators. Among the best-characterized transcriptional coactivators are TAFs (TATA binding protein-associated factors), which are subunits of TFIID and now believed to be the direct contacts and functional mediators for many enhancer-bound activators.

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TAFs apparently function as a bridge between enhancer-bound transcriptional activators and the basal transcription factors, and different TAFs mediate the activities of different transcriptional activators (10, 18, 39). NRs have been shown to interact with specific TAFs (23, 41), but no functional consequences of these interactions have been demonstrated. Transcriptional coactivators that are not associated with TFIID have also been identified for NRs (20, 35, 45, 48), as discussed below, and for other transcriptional activators (5, 26, 42).

SRs and class II NRs have two distinct transactivation domains: AF-1, located in the N-terminal AD, and AF-2, located in the HBD (19, 27, 28). While each of these AF domains can exhibit transactivation activity independently, in most cases both AF domains are required for full transcriptional activation by SRs. Differences in the promoter and cell type specificities of AF-1 and AF-2 and the fact that some partial agonists activate AF-1 but not AF-2 suggest that these two transactivation domains may function through different mechanisms (31, 44); i.e., each may interact with a different component of the transcription initiation complex, and/or each may need a different coactivator to mediate its transactivation activities. Little is known about the transactivation mechanism of the AF-1 domain; however, several possible transcriptional coactivators for the C-terminal AF-2 domain of NRs have been reported recently. While all of these proteins interact specifically and in a ligand-dependent manner with the HBDs of NRs (21), thus far only steroid receptor coactivator 1 (SRC-1), glucocorticoid receptor-interacting protein 1 (GRIP1), transcriptional intermediary factor 2 (TIF2), and androgen receptor-associated protein (ARA_{70}) have also exhibited a marked ability to enhance the transactivation activity of NRs (20, 35, 45, 48). GRIP1 proved to be partially homologous to SRC-1, and the subsequently identified TIF2 was found to be the probable human ortholog of mouse GRIP1. In addition to these putative coactivators, several putative corepressors have been shown to interact with some unliganded class II NRs and mediate the transcriptional repression exerted by these NRs in the absence of hormone (21). The ability of coactivators to bind specifically to NRs is presumably essential for their ability to enhance the transactivation function of NRs. Thus, it is surprising that in mammalian cells, TIF2 and SRC-1 strongly enhanced the activities of only a subset of the SRs and class II NRs with which they physically interacted, and the magnitudes of the observed enhancements by the coactivators were modest for most NRs (35, 45). The lack of correlation between physical interaction and coactivator activities may be due to the presence of endogenous coactivators and/or corepressors in mammalian cells (21, 35, 45), and it indicates the need for an experimental system that lacks the complications of endogenous coregulators.

We previously reported the isolation of a partial cDNA clone that codes for an 800-amino-acid fragment of a novel protein, GRIP1, from a mouse 17-day embryo cDNA library (20). This GRIP1 protein fragment interacted with the HBDs of the glucocorticoid receptor (GR), estrogen receptor (ER), and androgen receptor (AR) in a hormone-dependent manner. Furthermore, in the yeast *Saccharomyces cerevisiae*, the GRIP1 fragment dramatically potentiated the transcriptional activation activity of fusion proteins consisting of the GAL4 DBD and the HBD of any of these three SRs. Here we report the full-length coding sequence for GRIP1 and its ability to interact with and serve as a coactivator for the HBDs of all five SRs as well as class II NRs. SR-GRIP1 interaction was induced by SR agonists but not by antagonists. We also demonstrated the ability of GRIP1 to act as a transcriptional coactivator for intact SRs. The results of GRIP1 interaction and coactivator tests with individual AF-1 and AF-2 domains provides a molecular explanation for GRIP1's effect on the intact SRs and for the different transactivation mechanisms of the AF-1 and AF-2 domains. The ability of GRIP1 in our yeast system to enhance dramatically the transcriptional activation function of all of the NR HBD that it can physically interact with contrasts with the previous studies performed in mammalian cells with TIF2 (45) and SRC-1 (35), as described above. These contrasting results indicate the potential utility of the yeast system for studying NR AF-2 coactivators in an environment devoid of confounding endogenous coactivators and corepressors.

MATERIALS AND METHODS

Construction of plasmids. The yeast expression vector for the full-length GRIP1, pGRIP1/fl, was constructed by first using PCR to create an *Eco*RI site at nucleotide −9 relative to the first potential ATG initiation codon of the GRIP1 open reading frame and then inserting the resulting 4.7-kb *Eco*RI fragment containing the entire open reading frame into the new *Eco*RI site of a modified pGAD424 (Clontech) vector. This modified pGAD424 vector was created by replacing the *Hin*dIII fragment (containing the GAL4 AD and multiple cloning site) with AGCTTGGATCCCGGGAATTCTCG. To construct the yeast expression vector coding for an AD_{GAL4}-GRIP1/fl fusion protein, named pGAD424.GRIP1/fl, an *Eco*RI site was created in GRIP1 codon 5, and the *Eco*RI fragment coding for $GRIP1_{5-1462}$ was inserted in the proper reading frame in pGAD424. pGBT9.GRIP1/fl, coding for DBD_{GAL4}-GRIP1, was made by subcloning the same $GRIP1_{5-1462}$ coding fragment into the *Eco*RI site of pGBT9 (Clontech). pGBT9.HBD_{GR}, pGBT9.HBD_{ER}, and pGBT9.HBD_{AR}, yeast vectors coding for fusion proteins of the GAL4 DBD and SR HBDs, were described previously (20). pGBT9.HBD_{PR}, pGBT9.HBD_{MR}, and pGBT9.HBD_{VDR} were made by inserting PCR-amplified cDNA fragments coding for human progestone receptor amino acids 661 to 933 ($PR_{661-933}$) (34), rat mineralocorticoid receptor amino acids 699 to 981 ($MR_{699-981}$) (36), and human VDR amino acids 95 to 427 (VDR_{95-427}) (2) into *Eco*RI/*Sal*I, *Sma*I/*Sal*I, and *Eco*RI/*Bam*HI sites, respectively, of pGBT9. Yeast expression vectors coding for fusion proteins of GAL4 DBD and HBDs of human TRα, RXRα, and RARα, named pG6H/hTRα(LBD), pG6H/hRXRα(LBD), and pG6H/hRARα(LBD), were kindly provided by Ronald Evans (Salk Institute, La Jolla, Calif.). Yeast vectors coding for fusion proteins of GAL4 DBD and the HBD of mouse ER containing various AF-2 mutations were constructed by inserting PCR-amplified fragments coding for mouse $ER_{279-599}$ into the *Eco*RI/*Sal*I sites in pGBT9. The mutant ER coding sequences used in PCR, including pJ3.MOR (wild-type ER), pJ3.D542A, pJ3.L543A/L544A, pJ3.M547A/L548A, pJ3.D549A, and pJ3.D542N/E546Q/D549N (12), were kindly provided by Malcolm Parker (Imperial Cancer Research Fund, London, England). Yeast expression vectors pRS314-GR N795 (encoding full-length rat GR), pRS314-GR N556 (encoding rat GR AD and DBD), p2T/407–795 (encoding rat GR DBD and HBD), and reporter gene *GRE3-CYC-lacZ* were described previously (17, 40).

Screening of mouse brain cDNA library. The 2.4-kb GRIP1 fragment obtained by yeast two-hybrid system screening of a mouse embryo cDNA library (20) was used as a probe to screen a Lambda ZAP II mouse brain cDNA library (Stratagene catalog no. 936309) by following the manufacturer's protocols. After screening of 10^6 clones, two of the positive clones extended the GRIP1 sequence by 0.9 kb from the 5′ end of the original fragment and 0.7 kb from the 3′ end. Fragments from the newly extended 5′ and 3′ ends were used as probes to rescreen the same library; this resulted in a further 5′ extension of 0.3 kb and a 3′ extension of 0.6 kb. Each clone in this extensively overlapping set of cDNA clones, representing altogether 4.9 kb of GRIP1 mRNA, was sequenced in both directions with a Sequenase version 2.0 DNA sequencing kit (United States Biochemical). The full-length GRIP1 open reading frame, defined by multiple stop codons on the 5′ and 3′ ends, was reconstructed in pBluescript (Stratagene) from four overlapping GRIP1 cDNA clones by using strategically located restriction endonuclease sites.

Northern blotting. A Multiple Tissue Northern Blot (Clontech) was hybridized with the original 2.4-kb GRIP1 cDNA fragment by using ExpressHyb Hybridization Solution (Clontech) according to the manufacturer's protocols.

Analyses of GRIP1 and NR function and interactions. Yeast two-hybrid system interaction assays, GRIP1 coactivator assays with yeast, and glutathione *S*-transferase (GST) pull-down assays were performed as described previously (20). Hormone treatment of liquid yeast cultures was for 4 h at 30°C, at the following final concentrations of agonists: 10 μM deoxycorticosterone (DOC) for GR, 100 nM estradiol for ER, 100 nM dihydrotestosterone for AR, 10 μM progesterone for PR, 10 μM corticosterone for MR, 10 μM triiodothyronine for TRα, 1 μM 1,25-dihydroxy-vitamin D_3 for VDR, 10 μM 9-*cis* retinoic acid for RXRα, and 10 μM all-*trans* retinoic acid for RARα. β-Galactosidase (β-Gal) activities of extracts from the liquid yeast cultures are expressed as the means and standard deviations of results from three independent transformed yeast colonies. For in vitro SR-hsp90 interaction assays, translation of SRs in vitro and subsequent coimmunoprecipitation with hsp90, using the 8D3 monoclonal anti-

body against hsp90 (kindly provided by Gary Perdew, Purdue University, West Lafayette, Ind.), was performed as described previously (11).

Immunoblotting. Yeast protein extracts were prepared by a urea-sodium dodecyl sulfate (SDS) method (37). SDS-polyacrylamide gel electrophoresis (PAGE) and immunoblotting were performed with anti-GR antibody BuGR2 as described previously (33).

Nucleotide sequence accession number. The GRIP1 sequence has been deposited in the GenBank database under accession number U39060.

RESULTS

Sequence of the full-length GRIP1 coding region. To determine the complete coding sequence for GRIP1, the original 2.4-kb GRIP1 cDNA clone (20) was used to screen a mouse brain cDNA library, since GRIP1 is well expressed in mouse brain (see below). This and subsequent screens yielded four cDNA clones that overlapped extensively with each other and the original 2.4-kb embryo cDNA and altogether represented a total length of 4.9 kb. This sequence contains an open reading frame of 1,462 codons (Fig. 1A), preceded and followed by stop codons in all reading frames; the calculated molecular mass of the encoded protein is 158.5 kDa. A GenBank search with the National Institutes of Health BLAST program (1) indicated that GRIP1 has significant homology with SRC-1 (35), a human transcriptional coactivator for SRs (Fig. 1B). Although GRIP1 contains an N-terminal region that extends beyond the N terminus of the originally reported human SRC-1 sequence, the original SRC-1 sequence was subsequently found to be incomplete at the N-terminal end. The recently reported SRC-1a protein (24), one of the SRC-1 variants in mouse, contains a longer N-terminal region that has extremely high homology with GRIP1 (Fig. 1A and B). The amino acid identity between full-length GRIP1 and SRC-1a is 43% (excluding regions that are opposite gaps in the alignment). The extensive collinear sequence homology between GRIP1 and SRC-1 indicates that these proteins belong to a new transcriptional coactivator family. During preparation of this report, Voegel et al. (45) reported a human cDNA clone for TIF2, a 160-kDa transcriptional coactivator for the ligand-dependent AF-2 activation domains of some SRs. TIF2 has 94% amino acid identity with full-length mouse GRIP1, and thus TIF2 is the probable ortholog, i.e. the human version, of GRIP1.

An RNA blot probed with the original 2.4-kb GRIP1 cDNA showed that GRIP1 RNA is widely expressed in mammalian tissues, but the expression level varies considerably, with the highest levels in the testis, while expression in the spleen is lower (Fig. 1C). In most tissues, GRIP1 sequences were found in 9- and 7-kb transcripts, but testis contained an extra 5-kb transcript. Rehybridizing this blot with probes from the extreme 5′ and 3′ ends of the GRIP1 coding region produced the same pattern of bands (data not shown), suggesting that the different transcripts have a common coding region and may differ only in the lengths of their 5′ or 3′ untranslated regions.

Full-length GRIP1 interacts with and serves as a transcriptional coactivator for DBD_{GAL4}-HBD_{NR} fusion proteins in yeast. Tests in the yeast two-hybrid system indicated that full-length GRIP1 interacted specifically with all five SR HBDs; i.e., a fusion protein consisting of full-length GRIP1 and GAL4 AD bound to fusion proteins composed of the GAL4 DBD and the HBD of any of the five SRs. These interactions occurred in the presence but not in the absence of an appropriate agonist for each SR (Fig. 2A, columns 3 and 4). However, no interaction occurred when either GRIP1 or SR HBD sequences were replaced by an irrelevant protein (data not shown). GRIP1 also interacted specifically with the HBDs of several class II NRs (Fig. 2B), but the effects of agonists on these interactions were more complex than was the case for SRs. While GRIP1's interaction with the HBD of the VDR was strictly hormone dependent (as with SR HBDs), GRIP1 unexpectedly interacted with the HBDs of TRα, RARα, and RXRα to various degrees even in the absence of added ligand (Fig. 2B, columns 3). The interaction of GRIP1 with the TRα HBD was still stimulated strongly by addition of agonist (columns 3 and 4). In contrast, the RAR ligand all-*trans* retinoic acid reproducibly caused an apparent decrease in GRIP1 interaction with the RARα HBD. The DBD_{GAL4}-$HBD_{RXR\alpha}$ fusion protein activated the two-hybrid system reporter gene strongly in the presence of 9-*cis* retinoic acid, even in the absence of a second fusion protein (column 2); thus, we could not test for GRIP1-RXRα interaction in the presence of ligand in this system.

Transcriptional coactivators may contain a transactivation domain to allow them to interact with the transcription initiation complex. To test for a transcriptional activation activity in full-length GRIP1, we expressed the full-length GRIP1 sequence fused to GAL4 DBD and found that this fusion protein could activate a β-Gal reporter gene controlled by a GAL4 enhancer site in yeast, while either GAL4 DBD or GRIP1 alone failed to activate the same reporter gene (Fig. 2C). Finally, we directly tested the ability of the full-length GRIP1 to serve as a coactivator for NR HBDs fused to GAL4 DBD. Each DBD_{GAL4}-HBD_{SR} fusion protein by itself activated the reporter gene very weakly or not at all in the presence of an agonist (Fig. 2A, columns 2), but the coexpression of full-length GRIP1 dramatically activated the β-Gal reporter gene in a hormone-dependent manner (columns 5 and 6). GRIP1 also served as a coactivator for the HBDs of the class II NRs, including VDR, RARα, RXRα, and TRα (Fig. 2B, columns 5 and 6); i.e., GRIP1 strongly enhanced the transcriptional activation functions of these NR HBDs. The requirement for a cognate ligand in these coactivator tests was similar to that observed in the two-hybrid interaction tests (columns 3 and 4). Thus, the full-length GRIP1 can physically interact with and function as a transcriptional coactivator for the HBDs of all five SRs and all of the class II NRs tested.

In a preliminary effort to localize the functional domains of GRIP1, we found that a GRIP1 fragment containing amino acids 730 to 1121 (Fig. 1B) retains the ability to interact with the GR HBD, retains a transactivation activity, and retains the ability to serve as a transcriptional coactivator for the GR HBD in yeast (data not shown).

Interaction between GRIP1 and the GR HBD is stimulated by agonist but not antagonist. The fact that agonist-associated SRs activate transcription of target genes, while antagonist-associated SRs do not, has been attributed to a difference in the conformation of receptors associated with agonists and antagonists, and physical evidence has confirmed such a conformational difference (14, 47). The antagonist-associated receptors are presumably unable to interact productively with the transcription initiation complex; for example, this could be due to an inability to interact with a required transcriptional coactivator, such as GRIP1. Glucocorticoid agonists and antagonists were therefore tested in the yeast two-hybrid system for the ability to promote interaction between the GR HBD and the originally characterized GRIP1 fragment (amino acids 322 to 1121). The agonist DOC promoted a strong interaction between the GR HBD and GRIP1, but antagonists RU486, which sometimes acts as a partial agonist, and ZK98.299, which behaves as a pure antagonist (49), did not (Fig. 3A).

The ability of each of these ligands to promote GR-GRIP1 interactions was also tested in vitro, using a functional GRIP1 fragment (amino acids 730 to 1121) expressed as a GST-GRIP1 fusion protein. In this GST pull-down assay, the ability

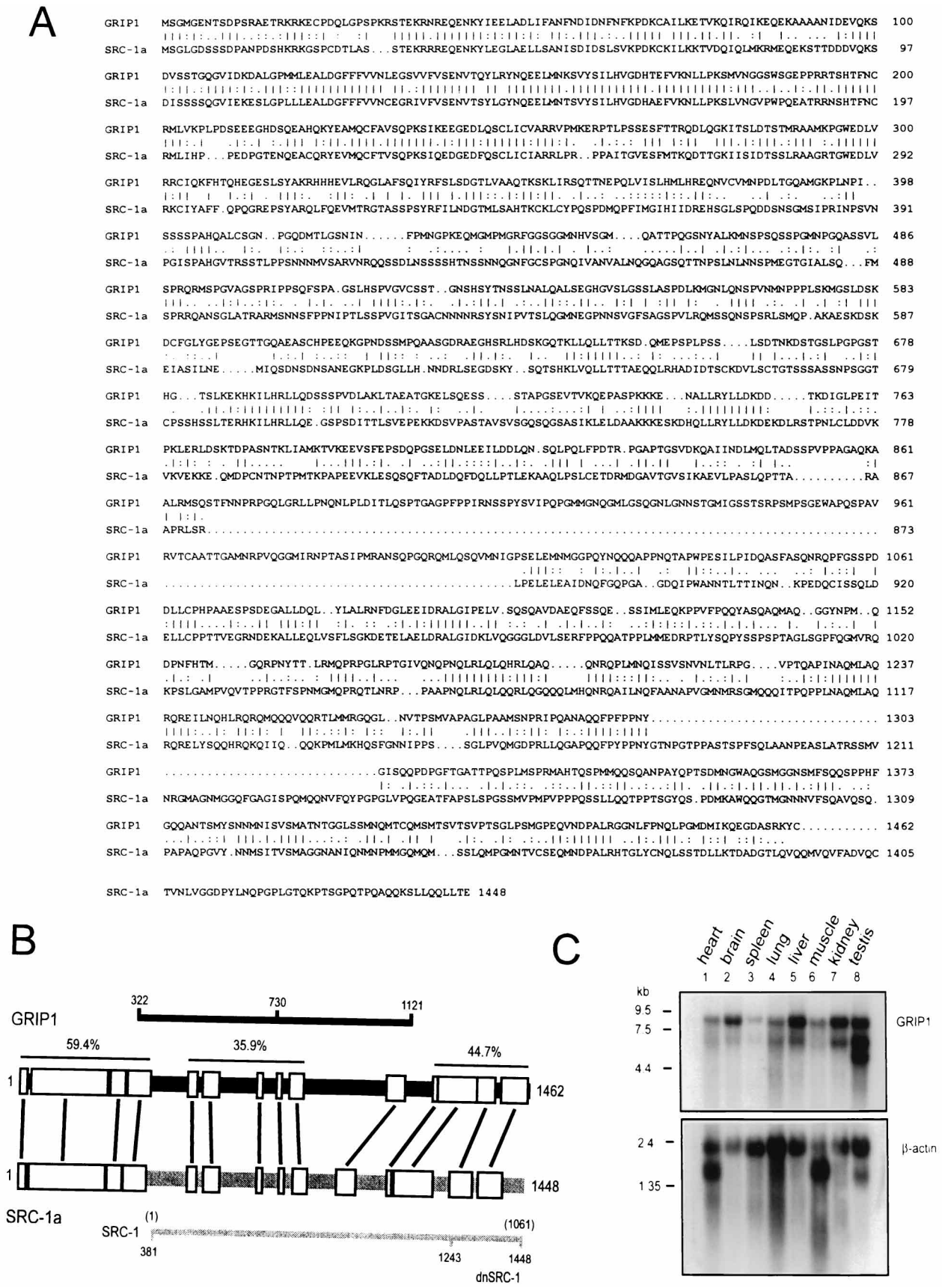
A
GRIP1 MSGMGENTSDPSRAETRKRKECPDQLGPSPKRSTEKRNREQENKYIEELADLIFANFNDIDNFNFKPDKCAILKETVKQIRQIKEQEKAAAANIDEVQKS 100
SRC-1a MSGLGDSSSDPANPDSHKRKGSPCDTLAS...STEKRRREQENKYLEGLAELLSANISDIDSLSVKPDKCKILKKTVDQIQLMKRMEQEKSTTDDDVQKS 97
GRIP1 DVSSTGQGVIDKDALGPMMLEALDGFFFVVNLEGSVVFVSENVTQYLRYNQEELMNKSVYSILHVGDHTEFVKNLLPKSMVNGGSWSGEPPRRTSHTFNC 200
SRC-1a DISSSSQGVIEKESLGPLLLEALDGFFFVVNCEGRIVFVSENVTSYLGYNQEELMNTSVYSILHVGDHAEFVKNLLPKSLVNGVPWPQEATRRNSHTFNC 197
GRIP1 RMLVKPLPDSEEEGHDSQEAHQKYEAMQCFAVSQPKSIKEEGEDLQSCLICVARRVPMKERPTLPSSESFTTRQDLQGKITSLDTSTMRAAMKPGWEDLV 300
SRC-1a RMLIHP...PEDPGTENQEACQRYEVMQCFTVSQPKSIQEDGEDFQSCLICIARRLPR..PPAITGVESFMTKQDTTGKIISIDTSSLRAAGRTGWEDLV 292
GRIP1 RRCIQKFHTQHEGESLSYAKRHHHEVLRQGLAFSQIYRFSLSDGTLVAAQTKSKLIRSQTTNEPQLVISLHMLHREQNVCVMNPDLTGQAMGKPLNPI.. 398
SRC-1a RKCIYAFF.QPQGREPSYARQLFQEVMTRGTASSPSYRFILNDGTMLSAHTKCKLCYPQSPDMQPFIMGIHIIDREHSGLSPQDDSNSGMSIPRINPSVN 391
GRIP1 SSSSPAHQALCSGN..PGQDMTLGSNIN.....FPMNGPKEQMGMPMGRFGGSGGMNHVSGM....QATTPQGSNYALKMNSPSQSSPGMNPGQASSVL 486
SRC-1a PGISPAHGVTRSSTLPPSNNNMVSARVNRQQSSDLNSSSSHTNSSNNQGNFGCSPGNQIVANVALNQGQAGSQTTNPSLNLNNSPMEGTGIALSQ...FM 488
GRIP1 SPRQRMSPGVAGSPRIPPSQFSPA.GSLHSPVGVCSST..GNSHSYTNSSLNALQALSEGHGVSLGSSLASPDLKMGNLQNSPVNMNPPPLSKMGSLDSK 583
SRC-1a SPRRQANSGLATRARMSNNSFPPNIPTLSSPVGITSGACNNNNRSYSNIPVTSLQGMNEGPNNSVGFSAGSPVLRQMSSQNSPSRLSMQP.AKAESKDSK 587
GRIP1 DCFGLYGEPSEGTTGQAEASCHPEEQKGPNDSSMPQAASGDRAEGHSRLHDSKGQTKLLQLLTTKSD.QMEPSPLPSS....LSDTNKDSTGSLPGPGST 678
SRC-1a EIASILNE.....MIQSDNSDNSANEGKPLDSGLLH.NNDRLSEGDSKY..SQTSHKLVQLLTTTAEQQLRHADIDTSCKDVLSCTGTSSSASSNPSGGT 679
GRIP1 HG...TSLKEKHKILHRLLQDSSSPVDLAKLTAEATGKELSQESS....STAPGSEVTVKQEPASPKKKE...NALLRYLLDKDD.....TKDIGLPEIT 763
SRC-1a CPSSHSSLTERHKILHRLLQE.GSPSDITTLSVEPEKKDSVPASTAVSVSGQSQGSASIKLELDAAKKKESKDHQLLRYLLDKDEKDLRSTPNLCLDDVK 778
GRIP1 PKLERLDSKTDPASNTKLIAMKTVKEEVSFEPSDQPGSELDNLEEILDDLQN.SQLPQLFPDTR.PGAPTGSVDKQAIINDLMQLTADSSPVPPAGAQKA 861
SRC-1a VKVEKKE.QMDPCNTNPTPMTKPAPEEVKLESQSQFTADLDQFDQLLPTLEKAAQLPSLCETDRMDGAVTGVSIKAEVLPASLQPTTA.....RA 867
GRIP1 ALRMSQSTFNNPRPGQLGRLLPNQNLPLDITLQSPTGAGPFPPIRNSSPYSVIPQPGMMGNQGMLGSQGNLGNNSTGMIGSSTSRPSMPSGEWAPQSPAV 961
SRC-1a APRLSR..... 873
GRIP1 RVTCAATTGAMNRPVQGGMIRNPTASIPMRANSQPGQRQMLQSQVMNIGPSELEMNMGGPQYNQQQAPPNQTAPWPESILPIDQASFASQNRQPFGSSPD 1061
SRC-1a .....LPELELEAIDNQFGQPGA..GDQIPWANNTLTTINQN..KPEDQCISSQLD 920
GRIP1 DLLCPHPAAESPSDEGALLDQL..YLALRNFDGLEEIDRALGIPELV.SQSQAVDAEQFSSQE..SSIMLEQKPPVFPQQYASQAQMAQ..GGYNPM..Q 1152
SRC-1a ELLCPPTTVEGRNDEKALLEQLVSFLSGKDETELAELDRALGIDKLVQGGGLDVLSERFPPQQATPPLMMEDRPTLYSQPYSSPSPTAGLSGPFQGMVRQ 1020
GRIP1 DPNFHTM.....GQRPNYTT.LRMQPRPGLRPTGIVQNQPNQLRLQLQHRLQAQ.....QNRQPLMNQISSVSNVNLTLRPG....VPTQAPINAQMLAQ 1237
SRC-1a KPSLGAMPVQVTPPRGTFSPNMGMQPRQTLNRP...PAAPNQLRLQLQQRLQGQQQLMHQNRQAILNQFAANAPVGMNMRSGMQQQQITPQPPLNAQMLAQ 1117
GRIP1 RQREILNQHLRQRQMQQQVQQRTLMMRGQGL..NVTPSMVAPAGLPAAMSNPRIPQANAQQFPFPPNY..... 1303
SRC-1a RQRELYSQQHRQKQIIQ..QQKPMLMKHQSFGNNIPPS....SGLPVQMGDPRLLQGAPQQFPYPPNYGTNPGTPPASTSPFSQLAANPEASLATRSSMV 1211
GRIP1 .....GISQQPDPGFTGATTPQSPLMSPRMAHTQSPMMQQSQANPAYQPTSDMNGWAQGSMGGNSMFSQQSPPHF 1373
SRC-1a NRGMAGNMGGQFGAGISPQMQQNVFQYPGPGLVPQGEATFAPSLSPGSSMVPMPVPPPQSSLLQQTPPTSGYQS.PDMKAWQQGTMGNNNVFSQAVQSQ. 1309
GRIP1 GQQANTSMYSNNMNISVSMATNTGGLSSMNQMTCQMSMTSVTSVPTSGLPSMGPEQVNDPALRGGNLFPNQLPGMDMIKQEGDASRKYC..... 1462
SRC-1a PAPAQPGVY.NNMSITVSMAGGNANIQNMNPMMGQMQM...SSLQMPGMNTVCSEQMNDPALRHTGLYCNQLSSTDLLKTDADGTLQVQQMVQVFADVQC 1405
SRC-1a TVNLVGGDPYLNQPGPLGTQKPTSGPQTPQAQQKSLLQQLLTE 1448
B
GRIP1
322
730
1121
59.4%
35.9%
44.7%
1
1462
1
1448
SRC-1a
(1)
SRC-1
(1061)
381
1243
1448
dnSRC-1
C
heart
brain
spleen
lung
liver
muscle
kidney
testis
1 2 3 4 5 6 7 8
kb
9.5
7.5
4.4
GRIP1
2.4
1.35
β-actin

FIG. 1. Amino acid sequence comparison of GRIP1 and SRC-1a, and expression pattern of GRIP1 mRNA. (A) The complete predicted amino acid sequences of GRIP1 and SRC-1a were aligned with the Gap program of Genetics Computer Group (University of Wisconsin) software (13). Sequence alignment is shown with amino acid identity (|), conservative substitutions (.) and highly conservative substitutions (:) indicated in the line between the two sequences; gaps introduced between amino acids for optimum alignment are indicated (. . .) within the sequences. (B) Corresponding regions of strong homology between GRIP1 and SRC-1a determined with the National Institutes of Health BLAST program (1) are indicated by open boxes connected by vertical or slanted lines. Percentages of amino acid identity for specified regions are indicated above the GRIP1 sequence. The following regions of GRIP1 are indicated at the top for reference: amino acids 322 to 1121, encoded by the originally isolated 2.4-kb GRIP1 cDNA clone (20); and amino acids 730 to 1121, which retained steroid receptor binding, transactivation, and coactivator activity. The following regions of SRC-1a (24) are indicated at the bottom: amino acids 381 to 1448, which correspond very closely (but not identically) to the originally reported SRC-1 sequence (35) (numbers in parentheses), now known to be a partial sequence; and amino acids 1243 to 1448, corresponding to a dominant negative protein fragment of SRC-1 (dnSRC-1) which interacts with nuclear receptors but lacks coactivator activity (35). (C) RNA blot analysis of GRIP1 and β-actin (control) expression in various mouse tissues was performed by hybridization first with the original 2.4-kb mouse GRIP1 cDNA and, after stripping, with human β-actin cDNA. Sizes of RNA markers (Clontech) are indicated on the left.

of a GR DBD-HBD fragment translated in vitro to interact specifically with GRIP1 was stimulated several-fold by agonist DOC (Fig. 3B, lanes 1 and 2) but not by antagonists RU486 or ZK98.299 (lanes 3 and 4). The failure of these antagonists to stimulate GR binding to GRIP1 could be due to their failure to cause GR dissociation from hsp90 in this experimental system; alternatively, the antagonists could cause dissociation of GR from hsp90 but fail to induce a GR conformation that can interact with GRIP1. To discriminate between these two possibilities, we examined the association between GR and hsp90 under the conditions used for the GST pull-down assay. A GR DBD-HBD fragment translated in the absence of ligand was specifically coprecipitated by an antibody against hsp90, compared with a control reaction lacking anti-hsp90 antibody (Fig. 3C, lanes 1). However, when the GR fragment was translated in the presence of agonist or antagonist, little if any GR specifically coprecipitated with hsp90 (lanes 2 to 4). Thus, all three ligands caused GR to dissociate from hsp90, but only the agonist promoted a GR conformation that could interact with GRIP1.

Point mutations that eliminate the transactivation activity of the ER HBD also prevented its interaction with GRIP1. Transcriptional activation domains of enhancer binding proteins, such as NRs, are believed to exert their effects through protein-protein interactions with the transcription initiation complex, either directly through contacts with basal transcription factors associated with RNA polymerase II or indirectly through intermediary proteins such as transcriptional coactivators (18, 20, 26, 43). The major transactivation activity in the HBD of NRs, called AF-2, has been defined by point mutations that eliminate the transactivation activity of this domain but do not affect hormone binding (12, 27, 28). This region,

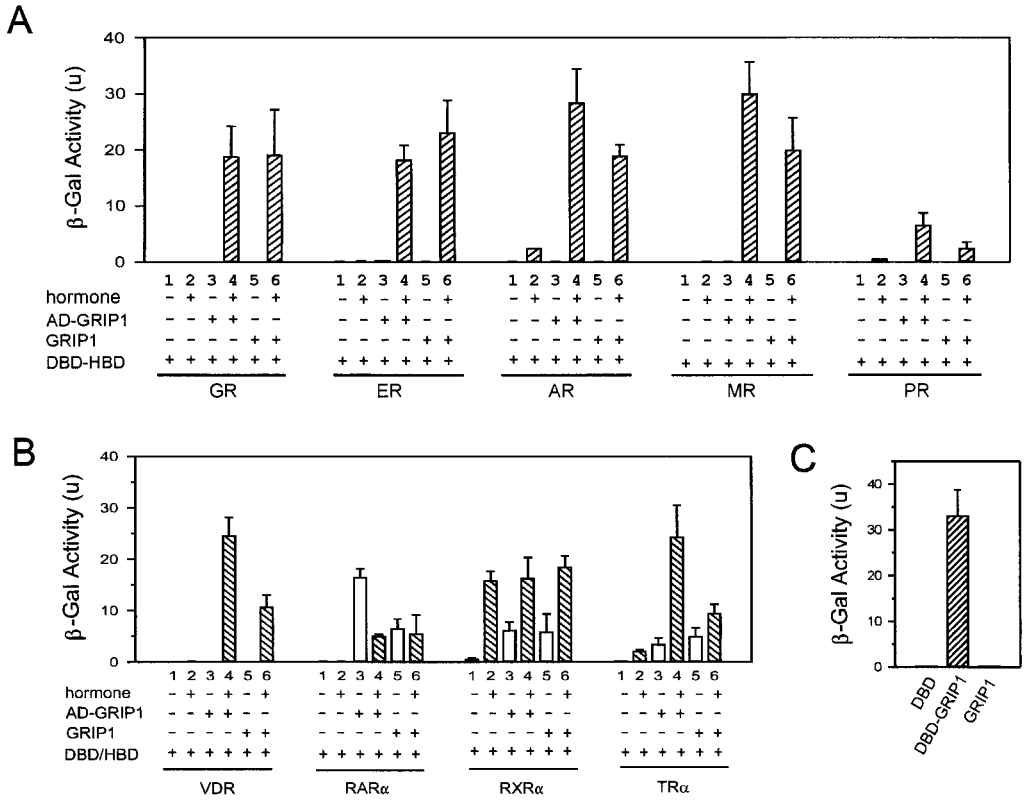

FIG. 2. Functional assays of full-length GRIP1 in yeast. The indicated proteins were expressed in yeast containing a β-Gal gene controlled by GAL4 enhancer elements. Where indicated, appropriate agonists for each nuclear receptor (see Materials and Methods) were added to liquid yeast cultures 4 h before harvest. Extracts of the cultures were assayed for β-Gal activity. DBD and AD indicate domains of GAL4; HBD indicates a domain of the specified NR. (A and B) Controls without GRIP1 (columns 1 and 2), GRIP1 interaction with NR HBDs in the yeast two-hybrid system (columns 3 and 4), and GRIP1 coactivator function for NR HBD transactivation activity (columns 5 and 6); (C) transactivation activity of GRIP1 when fused to the GAL4 DBD.

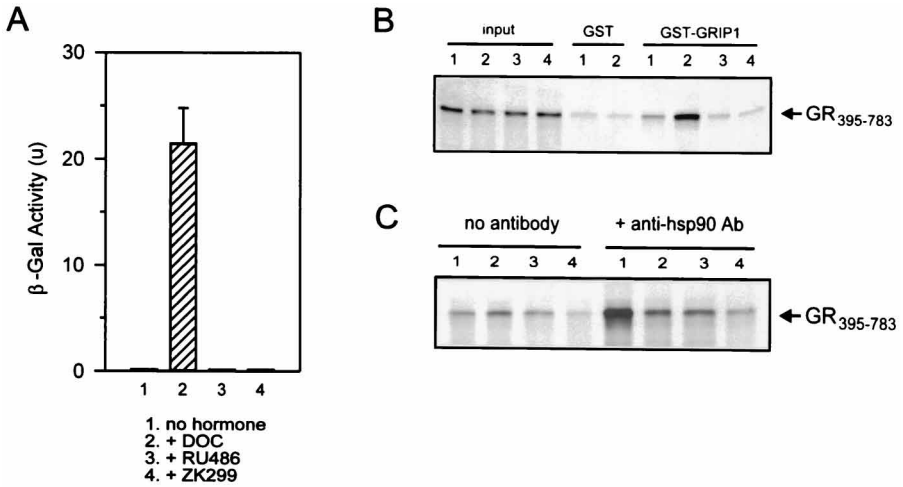

FIG. 3. Abilities of agonists and antagonists to stimulate GR interaction with GRIP1. (A) To examine GR-GRIP1 interactions in yeast, DBD_{GAL4}-HBD_{GR} and AD_{GAL4}-$GRIP1_{322–1121}$ fusion proteins were expressed in the yeast two-hybrid system in the presence of the indicated ligand (10 μM), and β-Gal activity was determined. DOC is a glucocorticoid agonist; RU486 and ZK299 are glucocorticoid antagonists. (B) To examine GR-GRIP1 interactions in vitro, a labeled mouse GR DBD-HBD fragment was translated in vitro in the presence of the indicated ligand (as indicated in the figure by the lane numbers) and incubated with Sepharose beads containing bound GST or GST-$GRIP1_{730–1121}$. Bound GR fragment was visualized by SDS-PAGE and autoradiography. Lanes: input, a sample equivalent to 1/10 of the original binding reaction; GST, the GR fragment bound to GST beads; GST-GRIP1, the GR fragment bound to GST-GRIP1 beads. (C) To examine the association of GR with hsp90, a labeled mouse GR DBD-HBD fragment was translated in vitro in the presence of the indicated ligand. The reaction products were incubated with antibody 8D3 against hsp90 or no primary antibody, and precipitation was achieved with a secondary antibody bound to protein A-Sepharose beads. Precipitated GR fragments were visualized by SDS-PAGE and autoradiography.

which corresponds to mouse ER amino acids 538 to 552, is highly conserved among many NRs.

We tested a panel of single and multiple point mutations in this region of mouse ER for the ability to interact with GRIP1. It was previously shown that two double mutations, L543A+L544A and M547A+L548A, completely eliminated transactivation by ER; the triple mutant, D542N+E546Q+D549N, caused substantial but not complete loss of function; and two single mutations, D542A and D549A, had little if any effect on ER activity (12). In the yeast two-hybrid system, GRIP1 did not interact with the two double mutants but interacted normally in a hormone-dependent manner with the other mutants (Fig. 4A, columns 3 and 4). GST pull-down assays produced identical results for all of the mutants except the triple mutant, which failed to interact with GRIP1 (Fig. 4B). The divergent behavior of the triple mutant observed here in the two different GRIP1 interaction assays may reflect the partial loss of function that it exhibited previously in reporter gene activation assays (12). Another putative coactivator, RIP140, also interacted weakly with this triple ER point mutant but not with the double mutants mentioned above (8). We used an hsp90-ER coimmunoprecipitation assay similar to that in Fig. 3C to demonstrate that all of these ER AF-2 mutants dissociated normally from hsp90 in response to estradiol (data not shown). Thus, lack of GRIP1 binding by some of the ER mutants was not due to failure to dissociate from hsp90. These studies demonstrated that the transactivation activity of various ER AF-2 mutants correlates strongly with the ability to interact with GRIP1 and that a functional AF-2 domain is apparently required for interaction with GRIP1.

GRIP1 was also tested for its ability to serve as a coactivator for the ER AF-2 mutants, by coexpressing GRIP1 in yeast with the DBD_{GAL4}-HBD_{ER} fusion proteins. These coactivator assay results (Fig. 4A, columns 5 and 6) were parallel with the ER-GRIP1 interaction data from the two-hybrid system assays (columns 3 and 4); i.e., GRIP1 served as a coactivator for those ER mutants that were fully or partially active in mammalian cells (the two single mutants and the triple mutant) but not for the ER mutants that lacked transactivation activity completely (the two double mutants). In the absence of GRIP1 or AD_{GAL4}-GRIP1 fusion protein, the DBD_{GAL4}-HBD_{ER} fusion proteins were inactive or only weakly active, even in the presence of estradiol (columns 1 and 2). Thus, whereas the ER HBD exhibits transactivation activity in most mammalian cells (6, 44), it was essentially inactive in yeast unless GRIP1 was coexpressed, indicating that yeast lack a coactivator for the transactivation activity of SR HBDs.

GRIP1 serves as a coactivator for intact GR in yeast by mediating the activity of the AF-2 domain but not the AF-1 domain. We demonstrated above the ability of GRIP1 in yeast to serve as an obligatory coactivator for the transactivation activity of DBD_{GAL4}-HBD_{SR} fusion proteins acting on a target gene controlled by a GAL4 enhancer site. Although this is a convenient test for the transactivation activity of SR HBDs, it would be more physiologically relevant to test the effect of GRIP1 on the ability of intact SRs to activate transcription through their cognate enhancer elements. When full-length GR was expressed in yeast and tested for its ability to activate a β-Gal reporter gene controlled by three tandem glucocorticoid response elements, the presence of GRIP1 resulted in a leftward shift of the dose-response curve obtained with the agonist deacylcortivazol (Fig. 5B) or DOC (not shown). Similar results were observed for full-length MR with the same reporter gene used for GR and for full-length ER with a reporter gene controlled by a single estrogen response element (data not shown). Thus, GRIP1 can enhance the activity of intact SRs in yeast.

The ability of intact SRs to function in yeast in the absence of GRIP1 was no surprise, since this result was demonstrated in many previous studies (16, 40). However, this activity of intact SRs in the absence of GRIP1 contrasted dramatically with the complete dependence on GRIP1 exhibited by the DBD_{GAL4}-HBD_{SR} fusion proteins. The difference in these two cases may be explained by the existence of multiple transacti-

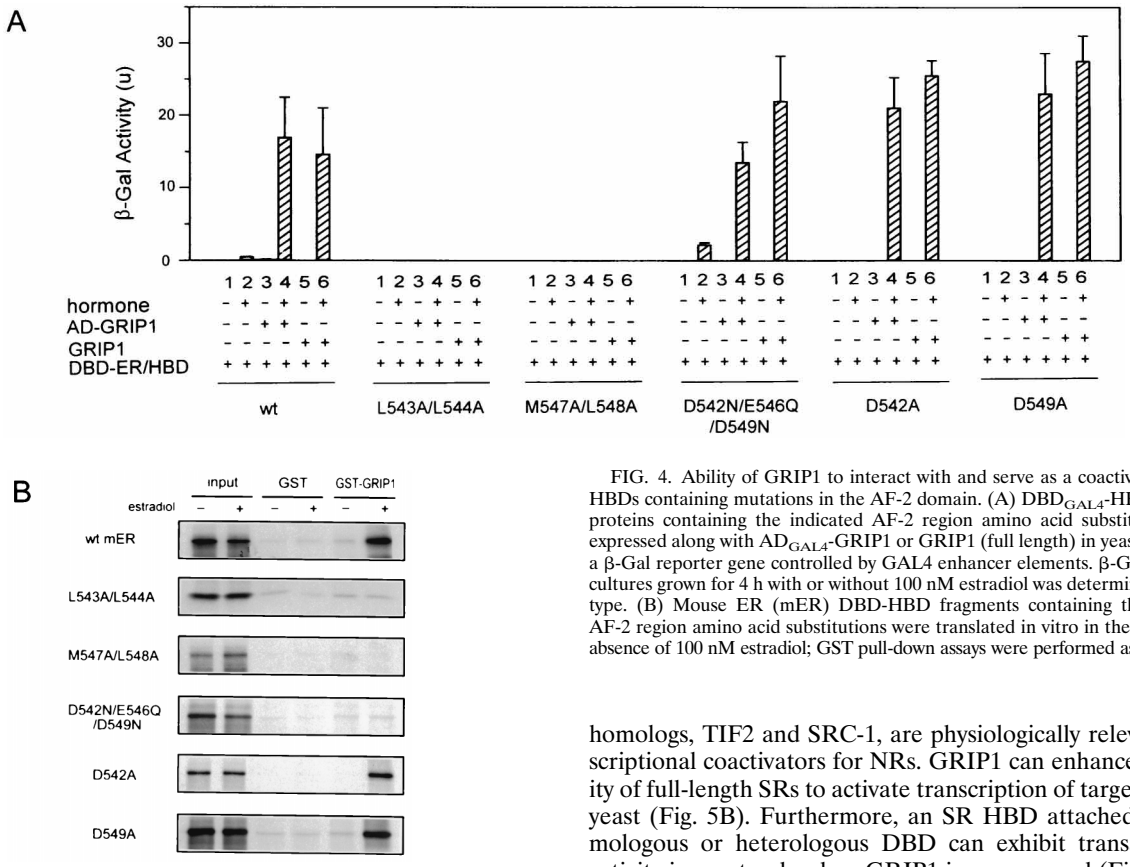

FIG. 4. Ability of GRIP1 to interact with and serve as a coactivator for ER HBDs containing mutations in the AF-2 domain. (A) DBD_{GAL4}-HBD_{ER} fusion proteins containing the indicated AF-2 region amino acid substitutions were expressed along with AD_{GAL4}-GRIP1 or GRIP1 (full length) in yeast containing a β-Gal reporter gene controlled by GAL4 enhancer elements. β-Gal activity of cultures grown for 4 h with or without 100 nM estradiol was determined. wt, wild type. (B) Mouse ER (mER) DBD-HBD fragments containing the indicated AF-2 region amino acid substitutions were translated in vitro in the presence or absence of 100 nM estradiol; GST pull-down assays were performed as for Fig. 3B.

vation domains in SRs (19, 28). The N-terminal AF-1 transactivation domain in intact SRs may be responsible for the GRIP1-independent transactivation activity in yeast. To investigate this hypothesis and the mechanism of GRIP1 coactivation of intact GR in yeast, we examined the ability of GRIP1 to activate the individual AF-1 and AF-2 activation domains of GR, with each linked in its native position to the GR DBD (Fig. 5A). The GR DBD-HBD fragment, like the previously examined fusion protein of the GAL4 DBD and GR HBD, was inactive in yeast by itself, but coexpression of GRIP1 restored its hormone-dependent activation of the glucocorticoid response element-controlled reporter gene (Fig. 5C). The enhancement by GRIP1 was not due to stabilization of the expression of the GR DBD-HBD fragment in yeast, since immunoblots with a GR-specific antibody indicated that the presence of hormone and GRIP1 did not affect the levels of the GR DBD-HBD protein in yeast (Fig. 5D).

In contrast, the GR AD-DBD fragment containing the AF-1 activation domain (Fig. 5A) was constitutively active in yeast, and this activity was not stimulated by hormone or GRIP1 (Fig. 5C). This result was consistent with results from GST pull-down assays, where no interaction between GRIP1 and the GR AD-DBD fragment was detected (Fig. 5E). Thus, GRIP1 acted as an obligatory transcriptional coactivator for the AF-2 transactivation domain but had no effect on the activity of the AF-1 domain when each domain was expressed independently from the other.

DISCUSSION

Evidence that GRIP1 is a coactivator for SRs and class II NRs. Current evidence argues strongly that GRIP1 and its homologs, TIF2 and SRC-1, are physiologically relevant transcriptional coactivators for NRs. GRIP1 can enhance the ability of full-length SRs to activate transcription of target genes in yeast (Fig. 5B). Furthermore, an SR HBD attached to a homologous or heterologous DBD can exhibit transactivation activity in yeast only when GRIP1 is coexpressed (Fig. 2A and 5C). In previous studies in mammalian cells, TIF2, the probable human ortholog of GRIP1, and the distinct but homologous protein SRC-1 stimulated the transactivation activity of some but not all NRs and thus served as coactivators, although the degree of enhancement by the coactivators was much more modest than in our yeast system (35, 45). When tested in mammalian cells, GRIP1 can also enhance ER function (30). GRIP1, TIF2, and SRC-1 all contain transactivation domains, which presumably allow them to interact with components of the RNA polymerase II transcription initiation complex. Another strong correlative argument for the physiological relevance of GRIP1 in SR function is the fact that it interacts with SRs only in their transactivationally active state. For example, the effect of ER AF-2 region mutations on ER transactivation activity in mammalian cells correlates closely with their effect on ER-GRIP1 interactions. This finding indicates that a functional AF-2 domain is required for interaction with GRIP1 and suggests that GRIP1 may interact directly with the AF-2 domain to mediate its activity. Furthermore, GRIP1 interacts with GR that is bound to agonist but not when GR is bound to antagonists or lacks bound ligand. Similar findings have been reported for SRC-1 (35) and TIF2 (45). These results finally may provide a molecular explanation of why some ligands serve as antagonists rather than agonists. Previous studies demonstrated that antagonists and agonists induce different conformations in SRs (14, 47), and it was thus proposed that the antagonist-induced SR conformation did not interact productively with the transcription initiation complex. The data presented here demonstrate directly that antagonist-associated SRs do not interact with required coactivators, such as GRIP1; this can explain why antagonist-associated SRs do not activate transcription.

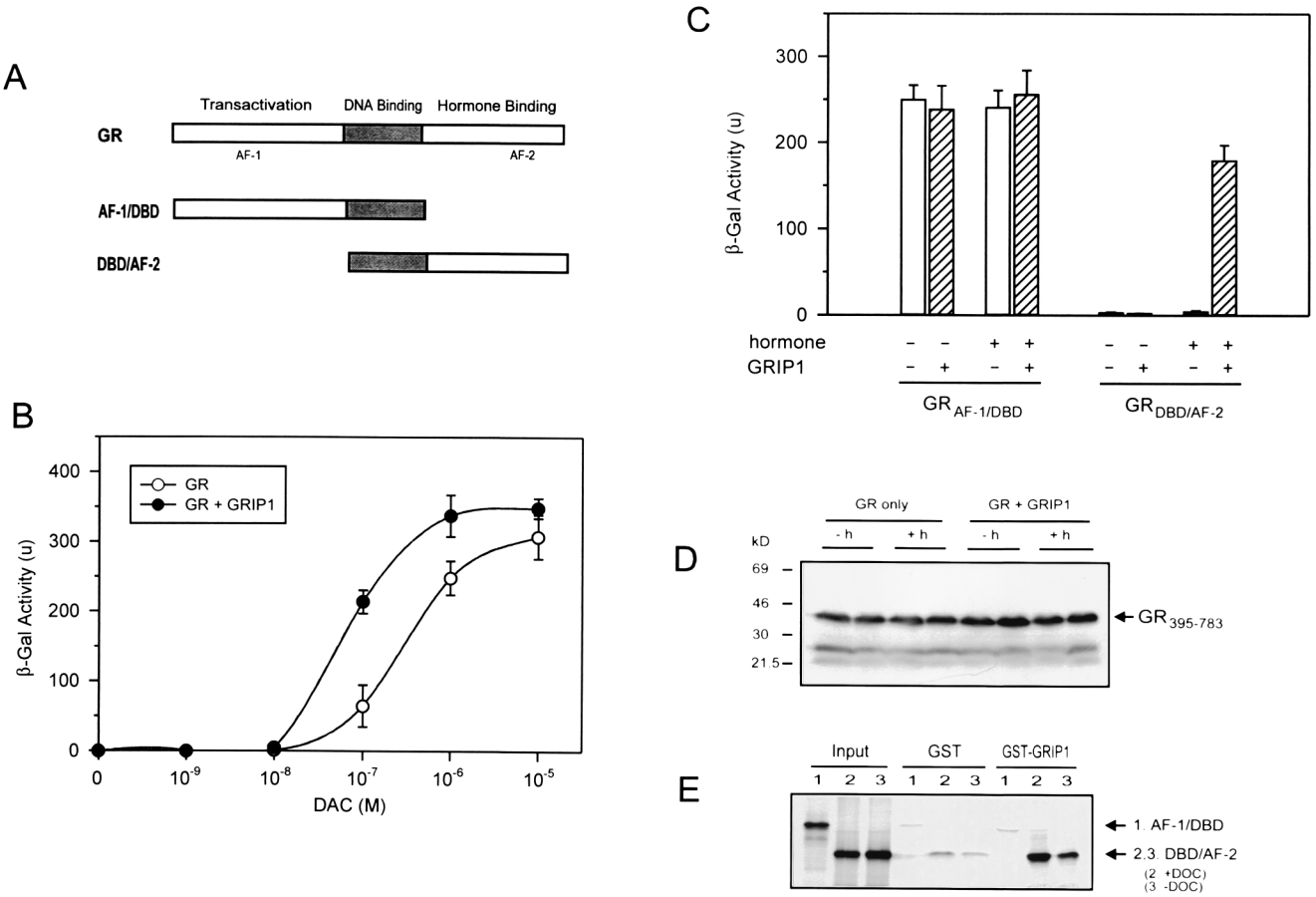

FIG. 5. GRIP1 serves as a coactivator for full-length GR or for the GR AF-2 domain but not for the GR AF-1 domain. (A) Rat GR species: full-length GR, equivalent to mouse GR amino acids 1 to 783; AF-1/DBD, equivalent to mouse GR amino acids 1 to 544; DBD/AF-2, equivalent to mouse GR amino acids 395 to 783. (B) Full-length rat GR was expressed in yeast containing a *GRE3-CYC-lacZ* reporter plasmid in the presence or absence of full-length GRIP1, and β-Gal activity was determined after 4 h of culture with the indicated concentration of deacylcortivazol (DAC). (C) The indicated truncated GR species was expressed in yeast with or without the $GRIP1_{322–1121}$ fragment. Cells were grown with or without 10 μM DOC for 4 h, and β-Gal activity was determined. (D) Yeast cultures expressing the GR DBD/AF-2 fragment with or without the $GRIP1_{322–1121}$ fragment were grown with or without DOC (h) as in panel C; extracts were analyzed by immunoblotting with BuGR2 antibody against GR. Results for duplicate cultures are shown for each condition. The faster-migrating band is presumably a degradation product of GR. No bands were observed in yeast lacking the GR expression plasmid (not shown). (E) GR AF-1/DBD or DBD/AF-2 fragments were translated in vitro with 10 μM DOC (reaction 2) or without DOC (reactions 1 and 3) and used in GST pull-down assays as in Fig. 3B.

This is the first report of a coactivator that can dramatically enhance the AF-2 transactivation activity of class II NRs. The ability of GRIP1 to interact with and serve as a coactivator for the transactivation domains in the HBDs of VDR, TRα, RARα, and RXRα suggests that GRIP1 is an important physiological mediator for a much larger group of transcription factors than just the SRs. In addition to the NR-GAL4 fusion proteins studied here, GRIP1 can also dramatically enhance the transactivation activity of full-length class II NRs, either as homodimers or as heterodimers with RXR (46). In contrast to the strictly hormone-dependent interaction with SRs, GRIP1 interactions with class II NRs exhibited variable degrees of hormone dependence in yeast (Fig. 2B). The differences for GRIP1 interaction with SRs and class II NRs may reflect the differences in biology of these two subfamilies of NRs. Unlike SRs, the class II NRs do not complex stably with hsp90 and can bind their response elements even in the absence of hormone. In contrast to our results, TIF2, the probable human ortholog of mouse GRIP1, interacted with the HBD of RARα in a hormone-dependent manner (45). This difference may be due to the different experimental systems used: we studied GRIP1-NR interactions in a yeast two-hybrid system, which apparently lacks coactivators and may also lack corepressors for NR HBDs; TIF2-NR interactions were examined in mammalian cells or their extracts which contain endogenous coactivators and corepressors, both known and unknown. The yeast system may represent an important tool for defining the specific roles and functional interactions of coactivators and corepressors in NR function.

Comparison of yeast and mammalian cell systems for studying transcriptional coactivators of NRs. The yeast system offers an important advantage for investigation of transcriptional activation by ligand-dependent NRs and their coactivators, because it provides an opportunity to study AF-2 coactivators under conditions where their expression from externally supplied plasmids is obligatory or strongly stimulatory for the AF-2 activity of all NRs tested to date. In contrast, GRIP1, TIF2, and SRC-1 are expressed relatively ubiquitously in mammalian cells (Fig. 1C) (35, 45), and the possible presence of additional currently unknown AF-2 coactivators, as well as corepressors (7, 9, 21, 25), must also be considered. This undoubtedly explains why detection of coactivator activity by overexpression in mammalian cells has thus far required overexpression of the NRs and why overexpression of coactivators

caused only a small enhancement of the transactivation activity of most NRs in mammalian cells (35, 45). Furthermore, the presence of endogenous coactivators in mammalian cells makes it difficult to interpret apparent differences in the ability of an overexpressed coactivator to enhance the activity of different NRs. For example, overexpression of TIF2 in HeLa cells enhanced the activity of PR, ER, and AR, but not GR or any class II NRs, although TIF2 could bind to all of these NRs (45). Likewise, overexpression of SRC-1 in mammalian cells caused relatively modest enhancements in the transactivation activity of class II NRs (35). In contrast, in yeast, GRIP1 dramatically stimulated AF-2 activity for all of the NRs tested, and the coactivator activity of GRIP1 in yeast correlated closely with its ability to interact physically with a wide spectrum of NRs (Fig. 2). Thus, the lack of stimulation of class II NR activity by coactivators in mammalian cells was not due to inability of TIF2 and SRC-1 to support the transactivation function of these NRs; instead, levels of endogenous coactivators in mammalian cells may be optimal for some NRs but suboptimal for others, due to different efficiencies of NR-coactivator interactions. The ability to reconstitute AF-2 activity cleanly with exogenously supplied coactivator, coupled with the genetic advantages of yeast, indicates the potential power and utility of yeast for studying this class of transcriptional coactivators.

Functional domains of the GRIP1/TIF2/SRC-1 coactivators. GRIP1, TIF2 (45), and SRC-1 (35) have all exhibited substantial coactivator activity. The fact that these three proteins have extensive sequence homology indicates that they belong to a new transcriptional coactivator family. Although GRIP1 and TIF2 have many regions of partial homology with SRC-1, current data suggest some possible differences in their functional domains. A small region of GRIP1 (amino acids 730 to 1121) retains SR binding activity, transactivation activity, and coactivator activity. However, this region of GRIP1 exhibits relatively low homology with SRC-1 (Fig. 1A and B). In addition, the dominant negative fragment dnSRC-1, which is reported to be the major NR interaction domain of SRC-1 (35), does not overlap with the originally identified fragments of GRIP1 (20) or TIF2 (45), which contain the only demonstrated NR interaction domains in those proteins (Fig. 1B). Thus, it remains to be determined whether SRC-1 has an NR interaction domain completely different from that of GRIP1 or TIF2 or, alternatively, there are multiple NR interaction domains in these proteins.

The originally reported sequence for SRC-1 protein (35) was incomplete, lacking 380 N-terminal amino acids. With the identification of SRC-1a (24), we found that the region of highest homology between GRIP1 and SRC-1a is located in the N terminus of each of those proteins (Fig. 1A and B), suggesting that an important conserved function resides in this region. It is also interesting that CREB-binding protein (CBP), which was initially identified as a coactivator for the CREB transcriptional activator protein (26), has subsequently been implicated as the core element of a large complex of coactivators, including SRC-1 (24). CBP was also shown to interact with the HBDs of NRs in a hormone-dependent manner and with SRC-1a at the same time, suggesting that the CBP coactivator complex may serve as an integrator of multiple signal transduction pathways within the nucleus (24). It will be interesting to test for an interaction between CBP and GRIP1 and the possible biological relevance of such an interaction.

Mechanism of coactivation by GRIP1. The results presented here indicate that yeast cells have an endogenous mechanism to support the transactivation activity of the AF-1 domain but not the AF-2 domain of SRs. This observation, together with the different promoter and cell type specificities exhibited by AF-1 and AF-2 transactivation domains in mammalian cells (31, 44), provides indirect evidence suggesting that these two domains utilize different mechanisms of transactivation. Our demonstration that AF-2 function in yeast requires GRIP1 as a coactivator, whereas AF-1 is GRIP1 independent, provides direct evidence to support this hypothesis and establishes a specific and distinct molecular mechanism for the AF-2 transactivation activity. While GRIP1 did not enhance the activity of an independent AF-1 domain, our results do not preclude the possibility that GRIP1 contributes to the demonstrated synergistic effect between AF-1 and AF-2 (19, 28, 32).

Possible mechanisms for GRIP1's enhancement of NR activity theoretically include stabilization of the NR protein or of the receptor-hormone complex. However, immunoblot data demonstrated directly that the presence of GRIP1 and hormone did not affect the levels of GR protein in yeast (Fig. 5D). Furthermore, while the leftward shift in the hormone dose-response curve for intact GR caused by GRIP1 (Fig. 5B) could theoretically be due to a stabilization of hormone binding to GR, other evidence argues against this interpretation. If GRIP1 were acting solely by stabilization of hormone binding to GR, we would expect to see similar effects by GRIP1 on intact GR and on GR DBD-HBD fragments. However, the activity of the DBD-HBD fragments was completely dependent on GRIP1, even at saturating concentrations of agonist (Fig. 5C). The failure of GRIP1 to enhance the activity of intact GR at high hormone concentrations (Fig. 5B) may be due to limiting amounts of other essential transcription components in this yeast reporter gene assay system, which impose an artificial ceiling on β-Gal gene activation. In conclusion, the transactivation activity of intact SRs in yeast in the absence of GRIP1 is apparently due entirely to AF-1, and the stimulatory effect of GRIP1 on intact SRs is due to restoration of AF-2 transactivation activity by GRIP1.

True transcriptional coactivators are thought to mediate transcriptional activation by interacting with an enhancer-bound transcription factor and with components of the basal transcription initiation complex (18). This interaction may promote the formation of a basal transcription initiation complex, stabilize such a complex after it is formed, or somehow convert the preformed complex to an active state. While the current studies have indicated the nature of the interaction between GRIP1 and NRs, further work will be required to determine the specific components of the basal transcription initiation complex that GRIP1 interacts with, and which of the above-described coactivator mechanisms pertains to GRIP1.

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